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Stimulation and production of some active compounds in the Chia plant with melatonin in vitro

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Abstract. A laboratory experiment was carried out in the tissue culture laboratories of the College of Agriculture \ University of Anbar \ Iraq from 1/2/ 2021 till 1/7/2021, to multiply and produce some active compounds in Chia in vitro. Nutrition medium (MS) (Murashige and Skoog) was used to propagate chia plants. The culture medium was enriched with benzyl adenine regulator at MS concentrations, 1, 2, and 3 mg $L^{-1}$with five replicates for each one, respectively, in order in the multiplication phase, to know its effect on some morphological characteristics of Chia plants such as branch length, nodes and leaflets number. Also, brassinolide was laid out of 0, 1 and 2 mg L^{-1}, while NAA added of 0, 1, 2 and 3 mg $L^{-1}$with five replicates for each one respectively, were added to the culture media at the rooting stage, in order to test its effectiveness in improving the formation of rootlets of Chia plants. In another experiment, the culture medium was enriched with different concentrations of the melatonin PGR 0, 1, 2, and 3 mg L^{-1}, with five replicates for each one, respectively, in order to study the effect of melatonin in the production of active compounds. The results showed that enrichment of the culture medium with benzyl adenine at a concentration of 1 mg L^{-1} increased the length, branches number and nodules number in plantlets (9.46 cm per branch, 5.45 branch per plantlet, and 9.57 nodes per shoot). The combined effect of Brassinolide 1 mg L^{-1} and 3 mg L^{-1} NAA improved both number of rootlets and their length. of 10.5 rootlets per plantlet and 5.7 cm, respectively. Also, the addition of melatonin to the culture medium significantly diversifying active compounds in term of quantity and quality characteristics. The concentration of 1 mg L^{-1} of melatonin gave the highest number of active compounds (25 compounds), however the etheric compounds and fatty acid derivatives were the highest. The percentage of etheric compounds was 80.0% by adding 3 mg L^{-1} of melatonin, and the percentage of fatty acid derivatives reached 16.36% of the total area ratio by adding 1 mg L^{-1}.

Key words: chia, melatonin, in vitro, active compounds.

INTRODUCTION

Medicinal plants have an important role in human life due to their abundance and multiple uses. The benefit of these plants lies in their ability to produce many organic compounds with medicinal properties and are listed as raw materials or co-factors in the pharmaceutical industry (Neamah & Almehemdi, 2018). The World Health Organization indicated that 80% of the world's population depends on medicines from plant sources in medical treatment (Hartmann, 2004).

Chia (*Salvia hispanica* L.) is one of the important and common plants used in medicine since ancient times and is widely used today in most countries of the world. Due to the interesting medicinal and nutritional properties of this plant (Jamboonsri et al., 2012). Its seeds represent the highest natural source of Ʊ-3 & Ʊ-6 fatty acids, which are important fatty acids in human nutrition as they reduce the risk of cardiovascular disease. Several bioactive chemicals in the Chia plant have also been identified as having a therapeutic ability to lower blood sugar and cholesterol and inhibit the growth of cancer cells (Wu et al., 2016; Gazem et al., 2017). Seeds are a good nutritional supplement, as they contain proteins, some vitamins (A, B, K, E, D), and minerals, and metals. Besides, it is an excellent source of polyphenols and oxidants, such as caffeic acid, rosmarinic acid, myristin, quercetin, and others (Lu & Foo, 2002). Reducing the risk of heart and liver disease, anticoagulant, antioxidant, and fungicidal (Yadav et al., 2019). Supporting the digestive system, strengthening bones and muscles and promoting skin health, it may enter curd that improves the composition of this product (Nadtochii et al., 2020). Seeds of Chia could be served for prolonging times occurring seed aging which caused various changes in that seeds such as alteration of taste, flavor and chemicals content (Caruso et al., 2018). From these chemicals, fatty acids when they degraded increase acidity in stored chia seeds at different temperatures (Cruz-Tirado et al., 2020). It also contains mucilage thereby effect on seeds hydration which indirectly effect on shelf-life of these seeds because of hydrophilic properties (Geneve et al., 2017). Therefore, concerted efforts have been made to evolve attainable and cost-effected sources of these plant-drugs. Biotechnological productivity of high-valued compounds that plant-synthesized via in vitro culture of plant cells is attracted and sustained alternative to extracting material from plant sources. Thus, it is paving the path for effecting the cost of production for therapeutically various constituents (Ajungla et al., 2009). Moreover, plant chemicals can be manufactured, multiplied, or converted into pharmaceutical preparations. So various methods have been developed using plant tissue culture technology such as root, organ and callus culture or suspended cell culture, as well as the use of bioreactor technology. Plant cell cultures compared to wild plants are distinguished by the year-round production of active compounds in sufficient quantities rather than from raw material and of high purity under controlled environmental conditions in a laboratory. So, tissue culture led to conserve many plant species when cultural media were strengthened by minimized the media for given periods. Some researchers have been able to increase the number of medicinal compounds by using some biological or non-biological stimuli. Auxins have a significant importance in the proliferation of calli and the emergence of roots. Plant species grown with tissue culture technology participate in many factors in the formation of rootlets, they may be external or they may be internal, including auxins, as they are the most important factors that have a major role in the emergence and occurrence of the rooting process and the weight of those emerging roots (Stefancic et al., 2005 and Sevik & Guney, 2013). Also, the external addition of this regulator increased the emergence of side radicals as a result of the transfer of this regulator (Salih & Al-Dabagh, 2020). As Marconi et al. (2013) conclude that the nutritional medium supplemented with the growth regulator NAA had a clear effect on the reproduction of rhizogenic roots. It is important in supporting the culture media prepared for the growth of chia plantlets (Schuelter et al., 2020). On the basil plant, Trettel et al. (2020) mentioned that the addition of the growth regulator NAA to the growing medium improved the number of leaflets, which was reflected in improving its other characteristics such as chlorophyll index, cotyledon length, fresh and dry weight, beside external factors include brassinosteroids, which are involved in cell division and the coding and production of new proteins (Hu et al., 2000). Improving growth, stem elongation and leaf formation under tensile conditions, and synthesis of carbon-representing pigments, antioxidant enzymes and electrolytes under tensile conditions (Niu et al., 2016). The addition of some nutritional components such as amino acids may contribute to improving the production of some active ingredients such as alkaloids that enter the metabolic pathways generating these compounds, as in the alkaloids of Datura produced in tissue culture (Neumann et al., 2009). Therefore, these PGRs have an important role in regulating the elongation of the petiole and hypocotyl, which contributes to increasing the stability of the root cells under the stress of depletion of nutrients in the culture medium. Consequently, the combination of these PGRs in proper method contributes into directly developed shoot calli.

Mohammed & Almehemdi (2021) showed that brassinolide regulator is the most effective and efficient in the formation and development of radicle of plantlets histologically developed, and that some PGRs and organic acids contribute to the gene expression of some enzymes when they are added to the food to the nutrient media as stress elicitors that control the construction of secondary compounds and then increase those compounds (Neamah & Almehemdi, 2017) in root plantations of the genus *Brugmansia* of the Solanaceae family (Zayed & Wink, 2004). This research aims to use melatonin in concentrations as a biostimulant for the production of some medicinal compounds in the Chia plant in vitro and to diagnose it quantitatively and qualitatively by using GC-MS technology.

MATERIALS AND METHODS

The research was carried out in the tissue culture laboratories of the College of Agriculture \ Anbar University \ Iraq. As a source of plant material, Chia seeds were used, the Malaysian variety grown in the Center for Desert Studies/University of Anbar.

Media preparation of seed germination

This was done by preparing a sterile medium for planting seeds that consisted of Murashige and Skoog medium (MS) (1962), to which sucrose was added at an amount of 30 g per liter, complete the volume to 1,000 mL of distilled water, after adjusting the pH to 5.7 by adding drops of sodium hydroxide (NaOH) or hydrochloric acid (HCl) solution and agar was added at an amount of 7 g L^{-1}. The components of the food medium were heated on the Hot Plate magnetic stirrer for the purpose of dissolving the agar with the components of the nutrient medium. After the medium became homogeneous, it was distributed into 300 mL jars, sealed with a lid, and then sterilized with an Autoclave at a temperature of 121 °C and a pressure of 1.04 kg cm^{-2} for 15 minutes. After the sterilization period was over, the media were taken out and transferred to the culture room at 25 °C and left to solidify

Sterilization and seed germination

Chia seeds were sterilized using 3% sodium hypochlorite for 15 minutes, then the seeds were washed with sterile distilled water three times for five minutes each time. The seeds were cultured after sterilization under cabinet of laminar airflow on the prepared nutritional medium and then transferred to the growth room under controlled environmental conditions at a temperature of 25 ± 2 °C and illumination for 16 hours daily at an intensity of 40–60 µE m^{-2} s^{-1}. Four weeks after the start of planting, seedlings were formed and ready for multiplying.

Growth and Multiplication stage

Four weeks after the start of planting, seedlings were formed and ready for multiplying via eradication of terminal buds. Here, concentrations of benzyl adenine were added viz., MS, 1, 2 and 3 mg L^{-1} enhanced by NAA of 0.5 mg L^{-1}, for each concentration with five replicates.

The same components of the nutrient medium were used in the stage of growing cultures added to the benzyl adenine regulator 2 mg L^{-1}, After that, the value of the pH value of the mentioned concentrations was adjusted to 5.7, then the agar was added 7 g per liter and the medium was sterilized by the aforementioned method. After solidification of the nutrient medium, the terminal buds with a length of 1 cm were removed and planted on the previously prepared nutrient medium. Then the cultures were incubated at 25 ± 2 °C and illuminated for 16 hours.

Rooting stage

The elongated shoots were planted on MS medium enriched with oxy naphthalene acetic acid at concentrations (1, 2 and 3) mg $liter^{-1}$ in combination with Brassinosteroid (1 and 2) mg $liter^{-1}$ as well as control without any addition of hormones with five replicates. and after four weeks, number and length of the initiated roots was calculated.

Stimulating the production of secondary compounds

Terminal buds resulting from the replication stage were excised and cultured on MS nutrient medium supplemented with the regulator benzyl adenine 2 mg. $liter^{-1}$ and with different concentrations of melatonin (0,1,2 and 3) mg L^{-1} with five replicates. After four weeks, the sample extracts were prepared for each treatment from the extracts from the leaves in order to separate and estimate the active ingredients using GS/MS technology.

Preparing the extracts:

After completing the growth period of the plantlets (4 weeks), these plantlets were taken, cleaned from the remnants of the culture medium, and then dried in the oven at a temperature of 68 °C. Samples were extracted after completion of the drying period. Weigh 100 mg of leaves emerging from dried vegetative branches and pulverized them using a ceramic mortar, add 100 mL of 99.99% ethanol alcohol. The extract was ultrsonicated for 20 min under 20–30 °C. Finally, it filtered via Whatman filter paper (no#1) under vacuum pump. The filtrates were served under 2 ± 2 °C till it took to be analyzed.

GC/MS consoles

The GC-MS profiling was established via GCMS-QP2010 plus device (Shimadzu, Kyoto, Japan) preparation-equipped with autoinjector and 5ms capillary column with dimensions of 30×0.25 mm with 0.25 µm film thickness. Gas of He was exploited as the carrier phase at rate- flown of 1.15 mL min^{-1}. Mass spectroscopic analysis was laid out via system- ionized of 70eV. The initial temperature was switched on at 80 °C for 2 min. gradually, it was risen at a rate of 10 °C per min. up to 280 °C for 5 min. The injecting the sample was relying on split mode at 250°C. Data-bases mass spectral National Institute of Standards and Technology (NIST11), advocated to identify the isolated constituents depended on time- retentive and mass spectra.

Statistical analysis

Means of the recorded data were statistically analyzed using the statistical analysis program Genstat (ed.12) under the probability of 0.05 to test the study parameters (Payne et al., 2009). The significant differences between the means were compared with the least significant difference test (LSD) as remembered in Al-Mohammadi & Al-Mohammadi, 2012.

RESULTS AND DISCUSSION

Effect of Benzyl adenine (BA) on the growth of Chia plants

The addition of benzyl adenine at a 1 mg L^{-1} achieved the highest average length of vegetative branches (9.46 cm) and the number of nodes in the shoot (9.57 nodes per shoot) of the chia plant (Table 1), while the concentration of 2 mg liter gave 5.54 vegetative branches per plantlet) and the average number of leaves (21.12 leaves of a per plantlet, while the addition of 3 mg L^{-1} to the medium gave the lowest averages for the length of vegetative branches (3.91 cm) and the number of vegetative branches (2.57 branches per plantlet in similarity with the control treatment (without any addition). The number of nodes in the shoot (5.51 nodes per shoot) and the number of leaflets (9.43 leaflets per plantlet).

Table 1. Effect of different concentrations of benzyl adenine on the length, of branches nodules and leaflets number of Chia plant in vitro

Benzyl adenine conc. mg L^{-1}	Branch length cm	Number of branches branch per plantlet	Number of nodes per shoot	Number of leaflets per plantlet
0.0	8.17	2.57	8.29	12.26
1	9.46	5.45	9.57	16.83
2	7.09	5.54	9.04	21.12
3	3.91	2.57	5.71	9.43
$LSD_{0.05}$	2.41	0.97	N.S	2.75

The appropriate concentration to improve the growth of induced Chia plantlets in vitro was effective in the propagation of vegetative buds and improving the length and number of branches, the number of shoots, shoot nodes and leaflets (Table 1). BA is the most effective plant growth regulator in inducing the multiplication of plantlets for most plant species, including the chia plant (Yadav et al., 2019). As this regulator has proven to be more effective in improving growth indicators, it has confirmed that the media of plant growth of Chia plants supplemented with benzyladenine improves the induction and multiplication of the vegetative parts, which depends on the concentration (Salih & Al-Dabagh, 2020). Which is reflected in the length and number of branches and the number of nodes and leaflets (Table 1). On the contrary, there are references that did not indicate this effect (Chen et al., 2005). Furthermore, given concentration of BA could increase multiplication factor (Egorova et al., 2021).

Effect of NAA and BR on the mean chia rootlets number

The effect of the PGR NAA and BR combinations between them on the average number of rootlets of Chia plants are shown in Fig. 1.

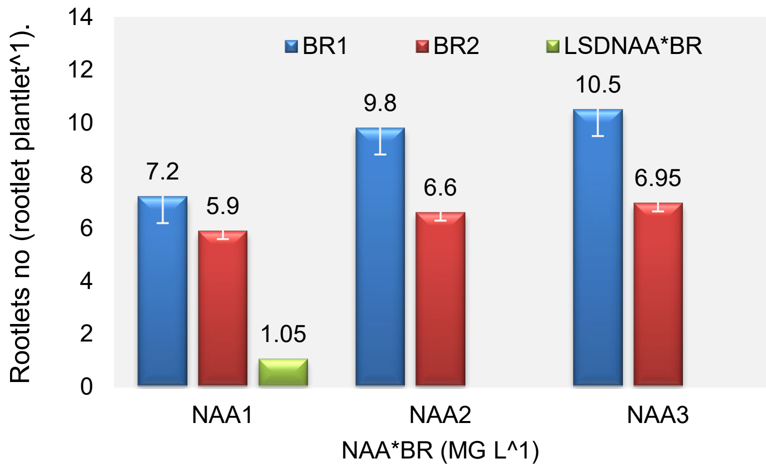

Figure 1. Effect of different concentrations of NAA and BR on the average number of rootlets of Chia plantlets in vitro.

The combinations of brassinolide and NAA has an important role in elevating the average number of roots in given plant. The treatment combination between 3 mg NAA L^{-1} and 1 mg BR L^{-1} gave the highest average number of rootlets in the plantlet reaching 10.50 rootlets per plantlet. While the addition of the combination 1 mg NAA L^{-1} × 2 mg BR L^{-1} reduced the average number of rootlets in the plantlet to 5.90 rootlets per plantlet.

Effect of Naphthalene acetic acid (NAA) and Brassinolide (BR) on rootlet length

The effect of the PGR NAA and BR combinations between them on the average length of roots of Chia plants shown in Fig. 2. The combinations of brassinolide and NAA has an important role in increasing the average lengths of rootlets in the Chia plantlets.

The combination between 3 mg NAA L^{-1} and 1 mg BR L^{-1} gave the highest mean length of the rootlets of the plantlet, which was 5.70 cm $rootlet^{-1}$. While the addition of a combination of 1 mg NAA L^{-1} × 1 mg BR L^{-1} reduced the average rootlet length of the plantlet to 2.60 cm $rootlet^{-1}$.

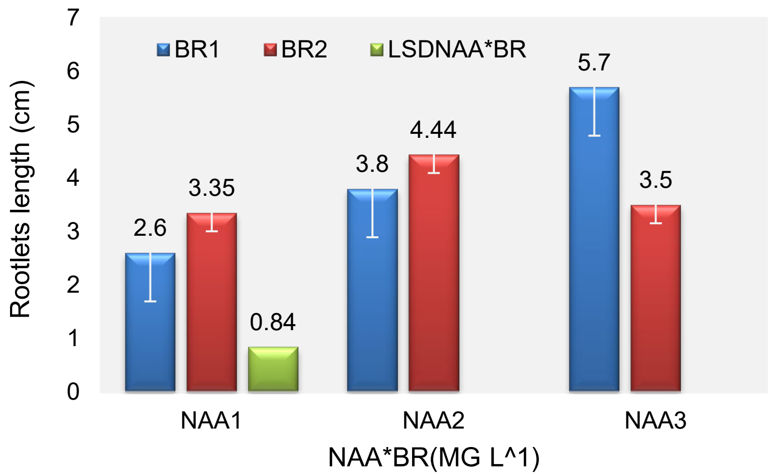

Figure 2. Effect of different concentrations of NAA and BR on the average rootlet length of Chia plants in vitro.

Many factors participate in the formation of roots, they may be external or internal, including auxins, as they are the most important factors that have a major role in the emergence and occurrence of the rooting process and the weight of these emerging roots (Stefancic et al., 2005 and Sevik & Guney, 2013) especially the NAA growth regulator. Also, the external addition of this regulator increased the emergence of side rootlets as a result of the transfer of this regulator (Salih & Al-Dabagh, 2020). Both the PGRs naphthalene acetic acid and prasinolide have proven their effectiveness in improving the average number and rootlets length of Chia plants growing under tissue culture conditions (Figs 1, 2). The reason may be attributed to the PGR naphthalene acetic acid, one of the auxins that contribute to the stimulation of root precursors. Marconi et al. (2013) showed that the nutritional medium supplemented with the growth regulator NAA was effective in the reproduction of rhizogenic roots (Schuelter et al., 2020), and the effectiveness of this regulator in rooting plantlets of plant species cultured in tissue culture (Yadav et al., 2019) was evident in culture media prepared to mutate with dimethyl sulfate to resist stress conditions (Sekhi et al., 2022), also, brassinosteroid PGRs may have important roles in root formation and reproduction, as they increase cell division and may be a substitute for cytokinin. This is what is observed from Figs 1, 2. The addition of 1 mg L^{-1} increased the number and length of the rootlets, and it may be attributed to the fact that the brassinolide regulator encouraged cell division by regulating the cloning of CycD3, which is involved in the manufacture of new proteins (Hu et al., 2000), increasing the production of chlorophyll pigments, antioxidant enzymes and some substances called osmolytes (Niu et al., 2016). Some of these compounds may move to new sites of influence, and may encourage the formation and development of roots growth, especially the lateral ones (Mohamed & Almehemdi, 2021).

The effect of Melatonin on the active compounds

Chia plantlets were significantly differed in their content of chemical compounds. The active ingredients in the melatonin-free culture medium of chia plants *in vitro* differ in the quantity and quality of those fortified with melatonin (Table 2).

The data of the GC/MS analysis indicated the predominance of the etheric components in these plants with an area of 65.16% of the total active ingredients. The active ingredient 9,12-octadecadienoic acid, methyl ether was more dominant with an area ratio of 45.47%, it was followed by the components hydrocarbonic with an area of 16.58%, and the compound Z-4-nonadecen-1-olacetate dominated with an area of 12.31%. Then the alkaloids represented by the alkaloid Pyrimidine-4,6(3H,5H)-dione with an area of 7.05%, and fatty acids by 4.19%, alcoholic compounds represented by 2-methyl-z,z-3,13-octadecadienol with an area of 3.07%, and oxidative compounds by an area of 2.25%.

Table 2. Retention time, area and totals of active ingredients separated by GC/MS technology in Chia plants in control treatment

RT	Area	Lib	Qual	Group	Identities
7.656	7.05	NIST11	83	Alkaloids	Pyrimidine-4,6(3H,5H)-dione
Σ	7.05				
7.962	1.84	NIST11	97	Oxides	dimethyl sulfoxide
8.200	0.61	NIST11	97	oxides	dimethyl sulfoxides
Σ	2.25				
20.467	12.30	NIST11	98	ethers	Hexadecenoic, methyl ether
22.761	45.47	NIST11	99		9,12-octadecadienoic acid, methyl ether
23.067	6.61	NIST11	18		11-octadecenoic acid, methyl ester
29.039	0.78	NIST11	12		1-monolinoleoylglyceroltrinmethylstylsilylether
Σ	65.16				
25.343	2.19	NIST11	89	Fatty acids	Cis-vaccenic acid
25.564	1.10	NIST11	76		Oleic acid
31.723	0.90	NIST11	49		6-octadecenoic acid, (Z)-
Σ	4.19				
27.450	3.07	NIST11	93	alcohol	2-methyl-z, z-3,13-octadecadienol
Σ	3.07				
30.976	12.31	NIST11	35	hydrocarbons	Z-4-nonadecen-1-olacetate
31.604	4.27	NIST11	25		propyleneglycerolmonoleate
Σ	16.58				

Active ingredients in culture media fortified with a 1 mg L^{-1} of the PGR Melatonin for Chia plantlets in vitro differ in these plantlets (Table 3). The GC/MS analysis data indicated the predominance of cycloorganometallic compounds in these plants with an area of 21.64% of the total active ingredients, then oleic acid with an area of 16.36%, followed by organometallic compounds with an area of 18.18%. Then the alkaloids represented by the alkaloid pyrimidine-4,6(3H,5H)-dione with an area of 7.05%, fatty acids by 4.19%, alcoholic compounds represented by 2-methyl-z,z-3,13-octadecadienol with an area of 3.07%, the compounds the oxide has an area of 2.25%.

Table 3. Retention time, area and totals of active ingredients separated by GC/MS technology in Chia plants at 1 mg L^{-1} melatonin.

RT	Area	Lib	Qual	Group	Identities
8.845	2.22	NIST11	5	Alkaloids	azetidine
28.708	2.20	NIST11	15		Benzo[h]quinolone,24-dimethyl
Σ	4.42				
11.403	3.61	NIST11	74	cycloorganometallic	cyclohexasiloxane
13.764	10.42	NIST11	95		cycloheptasiloxane
16.160	7.61	NIST11	83		cyclooctasiloxane
Σ	21.64				
18.403	3.91	NIST11	23	organometallic	hexasiloxane
20.493	4.53	NIST11	37		octasiloxane
24.281	3.00	NIST11	50		1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
30.432	5.02	NIST11	14		Octasiloxane,1,1,3,3..
31.868	1.72	NIST11	47		Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl
Σ	18.18				
24.281	3.00	NIST11	89	aldehydes	9-octadeccenal,(z)-
25.326	1.14	NIST11	53		13-octadecenal,(z)-
Σ	4.14				
3.723	1.77	NIST11	72	alkanes	n-hexane
3.867	1.00	NIST11	58		n-hexane
3.918	1.05	NIST11	72		n-hexane
4.063	1.15	NIST11	87		n-hexane
6.220	1.52	NIST11	47		n-hexane
6.263	2.16	NIST11	64		n-hexane
Σ	8.65				
27.450	16.36	NIST11	49	Fatty acids	Oleic acid
Σ	16.36				
26.991	1.12	NIST11	93	alcohol	2-methyl-z,z-3,13-octadecadienol
Σ	1.12				
28.572	1.18	NIST11	14	ketones	Benz[e]azulene-3,8-dione
27.527	10.46	NIST11	12		4,7,7-trimethylbicyclo [2-2-1] heptan-2-oneO-allyloxime
Σ	11.64				
29.022	5.03	NIST11	50	hydrocarbonic	Theocratic acid
Σ	5.03				

The active ingredients in culture media fortified with a concentration of 2 mg L^{-1} of the PGR Melatonin for Chia plantlets in vitro differ in these plantlets (Table 4). The GC/MS analysis data indicated that ethers compounds were dominant in these plants with an area of 63.88% of the total active ingredients, as the compound 9.12-octadecadienoic acid, methylether had the highest area ratio of 49.62%, followed by organometallic compounds with an area of 19.55%. Then fatty acids with an area of 8.22% of the total area. 17-octadecenoic acid dominated in it with an area of 5.33% of its area, then aldehydic compounds with an area ratio of 2.70%, ketogenic compounds (1.97%), and alcoholic compounds with an area ratio of 1.70%.

Table 4. Retention time, area and totals of active ingredients separated by GC/MS technology in Chia plants at 2 mg L^{-1} melatonin.

RT	Area	Lib	Qual	Group	Identities
31.205	19.55	NIST11	43	organometallic	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11, 13,13,15,15-hexadecamethyl
Σ	19.55				
27.858	2.70	NIST11	40	Cycloorgano metallic	Cyclodecasiloxane eicosamethyl
Σ	2.70				
20.441	14.26	NIST11	98	ethers	Hexadecanoic, methylether
22.744	49.62	NIST11	99		9,12-octadecadienoic acid, methylether
Σ	63.88				
23.058	5.33	NIST11	64	Fatty acids	17-octadecenoic acid
31.885	1.21	NIST11	18		Oleic acid
32.046	1.27	NIST11	41		6-octadecenoic acid, (Z)-
32.326	0.41	NIST11	15		Oleic acid
Σ	8.22				
25.352	0.39	NIST11	58	alcohol	2-methyl-z,z-3,13-octadecadienol
27.459	1.37	NIST11	87		2-methyl-z,z-3,13-octadecadienol
Σ	1.76				
29.260	2.70	NIST11	86	aldehydes	13-octadecenal,(z)-
Σ	2.70				
31.749	1.97	NIST11	10	ketones	Benz[e]azulene-3,8-dione
Σ	1.97				

The active ingredients in culture media fortified with a concentration of 3 mg L^{-1} of the PGR Melatonin for Chia plantlets in vitro differ in these plantlets (Table 5). The GC/MS analysis data indicated that ethers compounds were dominant in these plants with an area of 80.3% of the total active ingredients. The compound Trans-13-octadecenoic acid, methylester possessed the highest area percentage of it, reaching 58.51% of its area, followed by alkanes with an area ratio of 7.31%, represented by the compound 9,12-octadecadienoylchloride (linoleoyl chloride) with 6.60%. Then fatty acids with an area of 4.91% of the total area, it was dominated by 9,12-octadecadienoic acid, with an area of 2.58% of its area. Followed by cyclic organometallic compounds with an area of 3.53%, then alcoholic compounds had an area of 1.47%, and the nitrogen compounds (1.05%), however, the oxygenic compounds had the lowest area ratio of 0.64%.

The recognition of melatonin as an important plant growth regulator (Erland et al., 2015) has increased. Therefore, knowledge grew rapidly about this PGR, especially with regard to the ecosystem for growth, including tissue culture systems, which helped in strengthening the role of this PGR in the vital processes within the plant. However, the effective research area for him remained poorly understood, so tissue culture provides a valuable platform for researching this PGR (Lee & Back, 2016). Its importance has been proven through calcium indicators in the response to this PGR in many species by producing inhibitors in the plant medium (Ramakrishna et al., 2011). From Table 2–5, it is noted that the melatonin PGR is the cause of a difference in the number and type of compounds diagnosed by GC/MS technology, the reason may be attributed to the fact that this PGR is stable in aqueous solutions for a long time, regardless of the pH (Maharaj & Dukie, 2002),which keeps its effect for a longer period and this is the reason for the difference in the production of effective compounds in quantity and quality. Plants possess a complex plant environment (Tan et al., 2012). The content of melatonin and its three generators (tryptophan, tryptoamine and N-acetylserotonin) may have an important impact on biological processes within plant tissues. Erland et al. (2016) observed a clear difference in the content of melatonin and its three antigens. One of the roles that melatonin has, which stimulates the production of many compounds in most plants, is its importance in the formation of plants in the tissue culture environment (Erland & Saxena, 2018). It enters into the activation of the biochemical system inside the plant, as it interferes with the active types of oxygen and nitrogen and nitric acid, which reduces its harmful effect by activating other signaling pathways and increasing primary and secondary metabolic products (Fan et al., 2015). Other antioxidants that increased by increasing the addition of melatonin are phenolic and flavonoid compounds in callus culture (Neamah & Jdayea, 2022).

Table 5. Retention time, area and totals of active ingredients separated by GC/MS technology in Chia plants at 3 mg L^{-1} melatonins

RT	Area	Lib	Qual	Group	Identities
12.719	1.49	NIST11	72	cycloorganometallic	Cycloheptasiloxane, tetramethyl
15.905	2.04	NIST11	90		Cyclooctasiloxane, hexamethyl
Σ	3.53				
18.386	1.05	NIST11	38	Nitrogenous compounds	2H-1,4-Benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl-3-trimethylsilyl)oxy
Σ	1.05				
12.481	0.64	NIST11	81	Oxides	dimethylsulfoxide
Σ	0.64				
20.450	15.96	NIST11	99	ethers	Hexadecanoic, methylester
22.752	58.51	NIST11	97		Trans-13-octadecenoic acid, methylester
23.067	5.83	NIST11	62		13,16-octadienoic acid, methylester
Σ	80.3				
24.010	2.58	NIST11	98	Fatty acids	9,12-octadecadienoic acid
25.292	1.29	NIST11	92		Cis-vaccenic acid
25.326	1.04	NIST11	78		Oleic acid
Σ	4.91				
26.949	0.73	NIST11	90	alcohol	2-methyl-z,z-3,13-octadecadienol
27.527	0.74	NIST11	76		2-methyl-z,z-3,13-octadecadienol
Σ	1.47				
23.423	0.71	NIST11	93	Hydrocarbonic alkanes	9-methyl-z,z-10,12hexadecadienoic-1-olacetate
27.425	6.60	NIST11	89		9,12-octadecadienoylChloride (linoleoyl chloride)
Σ	7.31				
29.260	0.79	NIST11	90	ketones	Cyclopentadecanone,2-hydroxy
Σ	0.79				

They concluded that the moderate addition of melatonin is more suitable for the production of secondary compounds in *Hyoscymus pusillus* callus under water stress. This is reflected in the increase in the active compounds. The increase of phenols and organic acids bioaccumulation in plantlets had described in prior studies (Ramata-Stunda et al., 2020). Duran et al. (2019) observed a high increase of rosmarinic acid in the in vitro growing basil plant and some components of the volatile oil in it, including 3-methylbutanal, benzaldehyde, and 1,8-cineole. The increase in secondary metabolites may be a result of the increase in phenolic compounds due to the exogenous addition of the PGR melatonin (Liang et al., 2018),which generates chemical stress on the plant cell, which increases those outputs. The same happened in the rosemary plant growing in vitro, as the addition of melatonin changed its secondary components in the callus produced from it, such as the compounds linalool, styrene and methional, it has been found in the treatment of melatonin (Coskun et al., 2019), and that the growth inputs in the culture medium and its components and their integration may affect the diversity and difference of the active ingredients (Neamah & Almehemdi, 2017).

CONCLUSION

The results of the experiments showed that the used growth regulators were effective in improving root formation and some morphological characteristics of chia plants induced by tissue culture from their seeds. The enhancement of the plant media of chia plants with a concentration of 1 mg L^{-1} of benzyl adenine improved the length and number of shoots, nodes and leaflets. Also, addition of 1 mg L^{-1} of brassinolide and 3 mg L^{-1} of NAA was the best for rootlets formation and development. As for enhancing the culture medium with melatonin to produce the active compounds, it was effective in changing the active compounds quantitatively and qualitatively. The ether compounds increased (80.3%) when adding 3 mg L^{-1} to the culture medium and 63.88% at the concentration of 2 mg L^{-1}. Therefore, melatonin may be one of the generators for the production and increase of effective compounds using tissue culture technology.

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Fertilizing power evaluation of different mixtures of organic household waste and olive pomace

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Abstract. From the perspective of sustainable agriculture established by the Green Morocco Plan, it is interesting to direct research more towards the agronomic valorization of olive pomace, to give birth to a clean olive growing which leads to a viable economy thus respecting a pillar of sustainable development. Several studies have shown the effectiveness of using olive pomace as a soil amendment. Therefore, in this study we want to increase the agricultural performance of olive pomace by composting by mixing it with other waste.

Morocco is considered one of the major olive-producing countries with an annual production of 1.41 million tonnes (MT), part of it is dedicated to olive oil production. Morocco produces approximately 26.8 MT of waste annually, 8.3 MT are household waste, 70% are organic household waste (5.8 MT). The current production of organic household waste in urban areas is estimated at 4.8 million tonnes per year, or an average of 0.76 kg per hab per day, and in rural areas 1 million tonnes per year, or an average of 0.30 kg per hab per day (SNRVD, 2015). Agri-food industry waste is around 3 million tonnes with 600,000 to 700,000 tonnes of olive oil waste (pomace) (Agricultural Development Agency, 2018). The rejection of this waste without any prior treatment contributes to the environment deterioration. However, a large part of this waste remains recoverable, which would reduce both waste volume to be eliminated and the associated management cost. This; will contribute to reducing the negative impacts on receiving environments and the cost of restoring the environment state, and ensuring a transition towards a circular economy. Our work is part of the context of solid waste management and recovery, in particular organic waste from household and food-processing activities, and is oriented towards the pomace recovery by composting, mixing it with different percentages of organic household waste.

This work consists on composting olive pomace from the three phases system with another structural agent (organic household waste). Comparing the mixtures (6 treatments) with different concentrations in terms of composting process parameters (pH, electrical conductivity, organic matter temperature, etc.), organic matter evolution and composts quality, with manual aeration of the compost, in order to increase the agricultural yield of the olive pomace. Residues from the fermentation process can be used in agriculture. All the different mixtures of the different percentages are characterized at the initial state and at the end of the composting process in order to highlight their nutritional values.

Key words: olive pomace, compost, organic household waste, physico-chimical and bacteriological characterization.

INTRODUCTION

Morocco is one of the major olive-producing countries with an annual production of 1,414,000 tons. National production of olive oil is estimated at 142,000 t $year^{-1}$ on average between 2015 and 2019 (Ministry of Agriculture, Maritime Fisheries, Rural Development and Waters and Forests, 2022).

Olive waste valorization represents a great source of additional income for the olive farms (Messineo et al., 2019). It can be used as fertilizer, fuel, livestock and as a thermal insulator as a contraction material (Chouchene, 2010).

In recent decades, this increase in production and the introduction of modern oil extraction technologies have placed the olive tree in a delicate position as a potential polluter. The environmental problem of olive pomace remains unresolved in the olive-growing countries, particularly in the countries on the southern coast of the Mediterranean such as Morocco, which have begun to modernize the industrial sector through vast programs with the aim of increasing and improve the quality of olive production. It is therefore necessary to find effective and feasible solutions in these developing olive countries. Olive oil production generate by-products such as olive pomace and olive mill wastewater, also known as black water. They have a significant impact on the environment such as threat to the aquatic life, odors, impenetrable film causing negative impact for oxygen transfer, discoloring of natural waters and toxicity (Yaya et al., 2012). As an example, OWW spread on the soil could increase pollution risks because of the presence of phenolic compounds and other pollutants, especially when it is not evenly distributed on the soil and the correct doses are not applied (Montemurro et al., 2004).

On the other hand, intensive agricultural practices will lead to the soil organic matter depletion. This organic matter loss will lead to a decrease of the soil structure stability and a decrease in the retention of pollutants (organic or mineral). One of the ways to solve these different soil problems is to add external organic matter. To solve these environmental and agronomic problems, there are different solutions, including composting. Indeed, the organic matter abundance in olive pomace proves that this method is reasonable, and it can establish a link between the management of these olive by-products and agriculture. Human has started using the organic amendments; he has turned to the organic farming based on fertilizing soil with organic matter, which represents a nutrients reservoir, and plays a major role in the physical soil fertility, its aeration and its resistance to erosion and degradation (Girard et al., 2005).

The use of olive pomace in the agriculture as an organic amendment has shown some problems in plant growth, because of their high organic matter, mineral salts, the acidic pH and the presence of phytotoxic compounds (Del Buono et al., 2011; Gigliotti et al., 2012; Proietti et al., 2015). Therefore, composting is a better way to stabilize the high organic content and benefit from the pomace fertilizing power (Gómez-Muñoz et al., 2012). Nevertheless, no negative effects were observed when raw pomace was used as an amendment on soil fertility parameters (Ameziane et al., 2019). Similar studies have shown that composting olive pomace improves the physical and chemical characteristics of the soil, and provides the necessary nutrients for plant growth (nitrogen, potassium and phosphorus) (Sellami et al., 2008; Del Buono et al., 2011).

Composting is one of the solutions proposed to recycle waste (particularly pomace) from olive oil factories. Composting olive waste has been proven to produce very good quality compost. Composting aims to aerobically converting organic matter into humus while destroying pests and pathogenic microorganisms (Hay et al., 1996).

In addition, the use of compost in agriculture helps protect the soil by reducing the use of chemical fertilizers. Organic materials are increasingly used in agriculture as a fertilizer but also as a soil conditioner. Organic matter is a key component of soil, which affects its physical, chemical and biological properties (Hassink et al., 1997; Herold et al., 2014). Several studies showed the positive effect of organic product amendment or along with mineral fertilizer to cultivated soils (Alvarez et al., 1998; Goyal et al. 1999; Blair et al., 2006; Gong et al., 2009; Butler & Hooper, 2010; Evans et al., 2012; Zhou et al., 2013; Cannavo et al., 2014). It is in this context that this work focuses on the valorization of olive by-products. OP compost could be used as suitable soil amendment, ensuring at the same time an eco-friendly recycling of waste materials. It can optimize organic emmer yield production, sustain soil fertility, and reduce pollution risks linked to the landfill disposal (Diacono & Montemurro, 2019).

The objective is to valorize by composting the olive pomace; resulting from the three phases system to evaluate the agronomic quality of the pomace as a soil amendment; with organic household wastes in order to evaluate the composts quality.

For this, we made six mixtures of different composition based on olive pomace associated with different percentages of organic household waste. Physicochemical and bacteriological characterization of the different mixtures at the initial state called time zero (T_0) and once the compost is mature (T_f).

MATERIALS AND METHODS

Sampling

The pomace used in this study was collected from a crushing unit with a three phases extraction system located in Tiflet city (Tiflet, Morocco, latitude: 33°53′40″ North, longitude: 6°18′23″ West, altitude above sea level: 340 m), it is a city in the province of Khemisset, in the Rabat Salé Kénitra region (Fig. 1). For organic household waste, was generated from the wholesale market in the city of Salé (Salé, Morocco, latitude: 34° 1' 54.476" North, longitude: 6° 46' 17.494" West, altitude above sea level: 34 m). Organic household waste were cut into small pieces between 1 and 2 cm before starting the composting process (Fig. 2).

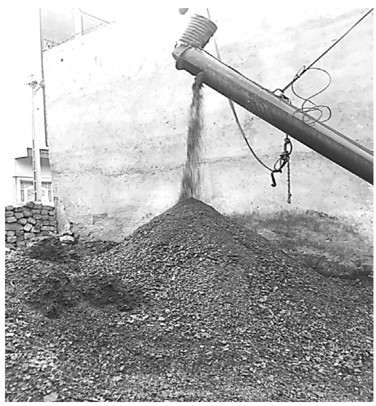


Figure 1. Olive pomace in the olive oil extraction unit (raw material).

The composting experiment consists on mixing olive pomace with another structural agent, which is organic household waste. Composting is carried out in 30 liters barrels (Ameziane et al., 2020), perforated to ensure a good aeration and placed in a sunny place (Manu et al., 2016). For the mechanical aeration, it had been performed many times, at the beginning of the composting process (the mesophilic phase) and at the end of the thermophilic phase and at the beginning of the cooling phase. Piles were supplied with air and turned periodically to ensure good aeration in order to promote aerobic fermentation and the humidity was monitored and adjusted.

Figure 2. Organic household waste from the wholesale market (raw material).

This method is based on forced aeration and mechanical turning devices, which aim to speed up the composting process. We chose to compost a sample well mixed and homogenized and then put into barrels in order to determine a better concentration at the level of the fertilizing elements (Manu et al., 2017). Then, the best concentration will be destined for application with large quantities. As a result, these results obtained at the laboratory scale can be reproduced in full-scale installations by adjusting the forced aeration according to the available equipment.

From the two raw materials, pomace (Gr) and organic household waste (D), four mixtures with different percentages were prepared: GD1; GD2; GD3 and GD4, ranging from 15% to 50% pomace and 85% to 50% organic household waste (Table 1). The composts stability and maturity are achieved after almost 120 days.

Table 1. Mixtures percentages

Mixtures	Percentages
Gr	100% olive pomace
D	100% household organic waste
GD1	15% olive pomace + 85% household organic waste
GD2	25% olive pomace + 75% household organic waste
GD3	43% olive pomace + 57% household organic waste
GD4	50% olive pomace + 50% household organic waste

Physico chemical characterization

In order to characterize the mixtures (raw materials and composts) from a physico-chemical perspective, a representative sample is dried in an oven in a temperature between 40 °C and 60 °C, ground and sieved to 2 mm to determine the pH and EC, and to 0.2 mm to determine the other mineral elements.

The pH is measured by a pH meter (Orion Star A111), while the electrical conductivity (EC) is measured by a conductivity meter (Orion Star A212). pH and EC are measured in the aqueous extract using the ratio 2:5 (w/v) for pH (Rodier et al., 2009), and the ratio 1:5 (w/v) for EC (ISO 11265, 1994).

The moisture content is determined by drying the sample at 105 °C until a constant mass is obtained (Rodier et al., 2009). Organic matter was characterized by ignition loss at 525 °C for 4 hours (Rodier et al., 2009). The measurement of Nitrate (NO_3^-), Ortophosphate (PO_4^{3-}) and Sulfate (SO_4^{2-}) were measured by the molecular absorption spectrometry method (Rodier et al., 2009).

However, the other minerals were obtained by calcination in the oven at 500 °C for 2 hours to 3 hours; the obtained ashes were dissolved to determine the other mineral elements (Pinta, 1979).

Total nitrogen and ammoniacal nitrogen were measured by the Kjeldahl method (Rodier et al., 2009), total organic carbon (Dabin, 1970). The macroelements (P_2O_5, K_2O, Na_2O, CaO, MgO, Cl^-…) were determined in the Research Unit on Environment and Natural Resource Conservation at the Institut National de Recherche Agronomique Rabat laboratory. P_2O_5 was obtained by the Olsen method (Olsen, 1954). Whereas for assimilable potassium (K_2O) and sodium (Na_2O) were measured by flame spectrometry (Bower et al., 1952). CaO and MgO were obtained by the atomic absorption spectrometry (Pinta, 1979).

The results of the physico-chemical analyses of the raw materials to be composted are presented in table (Table 2).

Table 2. Physical-chemical characterization of the raw materials used for the composting process (pomace and organic household waste)

Raw materials in the beginning of composting process (T_0)	Olive pomace	Organic household waste
pH	5.05 ± 0.53	6.18 ± 0.33
Electrical conductivity (EC), mS cm^{-1}	1.77 ± 0.10	0.72 ± 0.04
Moisture, %	26.52 ± 3.46	91.10 ± 3.16
Organic matter (OM), %	92.35 ± 2.46	89.87 ± 2.38
Ash, %	7.65 ± 2.46	10.13 ± 2.38
Total organic carbon (TOC), %	53.57 ± 1.43	52.13 ± 1.38
Total nitrogen (NTK), %	1.27 ± 0.04	1.57 ± 0.02
C/N ratio	42.23 ± 1.40	33.28 ± 0.46
Orthophosphate (PO_4^{3-}), %	0.0187 ± 0.0013	0.0655 ± 0.0044
Available phosphorus (P_2O_5), %	0.007 ± 0.001	0.014 ± 0.002
Calcium (CaO), %	0.22 ± 0.007	0.12 ± 0.005
Magnesium (MgO), %	0.08 ± 0.005	0.14 ± 0.005
Potassium (K_2O), %	0.46 ± 0.001	1.52 ± 0.002
Sodium (Na_2O), %	0.05 ± 0.001	0.27 ± 0.002
Chloride (Cl^-), %	0.0185 ± 0.001	0.0235 ± 0.001
Sulfate (SO_4^{2-}), %	0.0035 ± 0.0006	0.0042 ± 0.0007
Nitrate (NO_3^-), mg kg^{-1}	57.55 ± 10.37	127.32 ± 27.29
Ammonium nitrogen (NH_4^+), mg kg^{-1}	526.01 ± 41.78	728.29 ± 22.31

The values obtained represent the average of three repetitions.

Microbiological characterization

The bacteriological characterization of the different samples was carried out at the beginning and at the end of the composting process. For faecal pollution indicators (CT, CF and E. Coli), were counted by the 3 tube NPP method (Rodier et al., 2009).

While, the count of the total aerobic mesophilic flora (FMAT) and the thermophilic flora (FT) is made by counting colonies on PCA (Plate Count Agar) and incubated respectively) at 30 °C and 44 °C for 72 hours (Rodier et al., 2009).

The counting of fungi is done on Sabouraud agar with chloramphenicol at 5 µg mL^{-1} (yeasts and molds) and incubated at 25 °C in the dark for 72 hours (NM 08.0.123., 2004). The enumeration of lactic acid bacteria is carried out by counting on MRS and incubated at 30 °C. for 72 hours (Guiraud, 1998). Salmonella were observed by inoculation on S-S Agar to determine their presence at 37 °C for 24 to 48 hours (Rodier et al., 2009).

RESULTS AND DISCUSSION

Composts monitoring

Temperature

In this study, the temperature evolution shows different phases during the composting process. It shows in the beginning of the process that the initial temperature is almost the same for all the mixtures (Fig. 3). From the 28^{th} day, the temperature starts increasing for all the treatments to achieve a maximum values at the 84^{th} day, with a high temperature (61.22 °C) for the Gr compost. This temperature increase in the thermophilic phase due to the microbiological activity especially the intense thermophilic microorganism's activity (Mustin, 1987). However, these temperature values remain below 70 °C, which is necessary for a living organism's destruction (Bernal et al., 2009). In addition, heat production by microorganisms is proportional to the mass of the pile (Golden, 1986). However, after the thermophilic phase a temperature decrease was observed. We are talking about the maturation phase, which shows a decrease in temperature to stabilize close to the ambient temperature at the end of the composting process.

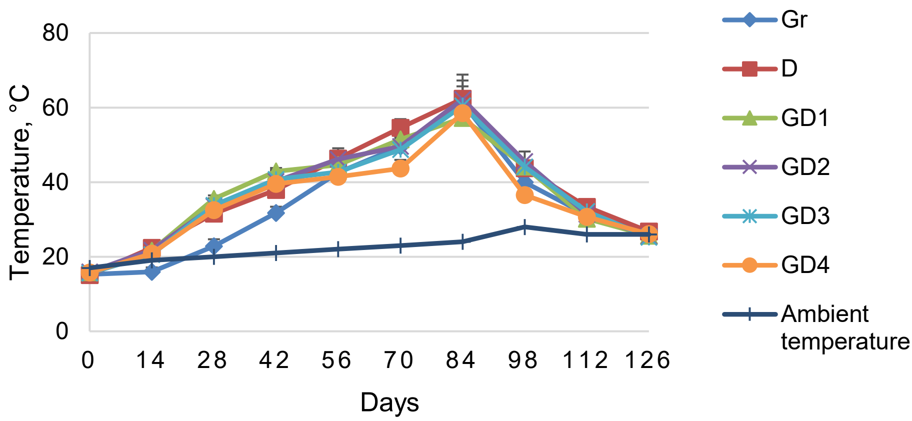


Figure 3. Evolution of the temperature during composting.

pH

The pH is an essential factor, which enters into the majority of the reactions, which allows the bioavailability of the nutrients and the solubility of the mineral elements for the microorganisms.

The pH of the two wastes used and the mixtures are acidic in the initial state with a pH ranging from 5.05 in olive pomace to 6.18 in organic household waste.

During the composting process, all mixtures have nearly the same movement, which they starts by an acidogenic phase then they move to an alkaline phase to stabilize at the end of the composting process near to a neutral pH.

At the beginning of the composting process, a minimum value was observed after 14 days for the GD1 (4.55 ± 0.35). Then, the pH of all mixtures has started increasing to reach a maximum value at the 84th day (8.77 ± 0.10 for the D compost). At the end of the composting process, the pH had a downtrend as to the neutrality for all the treatments with a minimum of (7.31 ± 0.17) in GD4 and a maximum of (7.9 ± 0.29) in olive pomace, this is the maturation phase (Fig. 4).

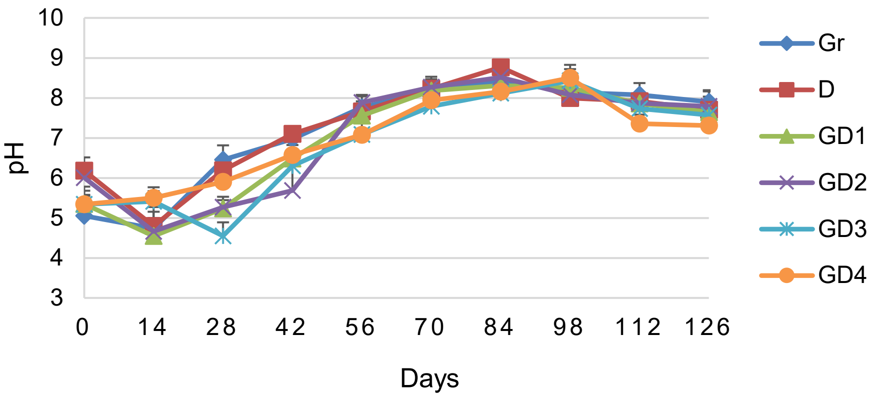


Figure 4. Temporal evolution of pH during composting.

A close pH values were observed at the end of the composting process in a study made by Ameziane et al. (2020) with an olive pomace from Fès-Meknès region mixed with poultry droppings (7.63 ± 0.12) and mixed with cow manure (8.16 ± 0.03).

However, organic household waste pH at T_0 (6.18 ± 0.33) remains lower than other household waste whose pH was neutral (pH = 7.5) (Chennaoui et al., 2016). The types of organic household waste related to seasonal vegetables and fruits can explain this difference in pH.

The pH of pomace at T_0 (5.05 ± 0.53) remains close to the pH of other olive pomace which have a pH of around 5.9 to 6.04 in Fez-Meknes region (Ameziane et al., 2018).

Similarly, a similar pH was observed in an Italian study on the use of solid waste from olive oil as a soil amendment (pH = 6.6) (Nasini et al., 2013). However, pH alkalinity towards the end of the process is characterized by ammonia production due to the degradation of protein amines in addition to the release of bases bound to organic matter (Kochtizky et al., 1969; Gray et al., 1971; Peters et al., 2000). A slightly alkaline pH of products or compost, at the end of the composting process, as a soil amendment is favorable for the assimilation of nitrogen, phosphorus and potassium, which is necessary for plant development (Nefzaoui, 1985). In addition, a pH close from 6 to 8 ensures the development of bacteria and fungi responsible for the organic matter degradation (Mennane et al., 2010).

In the acidogenic phase, the pH decrease can be explained by the organic acids production following the carbohydrates degradation, fats and other substances. During the aerobic degradation, the CO_2 produced contributes to the environment acidification, by dissolving it in water and producing the carbonic acid (Mustin, 1987). This pH decrease promotes the fungi growth and the lignin and cellulose degradation (Paredes et al., 1999).

Electrical conductivity

Electrical conductivity shows the compost salinity degree, and indicates its effectiveness in phytotoxicity tests and when used as a fertilizer for plant growth (Lin, 2008).

During the composting process, the electrical conductivity of the different piles fluctuates due to the organic matter degradation.

The conductivity underwent a decrease for the majority of the mixtures. However, organic household waste and the GD4 mixture showed a small increase (Fig. 5).

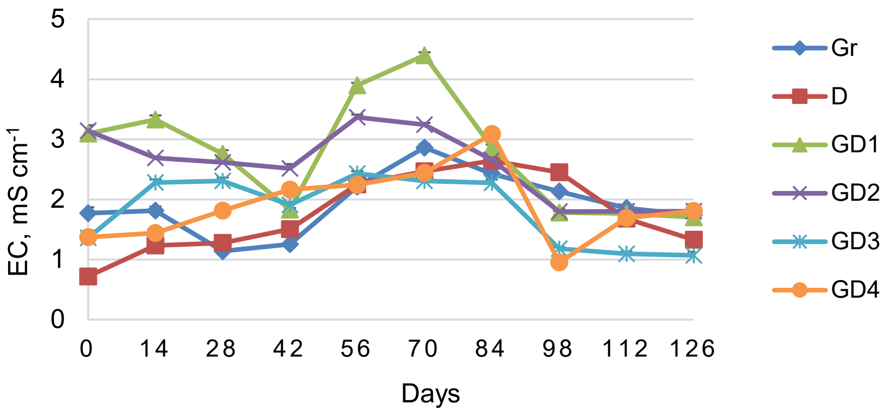


Figure 5. Electrical conductivity evolution during the composting process.

The lowest conductivity was recorded in organic household waste at T_0 from 0.72 mS cm^{-1} to 1.33 mS cm^{-1} in T_f. This value remains quite low compared to that found in other household waste (4.9 mS cm^{-1}) (Chennaoui et al., 2016) and in other waste such as olive pomace in T_0 (1.77 mS cm^{-1}). The latter remains close to that found in olive pomace (1.883 ± 0.56 mS cm^{-1}) from Fez-Meknes (Ameziane et al., 2020).

The electrical conductivities obtained in this study at the end of the composting process, were lower than those found by Ameziane et al. (2020) in a compost by olive pomace and poultry droppings (2.1 ± 0.45 mS cm^{-1}) and a compost by olice pomace and cow manure (2.06 ± 0.22 mS cm^{-1}).

The final product conductivity of all composts does not exceed the limit value of 3 mS cm^{-1} (Soumaré et al., 2002), so their use cannot harm plant growth. Generally, composts with low electrical conductivity can be used directly on the ground, while composts with high conductivity must be well mixed with other agents such as soil or other materials that have low electrical conductivity (Chen, 1999).

Moisture evolution

Humidity is a very important parameter for greater microbial activity, which accelerates the composting process (Chennaoui et al., 2016).

With the exception of olive pomace, which has a humidity equal to 26.5%, humidity shows a decrease from 92.1% at T_0 to 26.41% at T_f in organic household waste. These moisture contents underwent a significant decrease towards the end of the process with a minimum content of 12.36% in the GD1 mixture.

From the 42[sd] day, the thermophilic phase have already begun, which is manifested by a decrease in the humidity level of the composts (Jemali et al., 1996). During composting, moisture records the greatest rate of decrease (83.25%) in the GD1 mix. (Fig. 6).

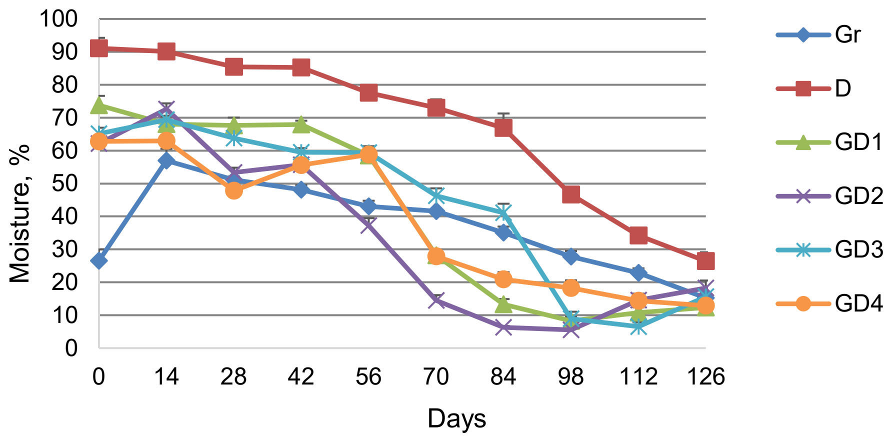


Figure 6. Moisture evoluuion during the composting process.

This decrease in humidity amounts to the decrease in water in all composts, either by leaching or by evaporation under the effect of the increase in temperature due to microbial activity during composting (Jemali et al., 1996).

These results have been much lower than those found in a compost by olive pomace and poultry droppings (30 ± 0.3%) and a compost by olive pomace and cow manure (30.4 ± 0.14%) (Ameziane et al., 2020).

Organic matter and ash evolution

Initially, the loss on ignition remains above 85% in all the mixtures with a maximum value of 95.80% observed in the GD3 mixture (Fig. 7).

The olive pomace value at T_0 in this study remains higher than that observed in pomace from Fez-Meknes region (77.02% ± 0.08%) at T_0 (Ameziane et al., 2020). However, the rate of organic matter recorded in household waste in T_0 (89.87%) remains very close to that of other household waste (92%) (Chennaoui et al., 2016).

Composting is an organic matter degradation, so a decrease in organic matter concentration is expected as the main result during composting (Ameziane et al., 2020), which is remarkable for all treatments.

During composting, the organic matter rate decreases with a rate that varies between 53.78% (GD1) and 43.02% (G). A close organic matter rates were observed at the end of the composting process in a study with an olive pomace from Fès-Meknès region mixed with poultry droppings (43.15 ± 0.15%) and mixed with cow manure (38.4 ± 0.76%) (Ameziane et al., 2020).

However, the mineral matter rate increases with a maximum rate in the GD3 mixture (91.16%) due to mineralization by microorganisms (Fig. 7).

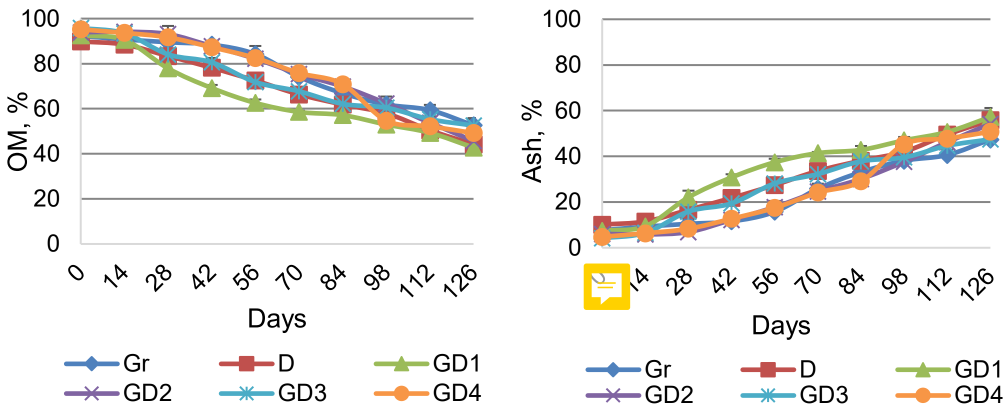


Figure 7. Organic matter and ashes evolution.

This significant organic matter loss can be reflected in a decrease in volatile solids and total organic carbon throughout the process (Ameziane et al., 2020 in Garcia-Gomez et al., 2003), which is due to the presence of relatively stable organic compounds probably represented by lipids, polyphenols, lignins, cellulose, hemicellulose and pectin (Aviani et al., 2010; Michailides et al., 2011; Tortosa et al., 2012).

The organic matter content diminution is ensured by different groups of microorganisms that work according to the temperature evolution and the compost mass (Keener et al., 2000). Furthermore, bacteria leads the composting phase early, while fungi are present throughout the process, but are very active at water levels below 35% and inactive at temperatures above 60 °C (Bernal et al., 2009). As well as fungi, actinomycetes predominate during the maturation phase, which have the ability to degrade very resistant polymers (Bernal et al., 2009; Federici et al., 2011; Agnolucci et al., 2013).

Macro-elements parameters

Total organic carbon (TOC), total Kjeldahl nitrogen (TKN) and C/N ratio

In this study, we observe a decrease in the C/N ratio for all the mixtures due to the mineralization of the organic matter with a strong decrease in the GD2 mixture whose C/N ratio went from 31.75 at the state initial to 18.56 in the final state (Fig. 8).

The decrease in the C/N ratio is due to the depletion of carbon compounds that constitute the main component of organic molecules than nitrogen for microorganisms (Chennaoui et al., 2016).

The mixtures (D, GD1 and GD2) have a C/N lower than 20 corresponding to a norm for a mature compost (Hirai et al., 1983) (Fig. 8).

At the end of the composting process, C/N values a little bit lower than those in this study, were observed in composts by olive pomace from Fes-Meknes region mixed with poultry droppings (16.72) and mixed with cow manure (17.16) (Ameziane et al., 2020).

Values very close to the olive pomace TOC in this study (53.57%) at the beginning of the composting process were found in pomace from Fes-Meknes region at T_0 with contents of 49.31% to 50.79% (Ameziane et al., 2018). Moreover, similar values were observed in Portugal 53.51% (Lopez-Pineiro et al., 2006) and in Italy 52.43% (Proietti et al., 2015).

At the end of the composting process, TOC values a little bit lower were observed in a composts by olive pomace from Fes-Meknes region mixed with poultry droppings (25.09 ± 0.76%) and mixed with cow manure (22.32 ± 0.89%) (Ameziane et al., 2020).

Values very far from the NTK of this study (1.27%) were found in pomace from the Fes-Meknes region at T_0 with a content of 0.001% and 0.004% (Ameziane et al., 2018). However, similar values were observed in Tunisia 1% (M'sadak et al., 2015) and in Italy 1.62% (Proietti et al., 2015).

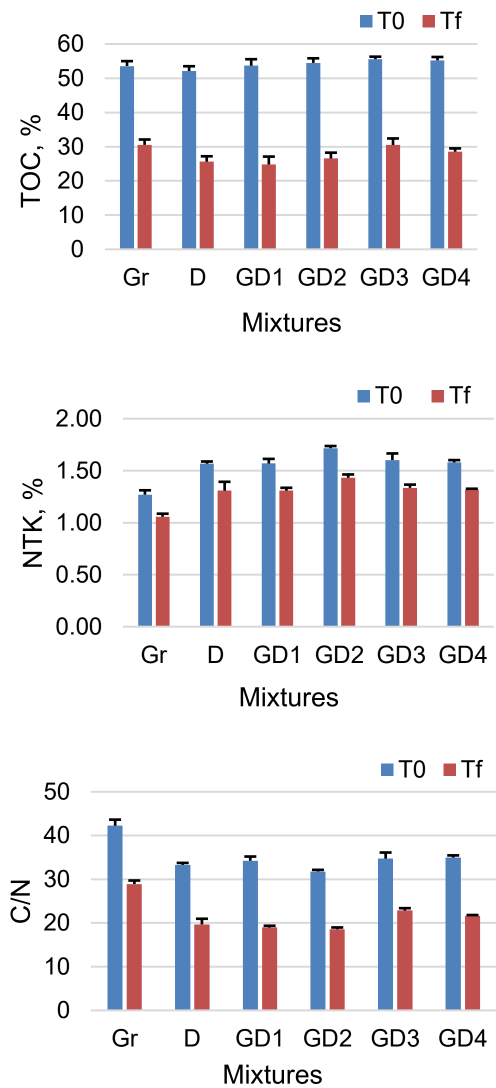


Figure 8. COT, NTK and C/N ratio characteristics.

At the end of the composting process, a close TNK values were observed in a composts by olive pomace from Fes-Meknes region mixed with poultry droppings (1.5 ± 2.43%) and mixed with cow manure (1.3 ± 1.89%) (Ameziane et al., 2020).

Nitrates (NO_3^--N) and ammonium nitrogen (NH_4^+-N)

The NO_3^- concentration increased during the composting process for all mixtures. The maximum NO_3^- content is recorded in the GD4 mixture at the beginning

(150.32 mg kg^{-1}) and at the end of the process (295.61 mg kg^{-1}). While the minimum content in the GD2 mixture at the beginning 44.42 mg $kg^{-1}$to be around 251.70 mg kg^{-1} at the end of the process (Fig. 9).

This increase in nitrates can be explained by the process of hardening of the compost by the temperature increase, which inhibited the growth of nitrifying bacteria (Chennaoui et al., 2016).

A similar increase was observed in a study carried out on composts prepared from pig manure and sawdust, which it has started increasing after the thermophilic phase (Huang et al., 2004). The high temperature and excessive amount of ammonia inhibit the activity and nitrifying bacteria growth in the thermophilic phase (Morisaki et al., 1989).

A decrease in NH_4^+ content was observed during the composting process between the beginning and the end of the process for all mixtures. A minimum value was observed in the GD2 mixture of 182.1 mg kg^{-1}. However, a maximum NH_4^+ content was observed in the GD1 mixture of 365.2 mg kg^{-1} at the end of the process (Fig. 9).

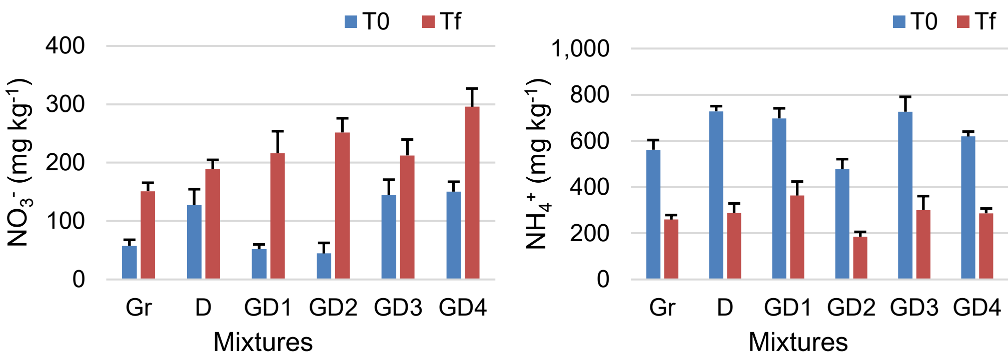


Figure 9. N–NH_4^+ & N–NO_3^- concentrations.

According to Huang et al. (2004), during composting, NH_4-N contents of both piles increased significantly and reached peak values due to ammonification with an increase in temperature and pH, as well as the mineralization of organic-N compound (Fang et al., 1999; Mahimairaja et al., 1994). After an initial increase, NH_4-N contents decreased by volatilization loss and immobilization by microorganisms (Huang et al., 2004). Final NH_4-N contents in two mixtures from pig manure and sawdust with different percentages (pile A 3/2 and pile B 4/1), pile A was close in term of NH_4–N (316 mg kg^{-1}) to the concentrations obtained in this study especially to the GD4 mixture. However, pile B (912 mg kg^{-1}) was so far.

This decrease can be explained by the organic matter decomposition containing nitrogen by microorganisms by converting it into ammonia (Chennaoui et al., 2016). Similarly, the decrease in NH_4^+ is an indicator of a good maturation process (Chennaoui et al., 2016). The absence of or decrease in NH_4–N is an indicator of a good composting and maturation process (Hirai et al., 1983; Riffaldi et al., 1986). Zucconi & de Bertoldi (1987) recommended a maximum NH_4^+ content of 400 mg kg^{-1} in mature compost.

Available phosphorus (P_2O_5) and Orthophosphate (PO_4^{3-})

Phosphorus is an essential element resulting from the decomposition of organic matter. The assimilable phosphorus content in this study increased in all mixtures with a maximum content in organic household waste (0.0654%) and a minimum content observed in pomace (0.0194%) at the end of the process (Fig. 10).

The P_2O_5 content of olive pomace at T_0 (0.014%) in this study remains close to those found for pomace from Fez-Meknes region at T_0 with 0.0001% to 0.0425% (Ameziane et al., 2018).

At the end of the composting process, P_2O_5 values too far were observed in a composts by olive pomace from Fes-Meknes region mixed with poultry droppings (0.3 ± 0.87%) and mixed with cow manure (0.42 ± 0.86%) (Ameziane et al., 2020).

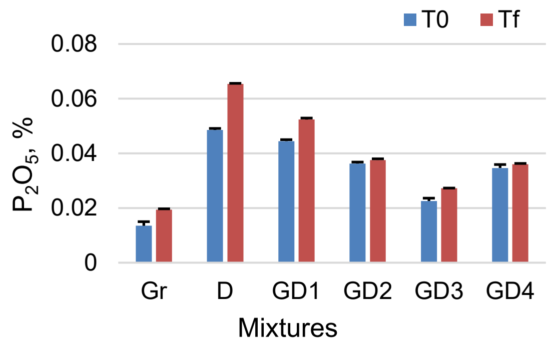


Figure 10. Available phosphorus P_2O_5 characteristics.

Orthophosphate concentrations increased during composting in all mixtures with maximum content in household organic waste (0.0876%) and minimum content in pomace (0.0260%) at the end of the process (Fig. 11).

The increase in electrical conductivity is explained by the release of mineral salts such as phosphorus (P) by the decomposition of organic substances (Gómez-Brandon et al., 2008). Moreover, the change of total P follows the same trend as total N with a gradual increase throughout the composting period, which is due to the net loss of dry mass (Huang et al., 2004). A low P value was observed at the end of the composting process in a compost of olive industry waste mixed with poultry manure (0.0258%) (Bargougui et al., 2019).

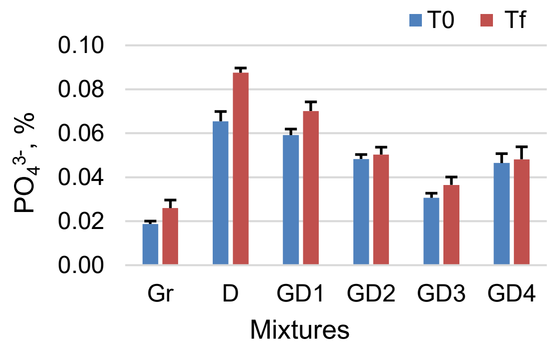


Figure 11. Orthophosphate PO_4^{3-} characteristics.

Sodium (Na_2O) and Potassium (K_2O)

Sodium Na^+ knew a significant increase in all mixtures with a maximum concentration in the GD2 mixture (0.70%) with an increase rate of 65.57% and a minimum content observed in olive pomace (0.19%) (Fig. 12).

The sodium content of olive pomace at T_0 (0.05%) remains quite far and much lower than those found for olive pomace in Fez-Meknes region at T_0 with 0.9% to 1% (Ameziane et al., 2018).

At the end of composting process, these values were too far and very high from those found by Bargougui et al. (2019) in a compost of olive industry waste mixed with poultry manure (0.029%).

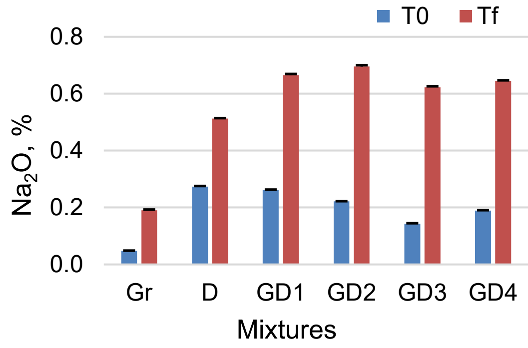


Figure 12. Sodium Na_2O characteristics.

Unlike Na^+ ions, the composts potassium characteristics during composting show that all mixtures undergo a reduction over time.

In terms of mixtures, organic household waste has a maximum K_2O content of 0.95% (this is due to the remarkable presence of this element in fruits and vegetables) and a minimum content in pomace of 0.40% at the end of the process (Fig. 13).

These values remain quite far and much lower than those found by (Ameziane et al., 2018) for olive pomace from Fez-Meknes region at T_0 with 4.6% to 9.4%.

At the end of the composting process, a higher K_2O values were observed in a composts by olive pomace from Fes-Meknes region mixed with poultry droppings (2.9 ± 1.22%) and mixed with cow manure (2.8 ± 0.36%). (Ameziane et al., 2020). Moreover, a low K value was observed in a compost of olive industry waste mixed with poultry manure (0.38%) (Bargougui et al., 2019).

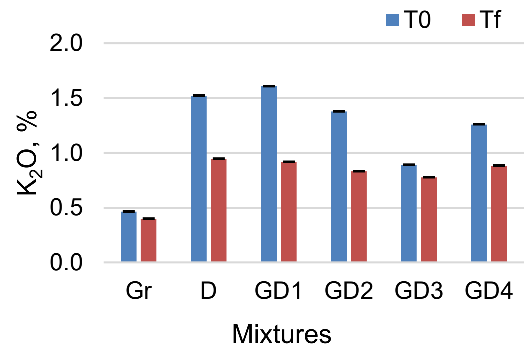


Figure 13. Assimilable potassium K_2O characteristics.

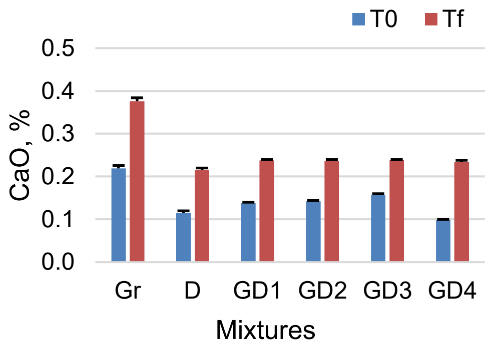
Calcium (CaO) and Magnesium (MgO)

The composting process has seen an increase in calcium concentrations in all mixtures with a high concentration in raw olive pomace (0.38%) and a minimum content (0.22%) in organic household waste (Fig. 14).

This value of olive pomace at T_0 (0.22%) is very close to that found for pomace in Fez-Meknes region at T_0 with 0.16% to 0.32% (Ameziane et al., 2018).

An excess of calcium can impair the absorption of some elements such as B, Cu, Mn and Fe (Ben Kheder, 1998). This increase from the second turning of the composts could be explained by the fact that all the mixtures enter the cooling phase (Znaidi, 2002). The presence of Ca^{2+} ions which increase during composting following humification and which play a role of buffer in the environment is the cause of the stability of the pH in the phase of maturation (Juste, 1980; Morel et al., 1986).

The magnesium concentrations evolution shows that there is a decrease in the different mixtures. With a high concentration in the GD4 mixture which is characterized by the highest concentration with a value of 0.07% and a minimum value 0.04% in the GD1 and GD3 mixtures towards the end of the composting process (Fig. 15).



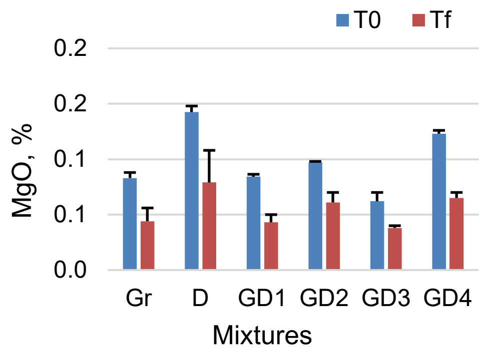


Figure 14. Calcium CaO characteristics.

Figure 15. Magnesium MgO characteristics.

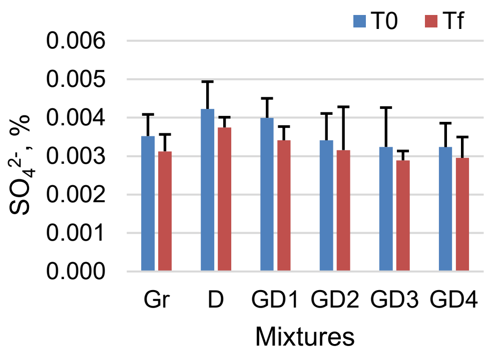
These values remain quite far and much higher than those found for olive pomace from Fez-Meknes region at T_0 with 0.192% to 1.776% (Ameziane et al., 2018) which are a bit far to that of olive pomace in this study at T_0 (0.080%).

Sulfate (SO_4^{2-}) and Chloride (Cl^-)

Sulfate content showed a small decrease during the composting process for all mixtures.

The compost of organic household waste which has a maximum concentration with a value of around 0.0037% and a minimum final content in the GD3 mixture with a content of 0.0029% towards the end of the process (Fig. 16).

For the chloride concentration during the composting process, it is revealed that the different mixtures have increased, with a maximum concentration observed in organic household waste with a value of 0.0301% and a minimum content of 0.0235% in the GD3 mixture towards the end of the process (Fig. 17).



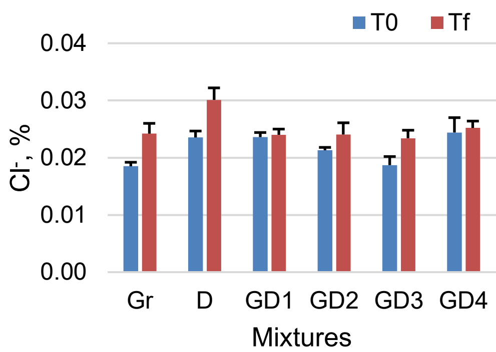


Figure 16. Sulfate SO_4^{2-} characteristics.

Figure 17. Chloride characteristics Cl^-.

This increase in chloride ions may be due to the increase in electrical conductivity during the composting process.

The table below summarizes the results obtained (Table 3):

Table 3. Physico-chemical properties of mature composts

Composts (T_f)	Gr	D	GD1	GD2	GD3	GD4
pH	7.90 ± 0.29	7.70 ± 0.33	7.65 ± 0.19	7.79 ± 0.37	7.57 ± 0.25	7.31 ± 0.17
EC, mS cm^{-1}	1.74 ± 0.08	1.33 ± 0.13	1.70 ± 0.06	1.80 ± 0.07	1.07 ± 0.06	1.81 ± 0.11
Moisture, %	15.22 ± 1.47	26.41 ± 2.70	12.36 ± 2.00	18.22 ± 2.31	15.65 ± 1.43	12.88 ± 1.85
OM, %	52.62 ± 2.71	44.18 ± 2.70	42.77 ± 3.95	45.79 ± 2.91	52.55 ± 3.31	49.21 ± 1.63
Ash, %	47.38 ± 2.71	55.82 ± 2.70	57.23 ± 3.95	54.21 ± 2.91	47.45 ± 3.31	50.79 ± 1.63
TOC, %	30.52 ± 1.57	25.62 ± 1.57	24.81 ± 2.29	26.56 ± 1.69	30.48 ± 1.92	28.54 ± 0.95
NTK, %	1.06 ± 0.03	1.31 ± 0.09	1.31 ± 0.03	1.46 ± 0.03	1.34 ± 0.03	1.32 ± 0.01
C/N ratio	28.90 ± 0.81	19.68 ± 1.28	18.97 ± 0.39	18.56 ± 0.41	22.84 ± 0.53	21.66 ± 0.14
PO_4^{3-}, %	0.0260	0.0875	0.0701	0.0503	0.0365	0.0482
P_2O_5, %	0.0088	0.0194	0.0656	0.0524	0.0376	0.0360
CaO, %	0.38	0.22	0.24	0.24	0.24	0.23
MgO, %	0.04	0.08	0.04	0.06	0.04	0.07
K_2O, %	0.40	0.95	0.92	0.83	0.78	0.88
Na_2O, %	0.19	0.51	0.67	0.70	0.62	0.65
Cl^-, %	0.0242	0.0301	0.0240	0.0241	0.0234	0.0252
SO_4^{2-}, %	0.0036	0.0035	0.0038	0.0043	0.0026	0.0035
NH_4^+, mg kg^{-1}	151.11 ± 14.44	189.29 ± 15.46	216.07 ± 37.85	251.78 ± 24.30	212.46 ± 27.29	295.70 ± 31.36
	259.25 ± 19.84	287.93 ± 41.32	363.43 ± 60.36	185.44 ± 20.36	300.74 ± 60.36	286.43 ± 20.59

The values obtained represent the average of three repetitions.

Microbiological parameters characteristics

In the composting process, microorganisms have a very important role. The presence of certain species reflects the qualities of mature compost (Ryckeboer et al., 2003).

Total aerobic mesophilic flora (TAMF) and thermophilic flora (TF)

The TAMF concentrations comparison at the initial state (T_0) and the final state (T_f) showed a decrease for all the mixtures.

Thus, the initial state of the mesophilic phase is dominated by a high bacterial concentration of TAMF varies between a maximum (1.26 10^{10} UFC g^{-1} of DM) observed in olive pomace and a minimum (2.23 10^9 UFC g^{-1} of DM) in organic household waste. (Fig. 18).

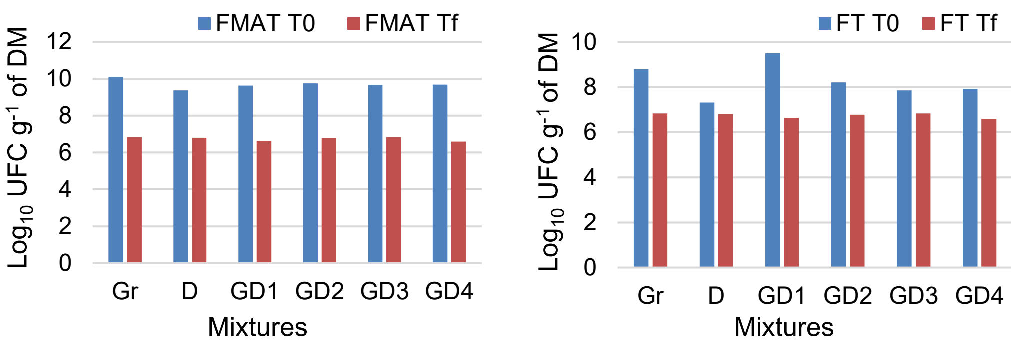


Figure 18. Mixtures characteristics in total aerobic mesophilic flora (TAMF) and thermophilic flora (FT).

These bacterial concentrations decreased at the end of the treatment (Tf), in order to stabilize respectively around 6.78 10^6 UFC g^{-1} of DM in olive pomace and 6.40 10^6 UFC g^{-1} of DM in organic household waste.

The total aerobic mesophilic flora of the mixtures reached high values in all the mixtures that is probably favored by the storage and the putting of this waste in contact with the air and the ground, which allows direct exposure to microbial contaminants.

TAMF olive pomace concentration at T_0 in this study (1.26 10^{10} UFC g^{-1} of DM) remains far superior to that of olive pomace from Jijel, Bejaia & Tizi-Ouzou in Algeria whose concentration varies between 0.45 10^6 UFC g^{-1} of DM and 1.36 10^8 UFC g^{-1} of DM (Boutiche et al., 2020).

By comparing the two states, a decrease in the thermophilic flora is observed for all the mixtures. Thus we observe a decrease in the GD1 mixture (15 % G & 85 % D) whose concentration increased from 3.21 10^9 UFC g^{-1} of DM (T_0) to 4.34 10^6 UFC g^{-1} of DM (T_f) and from 6.32 10^8 UFC g^{-1} of DM to 6.78 10^6 UFC g^{-1} of DM in olive pomace.

Fungal microflora

At the beginning of composting (T0) the different mixtures were characterized by a high concentration of fungal microflora. A high concentration at T0 was observed in the GD1 mixture with 4.59 1,010 UFC g^{-1} of DM in order to be around of 1.67 105 UFC g^{-1} of DM at the end of the process (Fig. 19).

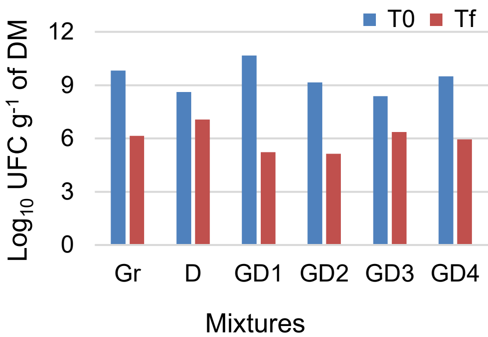


Figure 19. Mixtures characteristics of fungal microflora.

The concentration of fungi for olive pomace at T_0 in this study (6.61 10^9 UFC g^{-1} of DM) was much higher than those found during the olive pomace characterization with concentrations of 2.00 10^6 UFC g^{-1} of DM to 4.76 10^7 UFC g^{-1} of DM in Algeria (Boutiche et al., 2020).

At the end of the composting process (T_f) the fungal microflora has decreased. This decrease can be explained by the increase in temperature in the thermophilic phase, by creating unfavorable conditions for their growth, and by the decrease in humidity. (Greenberg et al., 1986; Guene, 2002).

Lactic acid bacteria

During composting, the lactic acid bacteria concentrations for all mixtures underwenta small increase with a maximum rate in organic household waste from 8.69 10^4 UFC g^{-1} of DM at T_0 and 2.23 10^7 UFC g^{-1} of DM at T_f (Fig. 20).

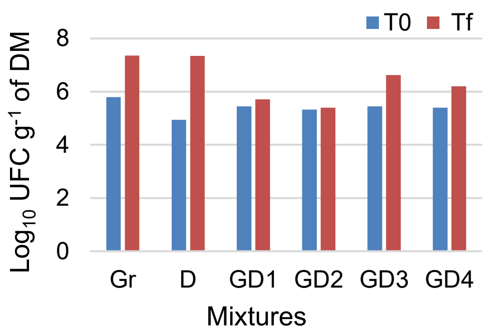


Figure 20. Mixtures characteristics of lacticacid bacteria.

The lactic acid bacteria concentration for olive pomace at T_0 in this study (6.17 10^5 UFC g^{-1} of DM) was slightly higher than those found in olive pomace in Algeria (0.13 10^4 UFC g^{-1} of DM to 3.0 10^5 UFC g^{-1} of DM) (Boutiche et al., 2020).

This increase is due to their growth during the acidogenic phase of the composting process.

Coliforms and E. Coli

The pathogenic micro-organisms presence in composted waste can represent a potential risk of contamination of harvested plants where compost has been spread. The use of composts in the agricultural sector requires the validation of their agronomic efficiency but above all the assurance of their environmental and health safety (Houot et al., 2009).

The mixtures studied characteristics show a high concentration of faecal coliforms at the beginning of the composting process and which suffered a reduction at the end of the treatment with a strong reduction in the GD3 mixture from 4.19 10^7 CF g^{-1} of DM à 63.3 CF g^{-1} of DM with a decrease rate (76.34%) is observed at the level of the GD3 mixture (Fig. 21).

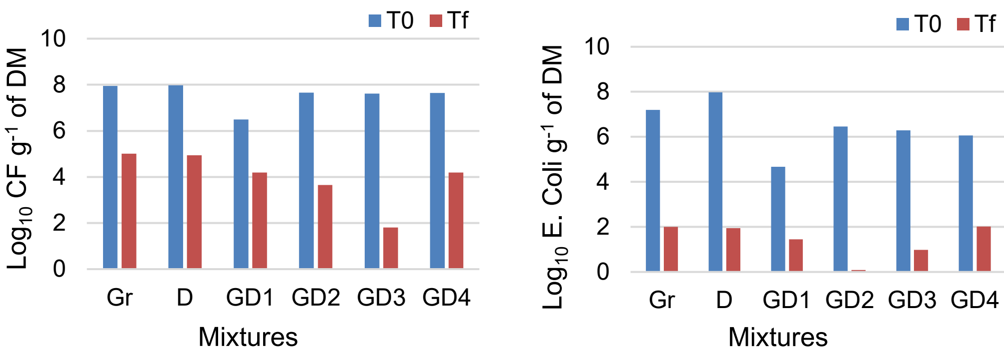


Figure 21. Mixtures characteristics of fecal coliforms and E. Coli.

The mixtures also all had a significant decrease in terms of E. Coli with a maximum concentration of 9.48 10^7 E. Coli g^{-1} of DM at T_0 in organic household waste towards 88.3 E. Coli g^{-1} of DM at T_f. All the treatments suffered a strong decrease, which means the effectiveness of composting in the elimination of pathogenic germs with a high decreasing rate in the GD2 mixture (98.62%) (Fig. 21).

To have a good compost quality in order to use it in agriculture, it is necessary to be aware of the standards concerning organic amendments (AFNOR, NF U 44-051, 2006) which set threshold values for certain pathogenic germs which are indicators of treatment such as *Escherichia Coli* (Houot et al., 2009).

Salmonella

During our study, we limited ourselves to the qualitative test (presence/absence) of Salmonella. The results obtained show that they were always present in all the mixtures throughout the composting process. This may be due to the lack of a high temperature in the thermophilic phase, which they did not exceed 61.22 °C as a higher temperature degree obtained at the 84^{th} day of composting (Gr compost), to ensure major or total elimination of Salmonella. This temperature limitation can be linked to the working period (winter period). These temperature values remain below 70 °C, which is necessary for a living organism's destruction (Bernal et al., 2009). As microbiological criteria, an organic amendment destined to the vegetable crops must not contain any pathogenic agent (Salmonella) in every 25 g as a limit value (NF V 08-052:1997).

The presence of Shigella and Salmonella is considered as the major and specific problem of the compost hygienic quality (Hussong & Burge, 1985; Brinton & Droffner, 1994; Yanko, 1995; Hay, 1996). This was probably because these bacteria are ubiquitous and have a very fast growth capacity. The United States Environmental Protection Agency (US-EPA) imposes for Salmonella a rate lower than three bacteria in 4 g of dry weight of compost and sludge (Hay, 1996). Salmonella comes from food wastes, especially from meats, poultry, milk and its derrivatives… (Hassen et al., 2001).

Brinton & Droffner (1994) reported that some mutant strains of Salmonella may withstand the high temperatures (42–54 °C), and could recontaminate windrows during compost storage.

Recapitulating what was said, the pH of the aqueous solution of the compost underwent an increase starting from the thermophilic phase. During the thermophilic phase, the alkalinization of the medium is linked to the ammonia (base) produced by the bacterial hydrolysis of protein and organic nitrogen. During maturation, the pH remains basic and then gradually decreases over time to reach neutralization. The pH stability is due to the slow maturation reactions and the buffering capacity of humus (Fauci et al., 1999).

The electrical conductivity is high at the start of composting then decreases as a function of composting time to reach values below the limit value of 3 mS cm^{-1} (Soumaré et al., 2002). During the composting process, the amount of extractable ammoniacal nitrogen gradually decreases with the age of the compost while the amount of nitrate increases. The transition from ammonia nitrogen to nitric nitrogen takes place through the mineralization of complex nitrogen compounds into ammonia and amino acids.

Ammonium can either be used directly in microbial metabolism or be oxidized to nitrates and nitrites by nitrogen-fixing organisms (Lhadi & Aylaj, 2008). The evolution of the C/N ratio is directly related to the biodegradation of organic matter, which results both in the elimination of carbon in the form of CO2 and in the apparent concentration of mineral elements (N, P, K, etc.).

On the other hand, there are losses of nitrogen, in the form of ammonia, during the thermophilic phase. These losses tend to attenuate the drop in the C/N ratio. Other authors (Bousselhaj et al., 1996 and Hafidi, 1996) have reported similar results. The C/N ratio is another indicator of compost maturity (Mathur et al., 1993; Ozores-Hampton et al., 1998; Tazi, 2001; Aylaj, 2002; Lhadi et al., 2004 and 2006).

The pathogenic microorganism's presence in composted waste can represent a potential risk of contamination of harvested plants where compost has been spread. The use of composts in the agricultural sector requires the validation of their agronomic efficiency but above all the assurance of their environmental and health safety (Houot et al., 2009).Composting is essentially a microbiological phenomenon that depends highly on temperature evolution within the windrows. The temperature within a composting mass determines the rate at which many of the biological processes take place and plays a selective role on the evolution and the microbiological communities succession (Mustin, 1987).

CONCLUSION

The composting process in the presence of oxygen (aerobic composting), micro-organisms need oxygen to decompose and degrade organic substances. In this study, the oxygen consumption was adjusted by injecting air while turning the composts. The results obtained show that the mixtures can be successfully composted in a period of 4 months and can give better results than other raw waste composts in terms of the C/N ratio... There are essential parameters (physico-chemical and agrochemical characteristics) such as pH, electrical conductivity, organic matter, P_2O_5, K_2O, CaO… to determine and to assess the quality and the compost maturity. The study of the parameters during the 4 months of composting revealed a strong biological activity during the phase bio-oxidant. This activity has been attributed to the mineralization of materials easily degradable.

The composts produced in this study were satisfactory for their agricultural application with a neutral pH in all the mixtures, an optimal C/N ratio around 20, an electrical conductivity that does not exceed the limit value of acceptance for use as support for the ground (3 mS cm^{-1}). In addition, the mixtures suffered a decrease in terms of pollution indicators. These composts contain nutrients that can allow them to play the role of fertilizer and support for soils poor in minerals...

At the end of the composting process, an optimal C/N was observed in the GD2 mixture (18.56) with a maximum content for Na^+ ions (0.70%) and a minimum content of ammoniacal nitrogen lower than the limit value (400 mg kg^{-1}).

The results we have achieved show the interest of composting this waste. Therefore, these laboratory scale results can be applied in full-scale installations by adjusting the forced ventilation according to the available equipment.

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Effects of shading on the growth of the purple pakchoy (*Brassica rapa* var. Chinensis) in the urban ecosystem

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Abstract. Open green spaces in urban area can be utilized in many ways. Recently, more of the open spaces have been cultivated for vegetable production, gradually shifted from aesthetical purpose to the need for fresh healthy foods. Urban vegetable farming can be conventionally practiced on a limited backyard. Our research was aimed to assess the effects of shading treatments on growth and yield of purple pakchoi (*Brassica rapa* var. *chinensis*). The research was arranged based on the randomized block design with 4 levels of shading treatment. The results showed that shading treatments at 0%, 45%, 55%, and 80% significantly affected morphological growth traits, including plant height, number of leaf, lengths of petiole, length of leaf midrib, width of leaf blade, canopy diameter, and leaf SPAD index, total leaf fresh and dry weights, total fresh and dry roots, and lengths of stem; but did not significantly affect the leaf thickness. In all affected traits, heavier shading inhibited growth, confirming that the purple pakchoi prefers full sunlight. The leaf of purple pakchoi reached its maximum size at less than 14 days counted from the first day of leaf blade was fully unfolded. Purple pakchoi can be harvested at 35 days after transplanting. The accurate leaf area estimation in purple pakchoi can be achieved by using LW as predictor and calculated using the power regression ($R^2 = 0.9806$).

Key words: aesthetic value, fresh vegetable, green space, healthy food, morphological trait, urban farming.

INTRODUCTION

The open green space in urban area is continuously decreasing due to socio-economic pressures, public policies, and the physical condition (Ustaoglu & Williams, 2017). Increase in economic value of urban land enhanced conversion of the green space to urban settlements (Rondhi et al., 2018). Essentially, the function of green space in urban area should be conserved in order to maintain comfortability, aesthetic value, and healthy environment. Intensification of the urban agricultural activities could ecologically and aesthetically improve living environment and also increase availability of fresh and healthy vegetables for urban community (Lakitan, 2021). Urban farming has been globally practiced and well accepted by city dwellers (Grebitus et al., 2020).

Urban farming opened opportunity for the knowledgeable community to apply science and technology in producing fresh foods (Orsini et al., 2013; Ferreira et al., 2018), especially fruits and vegetables (Lal, 2020). Walters & Midden (2018) suggested to grow vegetable crops with shallow roots. This farming activity may also improve community welfare (Surya et al., 2020).

Green pakchoi *(Brassica rapa* var. *chinensis)* has been globally recognized, including in Indonesia (Priadi & Nuro, 2017); however, purple pakchoi has not been widely known. Purple pakchoi and other colorful vegetables have attracted urban community to grow them for their aesthetic value (Zhang et al., 2014), encouraged the community to consume natural healthy substances, including anthocyanins (Zhu et al., 2017; Hao et al., 2020), and considered as a good selenium supplement for humans (Li et al., 2020). Leaf of pakchoi has a good tasted, high economic value, and easy to cultivate (Wahyuningsih et al., 2016).

In the tropical agroecosystem, shading was commonly practiced in the cultivation of purple pakchoi (Abdel–Ghany & Al–Helal, 2020). Shading application has been reported to increase number of leaf in pakchoi (Mansyur et al., 2014; Andini & Yuliani, 2020). However, it should be specified at what ranges or peak that the optimal shading percentage for the purple pakchoi can be achieved.

This research was aimed to specify optimum shading percentage for maximizing growth and yield of the purple pakchoi *(Brassica rapa* var. *chinensis)* in the tropical urban ecosystem.

MATERIALS AND METHODS

The study was carried out in the tropical lowland climate at the research facility in Jakabaring (104°46′44″E; 3°01′35″S), Palembang, South Sumatera, Indonesia. Average air temperature is 31 °C and relative humidity is 51% during period of cultivation. Seeds of purple pakchoi *(Brassica rapa* var. *chinensis)* were purchased from commercial seed producer. The seeds soaked in tap water for 15 minutes for breaking the dormancy. After soaked, seeds were sown in seedling trays filled with mixture of soil and chicken manure (1:1 v/v). Chemical characteristics of soil used include pH = 6.63, N-total 0.27%, P_2O_5-total 720.53 mg per 100 g, K_2O-total 63.94 mg per 100 g, Mg-total 123.09 mg per 100 g, and Available P Bray II 1306.4 ppm. Nutrient contents of the commercial chicken manure used were 3–5% nitrogen, 1.5–3.5% phosphorous, and 1.5–3.0% potassium.

The seedlings were transplanted to plastic pots at 14 days after sowing (DAS). The seedlings had two unfolded leaves at time of the transplantation. Each pot was filled with a mixture of soil and chicken manure at ratio of 3:1 v/v (Jaya et al., 2021). Fourteen days prior to transplanting, the growing mix was sterilized using bio-sterilization and aerobic decomposers (*Streptomyces* sp.; *Geobacillus* sp.; and *Trichoderma* sp.) at dose 200 mL plant^{-1}. The plants were daily watered at 5 p.m. for maintaining soil moisture near the field capacity (35%). The maximum wet-dry cycle was around 30 to 35 in the morning and 25 to 30 at late afternoon. Therefore, the purple pakchoi plants were never under water stress condition.

Experimental design

The experimental design used in this study was the randomized block design. Seedlings of the purple pakchoi were directly allocated in each of the 45%, 55%, and 80% shading house and on an open space for 0% shading at time of transplanting. Percentage of shading was calculated by substracting light intensity under full sunlight with the directly measured light intensity using a light meter (Krisbow KW06-291). Three shade houses were constructed for each shading treatment. Dimension of the house was 4 m length × 2 m width × 2 m height. The knockdown frames made of 1.5 inch PVC pipes were used in constructing the shading house. Each of the shading house was covered in all sides with black polyethylene net at different shading levels.

Data measurement

Collected data on plant growth included plant height, number of leaf, petiole length, leaf blade length, leaf blade width, leaf thickness, canopy diameter, and leaf chlorophyll index. The leaf chlorophyll index was measured using an instrument for soil plant analysis and development (SPAD) produced by the Konica Minolta (SPAD–502 Plus). The mature plants were harvested at 49 DAS or 35 days after planting (DAP). The measured traits at time of harvest included above ground organs, i.e., whole-plant weight, fresh and dry weight of leaf blade, fresh and dry weight of petiole, fresh and dry weight of stem; and under ground organs, i.e., root length, root fresh and dry weights. The measurement of all dimensional traits used the transparent regular ruler, except for leaf thickness that was measured using a digital caliper. Weight-related traits were measured using a digital scale.

Calculation of leaf growth rate was carried out based on directly measured length (L) and width (W) of the leaf blade for 18 consecutive days until the leaf was fully expanded. Leaf area (LA) estimation model was developed based on directly measured data on leaf length and width. Direct LA measurement used digital image analysis software (LIA32, developed by Kazukiyo Yamamoto, Nagoya University, Japan). The LIA32 digital measurement software recognizes all visible color spectrum (Kitao et al., 2022).These two morphological traits were used as predictors, individually (L or W) or in combination (LW). The zero-intercept linear model was used if LW was used as predictor. The power and polynomial regression was used if individual L or W was selected as predictor.

Data analysis

Analysis of variance (ANOVA) were carried out using statistical analysis software (SAS 9.0 for Windows, SAS Institute Inc., USA). Significant differences amongst treatments were tested using the Least Significant Difference *(LSD)* test at $P > 0.05$. Trend analysis between predictor and the leaf area was using the zero-intercept linear, power, and polynomial regressions (Meihana et al., 2017; Lakitan et al., 2021a). Leaf area estimation model was tested for accuracy based on the determination coefficient (R^2). The predictors used were leaf length (L), leaf width (W), and the multiplication of leaf length and width (LW).

RESULTS AND DISCUSSION

Growth responses to shading

The purple pakchoi has a short single stem without any branch; therefore, the shoot was dominated by leaf petioles and blades. Both stem growth and increase in number of leaves in the purple pakchoi were significantly halted if the sunlight received on the surface of the leaves is reduced as a result of being partially blocked by shading. The differences due to the effect of shading were increasingly noticeable as the purple pakchoi plant continued to grow (Fig. 1).

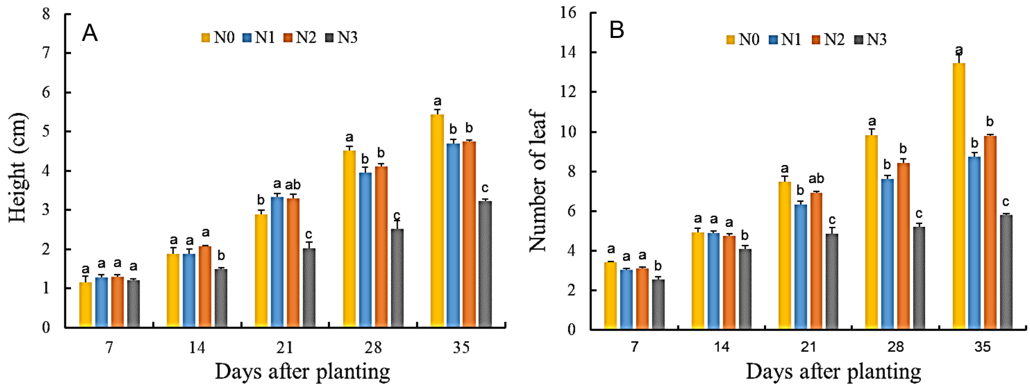

Figure 1. Stem height and number of leaf in purple pakchoi as affected by shading measured at 7 to 35 days after planting.

A common response of plants exposed to shade conditions is etiolation, i.e., excessive elongation of the stem (Armarego-Marriott et al., 2020). However, the symptoms of etiolation were not seen in the purple pakchoi plant in this study. On the contrary, the lengthening process of the stem was hampered in the shaded conditions. The incidence of etiolation resulting from lack of light occured only during the seedling phase in leafy vegetables, including green and purple pakchoi (Kong & Zheng, 2018).

The process of leaf formation was also hampered by shading treatments. Higher shading intensity (80%) suppressed stem lengthening and new leaf formation more strongly than moderate shading intensity (45% and 55%). Thus, the incidence of shading is believed to inhibit the overall growth of the purple pakchoi plant. The difference in stem length and number of leaves between plants that experienced different shade intensities became more noticeable as the purple pakchoi plant grew larger. Low light intensity (55% shading) significantly decreased the shoot and root growth in pakchoi (Yang et al., 2009).

Shading has direct and indirect effects on plant growth and development since shading alters microclimate condition underneath the shade screen. In addition to directly reducing the intensity of sunlight, the shading treatments also indirectly affects various climatic elements on the microscale at a position below the shade screen. Effects of shading on the length of leaf midrib, width of leaf blade, and length of petiole exhibited similar patterns to the effects on stem length and number of leaf. Higher shading intensity (80%) severely limit all measured components of leaf growth in purple pakchoi. Differences amongst 0, 45, 55, and 80% shading treatments were much clearer at 35 days after transplanting (Fig. 2).

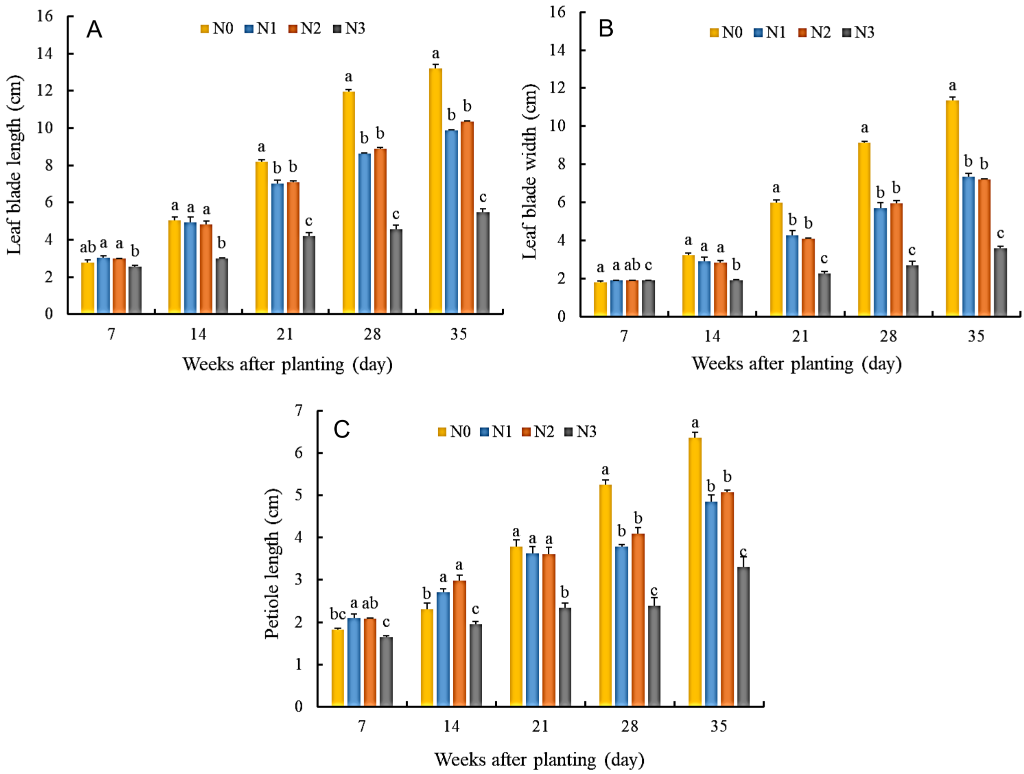

Figure 2. Leaf midrib length (A), leaf blade width (B), and petiole length (C) in purple pakchoi as affected by shading application measured at 7 to 35 days after planting.

Decrease in sunlight intensity would lower air and soil temperatures, increase relative humidity, decrease evapotranspiration, and increase soil moisture (Mahmood et al., 2018; Lakitan et al., 2021b). Furthermore, higher soil moisture could reduce oxygen transport into the soil matrix (Uteau et al., 2022), in turn, could decrease root metabolisms (António et al., 2016). In contrast, Budiarto et al. (2019) also reported that shading significantly improved the plant growth and number of leaves at early vegetatif growth.

The diameter of canopy also negatively affected by shading but the leaf thickness was not affected by shading (Fig. 3). The resistance to enlargement of the canopy area of purple pakchoi plant due to the influence of shading proportionally followed a pattern of resistance to all observed morphological traits, except for the thickness of the leaves. The leaf maintains the ability to form lateral cell additions throughout their life cycle and then develop into a flattened structure. The flattened leaf shape is advantageous for plants to capture more light needed for photosynthetic activity.

Du et al. (2018) explained that the flattened structures can optimally fulfill this function by precisely control the initiation, shape, and polarity of leaves. Leaf development underlies their morphogenesis to establish the three-dimensional leaf flattening forms.

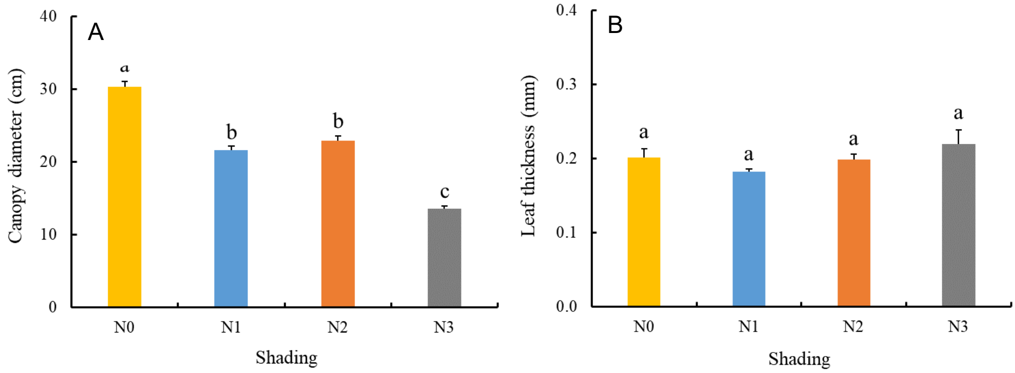

Figure 3. Canopy diameter (A) and leaf thickness (B) in purple pakchoi as affected by shading application.

Overall average of the mature leaf SPAD index was around 47.7 in purple pakchoi. The leaf SPAD index was above average in plant exposed to full sunlight intensity and reversely the index falled to below average in the heavily shaded purple pakchoi plant, i.e., exposed to 80% shading. The distributions of SPAD values on mature leaves were relatively the same at measurements on 21, 28, and 35 DAP. The highest SPAD value was always obtained in plants exposed to full sunlight, regardless of the age of the plant at the time of measurements (Fig. 4).

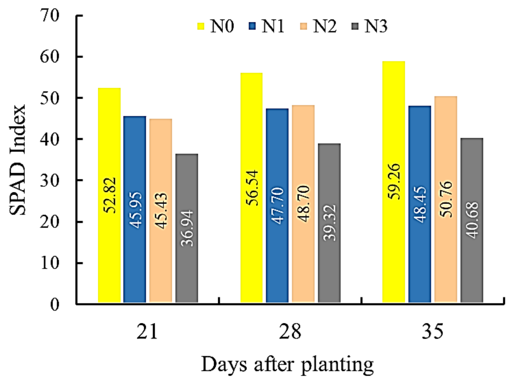

Figure 4. The effect of shading on the SPAD index on mature leaves in purple pakchoi.

The standard SPAD index measurement is on mature leaves that are characterized by their maximum size and have not shown symptoms of senescence. It should also be understood that the SPAD meter only measures the total chlorophyll concentration based on the optical principle and cannot distinguish between chlorophyll a and b. Technical details about the SPAD meter are provided by Süß et al. (2015). In addition addition to determining the total chlorophyll concentration, SPAD meter is also widely used as an indicator of leaf nitrogen status (Rongting et al., 2020). SPAD measurements in this study were used to monitor the effect of shading on the growth and health of pakchoi plants through changes in the green color intensity of the leaves.

Leaf surface area were measured daily for 18 consecutive days from the time the leaves began to unfold until they reached their full size. Knowledge of when the leaves cease to enlarge is a very useful in determining the right harvest time for leafy vegetables such as purple pakchoi. Leaf area monitoring based on leaf length and width separately is shown in Fig. 5 and monitoring based on the multiplication results between leaf length and width is presented in Fig. 6. The result of these two monitorings was consistent with each other.

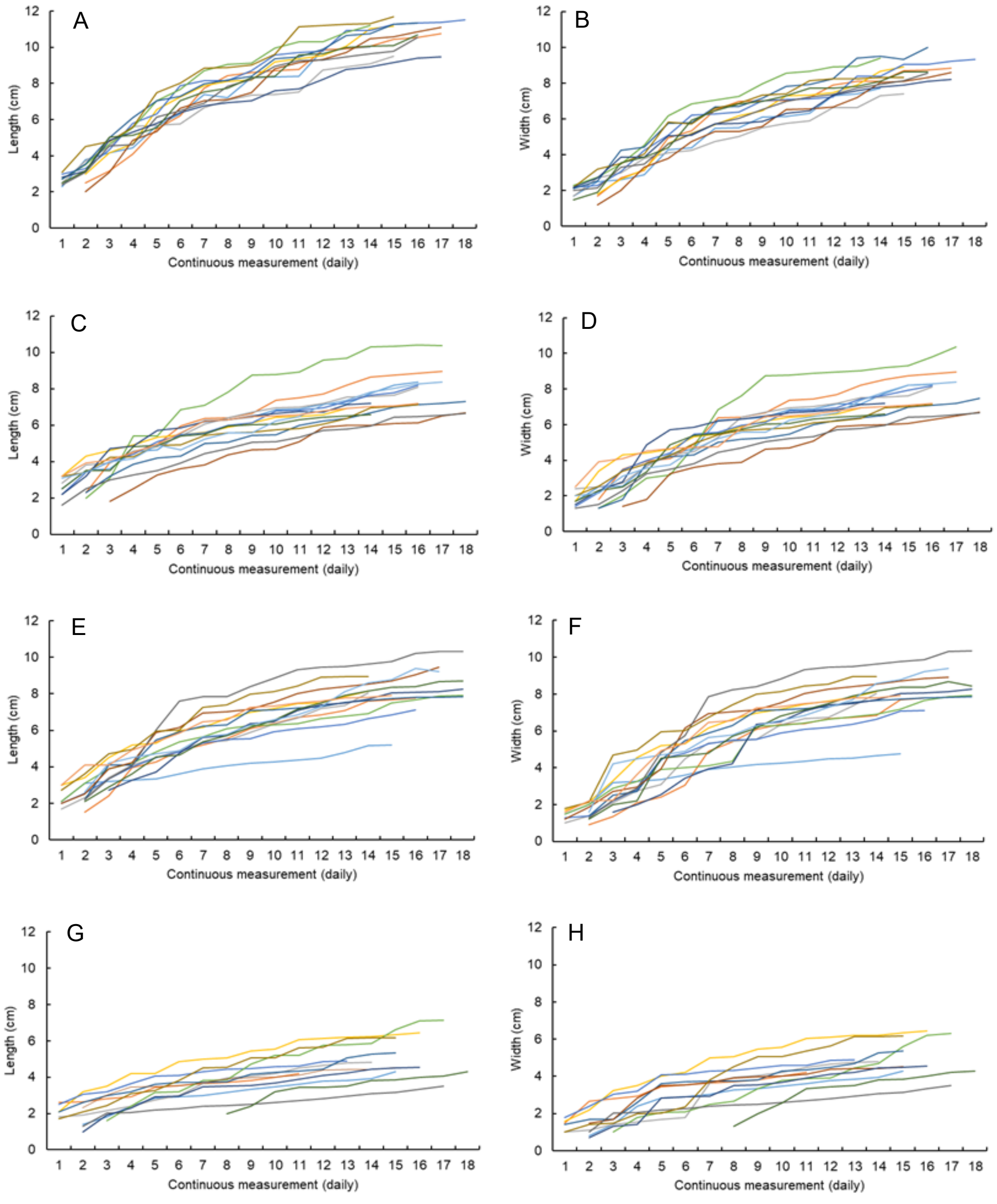

Figure 5. Continuous leaf growth based on their length and width in purple pakchoi exposed to full sunlight (A, B); 45% shading (C, D); 55% shading (E, F); and 80% shading (G, H).

Based on the results of this study, the final leaf area differed according to the percentage of shading treatment imposed to each group of the pakchoi plants. Plants exposed to full sunlight exhibited the largest leaf size, plants that were shaded 45% and 55% produced almost the same medium size leaves, and plants with 80% shade produced the smallest leaf size. However, the number of days it took to reach the full leaf size was about the same, which was between 13 to 15 days.

Healthy purple pakchoi has spoon-like leaves with distinctive elliptical shape of its leaf blade as indicated in plant exposed to full sunlight, i.e., the leaf length exceeds its width. Increase in shading treatment to 80% gradually alter the leaf shape of the purple pakchoi from elliptical to more rounded shape, i.e., measurement of the leaf length and its width are almost similar. Therefore, increase in shading exposure not only decreases the leaf size but also changing the shape of purple pakchoi leaf.

In this study, if leaf growth was represented by the length or width of the leaf, then the growth patterns was seen following an asymptotic curve, not fully following the Sigmoid curve (Fig. 5). This actually happened because the slow initial growth phase (as in an early segment of the full Sigmoid curve) occured when the leaf blade had not yet opened. The length or width of the leaves was still too small (< 5 mm), fragile, and had not been perfectly formed to be measured manually. In this phase, it is estimated that the leaf lengthening rate is still less than 1 mm per day. Meanwhile, if leaf growth was represented through the result of multiplication of leaf length by width, then the shape of the Sigmoid curve is slightly visible (Fig. 6). The technical information obtained from this study is that the growth of purple pakchoi leaves will start to grow rapidly when the leaf blade begins to unfold.

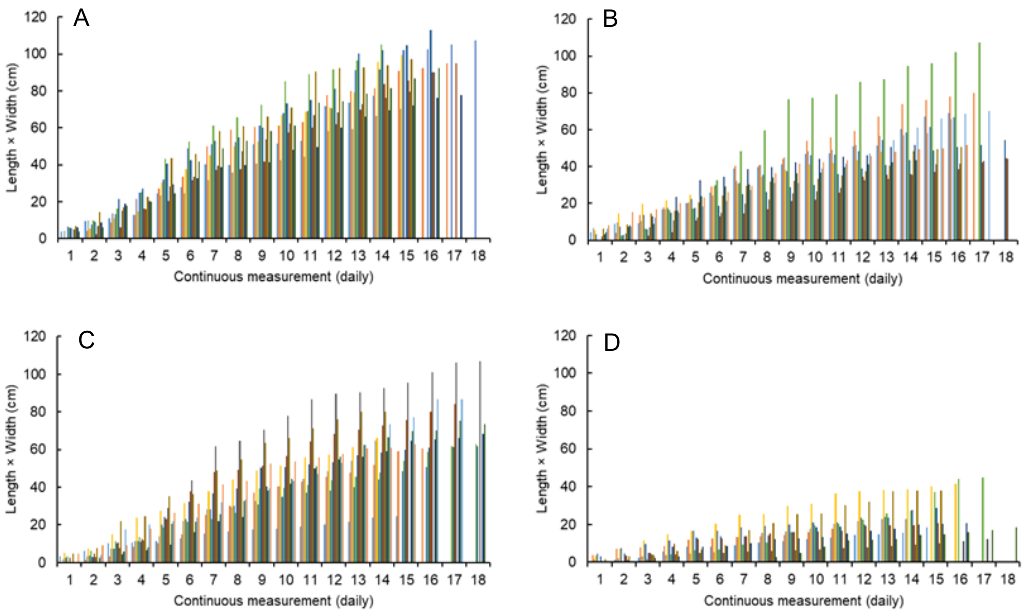

Figure 6. Growth of leaf blade length × width at 0% (A), 45% (B), 55% (C), and 80% (D) shading.

The growth pattern of leaves as well as individual plants generally follows the Sigmoid curve. Ding et al. (2022) reported that leaf growth (based on leaf length and width) and plant growth (based on plant height and canopy width) followed sigmoidal curves in three pakchoi cultivars. Similarly, Baker & Wang (2021) found that growth of bokchoy, measured based on image analysis of its canopy, consistently followed the Sigmoid curve.

Yield and biomass components

Leaf blade, petiole, and stem of purple pakchoi were negatively affected by shading (Table 1). Increase in shading intensity decreased fresh and dry weight of all above ground organs and also spesifically on the yield components, i.e., leaf blade and petiole. A sharp drop was observed at 80% shading treatment. Similar effects were also found in underground organs as indicated by decrease in root length, fresh weight, and dry weight (Table 2). More severe drop in roots of plant exposed to 45% shading than 55% shading indicated that the drops may not be solely due to shading treatment, it may confound with indirect effect of soil moisture condition. Heavy shading treatment caused low evaporation rate; therefore, increased soil moisture content.

Table 1. Effect of shading application on above-ground organs including leaf blade, petiole, and stem at fresh and dry weight conditions in purple pakchoi

	Leaf FW	Leaf DW	Petiole FW	Petiole DW	Stem FW	Stem DW
N0	39.9 ± 1.79^a	5.4 ± 0.32^a	0.5 ± 0.04^a	1.3 ± 0.11^a	5.4 ± 0.32^a	0.5 ± 0.04^a
N1	13.6 ± 2.93^b	1.8 ± 0.12^b	0.2 ± 0.02^b	0.2 ± 0.01^b	1.8 ± 0.12^b	0.2 ± 0.02^b
N2	13.2 ± 0.59^b	2.3 ± 0.10^b	0.2 ± 0.01^b	0.3 ± 0.02^b	2.3 ± 0.10^b	0.2 ± 0.01^b
N3	1.9 ± 0.10^c	0.5 ± 0.04^c	0.0 ± 0.01^c	0.0 ± 0.01^c	0.5 ± 0.04^c	0.0 ± 0.01^c
LSD	6.54	0.75	0.08	0.10	0.75	0.08

Data were presented as mean ± standard error. The means followed by different letters within each column indicate significant difference at the $LSD_{.05}$

Shading not only decreases the quantity of yield (fresh weight of leaf blade and petioles) but also has an impact on reducing the yield quality of purple pakchoi plants (the intensity of their attractive purple color becomes faded). In our study, the deep and blackish purple colors were found in all plant fully exposed to sunlight. Meanwhile, under heavy shading (80%), only a thin, uneven layer of soft purple color covered the surface of the leaf so that in some spots the green color was also visible. The result of the observations showed that exposure to shade for a long time inhibited the formation of a purple layer on young leaves and at the same time eroded the purple layer that had formed on the surface of mature leaves.

Table 2. Effect of shading application on under-ground organs including root length, root fresh weight and dry weight conditions in purple pakchoi

	Root Length	Root FW	Root DW
N0	26.27 ± 0.54^a	6.12 ± 0.24^a	0.616 ± 0.020^a
N1	13.87 ± 0.72^b	0.79 ± 0.06^c	0.078 ± 0.003^{cb}
N2	17.75 ± 0.07^b	1.69 ± 0.40^b	0.123 ± 0.018^b
N3	7.22 ± 0.55^c	0.20 ± 0.03^c	0.016 ± 0.001^c
LSD	4.55	0.73	0.07

Data were presented as mean + standard error. The means followed by different letters within each column indicate significant difference at the $LSD_{.05}$.

Song et al. (2020) proved that the purple color in pakchoi leaf was associated with the anthocyanin content. The ratio of anthocyanin to chlorophyll content was responsible for the color formation in purple pakchoi. If the ratio was high, the leaf appeared reddish purple. If the ratio was low, the leaf appeared deep purple or even blackish purple. Zhu et al. (2017) added that the purple color on the leaf surface decreased and turned green if the pakchoi plant was exposed to shading conditions for a longer period of time.

Estimating purple pakchoi leaf area

Leaf area can be estimated using appropriate regression models such as linear, polynomial, and power regression. Some dimensional traits can be used as predictors such as leaf length (L), leaf width (W), and multiplication of these two traits (LW). The accuracy can be very high if regular shape leaves are used. The purple pakchoi has a regular oval shape. Results of this study shown that if single predictor was used (L or W), the zero–intercept linear model was not suitable as indicated by lower coefficient of determination. Meanwhile, both polynomial and power models were very reliable in predicting LA if single predictor was used. Meanwhile, the simpler zero–intercept linear model was also very reliable in predicting LA if LW was used as predictor (Table 3).

Table 3. Regression models for leaf area estimation using individual and combination of the leaf length and width in purple pakchoi

Predictor	Regression model	Leaf Area (LA)	R^2
Leaf length (L)	Power	$LA = 0.3305\ L^{2.1412}$	0.9357
	Polynomial	$LA = 0.7135\ L^2 - 3.0651\ L + 6.8704$	0.9299
	Zero-intercept linear	$LA = 5.266\ L$	0.6933
Leaf width (W)	Power	$LA = 1.0348\ W^{1.8859}$	0.9477
	Polynomial	$LA = 0.469\ W^2 + 3.7209\ W - 6.6256$	0.9504
	Zero-intercept linear	$LA = 6.9926\ W$	0.8185
LW	Power	$LA = 0.5191\ LW^{1.0428}$	0.9806
	Polynomial	$LA = 0.0002\ LW^2 + 0.6213\ LW - 0.6181$	0.9784
	Zero-intercept linear	$LA = 0.6428\ LW$	0.9774

R^2 is the coefficient of determination used as the indicator for accuracy level of the leaf area estimation models.

Leaf is the most important and dominant organ in leafy vegetables, including purple pakchoi. Continuous measurement of leaf growth requires a non-destructive approach such that measurement of leaf morphological traits can be collected using the same individual leaf. Purple pakchoi has a spoon shape leaf which is relatively easy to estimate with high accuracy. Each leaf has three dimensions, i.e., length (L), width (W), and thickness (T). However, only L and W individually and in combination (LW) are commonly used as predictors. Leaf thickness is considerably negligible, compared to L or W (Walia & Kumar, 2016; Fascella et al., 2018).

Three most commonly used regression models are linear if LW is used as predictor and polynomial order–2 or quadratic and power regression are used if L or W is used separately as predictor. To ensure LA = 0 if L = 0 and W = 0 then zero-intercept linear regression is recommended (Lakitan et al., 2021a).

In this study, the best results were obtained with the power regression model on all predictors, with values of $R^2 = 0.9357$ (L), $R^2 = 0.9477$ (W), and $R^2 = 0.9806$ (LW). In previous studies, polynomial and zero-intercept linear regression were also used in estimating LA of tatsoi, a herbaceous leafy vegetable (Kartika et al., 2021). The power regression has been proven accurate for estimating the leaf area in many herbaceous vegetables, including leaf celery (Lakitan et al., 2021a), and *Talinum paniculatum* (Lakitan et al., 2021b). The zero–intercept linear regression is very accurate if LW is used as predictor ($R^2 = 0.9774$).

For future research, a study on the interaction effects of shading and split fertilizer applications in the pakchoi leaf area is proposed.

CONCLUSIONS

The full sunlight intensity produced the best results on growth and yield in purple pakchoi. Therefore, the purple pakchoi can be classified as sun plant. The shading density at 45–80% reduced growth and yield of the purple pakchoi plant and also affected leaf SPAD values. Shading was not only reduced yield quantity but also yield quality. The atractive purple color of pakchoi leaves was diminished after long shading exposure. Leaves of the purple pakchoi reached their maximum size at 13–15 days, counted after the young leaves were fully unfolded. The purple pakchoi can be harvested starting at 35 DAP. The power and polynomial regression model can be used for estimating leaf area in all predictors, including L, W, and LW. However, the zero–intercept linear regression is recommended only for LW as predictor. It is recommended to urban communities to choose a location that is directly exposed to sunlight in cultivating purple pakchoi vegetable.

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Role of acid phosphatase and enzymatic and non-enzymatic antioxidant systems in tolerance of alfalfa (*Medicago sativa* L.) populations to low phosphorus availability

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Abstract. This study aims at evaluating the tolerance of four alfalfa (*Medicgao sativa* L.) populations to low phosphorus (P) in rooting medium. The experiment was carried out under controlled conditions. The seedlings of 15 old days were subjected to P deficiency using Ca_3HPO_4, insoluble form and P sufficiency using KH_2PO_4, as soluble form, at a final concentration of 250 µmol P plant^{-1}. week^{-1}. After 60 days P deficit, several agro-physiological and biochemical traits were measured and determined in both conditions. The obtained results indicated that the P–starvation significantly ($P < 0.001$) reduced the agroeconomic traits evaluated such as plant dry weight and leaf area. The root and shoot P contents were found ($P < 0.001$) decreased by low–P availability in the rooting medium. This constraint induced significant ($P < 0.001$) increase in phosphatase acid activity and caused lipid peroxidation and oxidative damage to cells, evaluated through malondialdehyde and hydrogen peroxide contents. Our results showed also, that low P availability significantly ($P < 0.001$) increased the enzymatic antioxidant responses reflected by the activities of superoxide dismutase (SOD), guaiacol peroxidase and catalase. The non-enzymatic antioxidant molecule such as proline and total polyphenols were found significantly increased in alfalfa stressed plants. The behavior of alfalfa populations tested was significantly different ($P > 0.05$). The *OL* population was found to be the least affected and the *DEM* was most sensitive one, whereas the populations *TATA* and *RICH* showed a moderate tolerance. Our study advises that the tolerance of Moroccan alfalfa populations to low P–availability was associated with increased acid phosphate activity and ability to induce enzymatic and non-enzymatic antioxidant responses leading to cell detoxification from reactive oxygen species (ROS).

Key words: alfalfa, acid phosphatase, antioxidant activity, oxidative stress, phosphorus, ROS.

INTRODUCTION

Legumes are an important source of proteins for both humans and animal livestock. They contribute to the incorporation of nitrogen in agro–pastoral ecosystems with valuable economic influence, to reduce or limit expansive and eco–unfriendly chemical fertilizers (Farssi et al., 2021; Lazali et al., 2021). Legumes are also used in intercropping or rotation with many plant species because of their multiple beneficial functions (Mouradi et al., 2018). Indeed, when cultivated in intercropping, legumes showed multiple advantages such as biological control, nutrient cycling, and increase in total yield (Martins da Costa et al., 2018; Mouradi et al., 2018; Oukaltouma et al., 2021). Therefore, exploiting the N_2–fixing ability of legumes could be a good way to improve the growth and productivity in legume–cereal intercropping system.

However, Abiotic constraints accentuated in recent decades by climate change are the primary cause of crop losses in the Mediterranean region. In fact, the richness of the genetic heritage of many leguminous species including alfalfa (*Medicago sativa* L.) is threatened by several abiotic factors, such as prolonged periods of drought, soil and irrigation water salinity, and soil P deficiencies (Farissi et al., 2011; Bargaz et al., 2013b; Farissi et al., 2013) In fact, previous investigation on legumes showed that phosphorus limitations leading to an obvious reduction in different plant aspects including growth, photosynthesis, and nutrient use efficiency (Farssi et al., 2021; Lazali et al., 2021). In the same way, low P availability negatively affected P nutrition and atmospheric nitrogen fixation in forage and grain legumes, and importantly the effect was more aggravated under the combined effect of P and osmotic stresses (Oukaltouma et al., 2021). The culture of legumes is therefore concentrated in the northern regions with a favorable climate. Because of the climate change conditions, it is perceived that plant production globally, including legumes, is seriously threatened and the sensitivity of legumes to several abiotic stresses is a major obstacle to obtaining large quantity and quality products.

Low P availability, particularly in N_2–fixing symbiosis, has a significant effect on legumes' yield (Lazali & Drevon, 2018). Indeed, this symbiosis poses supplementary P demand (20% of total to nodules) and any P starvation may affect the activity of rhizobia and consequently the symbiosis efficacy (Drevon, 2017). However, soluble P enhances the growth of *Vigna unguiculata* L. plants, their total P content and nodulation (Benlahrech et al., 2018). In the same sense, Bekel et al. (2019) reported that the P fertilizers significantly influenced both total and effective number of nodules in *Arachis hypogaea* L. plants. Based on the positive correlations between acid phosphatase activity and P use efficiency, it would be worthwhile to take into account the role of acid phosphatases in building an effective legume-rhizobia symbiosis as a possible mechanism in P-deficiency tolerance (Lazali et al., 2021). In fact, Tran et al. (2010) reported that under low-P availability, acid phosphatases are thought to be crucial for the metabolism of organic P in both intracellular and extracellular plant tissues. It has been suggested that secreted or cell wall-associated acid phosphatases recycle Pi from endogenous phosphomonoesters that have leaked from the cytoplasm across the plasma membrane or scavenge Pi from organic-P compounds found in the rhizosphere (Shane et al., 2014).

Furthermore, the reactivity of P with some soil cations, such as iron, aluminum, and calcium, which results in the formation of insoluble compounds, limits its mobility in soil solutions. These interactions result in reduced P availability and low phosphate fertilizer efficiency in plants. As a result, the SNF process, root growth, photosynthesis, rhizobia proliferation, and nodule development are all limited (Neila et al., 2014; Boudanga et al., 2015).

In recent years, the most important technique for reducing the effects of environmental restrictions on legume production has been to select plant genotypes tolerating to abiotic stress (Latrach et al., 2014). In fact, the exploitation of the genetic diversity existing in local germplasm constitutes a promising approach to enhance plant productivity under unfavorable conditions. In this context, our study aims at evaluating the tolerance to low P availability in four Moroccan alfalfa (*Medicago sativa* L.) populations. The emphasis was on agro–physiological and biochemical properties related to the tolerance to this environmental constraint. The role of phosphatase and enzymatic and non-enzymatic antioxidant system were focused.

MATERIALS AND METHODS

Plant material and growth conditions

The biological material used in this study consists of four Moroccan alfalfa (*Medicago sativa* L.) populations; *OUED LMALEH*, *DEMNATE*, *TATA* and *RICH*. Seeds were supplied by The National Institute for Agronomic Research, Morocco. Local populations of alfalfa are commonly used in the Moroccan traditional agroecosystems, oasis and mountain, and powerfully involved in the socio-economic development chain of local families as the nutrition source for their livestock. They have been cultivated for many centuries and are still extensively used by farmers in these traditional agroecosystems. Continuous natural and human selection has led, by this time, to their adaptation to the local habitats with distinction in the agro–morphological traits of the landraces, which have reached Hardy–Weinberg equilibrium (Farissi et al., 2013). The seeds were germinated in 20 cm diameter and 15 cm height plastic pots containing sterilized perlite as a substrate. The experiment was conducted in a growth chamber at 28 ± 2 °C day/night, 60% – 80% relative humidity, and a photoperiod of 16 h. After 15 days of sowing, the young seedlings were irrigated by capillarity with a nutrient solution with two P forms, insoluble P using Ca_3HPO_4s (limited available P) *versus* soluble (KH_2PO_4). Both soluble and insoluble P forms were adjusted to reach 250 µmol P $plant^{-1}$ $week^{-1}$. The composition of the nutrient solution (Hoagland & Arnon, 1950) used was as follows: [KNO_3 (600 µmol), $MgSO_4$ (1,000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1,650 µmol), Fe-ethylenediaminetetraacetic acid (EDTA) (16 µmol), MnSO4 (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)]. By using 0.1 M HCl or 0.1 M NaOH, the pH of the nutrient solution was, respectively, reduced or raised to reach 7 before use. After 60 days of P stress, the plants were collected, measured, and several agro-physiological and biochemical traits were analyzed prevailing plant growth and development. Each pot was planted with five plants and each treatment was represented by three replicates, resulting in a total of 24 pots and 120 plants plants.

Plant biomass and leaf area

The shoot and root fresh weights (FW) were determined immediately after harvest. The dry weight (DW) was then measured using precision balance after their drying at 80 °C for 48 h. The leaf area was estimated using MESURIM software (version 3.4.4.0) using a digital scanner.

Phosphorus contents

The P contents were determined using 0.5 g of the dry matter of each plant parts after incineration at 600 °C for 6 h. The ash obtained was treated in 3 mL of HCl (10 N) and filtered. Then, the P concentration were measured using the molybdate blue colorimetric assay (Murphy & Riley, 1962). After color development at 100 °C for 10 minutes, the optical density was measured at 820 nm. A standard curve was established with KH_2PO_4 solutions.

Acid phosphatase activitiy

Samples of fresh matter (50 mg) were ground in mortar using 500 µL of sodium acetate extraction buffer (0.1 M, pH 5.5), 2.5% polyvinylpyrrolidone (PVP) and 5 µL of β mercaptoethanol. The homogenates were centrifuged at 12,000 × g for 30 min at 4 °C. The acid phosphatase activity was measured using 100 µL of enzymatic extract mixed with 200 µL of p–NPP (*p*–nitrophenyl phosphate) and incubated for 30 min at 37 °C. Then, 1 mL of 1N NaOH was added to stop the reaction. The acid phosphatase activity (µmol *p*–NPP min^{-1} mg^{-1} protein) was measured by a spectrophotometer at 410 nm wavelength (Araújo et al., 2008).

Protein content in all enzyme preparations was determined using bovine serum albumin (BSA) as standard (Bradford, 1976).

Oxidative stress markers and membrane cell intergity assessments

The lipid peroxidation was estimated by malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) accumulation. MDA was measured using 50 mg of fresh leaves added to 2 mL of 0.1% trichloroacetic acid (TCA). After centrifugation at 14.000 rpm for 15 min, 1 mL of supernatant was added to 2.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was placed at 95 °C for 30 min and cooled down by ice. The absorbance was measured at 532 and 600 nm. The MDA content was calculated by the extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ (Savicka & Škute, 2010).

The H_2O_2 content was determined as described by Velikova et al. (2000) 100 mg of fresh leaves were mixed with 5 mL of 0.1% TCA. After centrifuged at 12,000 × g for 15 min, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance was measured at 390 nm. The H_2O_2 content was expressed as µmol $H_2O_2.g^{-1}$ FW.

For the electrolyte leakage (EL), 0.1 g of young leaflets were washed three times with distillated water to eliminate surface–adhered electrolytes then placed in closed flasks filled with 10 mL of distillated water. The flasks were after that incubated for 24 h at 25 °C on a rotary shaker. The initial electrical conductivity (Li) was measured by using a conductivity meter. Then the samples were autoclaved at 120 °C for 20 min. The final electrical conductivity (Lf) was measured after 25 °C equilibration, The percentage of EL was calculated as follow (Lutts et al., 1996):

$$EL\ (\%) = (Li\ /Lf) \times 100$$

Enzymatic antioxidant actvities

100 mg of fresh leaves were crushed in 1 mL of phosphate buffer (20 mM, pH 7). After centrifugation at 15,000 × g for 20 min at 4 °C, the supernatant was used for the determination of the POD (EC 1.11.1.7) enzymatic activity according to (Beyer & Fridovich, 1987). The reaction mixture consisted of 200 µL of H_2O_2 at 0.3%, 300 µL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1 M, pH 6), 1 mL of distilled water and 10 µL of enzymatic extract. The POD activity was measured after 3 min, at 470 nm. The activity was calculated using the guaiacol extinction coefficient 26.6 mM^{-1} cm^{-1}, and expressed as µmol H_2O_2 min^{-1} mg^{-1} proteins.

SOD (EC 1.15.1.1) activity was determined as described (Chagas et al., 2008). 50 µL of crude enzymatic extract in phosphate buffer (20 mM, pH 7) was added to a solution containing 13 mM L–methionine, 75 µM *p*–nitro blue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The reaction was performed in assay tubes upon illumination using a 30 W fluorescent lamp at 25 °C for 15 min. The blue formazan produced by NBT photoreduction was spectrophotometrically measured at 620 nm. An enzyme unit was equal to the amount to inhibit 50% of NBT. SOD activity was expressed as enzymatic U min^{-1} mg^{-1} proteins.

CAT (EC 1.11.1.6) activity was determined using 250 µL of the extract was added to 2 mL of the assay mixture (50 mM Tris–HCl buffer pH 6.8, containing 5 mM H_2O_2) (Gong et al., 2001). Then after 10 min at 20°C, 250 µL of 20% titanic tetrachloride (v/v, in concentrated HCl) were added to stop the reaction. CAT activity was read at 415 nm and calculated by comparing the absorbance against a standard curve of 0.25 to 2.5 mM) H_2O_2. CAT activity was expressed as µmol H_2O_2 min^{-1} mg^{-1} proteins.

Protein content in all enzyme preparations was determined using BSA as standard (Bradford, 1976).

Non-enzymatic antioxidant molecules

Non-enzymatic antioxidant molecules were evaluated through proline and total polypenol contents. The proline content was determined using extract from 100 mg FW with 2 mL of 40% methanol: water. Afer incubation at 85 °C for 30 min, 1 mL extract was mixed with 1 mL of a mixture of glacial acetic acid andorthophosphoric acid at 6M (3: 2; v/v) and 25 mg ninhydrin. After 1 h incubation at 100°C, the tubes were cooled and 5 mL toluene was added. The optical density of the upper phase was measured at 528 nm. The proline content was determined using a standard curve obtained using reference proline solutions (Bates et al., 1973).

For total polyphenol content, 100 mg of fresh samples were grinded in 1 mL of methanol (80%). After centrifugation at 12,000 × g for 20 min at 4 °C, the supernatants were recuperated. The content of total polyphenols was determined through the Folin–Ciocalteu method and their concentration was described as mg gallic acid equivalents (GA) g^{-1} FW (Singleton & Rossi, 1965).

Statistical analysis

Statistical analysis was executed using SPSS version 22. A two-way analysis of variance (ANOVA) was adopted. Means comparison was performed using Tukey's test. XLSTAT software version 2014 (Addinsoft, Paris, France) was used to determine the correlations.

RESULTS

Effect on plant biomass and leaf area

The starvation of P availability in rooting medium caused significant ($P < 0.001$) reductions in plant shoot and root dry weights. The lowest reductions were noted in *OL* populations in comparison with other populations which showed the same behavior according to Tukey's test ($P > 0.05$). In fact, under low P availability, the shoot dry weight recorded in *OL* was 32.80 mg $plant^{-1}$ with a reduction of 28.90% comparatively to *OL* plants grown under P-sufficient conditions. However, the remaining populations, *DEM*, *TATA*, *RICH* showed the reductions of 52.80, 41.09 and 39.96% respectively for the same growth trait (Fig. 1, A). Regarding, the root dry weights (Fig. 1, B), the effect was more pronounced ($P > 0.05$) in *DEM* population with reductions of 41.44% relative to control. *OL* was found to be the least affected one ($P < 0.05$) with the reduction percentages did note exceed 19.39%. However, *TATA* and *RICH* showed an intermediate behavior.

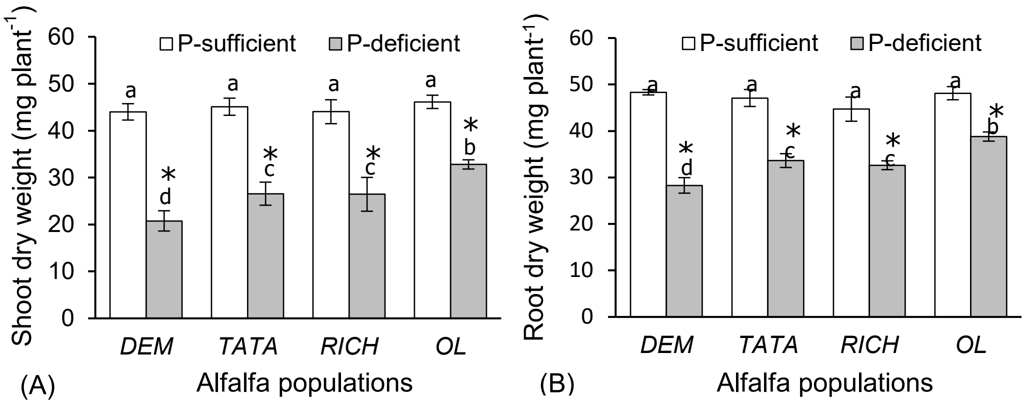

Figure 1. Shoot (A) and root (B) dry weights in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

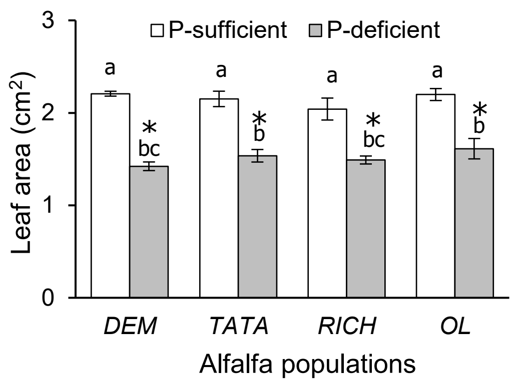

Figure 2. Leaf area of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

For leaf area (Fig. 2), the significant ($P < 0.001$) reductions were caused by the low P availability in rooting medium. OL population maintained the highest leaf area value

(1.61 cm^2), but with no significant differences ($P > 0.05$) in comparison with the remaining populations according to Tukey's statistical test.

Phosphorus contents

The Fig. 3 illustrates the shoot and root P concentration under the stressed and non-stressed conditions. The obtained results mentioned that the highest P contents of 238 and 333 mg g DW^{-1} were noted in shoots and roots of *OL* population respectively under P stress, followed by *TATA* and *RICH*, whereas the lowest P contents of 105 and 179 mg g DW^{-1} were noted in *DEM* population under the same conditions.

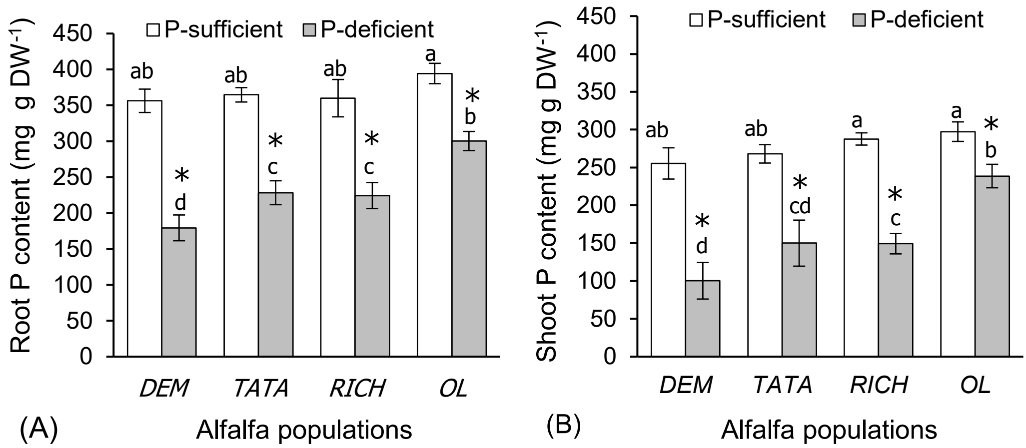

Figure 3. P contents in roots (A) and shoots (B) of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

Phosphatase acid activity

Low P availability induced significant ($P < 0.001$) increase in phosphatase acid activity in alfalfa roots. The highest activity ($P < 0.05$) was noted in *OL* population (19.85 µmol *p*–NPP min^{-1} mg^{-1} proteins). Nevertheless, the activities not exceeding 15.72 µmol *p*–NPP min^{-1} mg^{-1} proteins were recorded in *TATA* and *RICH* populations with non-significant differences ($P > 0.05$) between theme, according to the statistical grouping test considered. However, the lowest ($P < 0.05$) acid phosphatase specific activity was noted in *DEM* population 12.68 µmol *p*–NPP min^{-1} mg^{-1} proteins.

Effect on membrane cell integrity and oxidative stress markers

The EL contents reflecting the cell membrane integrity were found increased ($P < 0.001$) under low P availability (Table 1). The cell membrane damages were most prominent in the *DEM*, *TATA* and *RICH* populations with percentages of 22.98, 21.27 and 23.32% However, the lowest EL contents ($P < 0.05$) were noted in *OL* population 18.07%.

Table 1. EL, MDA and H_2O_2 contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

Alfalfa population	EL (%)		MDA (µmol g FW^{-1})		H_2O_2 (µmol g FW^{-1})	
	P-sufficient	P-deficient	P-sufficient	P-deficient	P-sufficient	P-deficient
DEM	10.22 ± 0.94*a*	22.98 ± 0.97*a*	13.32 ± 0.56*a*	48.72 ± 2.74*a*	0.45 ± 0.035*a*	1.19 ± 0.072*a*
TATA	09.19 ± 1.13*a*	21.27 ± 1.67*a*	15.04 ± 1.53*a*	47.45 ± 1.75*a*	0.29 ± 0.042bc	0.99 ± 0.051*b*
RICH	08.47 ± 1.21*a*	23.32 ± 1.73*a*	16.21 ± 0.82*a*	41.28 ± 3.11*b*	0.43 ± 0.028*a*	1.03 ± 0.061*b*
OL	11.33 ± 1.73*a*	18.07 ± 0.69*b*	17.93 ± 1.23*a*	39.11 ± 0.73*bc*	0.33 ± 0.063 bc	0.88 ± 0.073*c*

Table 2. Superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) specific enzymatic activities in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test

Alfalfa population	SOD U min^{-1} mg^{-1} proteins		POD µmol H_2O_2 min^{-1} mg^{-1} proteins		CAT µmol H_2O_2 min^{-1} mg^{-1} proteins	
	P-sufficient	P-deficient	P-sufficient	P-deficient	P-sufficient	P-deficient
DEM	21.08 ± 1.17*a*	32.11 ± 1.17*c*	9.32 ± 1.16*a*	20.63 ± 0.74*d*	14.25 ± 1.03*a*	32.19 ± 2.72*c*
TATA	18.13 ± 1.55*ab*	42.23 ± 1.55*b*	10.04 ± 1.33*a*	22.55 ± 1.75bc	15.08 ± 1.42a	36.99 ± 2.05*b*
RICH	23.32 ± 1.98*a*	39.09 ± 1.98*b*	12.21 ± 1.92*a*	23.21 ± 2.11*b*	14.43 ± 1.28*a*	37.03 ± 3.61*b*
OL	19.07 ± 1.62*ab*	48.16 ± 1.62*a*	8.93 ± 1.43*a*	26.19 ± 0.73*a*	13.33 ± 1.63*ab*	44.88 ± 1.73*a*

The oxidative markers, MDA and H_2O_2, were found significantly ($P < 0.001$) accumulated under low P availability in alfalfa stressed plants (Table 1). The significant accumulations were observed in *DEM* pollution followed by *TATA* and *RICH* populations. The contents ranged from 41.28 to 48.72 g FW^{-1} for MDA and from 0.99 to 1.19 g FW^{-1} for H_2O_2. Nevertheless, the *OL* population was significantly ($P > 0.05$) revealed to be the least affected one according to Tukey's test with the values of 39.11 µmol g FW^{-1} and 0.88 µmol g FW^{-1} for MDA and H_2O_2 respectively.

Low P availability induced significant ($P < 0.001$) increase of the enzymatic antioxidant activities in P–stressed alfalfa plants (Table 2). The highest activities were noted in OL population. The values recorded were 48.16 U min^{-1} mg^{-1} proteins, 26.19 µmol H_2O_2 min^{-1} mg^{-1} proteins and 44.88 µmol H_2O_2 min^{-1} mg^{-1} proteins respectively for SOD, POD and CAT. However, the lowest values were observed in DEM population (32.11 U $min^{-1\ mg-1}$ proteins, 20.63 µmol H_2O_2 min^{-1} mg^{-1} proteins and 32.19 µmol H_2O_2 min^{-1} mg^{-1} proteins for the mentioned enzymes respectively). Whereas, intermediate values were noted for the remaining alfalfa populations.

Effect on non-enzyamtic antioxidant molécules:

Starvation of P availability in rooting medium significantly ($P < 0.01$) increased the non-enzymatic antioxidant molecules in alfalfa plants (Table 3). The lowest proline and total polyphenol contents were noted in *DEM* population, 314 µg g^{-1} FW and 57.63 mg GA g^{-1} FW respectively. However, OL population accumulated the highest contents 534 µg g^{-1} FW and 77.19 mg GA g^{-1} FW in comparison with the other alfalfa populations ($P > 0.05$).

Table 3. Proline and total polyphenol contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

Alfalfa population	Proline, µg g^{-1} FW		Total polyphenols, mg GA g^{-1} FW	
	P-sufficient	P-deficient	P-sufficient	P-deficient
DEM	232 ± 23*b*	314 ± 23*c*	32.24 ± 1.16*b*	57.63 ± 2.54*d*
TATA	245 ± 21*b*	413 ± 15*b*	38.17 ± 1.33*ab*	61.55 ± 2.25*c*
RICH	276 ± 34*ab*	402 ± 21*b*	36.09 ± 1.92*ab*	65.21 ± 3.12*b*
OL	272 ± 64*ab*	534 ± 19*a*	32.11 ± 1.43*b*	77.19 ± 2.18*a*

DISCUSSION

Phosphorus (P) is a very important nutrient required for optimum crop production (Ibrahim et al., 2021). It involved in many physiological and biochemical process governing plant growth and development. The high reactivity of P with some cations such as iron, aluminum, and calcium, to form insoluble compounds, reduces its mobility in the soil solution. These reactions caused a very low–P availability and low efficiency of phosphate fertilizers used by plants (Farssi et al., 2021). As a consequence, the limitation of the growth plant and development. In fact, we report in the present study that the low-P availability in rooting medium significantly reduced the plant biomass and leaf area (Fig. 1 and Fig. 2). The comportment of the alfalfa populations in this study was significantly different. The OL population was found the least affected and the DEM population was the most sensitive one, whereas the two remaining alfalfa populations were found to be moderately affected. The effect of low–P availability was documented in many species. Indeed, the reduction in plant tillering and biomass barley (*Hordeum vulgare* 'Quench') was observed under P deficiency conditions (Carstensen et al., 2018). Same results were noted in leguminous species such as *Vicia faba* L. (Bargaz et al., 2012). The reduction in plant growth under low P availability was associated with the plant P contents. In fact, the lowest P contents were noted in the most sensitive population (Fig. 2). In fact, we observed significant and positive correlation between shoot biomass and their P contents ($r = 0.94$) and between root biomass their P contents ($r = 0.96$). Also, positive correlations were noted for leaf area and the contents of shoots and roots in P, $r = 0.87$ and $r = 0.93$, respectively.

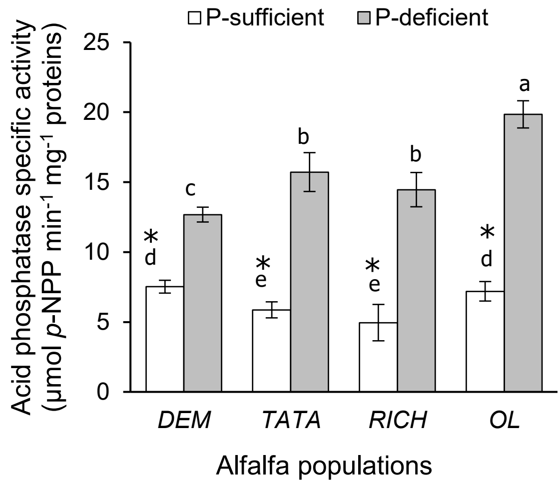

Figure 4. Acide phosphatase specific activity in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

In response to P deficiency, plants have several morphological, physiological, biochemical, and molecular adjustments to improve their P uptake (Plaxton & Tran, 2011; Farssi et al., 2021). In our study, the low P availability induced acid phosphatase activity in alfalfa stressed plants (Fig. 4). The activity was more pronounced in the least affected alfalfa population. Significant and negative correlations were noted for the alfalfa plant biomass and phosphatase activity ($r = -0.66$). The induction of phosphatase activity under low–P availability was documented in many species including *Medicoga sativa* L. In fact, under low soil P supply alfalfa roots released more phosphatases and carboxylates, principally tartrate, into the rhizosphere (He et al., 2020). Similarly, acid phosphatase activity of cell wall in leaves and roots of low-P tolerant stylo (Stylosanthes) mutant, TPRC2001-84, were 46.6% and53.6% higher than in non-mutant control (RY2) under P deficiency (Liu et al., 2018). The increase of activity in the mutant may contribute to increasing P use efficacy under P stress by cell wall P scavenging and recycling (Liu et al., 2018). An increase in this activity was reported in *Vicia faba* L. under low–P availability (Makoudi et al., 2018).

P is an essential in ATP, NADPH, nucleic acids, sugar phosphates, and phospholipids. These compounds have possessive role in cell membrane composition and integrity. Our findings indicate that low–P availability affects the membrane cell integrity and induced oxidative stress evaluated by the accumulation of EL, MDA and H_2O_2 (Table 1). The lowest contents ($P < 0.05$) were noted in the most tolerant population (OL), whereas the highest contents were noted in the most sensitive population (DEM). The generation of reactive oxygen species (ROS) is considered because of plant exposure to many stresses including P deficit (Mouradi et al., 2018a; 2018b). In the lack of effective protective mechanism, ROS can seriously damage plants by lipid peroxidation, protein degradation, breakage of DNA and programmed cell death (Bargaz et al., 2013a). To overcome with the increased ROS level, plants developed enzymatic and non-enzymatic antioxidant process leading to cell detoxification from ROS. The antioxidant systems involve SOD, POD, and CAT. In our study, significant increases in the activities of these enzymes were noted (Table 2). The tolerance of alfalfa populations tested to low–P availability is positively correlated with the induction of antioxidant enzymatic activity. Similar results were reported in *Phaseolus vulgaris* L. (Bargaz et al., 2013a) and in *Brassica napus* L. (Chen et al., 2015). SOD changes superoxide (O_2^-) into H_2O_2, POD and converts H_2O_2 to H_2O and O_2 using electron donors (Kapoor et al., 2019). In the same sense, our findings demonstrated that the P deficiency increase the non-enzymatic antioxidant molecules. The proline and total polyphenol contents were more accumulated in the least sensitive population (OL). Proline actions in plants may resume in chelation of metals, antioxidant ability and/or a signaling roles (Hayat et al., 2012). The proline accumulation can activate stress–responsive genes coding for other antioxidant compounds (El Moukhtari et al., 2021). Silva et al. (2018) reported that proline induces also the production of phenolic compounds in transgenic tobacco subjected to water deficit. This finding agrees with our results. In fact, the accumulation of the proline in alfalfa stressed plants was positively correlated with the total polyphenol contents. Previous findings on polyphenol accumulation were noted in other legume species such as common bean (Bargaz et al., 2013a). This increase suggests a possible role that phenols could play in during P–deficiency. Indeed, the accumulation of phenolic compounds has important antioxidant properties in protecting membranes by neutralizing lipid radicals (Takahama & Oniki, 2000). The statement we noted agrees with previous findings on proline and polyphenols that are triggered in alfalfa in response to abiotic stress such as salinity (El Moukhtari et al., 2020; 2021)

CONCLUSIONS

The present study suggests significant variability in Moroccan alfalfa populations against low–P availability. The OL population was found to be the least affected population and the DEM was the most sensitive one. However, TATA and RICH displayed a moderate tolerance to P–deficiency conditions. The alfalfa P stress tolerance was linked to the induction of acid phosphatase activity, the enhancement of P solubilization and uptake, the maintain of cell membrane integrity and the induction of non-enzymatic and enzymatic antioxidant responses against the accumulation of ROS.

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Rice growth and yield characteristics under elevated carbon dioxide and nitrogen management

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Abstract. The atmospheric carbon dioxide (CO_2) concentration is increasing and the on crop production needs to be investigated. A pot experiment was conducted in open top chambers (OTC) to determine the response of rice to elevated CO_2 (eCO_2) under varying time of nitrogen (N) application. The results revealed that photosynthesis, root and shoot dry matter production, yield components and nutrient absorption were favored at eCO_2 when N applied up to flowering stage (FT) of rice. However, the N application up to FT of rice also significantly improved percent filled grain, reduce spikelet sterility and rice yield increased by 18 to 20% under eCO_2. Rice plant absorbed higher amount of Zn, Ca, Mg, and Fe at eCO_2 when N was applied up to FT. Amylose was higher but protein percentage was lower at eCO_2. These results indicate that to maximize rice yield under eCO_2, it is important to supply N up to FT of rice in order to increase grain fertility and reduce spikelet sterility.

Key words: crop yield, gas exchange, grain fertility, plant nutrition, rice, spikelets sterility.

INTRODUCTION

Currently, the atmospheric CO_2 concentration is increasing much faster than previous (Bereiter et al., 2015; IPCC, 2019) and it would have a significant impact food production of the world through affecting plant growth, development, grain yield and quality (Raj et al., 2019). The C_3 crops respond more strongly to the eCO_2 than C_4 crop (Jablonski et al., 2002). The photosynthesis (Pn) of crops increased significantly under eCO_2 resulting increase in biomass and yield of C_3 plants (Chunwu et al., 2016). On the other hand, stomatal conductance (Gs) and transpiration rate (Tr) of plants reduced under eCO_2 condition (Chunwu et al., 2016). Rice (*Oryza sativa* L.) is one of the most important crops in Asia (FAOSTAT, 2017). As a C_3 plant, growth and yield of rice are markedly enhanced by eCO_2 (Pachauri et al., 2014). Previous studies have shown that leaf area, shoot dry matter (DM), panicle and grain number per unit area and grain yield of rice were enhanced due to eCO_2 (Yang et al. 2006; Sasaki et al. 2007; Satapathy et al., 2015). Increased growth of the crops under eCO_2 condition will require higher nutrient uptake and assimilation. Because, rice production is greatly influenced by nutrient management (Roy et al., 2015); and the nutrient absorption of a crop is determined by the time of nutrient application. The eCO_2 has a dilution effect on nutrient uptake by the crop and nutrient levels in plants is decreased (Li et al., 2013). On the other hand, plants removed greater amount of nutrients like P, K, Mg, Fe, B and Mo from soil and translocated to the grain under eCO_2. Rice crops exhausted soil minerals to great extent leading to very low available nutrient content in soil after harvesting of the crops (Wang et al., 2011). Therefore, the demand for nutrients by crops might also get changed in future under eCO_2 condition.

As mineral fertilizer, nitrogen (N) is the main nutrient associated with yield (Jing et al., 2016). For synchronizing plants growth and reducing loss, N is recommended to apply in splits starting from transplanting to flowering. The application of N at early growth stage will helps to produce sufficient shoot biomass, but application at panicle initiation (PI) stage increase rice grain yield (Mamun et al., 2016). Therefore, the time of application be adjusted to achieve high yield under eCO_2 condition and high proportion of N should be applied at the late growth stage of rice, especially after PI stage (Yang et al., 2007; Wang et al., 2011). Early studies also suggested that recommended N might not be enough and higher amount should be applied for getting high yield under eCO_2 condition (Razzaque et al., 2009). However, Pilipavicius et al. (2006) showed a negative effect of higher CO_2 concentration on the early growth of *Chenopodium album*. In this situation, this study was conducted to determine the effect of eCO_2 on photosynthetic traits, grain production and nutrient absorption in rice. Besides, this study will help to identify a suitable time of N application for higher grain production of rice under the world's climate change scenario especially, under the changing CO2 concentration.

MATERIALS AND METHODS

Experimental site and planting materials

A pot experiment was conducted inside OTC at Bangabandhu Sheikh Mujibur Rahman Agricultural University (24.09° N latitude and 90.26° E latitude), Bangladesh in boro. The OTCs (area 9 m^2) were constructed with iron frame that installed on the ground according to Uprety (1998). For growing rice plants, 96 pots (size: 24 cmin diameter and 27 cmin height) were filled up with wet soil. Where amount of soil per plot was almost 13 kg other than water and blank pot weight was 0.5 kg. After potting, triple super phosphate, muriate of potash, gypsum, $ZnSO_4$ were added @ 0.65, 0.72, 0.52, 0.065 g pot^1, respectively. Physic-chemical properties of initial soil was determined during pot preparation. Initial soil chemical parameters were pH 5.73, soil organic matter 1.70%, total N 0.082%, available P 10.22 µg g^{-1}, available K 0.41 meq per 100 g soil. Forty-day old seedlings of BRRI dhan28 were transplanted on 20 January 2018. Transplanting was done by hand with two seedlings $hill^{-1}$ pot^{-1}. All the fertilizers except N were applied before transplanting of rice seedlings.

Experimental design and setup

A randomized complete block design with eight replications was used for the experimentation. The treatments comprised of two factors. Factor A consisted of four growing conditions- i. OTC with 500 ppm (elevated CO_2) (eCO_2), ii. OTC with 450 ppm CO_2 (intermediate CO_2 concentration, iCO_2), iii. OTC with ambient CO_2 (400 ppm CO_2) (aCO_2) and iv. Open field (OF, 380 ppm CO_2). Factor B was the timing of N application, viz. i. N1 = 1/3rd N at early tillering (ET) + 1/3rd at active tillering (AT) + 1/3rd before PI, ii. N2 = 1/3rd N at ET + 1/3rd before PI + 1/3rd at booting stage, and iii. N3 = 1/3rd N at ET + 1/3rd before PI + 1/3rd at FT. The rate of N was 2.0 g plot^{-1} (320 kg ha^{-1}). The CO_2 gas was supplied to the OTC chambers from CO_2 gas cylinder using a blower from 7 days after transplanting to physiological maturity of rice. A portable Pn system (model: LICOR 6200, Lincoln, Nebraska) was used to determine the CO_2 concentration inside the OTC regularly. In eCO_2 treatment, the CO_2 concentration fluctuated from 490 to 510 ppm while it was 440 to 460 ppm in case of iCO_2. The present atmospheric CO_2 concentration in crop field is 380 ppm and it is increasing @ 1.5 ppm year^{-1}. Therefore, the CO_2 concentration was considered as 450 and 500 ppm, so that the we may predict the effect of eCO_2 on crop in near future. Month wise average temperatures were taken. The pots were irrigated after transplanting of rice seedlings. A floodwater depth of 2–3 cm was maintained in each pot until a week before maturity of the crop. Intercultural operations were done uniformly in each pot to ensure normal growth of the crop. Weeding was done in the experimental pot as and when necessary. Regular irrigation was also done to maintain a saturated condition in pot.

Experimental measurement

The Pn, Tr and Gs were determined during full flowering. A portable Pn system (model: LICOR 6200, Lincoln, Nebraska) was used to record Pn, Tr and Gs. Plant height, number of effective tillers hill^{-1}, filled and unfilled grains panicle^{-1}, grain size, grain yield, shoot and root biomass were taken during harvesting. Plant height was taken from base of the plant to the top of the plant by using a meter scale. Shoot and root weight was recorded at maturity stage. Samples were collected and oven dried at 70 °C for 72 hours and dry weight of each hill was recorded separately. In each case, the mean of eight hills was calculated. The total grain of each hill was weight and recorded as grain yield hill^{-1}. The grain yield was adjusted to 14% moisture content using the following formula:

$$\text{Adjusted weight} = \frac{W \times (100 - M_1)}{(100 - M_2)} \times 100$$

where, W, M_1 and M_2 were fresh weight, fresh and adjusted moisture percent of the grain, respectively.

Grain amylose and protein content as were measured after harvesting of the crop and the concentration of grain N, Zn, Ca, Mg and Fe were determined. The samples were dried at 70 °C for 72 hrs and ground. The ground sample was digested in concentrated H_2PO_4 and total N concentration was determined by Kjeldahl method (Kjeldahl, 1883). The concentration of other nutrients, samples were taken and digested using mixture of HNO_3: $HClO_4$ (Bhargava & Raghupathi, 1993). The amylose content as determined by following the procedure as described by Cruz & Khush (2000). Protein content in rice samples were determined by macro Kjeldahl procedures.

Statistical analysis

Data gathered on different parameters were statistically analyzed using computer software package CropStat, version 7.2 (IRRI, 2007). Analysis of variance of the data was calculated and the significance of the factor (growing condition and timing of N application) was tested at the 5% level of probability. Treatment means were separated with Duncan's Multiple Range Test at 5% level of probability (Gomez & Gomez, 1984).

RESULTS AND DISCUSSION

Photosynthetic parameters

The combined effect of CO_2 and N application on Pn rate, Tr and Gs was statistically significant (Fig. 1). The Pn increased gradually from OF to eCO_2 in all N treatments. A significant reduction in Tr and Gs were observed in OTC as compared to OF in all N treatments. These two parameters were decreased gradually with the increasing of CO_2 concentration. The highest Tr was measured from OF with N3 treatment. On the contrary, the lowest Tr was recorded from eCO_2 with N1. The eCO_2 improved leaf Pn during flowering, while Tr and Gs declined in all N application treatments (Fig. 1). Nitrogen is one of the most important constituents of chlorophyll (Chl). Application of N at FT increased the concentration of N in leaf. The eCO_2 concentration increased leaf Chl also reported by De Costa et al. (2003) and Wang et al. (2014). Exogenous supply of CO_2 augmented its concentration inside rice leaf. As it is the substrate of Pn, it was expected that increasing CO_2 level in cellular level would increase Pn rate. Higher Pn at and iCO_2 and eCO_2 implies that rice plants had adopted a beneficial acclimation strategy for growth in CO_2 enhancement. Higher Pn rate of crops grown under eCO_2 have been well documented in earlier studies (Chen et al., 2014). Both Tr and Gs of rice plants at eCO_2 conditions were significantly lower than OF condition at FT of rice. At high CO_2 level, the Tr is reduced because of a direct effect of Gs. The lower Tr and Gs of rice under eCO_2 conditions could be beneficial for the growth of rice (Chunwu et al., 2016). The decreased Gs under eCO_2 was also reported by Wang et al. (2020) and Cai et al. (2018). Reduction in Gs might increase resistance to CO_2 diffusion into the leaf, thus partially offsetting the maximum stimulation of carboxylation rate.

Plant growth parameters

Plant height, shoot and root DM were significantly influenced by the interaction of CO_2 and N (Fig. 1). The tallest plant was measured in eCO_2 condition in all N application treatments. Significantly the shortest plant height was recorded from OF with all N treatments. Significantly the highest shoot DM production was recorded from eCO_2 with N1 (42.22 g plant^{-1}). The shoot DM obtained from N2 and N3 under eCO_2 were statistically similar. Though, shoot DM production under aCO_2 was numerically higher than OF, but they were not statistically different in all N treatments. The root DM of rice plant also increased gradually from OF to eCO_2 as well as N3 to N1 (Fig. 1). The highest root mass was recorded under eCO_2 with N1 (15.02 g plant^{-1}) which was followed by N2 (12.87 g plant^{-1}) and N3 (12.20 g plant^{-1}) under same environment. The lowest root mass was obtained from OF in all N treatments. The eCO_2 enhanced plant height, shoot and root DM of rice (Fig. 1). The result is in agreement with the findings of Haque et al. (2005). The eCO_2 increased the Pn rate of rice (Fig. 1) and the high Pn rate might have contributed to production of taller plants under such condition. The shoot DM of the crop grown under eCO_2 increased as compared to that under OF (Satapathy et al., 2015).

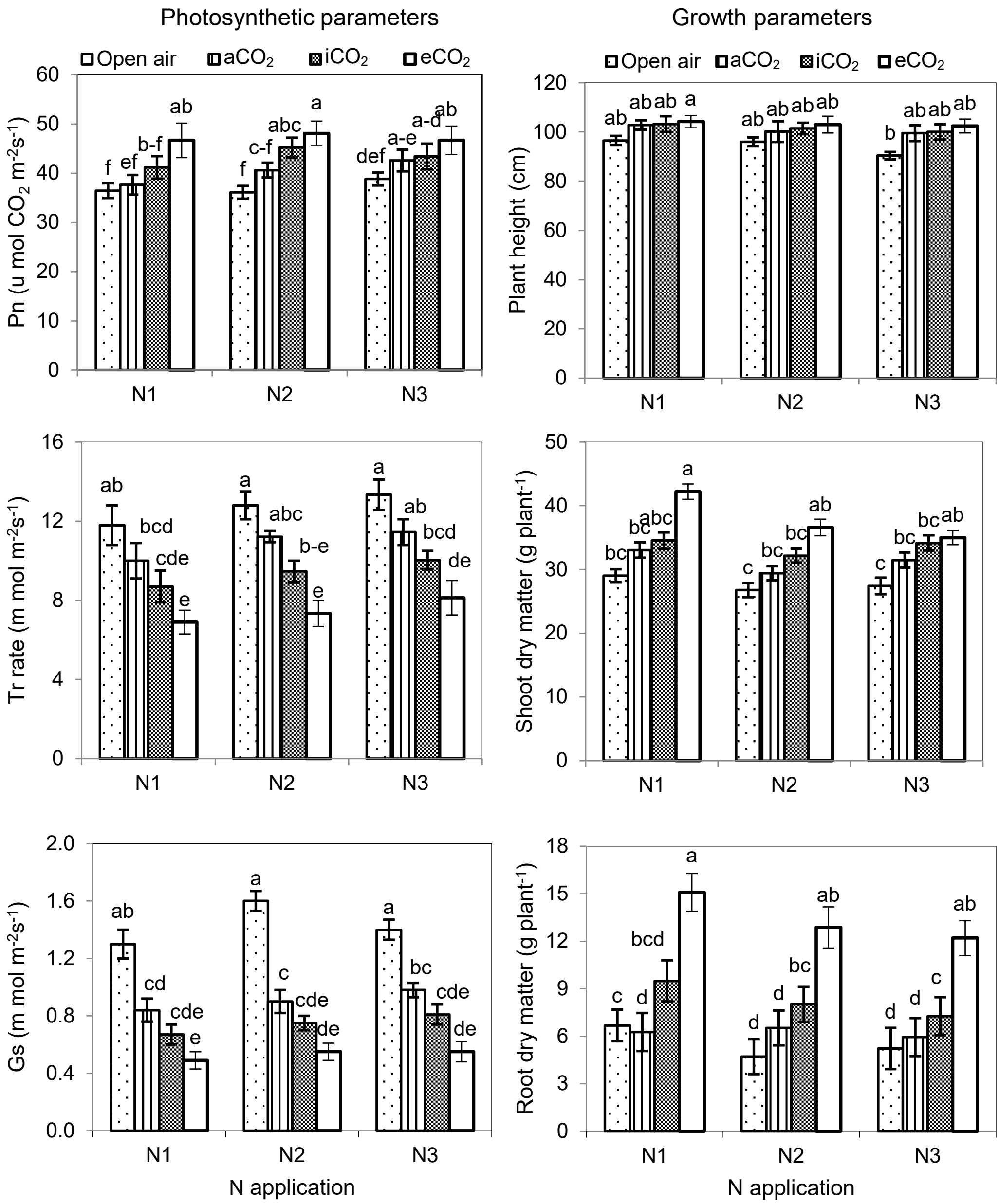

Figure 1. Photosynthetic and growth parameters of rice as affected by N fertilization under eCO_2 at FT. Bar graphs indicate mean value ± standard error and bars with similar letters did not vary significantly at 5% level of probability; eCO_2, iCO_2 and aCO_2 indicate 500, 450 and 400 ppm CO_2, respectively; N1 = 1/3rd of N at ET + 1/3rd at AT + 1/3rd before PI; N2 = 1/3rd of N at ET + 1/3rd before PI + 1/3rd at booting; and N3 = 1/3rd of N at ET + 1/3 N before PI + 1/3rd at flowering.

In this experiment, taller plants were measured under eCO_2 condition, thus the assimilated DM in plants under this condition enhanced the height of the plants. The eCO_2 induced increase in biomass was also observed in mungbean (Chowdhury et al., 2005) and rice (Razzaque et al., 2009). On the other hand, Pilipavicius et al. (2006) showed that eCO_2 (700 ppm) had detrimental effect on the early growth of *Chenopodium album*. However, they also showed that the increase of air temperature and CO_2 concentration had damaging tendency for above and below ground fresh and dry weight of *Chenopodium album*.

Yield characteristics

Production of panicle hill^{-1} varied due to interaction of CO_2 concentration and timing of N application (Fig. 2). The highest panicles production was recorded at OF with N2 which was statistically identical with the panicle production at eCO_2 with N1 as well as at eCO_2 with N3. The sterility of rice spikelets varied statistically due to combined effect of CO_2 and N application. Rice grown in N1 treatment produced higher percentage of unfilled grains in all growing conditions and lowest in N3 treatment. In case of N3 treatment, production of unfilled grains were 20, 19, 14, and 20 under OF, aCO_2, iCO_2 and eCO_2, respectively. The highest number of filled grains were obtained from N2 (143) under eCO_2 condition which was statistically identical with N1 under iCO_2 and N3 under aCO_2 conditions (142). The production of total number of grains hill^{-1} was the highest under eCO_2 with N1 (Table 1). However, the percent filled grain was the highest in iCO_2 with N3, where the spikelet sterility was the lowest. The grain size did not affect significantly due to the interaction of eCO_2 and N application timing. Weight of 1,000-grains were numerically higher under iCO_2 condition with N3 followed by treatment N1 for aCO_2. A lower weight of 1,000-grain was recorded in the conditions of aCO_2 and eCO_2 with N2 treatment. The grain yield of rice varied significantly due to interaction of time of N application under eCO_2. The highest rice yield was recorded under eCO_2 (66.47 g) with N1 which was statistically identical with N3 treatment under same environmental condition.

Table 1. Effect of eCO_2 and N fertilization on grain production of rice

Growing conditions	N application	Total spikelets (no. hill^{-1})	Filled grains (%)	1,000-grain weight (
Open field	N1	3,490^{def}	74^{e}	20.31
	N2	3,991^{abc}	75^{e}	20.67
	N3	3,173^{fg}	85^{abc}	20.80
aCO_2	N1	3,453^{def}	80^{cde}	20.88
	N2	3,570^{c-f}	83^{bcd}	19.31
	N3	3,375^{ef}	88^{ab}	19.45
iCO_2	N1	3,633^{b-f}	80^{cde}	20.41
	N2	3,368^{ef}	86^{abc}	20.61
	N3	2,832^{g}	90^{a}	21.56
eCO_2	N1	4,205^{a}	77de	20.51
	N2	3,900^{a-d}	80^{cde}	19.31
	N3	3,726^{a-e}	87^{ab}	20.40
CV (%)		11.8	6.4	3.2

Figures with similar letters did not vary significantly at 5% level of probability; eCO_2, iCO_2 and aCO_2 indicate 500, 450 and 400 ppm CO_2, respectively; N1 = 1/3rd of N at ET + 1/3rd at AT + 1/3rd before PI; N2 = 1/3rd of N at ET + 1/3rd before PI + 1/3rd at booting; and N3 = 1/3rd of N at ET + 1/3 N before PI + 1/3rd at flowering.

In N3 treatment, rice grown under eCO_2 produced 65.85 g grain plant^{-1}, which was 18, 14 and 20% higher than that of OF, aCO_2 and iCO_2 conditions, respectively. Similarly, in both N1 and N2 treatments, significantly higher grain yield also recoded from eCO_2 as compared to other environmental conditions. The highest grain under

eCO_2 with N3 treatment due to production maximum number of productive panicles and a greater number of grains panicle^{-1}. The lowest grain yield was obtained from iCO_2 with N3 (54.82 g). Rice yield is the product of number of panicles hill^{-1}, number of grains panicle^{-1} and weight of individual grain. In this experiment, the production of bearing tillers increased at higher level of CO_2. Higher shoot DM was recorded from eCO_2; thus, it was expected to increase number of panicles from this treatment. In general, growth and yield of rice were expected to increase with CO_2 elevation. Previous experiments have demonstrated that rice produced higher number of tillers and panicle at eCO_2 condition (Yang et al., 2006). The main reason for the increases in yield with rising CO_2 were due mainly to the increased panicle number per unit area, as was also observed for rice grown in CO_2 enriched enclosures (Moya et al., 1998). Our results indicated that greater panicle number hill^{-1} was clearly due to the increases in maximum shoot DM (tiller number) with eCO_2 (Fig. 2). Mamun et al. (2017) also found that the panicles production rice was also increased due to late application of N fertilizer. However, application of N before panicle application increased grain number panicle^{-1} as reported by Abedin et al. (2015).

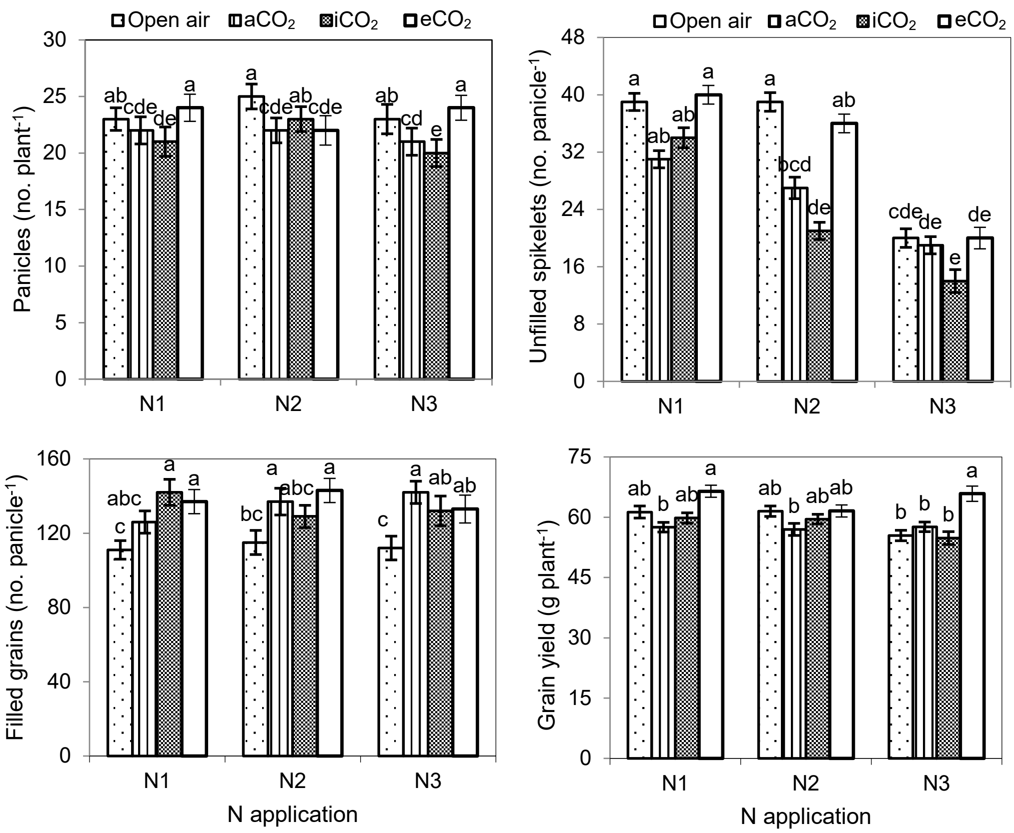

Figure 2. Panicles and grain production of rice as affected by N fertilization under eCO_2.

Bar graphs indicate mean value ± standard error and bars with similar letters did not vary significantly at 5% level of probability; eCO_2, iCO_2 and aCO_2 indicate 500, 450 and 400 ppm CO_2, respectively; N1 = 1/3rd of N at ET + 1/3rd at AT + 1/3rd before PI; N2 = 1/3rd of N at ET + 1/3rd before PI + 1/3rd at booting; and N3 = 1/3rd of N at ET + 1/3 N before PI + 1/3rd at flowering.

Nutrient accumulation and grain quality of rice

Rice plants accumulated higher amount of N in grain at eCO_2 condition as compared to aCO_2, iCO_2 and OF (Table 2). Similarly, N3 facilitated in absorption of more N in grain followed by N2. For Zinc, rice plant uptake more Zn at eCO_2 as compared to iCO_2 and aCO_2. Similarly, N3 facilitated in absorption of more Zn in grain followed by N2. In case of Calcium, rice plant uptake more Ca at eCO_2 and N3 facilitated in absorption of more Ca in grain followed by N2. In case of Magnesium, rice plant uptake more Mg at eCO_2 and N3 facilitated in absorption of more Mg in grain followed by N2. In case of iron (Fe), rice plant uptake more Fe at eCO_2 and N3 facilitated in absorption of more Fe in grain followed by N1 (Table 2).

Table 2. Effect of eCO_2 and nitrogen application on nutrient accumulation in rice grain

Growing conditions	Nitrogen application	Nitrogen (%)	Zinc (ppm)	Calcium (%)	Magnesium (%)	Iron (ppm)
Open field	N1	1.08^{h}	12.15	0.09	0.28	71.66^{f}
	N2	1.35^{ef}	13.10	0.11	0.30	78.87^{f}
	N3	1.50^{cd}	13.48	0.10	0.29	74.96^{f}
aCO_2	N1	1.29^{fg}	13.16	0.09	0.29	111.40^{cde}
	N2	1.39^{e}	14.18	0.10	0.31	100.43^{e}
	N3	1.43^{d}	14.52	0.11	0.30	104.94^{de}
iCO_2	N1	1.25^{g}	14.17	0.11	0.30	120.24^{cd}
	N2	1.47^{cd}	15.32	0.11	0.31	124.28^{bc}
	N3	1.54^{bc}	15.56	0.12	0.32	119.93^{cd}
eCO_2	N1	1.50^{cd}	16.33	0.11	0.33	142.22^{a}
	N2	1.60^{ab}	16.65	0.12	0.34	139.23^{ab}
	N3	1.72^{a}	17.63	0.13	0.36	149.92^{a}
CV (%)		8.6	9.8	8.7	6.9	12.3

Figures with similar letters in a column did not vary significantly at 5% level of probability; eCO_2, iCO_2 and aCO_2 indicate 500, 450 and 400 ppm CO_2, respectively; N1 = 1/3rd of N at ET + 1/3rd at AT + 1/3rd before PI; N2 = 1/3rd of N at ET + 1/3rd before PI + 1/3rd at booting; and N3 = 1/3rd of N at ET + 1/3 N before PI + 1/3rd at flowering.

Rice grain quality was influenced by the interaction of various CO_2 concentrations and time of N application (Fig. 3). Amylose percentage was found higher at eCO_2 concentration i.e., iCO_2 with N3 followed by eCO_2. The aCO_2 with N2 showed the lowest percentage of amylose. Protein percentage was found lowest at eCO_2 with N1 and the highest amount of protein percentage was found in OF condition with N3. The pants grown under iCO_2 condition accumulated the highest amount of N in grain, while the lowest at eCO_2 in all N treatments. Under eCO_2 condition, enhanced carbohydrate accumulation and accelerated growth would dilute the nutrient concentration in plants (Chunwu et al., 2016). Similarly, lower Tr and Gs may limit the Tr-mass flow of nutrients, lowering the nutrient accumulation in shoot (McGrath & Lobell, 2013; Houshmandfar et al., 2015). The N3 facilitated in absorption of more N, Zn, Ca, Mg and Fe in grain. Decreased N concentration under eCO_2 by 9.8% and 14.62% in leaf and 7.38% in roots as compared to ambient CO_2 and field condition (Razzaque et al., 2009). Similarly lower N concentration under eCO_2 has been reported in wheat and soybean (Pal et al., 2003). Under late application of N, higher grain N content was also found by Mamun et al. (2020). Amylose content slightly increased under both iCO_2 and eCO_2

condition, though protein percent was reduced. However, protein content at iCO_2 slightly increased with N2 treatment. Moreover, the percentage of the both characters decreased under aCO_2 condition. Amylose content determines the cooking and eating quality of rice. Khanam et al. (2004) reported that eCO_2 adversely affect the amylose content of rice. Although eCO_2 causes a decrease in grain protein concentrations of rice, grain protein yield remains stable or even increases in future high CO_2 environment because of the significant increase in grain yield due to CO_2 enrichment with N2 treatment (Yang et al., 2007). Under control condition, the grain yield of rice increased when N applied at PI stage of rice (Mamun et al., 2018).

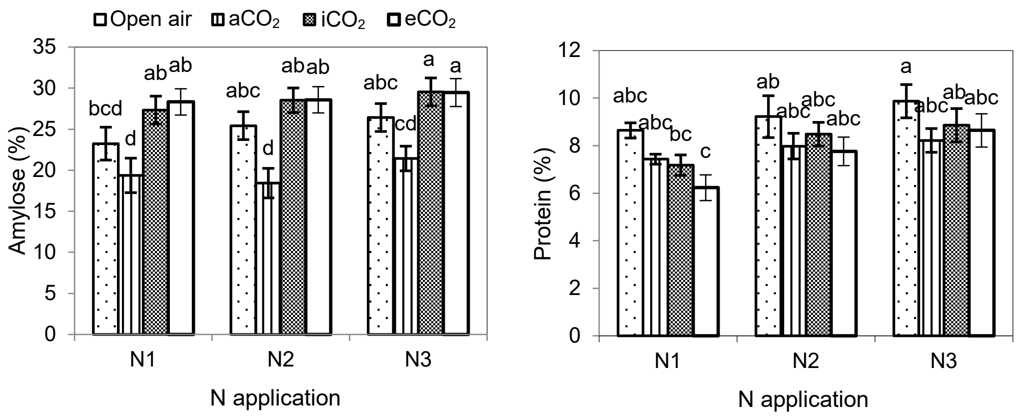

Figure 3. Grain quality properties of rice as affected by N fertilization under eCO_2.

Bar graphs indicate mean value ± standard error and bars with similar letters did not vary significantly at 5% level of probability; eCO_2, iCO_2 and aCO_2 indicate 500, 450 and 400 ppm CO_2, respectively; N1 = 1/3rd of N at ET + 1/3rd at AT + 1/3rd before PI; N2 = 1/3rd of N at ET + 1/3rd before PI + 1/3rd at booting; and N3 = 1/3rd of N at ET + 1/3 N before PI + 1/3rd at flowering.

CONCLUSIONS

The results of this study revealed that the eCO_2 (450 to 500 ppm) with N application up to flowering stage favored Pn, shoot and root biomass production. Rice produced higher number of panicle and grains, gave better grain size and accumulated greater amount of nutrient in plants under eCO_2 with application of N up to flowering stage, resulting higher grain yield. Therefore, under future elevated eCO_2 conditions, rescheduling of N for rice is need. In addition, the amount of N supplied after initiation stage should be sufficient to maintain the extra dry matter produced under the eCO_2 conditions.

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Qualitative traits of various Egyptian clover varieties under efficiency of bio-chemical phosphorus fertilization and lithovit regimes

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Abstract. This investigation was performed at the Agriculture Research Station, Faculty of Agric. Zagazig Univ., Sharkia Governorate, Egypt, throughout the winter seasons of 2019/2020 and 2020/2021. The study aimed to evaluate the influence of ten bio-chemical fertilization and lithovit regimes on forage quality of 1st and 3rd cuts of various Egyptian clover varieties (Berseem, *Trifolium alexandrinum* L.) q.e. Helaly, Gemmeza 1, Sakha 4, Serw 1, Giza 6 and local variety. The ten fertilization regimes were F_1, control; F_2, chemical phosphorus 15.5 P_2O_5 kg fad^{-1}; F_3, bio-phosphorus fertilizer 'phosphorein'; F_4, 50% of F_2 + phosphorein; F_5, 25% of F_2 + phosphorien; F_6, F_2 + lithovit; F_7, F_3 + lithovit; F_8, F_4 + lithovit; F_9, F_5 + lihtovit; F_{10}, sole lithovit. Giza 6 Egyptian berseem variety ranked first in forage quality because of its high content of each CP (%), NFE (%), TDN (%), and DP (%) in the 3rd cut. The local berseem variety ranked the least over the six Egyptian clover varieties with the highest ash (%) in the 1st cut. Other varieties were in between. Allusion to the bio-chemical phosphorus fertilization and lithovit regimes, application of any phosphorus fertilization regimes surpassed the control treatment in most nutritive values. Application of F_9 fertilization regime (25% chemical P + bio-fertilizer 'phosphorien' + nano-fertilizer 'lithovit') tended to produce high forage quality with high content of CP (%) in the 1st cut, TDN (%) in both 1st and 3rd cuts as well as DP (%) in the 1st cut.

Key words: Egyptian clover, fertilizer, phosphorus, lithovit, quality.

List of abbreviations: CP (%) is crude protein content, CF (%) is crude fiber content, EE (%) is ether extract content, NFE (%) is nitrogen free extract content, TDN (%) is total digestible nutrients, and DP (%) is digestible protein.

INTRODUCTION

Genus *Trifolium* belongs to the Leguminosae family, it contains about 240 species. White clover (*T. repen*), Red clover (*T. pratense* L.), Alsike clover (*T. hybridum* L.), and Egyptian clover (*T. alexandrinum* L.) are the most important species (Zayed et al., 2012).

Egyptian clover is of great moment forage crop, and inshore is cultivated as a winter annual forage crop in many countries e.g., Egypt, India, Pakistan, Turkey, and Mediterranean countries. The distinction of the crop ascribed to its multi-cut nature, long duration of green fodder availability, and above all its high yield as well as good quality, palatability, and digestibility (Malaviya et al., 2005; Roy et al., 2005; Zayed et al., 2011).

Egyptian clover 'Berseem' is the main widely cultivated multi-cut winter leguminous forage crop in Egypt. Berseem is the main added value in the Egyptian economy, the cycle of berseem capital equals tens billions of American dollars. More and above berseem crop is the guard on Egyptian soil fertility. Berseem is the Arabic and Coptic word, it grows since 6,000 years BC in Egypt (Tarrad & Zayed, 2009; Zayed, 2013). Berseem is a multi-cut leguminous fodder crop and fixes atmospheric 'N' in the soil which is valued as much as (33–66 N ha^{-1}) for the following crops (Knight, 1985; Williams et al., 1990; Govindasamy et al., 2021).

Forage quality is determined pointedly by its content of various nutrients such as minerals, Protein components, Crude fiber, etc. Forage content of mineral and nutritional components varied respecting various and many factors such as fertilization regimes (Türk et al., 2007; Seif & Saad, 2014), Soil properties, and different environmental variables (Kulik, 2009). Berseem is highly nutritive forage crop, contains 14.9–28.3% crude fiber (CF), 15.8–26.7% crude protein (CP), 1.4–3.0% ether extract (EE), 1.4–2.58% calcium and 2.22–2.46% phosphorus (Mohsen et al., 2011b).

Raising levels of phosphorus fertilizers tended to decrease berseem contents of both dry matter (DM) and Nitrogen Free Extract (NFE), while crude protein (CP), crude fiber (CF), ether extract (EE), ash, and Phosphorus content (P) were increased due to increasing phosphorus fertilizer levels (Mohsen et al., 2011a; Seif & Saad, 2014). Roy et al. (2015) attested that increasing phosphorus level from 40 to 80 kg P_2O_5 ha^{-1} gradually increased crude protein (%), crude protein yield, dry matter (%), ether extract, and nitrogen free extract (NFE).

Phosphorus (P) after nitrogen, is the second major nutrient deficient in Egyptian soils. Phosphorus displays a dogmatic role in photosynthesis, as well phosphorus is of great moment in the synthesis of proteins, lipids, nucleic acids, and various important compounds (Ayub et al., 2013; Khattab et al., 2019). Phosphorus fertilization is more important for leguminous field crops than nitrogenous fertilization, nutrient movement within the plant and energy transport as well as the transfer of genetic characters to the following generation are controlled by phosphorus (Abdelsalam & El-Sanatawy, 2022). Phosphorus displays a dogmatic role in legume seedling establishing its roots and shoots (Fathy, 2014).

Phosphorus in soil solution is the immediate source of plant-available P (Holford, 1989), but Phosphorus solubility is inhibited due to the existence of alumunium and iron in acidic soils and calcium in alkaline and neutral soils (Ponmurugan & Gopi, 2006). According to Hamid & Sarwar (1977), the crop uses only about 15–33% of the supplied chemical phosphorus.

The usage of bio-fertilizers is a critical component to crop production in sustainable farming systems (Canbolat et al., 2006). Bio-fertilizers include many types of free-living microorganisms that convert nutrients from unavailable forms through some biological processes which enhance plant growth, development, and productivity (Chaichi et al., 2015). There is a noteworthy and momentary trend to replace chemical fertilizers with eco-friendly biofertilizers. The practice of microbial inoculation became popular and of great moment globally ascribed to being simple to use and safe for the environment. Phosphorus unavailability is the imperative factor limiting crop yield. Phosphorus availability increase through the usage of phosphate solubilizing bacteria (PSB), and it may enhance berseem forage yield and quality (Roy et al., 2015). Phosphate solubilizing bacteria (PSB) produce some organic acids such as succinate, oxalate, lactate, citrate, etc., in the soil that decrease the pH and thus solubilize calcium phosphate complexes. Because of these organic acids, PO_4^{-2} is exchanged by acid anion or is chelated and becomes dissolved and available mineral phosphate (Bajpal & Rao, 1971).

Lithovit (manufactured by Zeovita- GmbH, Berlin, Germany) and quantitatively analyzed by Wichmann & Basler (2006), Wedad Kasim et al. (2020), is the first CO_2 foliar nano-fertilizer that can be used successfully outdoors as well as under glass. It consists of calcium carbonate [($CaCO_3$), 80%] supplemented by numerous important micro-nutrients. Most lithovit particles are so small (< 10 µm) that they can be absorbed directly through the stomata of the plant's foliage. In leaves stomata, calcium carbonate decomposes to calcium oxide (CaO) and carbon dioxide (CO_2), this CO_2 increases the photosynthesis process causing an increase in carbon uptake and assimilation, thereby promoting plant growth and productivity (Carmen et al., 2014; Abdelkader et al., 2018; Mostafa, 2019).

The aim of the study

The study aims to investigate the interactive impacts of bio-chemical phosphorus fertilization and lithovit regimes on the forage quality of some Egyptian clover (Berseem) varieties.

MATERIALS AND METHODS

The study was conducted in a bare field at the Agricultural Research Farm, Faculty of Agriculture, Zagazig University, Zagazig District, Sharkia Governorate, Egypt (Lat. 30°34'59.3" N, long 31°31'03.3" E, 9 m above the sea level) during two consecutive winter growing seasons(2019/2020; 2020/2021) to investigate the impact of bio-chemical phosphorus fertilization and lithovit regimes on forage quality of various Egyptian clover varieties (Berseem, *Trifolium alexandrinum* L.). Certified seeds of the investigated varieties q.e. Helaly, Gemmeza 1, Sakha 4, Serw 1, and Giza 6 were obtained kindly from the production unit, Agriculture Research Center, Giza, Egypt. Mixture of *Rhizobium trifolii* with sand was broadcasted and incorporated with the field soil before planting for promoting N fixation. A local variety commonly planted by farmers was also studied. Seeds were broadcasted in 2×5 m plots (10 m^2) with a seeding rate of 20 kg fad^{-1} (fad. = 4,200 m^2).

Bio-chemical phosphorus fertilization and lithovit treatments included sole chemical phosphorus fertilization and sole biological fertilization (Phosphorien) as well as bio-chemical fertilization in addition to lithovit foliar application. The ten fertilization regimes were (F_1–F_{10}) as follows:

F_1, control; F_2, chemical phosphorus, 15.5 kg P_2O_5 fad^{-1}; F_3, bio- phosphorus fertilizer 'Phosphorien'; F_4, 50% of F_2 + Phosphorien; F_5, 25% of F_2 + Phophorien; F_6, F_2 + litovit; F_7, F_3 + litovit; F_8, F_4 + lithovit; F_9, F_5 + lithovit; F_{10}, sole lithovit.

Fertilizers used

Calcium superphosphate (15.5% P_2O_5) as a chemical phosphorus fertilizer was supplied at sowing, the amounts of the commercial fertilizer were calculated according to each phosphorus level in the fertilization regimes F_2, F_4, F_5, F_6, F_8, and F_9. The biofertilizer 'phosphorien' is phosphate dissolving bacteria (*Bacillus megatherium* var. *phosphaticum*) commercially produced by the General Organization for the Agricultural Equalization Fund (GOAEF), Ministry of Agriculture, Egypt. Phosphorien was used at the rate of 600 g seeds per fad. to detect the fertilization regimes F_3, F_4, F_5, F_7, F_8, and F_9. Inoculation by phosphorien was done by mixing with berseem seeds using Arabic gum 5% as an adhesive substance, just before sowing. Berseem plants were sprayed with the aqueous solution of the nano-fertilizer Lithovit (100 g L^{-1} tap water) using hand operated compress air sprayer. Welting agent Kinzo (100 cm^3 20 L^{-1} tap water) was applied with the spraying solution. Lithovit application was done in the fertilization regimes F_6, F_7, F_8, F_9, and F_{10}. Lithovit foliar spray was applied 30 days after sowing, then after 15 days from each cut at the same rate. It is worth noting that, the weather was not rainy for many days after lithovit foliar spray application. Rains in Egypt is drizzling during the winter season.

The experiment included three replicates in a split-plot design in both seasons. Berseem varieties were allotted to the main plots, the ten regimes of bio-chemical phosphorus fertilization and lithovit were assigned to the sub-plots. A composite soil sample up to 30 cm depth was used for physical and chemical analyses. The soil texture of the experimental site was sandy loam. Fodder maize was the preceding summer crop in both seasons. Egyptian clover varieties were sown on 4th and 7th November in the first and second seasons, respectively, surface flood irrigation system was used.

Four cuts were taken each season, the first cut was attained 65 days post planting date, 50 days later, the second cut was achieved, the third cut was done 40 days after the second one, finely the fourth cut was obtained 30 days later to the third one. A representative subsample of approximately 0.5 kg of the whole plant material per plot in the 1st and 3rd cuts of the two seasons were oven dried at 70 °C for 72 hr. The dried subsamples were ground, bagged, and stored until analyzing and determining quality parameters. Crude protein (CP), crude fiber (CF), ether extract (EE), and ash were determined according to the Association of the Official Analytical Chemist (AOAC, 1995). Nitrogen free extract (NFE) was calculated as follows, NFE = 100 - (CP% + CF% + EE% + Ash%), total digestible nutrients (TDN) was calculated according to Adams et al. (1964) using the equation, TDN = 74.43 + 0.35 CP% - 0.73 CF%, digestible protein (DP) content was calculated according to the following equation, DP (%) = 0.9596 CP - 3.55 (Bredon et al., 1963). Chemical analyses were conducted and presented on a dry matter basis. Data were analyzed by analysis of variance based on split plot design according to the procedures outlined by Snedecor & Cochran (1990). Homogeneity of variance between the two growing seasons was tested using bartellet's test (Steel et al., 1997) which revealed insignificant variation, for all investigated traits; thus data of the two seasons were presented and discussed in a combined analysis. Mean comparing was done using the least significant differences (LSD) at 1 and 5% probability levels according to Duncan (1955). In the interaction tables, capital and small alphabet were used for mean comparison in rows and columns, respectively.

RESULTS AND DISCUSSION

Worthy to be noted that chemical analyses were conducted for the first and third cuts in both growing seasons. Withal, the chemical constituents for the whole plant material of the various berseem varieties were analyzed and presented on a dry matter basis.

Crude Protein Content (CP, %)

Results outlined in Table (1) clear that crude protein content (CP, %) recorded significant variation among the Egyptian clover varieties in both the 1st and 3rd cuts. According to the descending ranking order, CP (%) in the 1st cut valued as much as 25.54, 24.76, 24.60, 24.41, 24.25, and 22.72%, for Sakha 4, Helaly, Serw 1, Gemmeza 1, Giza 6 and the local variety, respectively. In the 3rd cut, there was diverse ranking order concerning the varietal content of CP as follows; 21.71, 21.65, 21.13, 20.97, 20.67, and 20.22% for Egyptian clover varieties Giza 6, local, Gemmeza 1, Sakha 4, Helaly and Serw 1, in respective order. In the 1st cut, Sakha 4 had the highest CP content with a slight significant magnitude. Diverse results were noted in the 3rd cut, wherein, Giza 6 clover variety recorded the highest content of crude protein while the lowest content of CP was recorded by serw 1 clover variety. Varietal differences in the crude protein content of Egyptian clover were also corroborated by Lannucci (2001), De Santis et al. (2004), Mohsen et al. (2011a; 2011b), Seif & Saad (2014) and Salama et al. (2020). The variation in CP content among the tested varieties may be ascribed to the slight differences in their specific genetical makeup and their response to the environment.

Allusion to the crude protein content of berseem varieties for the earlier than the later cuts results in Table (1) asserted that the 1st cut has a relatively high CP content than the 3rd cut for all Egyptian clover varieties under study. The average value of CP over the 6 varieties in the 1st cut was 24.38% vs 21.06 in the 3rd cut. This suggestion matched well with that of Mohsen et al. (2011b), Seif & Saad (2014), Bakhoum et al. (2016). Such findings are more appropriate along more leafy plants alongside shorter stems of higher biophysiological activities forming more crude protein for earlier than the later cuts. More and above, the later cuts are accompanied by a relatively warmer environment and its limited assimilation rate causing decreases in CP content.

Effective and significant impact of the bio-chemical phosphorus fertilization and lithovit regimes on CP (%) was appreciable in 1st and 3rd cuts (Table 1). In the first cut, the highest CP (%) was achieved when the F_9 regime was applied. Fertilization regime F_9 comprised bio P-fertilizer phosphorien and the nano-fertilizer lithovit in addition to 25% of the recommended chemical P fertilizer. In the 3rd cut, the highest CP (%) in Egyptian clover was attained due to the application of the F_5 regime which included duality of 25% of the recommended P chemical fertilizer and the bio-fertilizer 'phosphorien' (Phosphorus solubilizing bacteria, PSB). In both cuts, application of either sole chemical P with the recommended level (15.5 kg P_2O_5 fad^{-1}) i.e. F_2 regime, or application of sole chemical P (recommended level) accompanied with foliar spray of the nano-fertilizer lithovit (F_6) resulted in the lowest CP content (23.28, 22.98 in the first cut; 19.86, 20.03 in the 3rd cut) in the same respective order. Crude protein is distinctly diverse between the 1st and 3rd cuts, wherein the early cut has higher CP (%) than the latter one. Seif & Saad (2014) purported that increasing phosphorus levels from the control to 22.5 and 45 kg P_2O_5 fad^{-1} caused a significant increase in leaves CP content (over 5 Egyptian clover varieties). Roy et al. (2015) avowed that Phosphate Solubilizing Bacteria (PSB) inoculation increased CP (%) of berseem compared to uninoculated treatment. They also added that raising the chemical P fertilizer level from 40 kg P_2O_5 ha^{-1} to 80 kg ha^{-1}, gradually increased the crude protein content of berseem. Obtained results go along with those of Türk et al. (2007), Mohsen et al. (2011a), and Chaichi et al. (2015) on Egyptian clover.

Table 1. Varietal differences as well as bio-chemical phosphorus fertilization and lithovit regimes effect on crud protein (CP, %), Crude fiber (CF, %) and ether extract (EE, %) of Egyptian clover varieties

Main effects and interaction	CP (%)		CF (%)		EE (%)	
	Combined		Combined		Combined	
	1st cut	3rd cut	1st cut	3rd cut	1st cut	3rd cut
	Egyptian clover variety (V)					
Helaly	24.76 b	20.67 e	28.61 a	27.72 b	1.46 f	2.87 d
Gemmeza 1	24.41 d	21.13 c	24.59 f	26.58 d	2.29 b	3.13 a
Sakha 4	25.54 a	20.97 d	26.26 b	27.04 c	1.69 c	2.93 c
Serw 1	24.60 c	20.22 f	26.13 c	28.66 a	2.32 a	1.97 f
Giza 6	24.25 e	21.71 a	25.58 d	24.78 f	1.57 e	2.53 e
Local	22.72 f	21.65 b	25.25 e	26.00 e	1.64 d	3.01 b
F-test	**	**	**	**	**	**
	Bio-chemical phosphorus fertilization and lithovit regime (F)					
F_1	25.10 c	20.14 h	27.13 b	26.90 d	2.46 a	2.93 c
F_2	23.28 i	19.86 j	26.34 d	26.28 g	1.77 d	2.56 g
F_3	24.75 d	20.97 g	28.74 a	26.60 f	1.71 f	2.71 e
F_4	23.48 h	21.33 e	26.36 c	25.69 i	1.60 i	2.49 h
F_5	23.58 g	22.38 a	26.11 e	28.75 a	2.03 b	2.58 f
F_6	22.98 j	20.03 i	24.19 j	28.50 b	1.87 c	2.96 b
F_7	24.07 f	21.18 f	25.38 h	26.72 e	1.71 g	2.80 d
F_8	24.22 e	21.52 c	25.92 f	27.14 c	1.70 h	2.96 b
F_9	27.17 a	21.44 d	25.72 g	25.41 j	1.73 e	2.34 i
F_{10}	25.16 b	21.72 b	24.81 i	25.98 h	1.71 g	3.04 a
F-test	**	**	**	**	**	**
Interaction						
V×F	**	**	**	**	**	**

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

The interaction between Egyptian clover varieties and fertilization regimes acted significantly on crude protein content (CP, %) of the first and third cuts (Tables 1-A; 1-B), respectively. Allusion to the 1st cut, each of Helaly and the local variety of Egyptian clover produced the highest CP (%) under sole bio-fertilizer ‘phosphorien’ i.e. the under F_3 fertilization regime. Berseem variety ‘Serw 1’ has the highest CP (%) under F_4 fertilization regime (50% chemical P + phoaphorien). Each of Gemmeza 1, Sakha 4 and Giza 6 Egyptian clover varieties produced the highest CP (%) under fertilization regime F_9 which included 25% of the recommended P level + bio-fertilizer ‘phosphorein’ + the nano fertilizer foliar spray 'lithovit'. The highest CP (%) content (29.7%) was the resultant of the interaction effect between Giza 6 berseem variety and fertilization regime F_9. The lowest value of CP (%) (19.31%) was obtained under the interaction between Giza 6 berseem variety and fertilization regime F_6 (chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit). In respect to the third cut, each of the following highest CP (%) 24.61, 26.48, 22.95, 23.31, 24.72, and 23.38% was the resultant of the following interactions, Helaly × F_{10}, Gemmeza 1 × F_5, Sakha 4 × F_2, Serw 1 × F_{10}, Giza 6 × F_9 and local variety × F_6, in the same order. The highest (26.48%) and the lowest CP (%) (14.39%) were obtained under the interaction effect of Gemmeza 1 berseem variety × F_5, and Serw 1 × F_2, respectively. Seif & Saad (2014) reported the interaction effect for berseem varieties and the supplied phosphorus fertilizer levels on CP (%) of leaves, were significant. They added that the highest leaf CP content (19.45%) was the resultant of the interaction effect between berseem variety Gemmeza 1 and phosphorus level 45 kg P_2O_5 fad^{-1}, while Giza 6 variety produced the lowest CP content (10.94%) under phosphorus deficiency in the control treatment. Analogues findings were noted by Roy et al. (2015) as well as Ismail & Hassanen (2019).

Table 1-A. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on crude protein content (CP, %) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	F	I	E	B	C	J	H	D	G	A
	20.66 c	19.46 d	20.84 d	22.15 b	21.47 d	16.01 f	19.99 e	21.00 e	20.47 e	24.61 a
Gemmeza 1	J	B	I	C	A	H	F	D	E	G
	17.97 e	22.72 b	18.97 f	22.44 a	26.48 a	19.98 d	20.73 d	21.05 d	20.80 d	20.14 e
Sakha 4	I	A	B	H	F	C	E	B	G	D
	17.33 f	22.95 a	21.77 a	20.04 f	21.22 e	21.53 c	21.28 c	21.77 c	20.31 f	21.50 d
Serw 1	G	J	E	C	F	B	I	H	D	A
	20.32 d	14.39 f	20.78 e	21.11 d	20.53 f	21.54 b	19.01 f	20.11 f	21.06 c	23.34 b
Giza 6	G	I	E	H	D	J	B	C	A	F
	21.37b	19.25 e	21.76 b	20.99 e	22.45 b	17.75 e	23.76 a	23.37 a	24.72 a	21.69 c
Local	B	I	F	H	D	A	C	E	G	J
	23.16 a	20.41 c	21.72 c	21.27 c	22.12 c	23.38 a	22.27 b	21.81 b	21.30 b	19.01 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Crude fiber content (CF, %)

The Egyptian clover (berseem) varieties under study displayed significant variation in their crude fiber content (CF, %) in first and third cuts as compiled in Table (1). In the 1^{st} cut, Helaly variety has the highest content of crude fiber (28.61) followed by Sakha 4 berseem variety, while Gemmeza 1 variety was the lowest (24.59). Conflicting results were observed in the 3^{rd} cut, wherein Serw 1 berseem variety ranked first in CF (%) and valued about 28.66%, Helaly placed in the second rank (27.72) with an operative difference. The berseem variety Giza 6 has the lowest crude fiber content (24.78%). The average value of crude fiber content tended to increase slightly from 1^{st} to 3^{rd} cut (26.07 to 26.80) Bakhoum et al. (2016) alluded to the gradual increase of crude fiber content from 1^{st} cut to 3^{rd} cut of the Egyptian clover. Seif & Saad (2014) highlighted that crude fiber content varied significantly among five Egyptian clover varieties. They added that CF (%) of the fourth cut was relatively higher than the second cut. Varietal variation in crude fiber content may be ascribed to the slight differences in their genetic specific and/ or their interaction with the environmental seasonal condition.

Table 1-B. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on crude protein content (CP, %) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	B 26.27 a	I 23.03 e	A 27.34 a	E 24.80 b	G 24.07 c	J 22.28 e	F 24.62 b	H 23.34 e	D 25.79 d	C 26.10 c
Gemmeza 1	D 24.80 d	E 24.63 a	C 25.05 c	I 23.26 d	J 20.22 f	G 23.76 c	H 23.57 d	B 25.22b	A 29.34 b	F 24.18 d
Sakha 4	E 25.94 c	H 23.26 c	G 24.17 d	J 20.52 f	C 27.87 a	F 25.76 a	I 22.92 f	B 28.80 a	A 29.24 c	D 26.95 b
Serw 1	B 26.18 b	E 24.57 b	J 22.65 f	A 27.17 a	F 24.15 b	H 23.81 b	D 24.59 c	I 23.55 d	C 25.26 e	G 24.01 e
Giza 6	G 23.21 f	H 23.06 d	F 23.22 e	E 23.98 c	I 22.75 d	J 19.31 f	C 25.47 a	D 24.58 c	A 29.47 a	B 27.45 a
Local	B 24.18 e	I 21.11 f	A 26.07 b	H 21.16 e	F 22.42 e	E 22.93 d	D 23.25 e	J 19.81 f	C 23.94 f	G 22.28 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

The bio-chemical phosphorus fertilization and lithovit regimes influenced significantly the crude fiber content of the 1^{st} and 3^{rd} cuts of the Egyptian clover variety. The highest CF (%) in the 1^{st} cut (28.74%) was the resultant of the fertilization regime F_3 i.e. under sole phosphorus bio-fertilizer 'phosphorien'. The fertilization regime F_{10} comprised a sole foliar spray of nano-fertilizer lithovit lessen crude fiber content to the lowest value (24.81%). Regarding the 3^{rd} cut, crude fiber content ranged between 25.41% to 28.75%. Seif & Saad (2014) in Egypt deposed that increasing levels of phosphorus fertilizer applied caused a slight nidgin increase in the crude fiber content of five Egyptian clover varieties. Fertilization treatment comprised dual application of PSD and 80 kg P_2O_5 ha produced the lowest crude fiber (20.08) as ratified by Roy et al. (2015) who worked on Egyptian clover in India.

The crude fiber content (CF, %) of the first cut was significantly influenced by the interaction between the fertilization regimes and the Egyptian clover varieties (Table 1-C). Each of the following interactions has the highest CF (%); Helaly variety × F_5, Gemmeza 1 × F_1, Sakha 4 × F_3, Serw 1 × F_3, Giza 6 × F_9, and the local variety × F_4. Under five out of the ten fertilization regimes i.e., F_1, F_4, F_5, F_6, and F_7; Helaly berseem variety was superior in CF (%). Sakha 4 outclassed the other varieties under fertilization regimes F_2 and F_3, Giza 6 berseem variety produced the highest CF (%) under each of F_8, F_9, and F_{10} fertilization regimes. The uppermost crude fiber content (34.98%) has been observed under the interaction effect between berseem variety 'Helaly' and F_5 fertilization regime, whereas the lowermost CF (%) amounted to 20.80% was the resultant of the interaction between Giza 6 berseem variety and the sole chemical P fertilization (15.5 kg P_2O_5 fad^{-1}).

Table 1-C. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on crude fiber content (CF, %) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	C	D	A	E	B	G	H	F	J	I
	29.53 b	28.91 a	31.31 a	27.98 b	30.53 b	26.48 d	26.43 c	26.78 c	24.56 d	24.68 f
Gemmeza 1	I	H	J	B	D	E	F	A	C	G
	25.07 e	25.33 e	23.95 f	28.91 a	26.65 e	26.44 e	26.27 d	29.28 b	27.70 b	26.22 d
Sakha 4	F	C	E	H	A	D	B	I	J	G
	27.04 c	27.72 b	27.51 b	26.52 d	28.82 c	27.65 c	28.75 b	26.21 d	23.46 e	26.73 b
Serw 1	J	I	G	F	E	A	C	B	D	H
	21.62 f	23.04 f	27.00 c	27.28 c	28.80 d	34.80 a	34.07 a	34.35 a	30.57 a	25.06 e
Giza 6	A	B	E	H	J	F	I	G	D	C
	32.41 a	26.66 c	24.08 e	22.24 e	21.77 f	23.91 f	22.17 f	23.12 e	25.08 c	26.36 c
Local	F	D	E	I	A	B	H	G	J	C
	25.70 d	26.03 d	25.78 d	21.23 f	35.92 a	31.71 b	22.63 e	23.10 f	21.12 f	26.81 a

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Table 1-D. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on crude fiber content (CF, %) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	D	F	E	B	A	I	C	H	G	J
	29.47 a	27.68 c	29.33 c	31.16 a	34.98 a	25.72 a	29.83 a	27.06 b	27.59 b	23.31 e
Gemmeza 1	A	I	G		F	B	H	E	D	C
	28.57 b	22.57 e	24.03 f	22.30 f	24.38 c	25.65 b	23.40 f	24.51 e	25.09 c	25.37 b
Sakha 4	D	B	A	C	G	J	F	H	I	E
	25.36 e	32.30 a	34.49 a	30.62 b	23.14 e	22.35 e	23.65 d	23.08 f	22.71 f	24.88 c
Serw 1	C	B	A	I	H	J	D	E	G	F
	27.33 d	31.15 b	33.68b	22.51 e	22.57 f	22.13 f	27.16 b	26.11 d	23.82 e	24.86 d
Giza 6	C	J	E	I	H	F	G	B	A	D
	27.56 c	20.80 f	25.40 e	23.20 d	23.40 d	25.06 c	24.73 c	28.12 a	30.36 a	27.15 a
Local	F	H	D	A	B	G	I	C	E	J
	24.51 f	23.53 d	25.50 d	28.40 c	28.17 b	24.21 d	23.51 e	26.65 c	24.75 d	23.29 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

The crude fiber content of the 3rd cut was affected significantly by the interaction between Egyptian clover varieties and the fertilization regimes of both bio-chemical phosphorus and lithovit (Table 1-D). Under F_1 fertilization regime, Giza 6 berseem variety ranked first in CF (%); under each of F_2 and F_3 fertilization regimes, Helaly berseem variety has the highest CF (%); under F_4 fertilization regime, Gemmeza 1 variety ranked first in CF (%), under each of F_5 and F_{10}, the highest CF (%) was obtained by the local berseem variety; while under each of F_6, F_7, F_8, and F_9 fertilization regimes, Serw 1 berseem variety has the ultra most value of crude fiber content. The uppermost value of crude fiber content (35.92%) was the resultant of the interaction of F_5 fertilization regime and the local berseem variety, while the lowest value of crude fiber content (21.12%) was achieved under the interaction of F_9 fertilization regime and the local berseem variety. Seif & Saad (2014) corroborated the significant interaction impact of the berseem varieties × phosphorus levels, their results indicated that each of the berseem varieties was differently affected by the phosphorus application level. They added that the berseem variety Sakha 4 produced the highest leaf CF content (32.35%) when fertilized with 45 kg P_2O_5 fad^{-1}.

Ether extract content (EE, %)

Varietal differences among the six Egyptian clover varieties concerning ether extract content were verified in both 1st and 3rd cuts (Table 1). Ether extract content recorded the highermost value (2.32%) in the 1st cut for the berseem variety 'Serw 1' while the lowermost EE, % (1.46%) was reported for the berseem variety 'Helaly' in the 1st cut. Diverse varietal differences in EE (%) of the 3rd cut were disclosed. The highest ether extract content value (3.13%) was observed in the berseem variety 'Gemmeza 1', while the lowest one (1.97%) was recorded in berseem variety 'Serw 1'. The average EE (%) value over the six berseem varieties increased from 1.83% in the 1st cut to 2.74% in the 3rd cut. These results resemble those ratified by Seif & Saad (2014), who pointed out that the highest EE content was for Helaly berseem variety, whereas the lowermost EE (%) was for Giza 6 berseem variety. They added that the 2nd cut was relatively higher in EE content than the 4th cut in each of the five Egyptian clover varieties studied. Bakhoum et al. (2016) deduced that ether extract content of 'Meskawy' Egyptian clover variety relatively decreased from 1st cut to the third one.

Application of various bio-chemical phosphorus fertilization and lithovit regimes caused significant variation in ether extract content of berseem (all over the six varieties) in the 1st and 3rd cuts as elucidated in Table 1. Ether extract was differently affected in 1st cut than the 3rd one by the bio-chemical phosphorus fertilization and lithovit regimes, wherein in the 1st cut the highest EE content (2.46) was obtained under control treatment, while the lowest EE content (1.60) was the resultant of application F_4 fertilization regime (50% chemical P + bio-fertilizer 'phosphorien'). Results of EE content in 3rd cut were differ from those of the 1st cut, wherein, the fertilization regime F_{10} (sole foliar application with lithovit) produced the highest content of EE content (3.04) and the fertilization regime F_9 (25% chemical P + bio-fertilizer 'phosphorien' + lithovit) produced the lowest EE content (2.34%). The increment of ether extract content of the 3rd cut over the 1st one was noted under each fertilization regime studied. Increasing EE content of Egyptian clover due to raising chemical phosphorus fertilizer levels was in place as previously confirmed by results of Mohsen et al. (2011a), Seif & Saad (2014), and Roy et al. (2015).

The interaction between the Egyptian clover varieties and the bio-chemical phosphorus fertilization and lithovit regimes was influential on EE content of both 1st and 3rd cuts (Tables 1-E; 1-F). In the 1st cut and under the control fertilization regime (F_1), the highest EE content was recorded for each of Gemmeza 1, Sakha 4, Serw 1, and the local berseem varieties. The highest EE content was also achieved under F_2 fertilization regime by Helaly berseem variety while under F_5 fertilization regime, Giza 6 berseem variety has the highest EE content. In the other direction, Gemmeza 1 berseem variety was supreme in EE content under each of F_1, F_2, F_6, and F_7 fertilization regimes. Withal, Serw 1 berseem variety outrank the other varieties in EE content under each of F_3, F_4, F_5, F_8, F_9, and F_{10} fertilization regimes. The utmost EE content value (3.05%) was obtained under the interaction impact of F_1 fertilization regime × Gemmeza 1 berseem variety. The lowermost EE content value (0.81%) was achieved under the impact of the interaction between F_4 fertilization regime and Giza 6 berseem variety. Oscillatory results were observed in the 3rd cut, wherein under the fertilization regimes F_1, F_2, F_3, F_4, F_6, and F_8, the highest EE content was achieved from Gemmeza 1, Serw 1, Helaly, Sakha 4, Giza 6 and the local variety, in the same respective order. In the other direction, Helaly berseem variety was superior in EE content under each of F_2 and F_3 fertilization regimes. Likely, Gemmeza 1 variety content of EE (%), surpassed the other varieties under F_1 and F_9 fertilization regimes, while Sakha 4 has the highest EE content under each of F_4, F_5, F_7, and F_{10} fertilization regimes. Giza 6 under F_6 as well as the local variety under F_8 fertilization regimes produced the highest EE content.

Table 1-E. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on ether extract (%) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	B 2.08 e	A 2.10 b	G 0.99 f	F 1.24 c	F 1.23 f	D 1.69 d	C 1.81 b	E 1.65 c	H 0.92 f	I 0.86 f
Gemmeza 1	A 3.05 a	B 2.65 a	H 1.68 e	C 2.50 b	F 2.21 c	B 2.66 a	D 2.44 a	I 1.30 d	E 2.26 b	G 2.13 b
Sakha 4	A 2.64 c	I 1.00 f	D 1.92 c	G 1.23 d	B 2.50 b	F 1.47 e	J 0.92 f	C 2.48 b	E 1.66 d	H 1.08 e
Serw 1	A 2.97b	H 1.94 c	G 1.98 a	D 2.54 a	C 2.68 a	I 1.80 c	J 1.74 c	E 2.50 a	F 2.33 a	B 2.72 a
Giza 6	CD 1.75 f	CD 1.76 d	B 1.96 b	I 0.81 e	A 1.99 d	G 1.37 f	F 1.65 e	H 1.00 f	E 1.69 c	C 1.76 c
Local	A 2.27 d	G 1.17 e	C 1.75 d	F 1.24 c	E 1.55 e	B 2.23 b	D 1.69 d	F 1.25 e	E 1.54 e	D 1.69 d

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Table 1-F. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on ether extract (%) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	D 3.11 b	B 3.44 a	A 3.76 a	H 2.25 d	G 2.75 b	C 3.40 b	F 2.88c	J 1.86 f	I 2.20 d	E 3.04 c
Gemmeza 1	A 4.06 a	J 2.16 e	C 3.56 c	I 2.57 c	H 2.61 e	E 3.04 d	B 3.93 b	D 3.46 b	G 2.91 a	F 2.96 d
Sakha 4	F 2.57 e	G 2.50 c	J 1.23 f	A 4.16 a	D 3.36 a	I 2.32 f	C 3.95a	E 2.60 d	H 2.44 b	B 4.15 a
Serw 1	D 2.39 f	A 2.68 b	H 1.44 e	G 1.61 e	J 1.32 f	C 2.43 e	I 1.39 f	B 2.57 e	E 2.15 e	F 1.67 f
Giza 6	C 2.84 c	I 2.12 f	E 2.55 d	J 1.40 f	D 2.75 c	A 3.48 a	G 2.33 d	B 3.14 c	H 2.29 c	F 2.36 e
Local	G 2.64 d	H 2.48 d	C 3.69 b	E 2.96 b	F 2.71 d	D 3.08 c	I 2.31 e	A 4.14 a	J 2.03 f	B 4.05 b

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Nitrogen Free Extract (NFE, %)

Table (2) expose the varietal differences and the impact of bio-chemical phosphorus fertilization and lithovit regimes on nitrogen free extract (NFE%) of Egyptian clover (1st and 3rd cuts). Results of the first cut exhibited that NFE (%) value ranged between 27.77% for Helaly berseem variety and 33.46% for Giza 6 berseem variety. The same trend was reaffirmed in the third cut, wherein, the highest NFE content (39.11%) was recorded by Giza 6 berseem variety, while the lowest NFE (%) value (35.16) was for Helaly berseem variety. Combined results clarified that the 3rd cut has higher content of nitrogen free extract than the 1st cut. Obtained results go along with those of Kulik (2009) as well as Seif & Saad (2014).

Table 2. Varietal differences as well as the bio-chemical phosphorus fertilization and lithovit regimes effect on nitrogen free extract (NFE, %), ash (%), and total digestible nutrients (TDN, %) of Egyptian clover varieties

Main effects and interactions	NFE (%)		Ash (%)		TDN (%)	
	Combined		Combined		Combined	
	1st cut	3rd cut	1st cut	3rd cut	1st cut	3rd cut
	Egyptian clover variety (V)					
Helaly	27.77 f	35.16 f	17.39 b	13.58 a	62.21 f	61.43 e
Gemmeza 1	32.30 c	36.79 c	16.42 e	12.38 d	65.02 a	62.42 c
Sakha 4	29.87e	36.47 e	16.64 d	12.59 b	64.20 c	62.03 d
Serw 1	30.15 d	36.68 d	16.80 c	12.47 c	63.96 d	60.58 f
Giza 6	33.46 a	39.11 a	15.14 f	11.88 f	64.25 b	63.94 a
Local	32.73 b	37.09 b	17.67 a	12.25 e	63.95 e	63.02 b
F-test	**	**	**	**	**	**
	Bio-chemical phosphorus fertilization and lithovit regime (F)					
F_1	28.34 i	37.14 e	16.97 c	12.89 b	63.41 g	61.84 h
F_2	31.73 d	38.71 a	16.88 f	12.59 d	63.35 i	62.20 f
F_3	27.73 j	37.37 d	17.07 b	12.35 g	62.11 j	62.35 d
F_4	30.99 g	38.30 b	17.57 a	12.18 j	63.40 h	63.14 b
F_5	31.64 e	34.05 j	16.65 g	12.24 h	63.62 f	61.28 i
F_6	35.05 a	36.08 h	15.92 i	12.43 f	64.82 c	60.64 j
F_7	33.16 b	37.10 f	15.68 j	12.21 i	64.33 d	62.34 e
F_8	32.00 c	35.50 i	16.16 h	12.88 c	63.98 e	62.15 g
F_9	28.48 h	37.82 c	16.89 e	12.99 a	65.16 a	63.38 a
F_{10}	31.36 f	36.78 g	16.96 d	12.49 e	65.13 b	63.07 c
F-test	**	**	**	**	**	**
Interaction						
V×F	**	**	**	**	**	**

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Nitrogen free extract content which represents the carbohydrate content was significantly influenced by bio-chemical phosphorus fertilization and lithovit regimes in both 1st and 3rd cuts of Egyptian clover. It is worth mentioning that the duality application of chemical phosphorus (15.5 kg P_2O_5 fad^{-1}) and lithovit (F_6) increased NFE (%) of the first cut of Egyptian clover to the uttermost value (35.05). Availability of phosphorus through the application of sole chemical phosphorus fertilizer (15.5 kg P_2O_5 fad^{-1}) i.e. F_2 fertilization regime increased NFE content of the 3rd cut of berseem to the highermost value (38.71). Unfertilized berseem plants under control fertilization regime F_1 produced the lowest value of NFE content (28.34) in the 1st cut, whereas the lowest content of NFE (34.05) in the 3rd cut was the resultant of supplying the fertilization regime 25% chemical P + bio phosphorien (F_5). The present results resemble those noticed by Türk et al. (2007) on narbon vetch, Mohsen et al. (2011a) on berseem, Seif & Saad (2014) as well as Roy et al. (2015) on Egyptian clover. Bakhoum et al. (2016) imparted that, the highest content of NFE was obtained by applying the bio-organic + mineral phosphorus fertilization treatment.

The interaction between the two main studied factors affected significantly on NFE content of Egyptian clover in the 1st and 3rd cuts. Allusive to the first cut results (Table 2-A), F_2 fertilization regime produced the highest NFE content (36.23%) for the local berseem variety. Withal, fertilization regime F_6 was superior in NFE content when supplied to any of the following berseem varieties q.e., Helaly, Serw 1, and Giza 6. Whereas F_7 fertilization regime exhibited the uppermost NFE content when applied to either Gemmeza 1 or Sakha 4 berseem varieties. In the other direction, Giza 6 berseem variety represents superiority in NFE content under six out of ten phosphorus fertilization regimes (F_1, F_2, F_3, F_4, F_5, and F_6) while the local berseem variety represents superiority in NFE content under three out of ten fertilization regimes (F_8, $F_{,9}$ and F_{10}). Availability of phosphorus fertilizer in addition to the nano fertilizer 'lithovit' through the application of F_6 fertilization regime produced the uppermost NFE content for Giza 6 berseem variety (40.16%). But the same variety 'Giza 6' produced the lowest NFE content (22.10%) under the application of F_9 fertilization regime *i.e.,* under a limited mineral phosphorus fertilizer (25% chemical P of the recommended level).

Table 2-A. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on nitrogen free extract (NFE, %) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	J	D	H	G	I	A	E	C	F	B
	23.87 f	29.63 d	24.38 d	24.44 f	24.08 f	33.38 e	27.74 f	30.81 e	27.31 d	32.08 b
Gemmeza 1	I	E	G	D	B	C	A	F	J	H
	26.66 e	33.77 c	32.24 b	33.83 b	35.51 b	34.39 c	35.80 b	33.10 b	26.41 e	31.31 d
Sakha 4	H	I	J	E	G	B	A	F	D	C
	29.15 c	26.30 e	22.58 f	29.97 d	29.43 e	33.58 d	36.83 a	29.79 f	30.38 c	30.71 e
Serw 1	H	I	J	G	B	A	F	E	D	C
	27.45 d	24.98 f	24.25 e	29.67 e	33.84 c	36.34 b	30.44 e	31.47 d	31.48 b	31.58 c
Giza 6	G	B	E	D	C	A	F	H	J	I
	32.05 a	39.46 a	34.35 a	36.84 a	37.03 a	40.16 a	34.29 c	31.76 c	22.10 f	26.58 f
Local	H	A	J	G	I	F	D	C	E	B
	30.84 b	36.23 b	28.56 c	31.19 c	29.95 d	32.432 f	33.88 d	35.08 a	33.22 a	35.88 a

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Regarding the third cut (Table 2-B), the highest NFE content value was the resultant of the interaction effect of any of the following. F_1 ×Sakha 4, F_2 × Serw 1, F_3 × Gemmeza 1, F_4 × Giza 6, F_6 × Helaly, and F_9 × local variety. In the other direction, Serw 1 berseem variety was superior in NFE content under F_1, F_2, and F_{10} fertilization regimes, withal, the local berseem variety exhibited the highest NFE content under F_7, F_8, and F_9 fertilization regimes. The highest NFE content (47.72%) and the lowest NFE content (27.51%) were obtained under the interaction effect of F_2 × Serw 1 berseem variety and F_5 × the local berseem variety, consecutively. These results of the interaction are in harmony with those clarified by Seif & Saad (2014).

Table 2-B. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on nitrogen free extract (NFE, %) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	H	E	J	G	I	A	C	D	B	F
	32.94 e	35.08 e	31.12 f	33.61 a	31.31 e	40.41 b	37.84 c	36.95 c	38.57 c	33.80 f
Gemmeza 1	B	E	A	H	J	C	F	I	G	D
	39.96 c	37.38 d	41.58 a	33.93 e	32.72 d	38.51 c	37.30 d	33.20 e	35.49 e	37.83 c
Sakha 4	A	J	D	C	G	E	I	F	B	H
	40.30 b	33.69 f	36.65 d	37.82 d	35.48 c	36.43 d	33.89 e	35.82 d	39.79 d	34.83 e
Serw 1	B	A	C	E	F	J	H	I	G	D
	42.89 a	47.72 a	38.55 c	38.26 c	36.29 b	28.40 f	33.39 f	29.53 f	33.45 f	38.38 a
Giza 6	J	D	E	A	C	B	F	G	I	H
	31.35 f	40.26 b	40.05 b	44.04 f	41.00 a	42.76 a	39.41 b	38.52 b	36.14 d	37.52 d
Local	H	F	G	B	J	I	C	D	A	E
	35.41 d	38.11 c	36.28 e	42.16 b	27.51 f	29.96 e	40.77 a	38.99 a	43.44 a	38.29 b

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Ash content (Ash, %)

Ash content exhibited significant differences among the six Egyptian clover varieties in 1st and 3rd cuts (Table 2). The local variety has the highest ash content (17.67%) in the first cut, while the uttermost ash content (13.58%) for the 3rd cut was obtained by Helaly berseem variety. Allusion to the lowest ash content for the 1st cut (15.14%), as well as for the 3rd cut (11.88%), it was achieved by berseem variety 'Giza 6'. The decrement of ash content for the third cut compared with the 1st cut was assured all over the six Egyptian clover varieties investigated. Ash content of different Egyptian clover varieties was of slight significant differences in both the 2nd and 4th cuts, that was the finding reported by Seif & Saad (2014) in Egypt. Bakhoum et al. (2016) postulated that ash (%) for berseem clover decreased gradually from 1st cut to the 3rd one.

The bio-chemical phosphorus fertilization and lithovit regimes exhibited operative impact on the ash content of berseem for 1st and 3rd cuts (Table 2). F_4 fertilization regime (50% chemical P + bio-fertilizer phosphorien) outstrips the other fertilization regimes allusive to ash content for the 1st cut (17.57%), the same fertilization regime (F_4) has the lowest ash content for the 3rd cut (12.18%). The highest ash content for the 3rd cut (12.99%) was obtained when F_9 (25% chemical P + bio-fertilizer phosphorien + lithovit) was applied. The effectual promoter impact of phosphorus on the ash content of forage was assured by each of Mohsen et al. (2011a), Seif & Saad (2014) as well as Bakhoum et al. (2016). Conversely, Roy et al. (2015) denoted that phosphorus application impact on the ash content of Egyptian clover was nonentity.

Table 2-C. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on ash (%) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	C	F	D	B	J	H	I	G	A	E
	18.31 a	17.56 b	17.95 b	18.36 a	15.64 e	16.93 b	16.00 c	17.14 b	18.39 a	17.64 a
Gemmeza 1	D	F	C	A	B	I	H	G	E	C
	16.92 c	16.38 e	17.00 d	18.10 b	17.68 b	13.54 f	14.79 e	15.87 d	16.90 c	17.00 c
Sakha 4	D	B	E	A	C	E	I	H	G	F
	16.91 c	17.14 d	16.84 e	17.66 d	17.06 c	16.84 c	15.69 d	15.85 e	16.01 f	16.38 f
Serw 1	H	C	B	A	F	I	H	G	D	E
	16.06 d	17.35 c	17.44 c	18.10 b	16.76 d	15.92 d	16.07 b	16.36 c	17.10 b	16.84 e
Giza 6	C	F	E	D	G	I	J	H	B	A
	15.42 e	14.92 f	15.07 f	15.16 e	14.84 f	14.10 e	13.86 f	14.54 f	16.38 e	17.06 b
Local	A	E	C	D	F	B	G	H	J	I
	18.21 b	17.95 a	18.12 a	18.00 c	17.92 a	18.20 a	17.67 a	17.21 a	16.55 d	16.85 d

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

The interaction between the ten phosphorus fertilization regimes and the six Egyptian clover varieties significantly impacted the ash content of 1st and 3rd berseem cuts. Regarding to the 1st cut, Table (2-C). The control fertilization regime (F_1) surpassed other fertilization regimes in ash content (18.32%) when supplied to the local berseem variety. Application of F_4 (50% chemical P + bio-fertilizer phosphorien) caused supereminence each of Gemmeza 1, Sakha 4, and Serw 1 in ash content. F_9 × Helaly berseem variety and F_{10} × Giza 6 interaction had the highest ash content. In the other direction, Helaly berseem variety outbrave the other berseem varieties in ash content under each of F_1, F_4, F_9, and F_{10} fertilization regimes, while the local variety has the utmost ash content under each of F_2, F_3, F_5, F_6, F_7, and F_8 fertilization regimes. The interaction effect between F_9 fertilization regime and Helaly berseem variety produced the uppermost ash content (18.39%), while the lowermost ash content (13.54%) was the resultant of the interaction effect between F_6 (chemical P 15.5 P_2O_5 fad^{-1} + lithovit) and Gemmeza 1 berseem variety. As for the third cut, results in Table (2-D) exhibited that the control fertilization regime (F_1) surpassed other fertilization regimes in ash content (13.10%) when applied to the local berseem variety. F_7 × Giza 6 berseem variety as well as F_8 fertilization regime × Serw 1 berseem variety had the highest ash content. Supplementation of F_9 (25% chemical P + bio-fertilizer phosphorien + lithovit) fertilization regime caused supereminence in each of Helaly, Gemmeza 1, and Sakha 4 berseem varieties in ash content. In other direction, Helaly berseem variety ranked first in ash content compared with other berseem varieties under eight out of ten fertilization regimes (F_1, F_3, F_4, F_5, F_6, F_7, F_9, and F_{10}). Sakha 4 ranked first under F_2 and F_8 fertilization regimes. The uppermost ash content (14.19%) was the resultant of the interaction effect between F_9 (25% chemical P + bio-fertilizer phosphorien + lithovit) and Helaly berseem variety. The lowermost ash content (11.11%) was obtained due to the interaction impact of F_5 fertilization regime and Sakha 4 Egyptian clover variety.

Table 2-D. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on ash (%) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	E	H	I	B	C	F	J	G	A	D
	13.75 a	13.11 b	12.96 a	14.01 a	13.94 a	13.70 a	12.87 a	13.40 c	14.19 a	13.87 a
Gemmeza 1	C	E	H	F	J	G	I	B	A	D
	12.94 c	12.41 d	11.94 e	12.16 c	11.54 e	12.03 e	11.77 f	13.01 d	13.10 c	12.85 b
Sakha 4	F	C	D	I	J	H	G	B	A	E
	12.76 e	13.15 a	12.84 b	11.45 e	11.11 f	12.07 d	12.14 d	13.60 a	14.00 b	12.78 c
Serw 1	D	F	E	H	B	C	G	A	D	I
	12.77 d	12.18 e	12.24 d	11.74 d	13.06 b	12.83 b	12.15 c	13.44 b	12.77 d	11.55 f
Giza 6	D	G	H	J	D	B	A	E	F	C
	12.03 f	11.71 f	11.56 f	11.32 f	12.03 c	12.10 c	12.33 b	11.84 f	11.76 f	12.07 d
Local	A	B	C	D	J	H	F	G	E	I
	13.10 b	12.97 c	12.54 c	12.39 b	11.74 d	11.87 f	12.01 e	11.96 e	12.11 e	11.83 e

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Results announced by Seif & Saad (2014) referred to a significant interaction effect between Egyptian clover varieties and phosphorus fertilization treatment on the ash content of berseem leaves.

Total Digestible Nutrient Content (TDN, %)

TDN is a calculated figure that represents the sum of all digestible organic nutrients in the feed (Torell et al., 2005). The results in Table (2) point to the influence of Egyptian clover varieties as well as bio-chemical phosphorus fertilization and lithovit regimes on total digestible nutrient content (TDN%). Despite the narrow range of TDN (%) between the highest forage content (62.05%) for Gemmeza 1 berseem variety and the lowest one (62.21%) for Helaly berseem variety in the 1st cut, the varietal differences in TDN (%) were significant. Withal, findings in the 3rd cut had analogous trend wherein, Giza 6 has the highest TDN content (63.94%) while Serw 1 berseem variety has the lowest TDN content (60.58%). It is worth mentioning that, as crude protein content and TDN (%) increase, forage quality also rises (Mohsen et al*.,* 2011a). So, forage quality of the 1st cut tended to outclass that of the 3rd cut in the present study (Tables 1 and 2). Salama et al. (2020) purported that some berseem varieties and/ or cuts appeared to be of better quality than others because of the association between high CP (%) and TDN (%) and low NFE and fiber content. Varietal differences in TDN content were also reported by Seif & Saad (2014).

The bio-chemical phosphorus fertilization and lithovit regimes acted significantly on the digestible organic nutrients of Egyptian clover 1st and 3rd cuts. Application of F_9 fertilization regime (25% chemical P + bio-fertilizer 'phosphorien' + the nano-fertilizer 'lithovit') improved berseem quality *via* raising TDN (%) to the uppermost value in each of 1st and 3rd cuts (65.16 and 63.38%, respectively). The fertilization regime F_8 recorded

TDN (%) to the lowest value (62.11%) in the 1st cut of Egyptian clover. Whereas the lowest TDN (%) value (60.64%) in the 3rd cut was obtained under F_6 fertilization regime (chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit). Mohsen et al. (2011a) divulged that as the percentages of CP and TDN increase berseem quality likely rises. They added that TDN (%) for both berseem 3rd and 4th cuts increased significantly with phosphorus fertilization. Seif & Saad (2014) discerned that the TDN content of berseem leaves (over 5 Egyptian varieties) increased as phosphorus level increased up to 45 kg P_2O_5 fad^{-1}. Bakhoum et al. (2016) evinced that the total digestible nutrient (%) of berseem clover forage increased consistently in the bio-organic mineral fertilization treatments of both phosphorus and nitrogen.

Table 2-E. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on total digestible nutrients (TDN%) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	G	F	E	I	J	B	H	D	C	A
	62.11 e	62.28 d	62.59 d	60.36 e	57.32 f	63.45 e	61.27 f	62.84 d	63.31 e	66.55 a
Gemmeza 1	J	A	D	C	I	H	E	F	B	G
	62.25 d	66.58 b	65.66 a	66.30 b	63.71 d	64.02 d	65.60 a	65.36 b	66.39 b	64.37 e
Sakha 4	G	I	J	H	C	D	F	B	A	E
	65.00 a	58.99 f	57.71 f	59.26 f	67.29 a	67.13 a	65.19 d	67.66 a	68.08 a	65.70 b
Serw 1	F	I	J	A	C	B	H	G	D	E
	63.64 b	60.29 e	57.77 e	67.51 a	66.40 b	66.61 b	63.21 e	63.61 c	65.88 c	64.69 d
Giza 6	J	A	F	B	C	G	D	I	H	E
	62.43 c	67.31 a	64.02 c	65.88 c	65.31 c	62.90 f	65.29 c	62.50 e	62.58 f	64.22 f
Local	C	G	D	J	I	E	A	H	F	B
	65.00 a	64.64 c	64.94 b	61.11 d	61.71 e	64.78 c	65.41 b	61.91 f	64.74 d	65.23 c

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

The effective and significant influence of the interaction between the bio-chemical phosphorus fertilization and lithovit on one side, and the Egyptian clover varieties on the other one was appreciable on TDN content in 1st and 3rd cuts (Tables 2-E; 2-F). Allusion to the 1st cut, and under F_2 fertilization regime (chemical P 15.5 kg P_2O_5 fad^{-1}), the highest TDN (%) was achieved by each of Gemmeza 1 and Giza 6 berseem varieties. The highest TDN (%) was also reported under each of the following interactions, F_4 × Serw 1; F_7 × Local variety; F_9 ×Sakha 4, and F_{10} × Helaly. Regarding the other direction, Helaly berseem variety was supreme in TDN (%) under F_{10} (sole foliar spray with lithovit) fertilization regime. Withal Gemmeza 1 berseem variety outrank the other varieties in TDN (%) under each of F_3 and F_7 fertilization regimes. Sakha 4 berseem variety has the highest TDN (%) value under each of F_1, F_5, F_6, F_8, and F_9 fertilization regimes. Each of Serw 1 × F_4; Giza 6 × F_2 and the local variety × F_1 has the highest TDN (%). The utmost TDN content value (68.08%) was achieved under the interaction influence of F_9 fertilization regime ×Sakha 4 berseem variety. The lowermost TDN (%) value (57.32) was obtained under the interaction effect between F_5 fertilization regime and Helaly berseem variety. Oscillatory results were noted in the 3rd cut, wherein under fertilization regimes F_1, F_5, F_7, F_9, F_{10}, the highest TDN (%) was obtained from Serw 1, Gemmeza 1, Giza 6, both Sakha 4 and the local variety as well as Helaly varieties, in the same order. Allusive to the other direction, Helaly berseem variety has the highest TDN (%) under F_{10} fertilization regime. Withal, Gemmeza1 variety content of TDN (%), outranks other berseem varieties under F_2 fertilization treatment, while berseem variety Serw 1 has the highest TDN (%) under F_1 fertilization regime. It's worth noting that Giza 6 berseem variety can produce high forage quality as TDN (%) was the highest under each of the following fertilization regimes i.e., F_3, F_5, F_6, F_7, and F_8. The local berseem variety has the highest TDN (%) under F_4 fertilization regime. The uppermost value of TDN content (66.46%) was the resultant of the interaction of F_9 fertilization regime and the local berseem variety, it is worth noting that the same interaction lower the crude fiber content to the lowest value (21.12%, Table 1-D) so the forage quality raise under this interaction effect. The lowest TDN content (55.95%) was obtained under the interaction effect of the local variety × F_5 which produced also the highest crude fiber content (35.92, Table 1-D) so, the forage quality was reduced under this interaction effect. Significant interaction influence of Egyptian clover varieties and phosphorus fertilization treatments on TDN content was also reported early by Seif & Saad (2014) in Egypt.

Table 2-F. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on total digestible nutrients (TDN%) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	H	G	J	E	I	F	D	C	B	A
	60.10 e	60.14 f	58.87 f	61.76 e	59.66 e	60.70 d	62.14 d	62.23 d	63.66 d	65.03 a
Gemmeza 1	E	B	C	I	A	G	D	J	H	F
	62.42 c	63.89 a	63.59 b	61.18 f	64.25 b	62.12 b	62.51 c	60.43 e	61.49 e	62.34 e
Sakha 4	J	D	F	E	I	G	H	B	A	C
	60.76 d	62.23 d	61.97 e	62.09 c	60.82 c	61.78 c	60.89 e	62.91 c	64.42 c	62.44 d
Serw 1	A	C	D	E	F	H	J	I	G	B
	65.76 a	62.65 b	61.99 d	61.90 d	60.59 d	56.56 f	56.21 f	56.39 f	59.48 f	64.30 b
Giza 6	J	I	F	D	B	G	A	C	E	H
	58.25 f	61.71 e	64.46 a	65.54 b	66.39 a	63.19 a	66.56 a	65.73 a	64.78 b	62.78 c
Local	E	G	F	B	J	I	C	D	A	H
	63.78 b	62.57 c	63.21 c	66.38 a	55.95 f	59.47 e	65.70 b	65.20 b	66.46 a	61.51 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Digestible protein content (DP, %)

The results compiled in Table (3) clearly show that the Egyptian clover varietal differences in the content of digestible protein (DP, %) as well as the bio-chemical phosphorus fertilization and lithovit regime impact on digestible protein content were analogous to those of crude protein content previously shown in Table (1) and previously discussed. Also, results of the interaction effect between Egyptian clover varieties and fertilization regimes on DP (%) Tables (3-A; 3-B) behaved in a parallel trend almost like to crude protein content previously presented and discussed (Tables 1-A and 1-B). Analogous findings were ascertained by Seif & Saad (2014) as well as Bakhoum et al. (2016).

Table 3. Varietal differences as well as the bio-chemical phosphorus fertilization and lithovit regimes effect on digestible protein (DP%) of Egyptian clover varieties

Main effects and interactions	DP (%)	
	Combined	
	1st cut	3rd cut
	Egyptian clover variety (V)	
Helaly	20.21 b	16.28 e
Gemmeza 1	19.87 d	16.73 c
Sakha 4	20.96 a	16.57 d
Serw 1	20.05 c	15.85 f
Giza 6	19.72 e	17.28 a
Local	18.25 f	17.22 b
F-test	**	**
	Bio-chemical phosphorus fertilization and lithovit regime (F)	
F_1	20.53 c	15.77 h
F_2	18.79 i	15.51 j
F_3	20.20 d	16.57 g
F_4	18.98 h	16.92 e
F_5	19.08 g	17.93 a
F_6	18.50 j	15.67 i
F_7	19.55 f	16.77 f
F_8	19.69 e	17.10 c
F_9	22.53 a	17.03d
F_{10}	20.60 b	17.29 b
F-test	**	**
Interaction		
V×F	**	**

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Table 3-A. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on digestible protein (DP %) of the first cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	B	I	A	E	G	J	F	H	D	C
	21.66 a	18.55 e	22.69 a	20.25 b	19.55 c	17.83 e	20.07 b	18.84 e	21.20 d	21.50 c
Gemmeza 1	D	E	C	I	J	G	H	B	A	F
	20.25 d	20.09 a	20.49 c	18.77 d	15.86 f	19.25 c	19.07 d	20.65 b	24.61 b	19.66 d
Sakha 4	E	H	G	J	C	F	I	B	A	D
	21.34 c	18.77 c	19.65 d	16.14 f	23.20 a	21.17 a	18.44 f	24.09 a	24.51 c	22.31 b
Serw 1	B	E	J	A	F	H	D	I	C	G
	21.58 b	20.03 b	18.18 f	22.53 a	19.62 b	19.30 b	20.05 c	19.05 d	20.69 e	19.49 e
Giza 6	G	H	F	E	I	J	C	D	A	B
	18.72 f	18.58 d	18.73 e	19.46 c	18.28 d	14.98 f	20.89 a	20.03 c	24.73 a	22.79 a
Local	B	I	A	H	F	E	D	J	C	G
	19.65 e	16.71 f	21.46 b	16.76 e	17.96 e	18.45 d	18.76 e	15.46 f	19.43 f	17.83 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

Table 3-B. Interaction effect between Egyptian clover varieties and bio-chemical phosphorus fertilization and lithovit regimes on digestible protein (DP %) of the third cut

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Helaly	F	I	E	B	C	J	H	D	G	A
	16.28 c	15.13 d	16.45 d	17.71 b	17.05 d	11.81 f	15.64 e	16.60 e	16.10 e	20.07 a
Gemmeza 1	J	B	I	C	A	H	F	D	E	G
	13.70 e	18.25 b	14.65 f	17.98 a	21.86 a	15.62 d	16.34 d	16.65 d	16.41 d	15.78 e
Sakha 4	I	A	B	H	F	C	E	B	G	D
	13.08 f	18.47 a	17.34 a	15.68 f	16.81 e	17.11 c	16.87 c	17.34 c	15.94 f	17.08 d
Serw 1	G	J	E	C	F	B	I	H	D	A
	15.95 d	10.26 f	16.39 e	16.71 d	16.15 f	17.12 b	14.69 f	15.75 f	16.66 c	18.84 b
Giza 6	G	I	E	H	D	J	B	C	A	F
	16.96 b	14.93 e	17.33 b	16.59 e	17.99 b	13.48 e	19.25 a	18.88 a	20.17 a	17.27 c
Local	B	I	F	H	D	A	C	E	G	J
	18.67 a	16.04 c	17.29 c	16.86 c	17.68 c	18.88 a	17.82 b	17.38 b	16.89 b	14.69 f

F_1 control; F_2 chemical P 15.5 kg P_2O_5 fad^{-1}; F_3 bio-fertilizer phosphorien; F_4 50% chemical P + bio (phosphorien); F_5 25% chemical p + bio (phosphorien); F_6 chemical P 15.5 kg P_2O_5 fad^{-1} + lithovit; F_7 Phosphorien + lithovit; F_8 50% chemical P + bio (phosphorien) + lithovit; F_9 25% chemical P + bio (phosphorien) + lithovit; F_{10} Lithovit (foliar).

CONCLUSION

Giza 6 Egyptian berseem variety ranked first in forage quality because of its high content of each CP (%), NFE (%), TDN (%), and DP (%) in the 3rd cut. The local berseem variety ranked the least over the six Egyptian clover varieties with the highest ash (%) in the 1st cut. Other varieties were in between. Allusion to bio-chemical phosphorus fertilization and lithovit regimes, application of any phosphorus fertilization regimes surpassed the control treatment in most nutritive values. Application of F_9 fertilization regime (25% chemical P + bio-fertilizer 'phosphorien' + nano-fertilizer 'lithovit') tended to produce high forage quality with high content of CP (%) in the 1st cut, TDN (%) in both 1st and 3rd cuts as well as DP (%) in the 1st cut. It is worth mentioning that, as crude protein content and total digestible nutrient content (TDN %) increases, forage quality also rises, so, forage quality of the 1st cut tended to outclass that of the 3rd cut in the present study.

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Growth-yield performances of two chilli varieties under different agronomical components applied and their partial economic analysis

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Abstract. The present study had main objective to improve growth-yield performances of two curly red chilli varieties under different agronomical components (ACs) and their partial economic analysis. The research was conducted at Tampirkulon Village, Candimulyo Subdistrict, Magelang Regency, Central Java-Indonesia. The experiment was arranged in completely randomized design with six combination treatments and four replications. Two curly red chilli varieties tested were *Capsicum annum* L. 'Kencana' and 'OR Twist 42'. Three different ACs investigated were (1) Indonesian Vegetable Research Institute (IVegRI) ACs, (2) farmer ACs, and (3) low-agro input and environmental-friendly (Lai-ef) ACs. Results of the study reveal that growth-yield of chilli were significantly improved due to utilization of healthy seedlings, 'OR Twist 42' variety and IVegRI ACs. The seedlings increased number of flowers and fruit plant^{-1} up to 24.9% and 14.2%; 23.6% and 19.5% induced by 'OR Twist 42'; and 54.5% and 25.5% stimulated by IVegRI ACs. Combination of 'OR Twist 42' and IVegRI ACs was the optimal combination treatment in improving growth-yield performances of the curly red chilli with as high as 279.2 fruits per plant, 1,111.4 g chilli productivity fruit plant^{-1}, 167 kg chilli productivity plant^{-1} with 40.6, 39.9, and 33.3% improvement compared to farmer ACs. The combination had Return Cost (R/C) ratio with as high as 2.04. Lai-ef ACs was another interesting result with 1.90 R/C ratio due to low agro-input cost compared to the farmer ACs with as low as 1.64. These results clearly contributed to a better understanding the effect of utilizing healthy seedlings, high adaptive-productive variety and optimal ACs individually or in combination on improving growth-yield of chilli.

Key words: agronomical component, chilli, growth, variety, and yield.

Used abbreviations: ACs – agronomical components; ANOVA-analysis of variance; *CV* – coefficient of variation; DAP – day after planting; IVeGRI – Indonesian Vegetable Research Institute; Lai-ef – low-agro input and environmental-friendly; KCl – potassium chloride; KNO_3 – potassium nitrate; NPK – nitrogen phosphor potassium; SP36 – super phosphate; R/C ratio – Return cost ratio; TSP-triple super phosphate, V1T1, ‘Kencana’ planted using IVegRI ACs; V1T2, ‘Kencana’ cultivated using farmer ACs; V1T3, ‘Kencana’ grown under lai-ef ACs; V2T1, ‘OR Twist 42’ planted using IVegRI ACs; V2T2, ‘OR Twist 42’ cultivated using farmer ACs; V2T3, ‘OR Twist 42’ grown under lai-ef ACs.

INTRODUCTION

Chilli (*Capsicum annum* L.) are strategic and important vegetable commodity in Indonesia due to each alteration of the chilli price affecting the national economy growth. In October 2020 due to rainy season effect, increasing chilli price induced national inflation as high as 0.07% (Pryanka, 2020). The commodity is widely cultivated in all Indonesian provinces with Central Java as main production areas (Statistic Indonesia, 2021a) with 2.6 million tons total production in 2019 (Statistic Indonesia, 2021b). Return Cost ratio (R/C ratio) of the chilli agribusiness varied from 1.93 to 2.83 (Maharti et al., 2019; Astining & Bangun, 2020) with selling product from 4,000 to 60,000 rupiahs in farmer level (Mahardhika, 2020). Though the commodity was important and has high economic value, low productivity is still serious problem faced (Statistic Indonesia, 2021c), especially noted in Central Java province with as low as 6.45 tons ha^{-1} (Statistics of Jawa Tengah Province. 2020). Therefore, improving chilli growth and yield performances shall be addressed.

In chilli cultivation, optimal growth and yield performances of the plants are significantly affected by various factors *viz*, the use of varieties and their seed nurseries (Kesumawati et al., 2019; Syafruddin et al., 2019), plant spacing and density (Sharma & Kumar, 2017; Ansa & Woke, 2018), soil processing and application of basic and supplement fertilizers both organic and inorganic materials (Islam et al., 2018; Mahmud et al., 2019; Valenzuela-García et al., 2019), plastic mulch (Yartawi & Yahumri, 2015), and physical barrier (Salas et al., 2015). Combination of two factors as reported by (Bhuvaneswari et al., 2014; Salas et al., 2015; Yartawi & Yahumri, 2015; Sharma & Kumar, 2017; Kesumawati et al., 2019; Valenzuela-García et al., 2019; Putra et al., 2020) were generally applied to increase the chilli growth and productivity, while combining several factors to establish optimal results are not researched yet previously.

Combining several factors to stimulate maximal growth and yield of chilli and application of low agro-input and environmentally friendly technology or Lai-ef/organic farming were a few reported. Raziliano et al. (2015) reported that application of 12 g plot^{-1} sawdust ash in combination with 100 g plot^{-1} Urea, 200 g plot^{-1} triple super phosphate or TSP and 160 g plot^{-1} potassium chloride or KCl resulted in optimal growth and yield of ‘TM999’ variety as high as 1,180.3 g plot^{-1}. Combination treatment of Lado F1 variety fertilized with 75 kg ha^{-1} urea, 75 kg ha^{-1} super phosphate 36 or SP36, 75 kg ha^{-1} KCl with addition of 1.35 kg compost per planting hole induced the highest results of chilli fruits up to 253.4 g plot^{-1} (Anggraheni et al., 2019). Through the organic farming, ‘Lado F1’ variety gave higher productivity up to 627.9 g plot^{-1} than ‘Perintis’ variety (Syafruddin et al., 2019), ‘Bemeri’ variety had high number of fruits per plant till 8.4 fruits compared to ‘Kopay’ and ‘Perintis’ variety (Kesumawati et al., 2019). Utilization of 3 tons ha^{-1} vermicompost increased productivity of ‘Pusa Jawala’ variety up to 20.1 tons ha^{-1} (Bilal et al., 2019). Using mountain microorganisms as bio-activator in horse manure decomposition and biopesticide successfully improved chilli yield up to 21.5 tons ha^{-1} (Liferdi et al., 2020). While application of IVegRI technology in comparison to farmer and lai-ef ACs which emphasize on utilizing different varieties such as ‘Kencana’ and ‘OR Twist 42’ is not reported yet.

Valuable information’s derived from research on improving growth-yield performances of two churly chilli varieties under different ACs and their partial economic analysis in Central Java Indonesia as main objective were successfully proved. Application of IVegRI and lai-ef ACs for ‘Kencana’ and ‘OR Twist 42’ variety promoted interesting and significant results compared to the farmer ACs in all aspects. The interesting results were discussed in detail in the paper.

MATERIALS AND METHODS

Experimental site and soil condition

The experimental site was in Tampirkulon Village, Candimulyo Subdistrict, Magelang Regency, Central Java Province-Indonesia from March to August 2020. The location altitude was 327 meters above sea level with fluctuated amount of precipitation from 0 to 516 mm per month and 131 mm in average per month; air temperature and humidity were 28–34 °C and 70–95% during the day and 22–26 °C and 47–68% during the night, respectively. Soil characteristics of research farm had loam texture class with 41.06% sand, 41.64% silt, 17.30% clay and 5.85 soil pH. Application of dolomite 2.8 kg m^{-2} was carried out to increase soil pH suitable for chilli growth. The soil characteristics above were used to investigate three different ACs either IVegRI, farmer or lai-ef ACs in combination with utilization of two different varieties of ‘Kencana’ and ‘OR Twist 42’. Different responses in conjunction to find the best treatment derived from the two combination treatments between ACs and varieties expected would be established in the research.

Nursery practice

Healthy seedlings were prepared by immersing seeds of ‘Kencana’ and ‘OR Twist 42’ variety in warm water at 60 °C in closed-jar added by 1 mm L^{-1} propamocarb hydrochloride for 2 hours. After 2 hours, the seeds were air-dried on the paper, then planted in mixture media of fine bamboo soil and organic manure (1:1, v/v) in small plastics watered sufficiently and placed in bamboo trays (± 400 seeds). Each hole was planted with one seed, covered by fine organic manure in 1 cm in thickness, placed in sterile insect proof screen from pests and diseases, covered by plastic net and mulch for 7 days, watered regularly and fertilized after 20 days after planting (DAP) in half dosage. Three weeks after seed sowing, the chilli seedlings were pruned and left 2 cotyledon leaves. After lateral shoots growth with one normal leaf, the seedlings were treated by organic inducer containing pagoda leaf extract to protect against Gemini virus and 5 days later, the seedlings were then planted in the planting beds. While farmer seedlings, the seeds were directly planted in planting medium as previously mention without any treatments and maintained under plastic screen by watering and fertilizing till the seedlings ready planted in the similar old as healthy seedlings.

Land and planting bed preparation

Whole experimental area was cleared from rest of rice straw and grasses using lawn mower. The cut rice straw and grasses were moved from the experimental area. The experimental land was then tilled using hand tractor and left for 2 weeks. After two weeks, the research farm was processed and fertilized manually as each ACs tested using hoes. In application of fertilizers, all fertilizers in each ACs were homogeny mixed maximally, then poured evenly in two long holes with 50 cm distance between them in each plant bed. Farmer ACs beds with 1×16 m in size and 1×12 m beds for IVegRI and lai-ef ACs were then constructed. In farmer ACs, each plot consisted of 6 beds and 4 beds with bed borders for IVegRI and lai-ef ACs. Distance between beds was 50 cm, 100 cm between experimental plots and 200 cm between replications.

Preparing plant border

Plant border used for IVegRI and lai-ef ACs was corn plants. The border was prepared by culturing corn seeds with 20×20 cm planting distance in zig-zag model. The seeds were planted in 2 cm in depth. Each hole was put one seed then covered again using media. During the experiment, the plant border was cultured twice. Meanwhile, in farmer ACs did not use the plant borders.

Planting chilli seedlings

In farmer ACs with 50×50 cm in plant spacing, each hole with ± 5 cm in depth and diameter put one seedling and covered again by media was applied. While for IVegRI and lai-ef ACs, 50×70 cm in plant spacing was utilized. In 50 cm the plant spacing, one hole one seedling was practiced, but in 70 cm spacing, one wider hole with two seedlings cultured was applied.

Plant maintenance

The chilli plants were watered regularly and sufficiently. Regular weed clearing and pest-disease controlling were carried out regularly. For farmer ACs, pests and diseases attacking chilli plants were totally controlled using synthetic pesticides; 50% synthetic pesticides; 50% biopesticides for the IVegRI ACs; and 100% biopesticides for lai-ef ACs.

Experimental design and treatments

The experiment was arranged in completely randomized design with six combinations of two curly red chilli varieties and three ACs and four replications. The chilli varieties i.e., (1) *C. annum* 'Kencana' (V1) and (2) 'OR Twist 42' (V2) and three ACs (1) IVegRI ACs (T1), (2) farmer ACs (T2) and (3) Lai-ef ACs (T3) were used in the study (Table 1).

Experiment variables

The variables observed in the experiment were (1) plant height (cm); measured from base of stem till growth point (2) main branching height of plant (cm); quantified from the base of the stem till main branching position determined, (3) stem diameter (mm); calculated in the base of the stem using digital caliper, (4) canopy width (cm); assessed in the middle position of canopy, (5) number of flowers plant^{-1}, (6) weight of fruit fruit^{-1} (g); weighed using digital balance, (7) yield of chilli plant^{-1} (g), (8) chilli productivity plant^{-1} (g); counted from average of fruit weight timed by number of fruits harvested plant^{-1}, (9) chilli productivity plot^{-1} (kg); calculated by multiplying average productivity plant^{-1} with total number of plants plot^{-1}. Periodical observation was conducted to know growth responses of chilli plants starting from initial planting till final harvest.

Table 1. Different agro-inputs of the three ACs tested in the research

No.	Agro-input	IVegRI ACs (T1)	Farmer ACs (T2)	Lai-ef ACs (T3)
1.	Type of seedling	- Healthy seedlings	- Farmer seedlings	- Healthy seedlings
2.	Plant population	- 30,000 plants ha^{-1}	- 20,000 plants ha^{-1}	- 30,000 plants ha^{-1}
3.	Plant spacing	- 50×70 cm	- 50×50 cm	- 50×70 cm
4.	Basic fertilizers	- 20 tons ha^{-1} of fermented-chicken manure, 200 g ha^{-1} Bionutrient, 500 tons ha^{-1} NPK 16:16:16, 200 tons ha^{-1} ZA, and 0.5 kg per 100 liter humic acid	- 12.5 tons ha^{-1} of fermented chicken manure, 750 black NPK, 750 TSP/SP 36	- 20 of fermented-chicken manure, 200 g ha^{-1} Agrimeth, 20 kg ha^{-1} Gliocompost, and 10 mL L^{-1} per m^2 PGPR
5.	Supplement fertilizer	- 0.5 kg humid acid; dissolved in 100 liters of water; applied 200 mL $plant^{-1}$ in 20 days after planting (DAP) - 1 kg NPK 16:16:16; dissolved in 100 liters of water; applied 200 mL $plant^{-1}$ every 10 days periodically after 1 month of planting till final harvest times - 0.5 kg red KNO3; dissolved in 100 liters of water; applied 200 mL $plant^{-1}$ twice at 30 and 50 DAP - 0.5 kg red KNO3; dissolved in 100 liters of water; applied 200 mL $plant^{-1}$ twice at 60 and 80 DAP	- 1 kg NPK Maroke and 1 kg white KNO_3; dissolved in 200 liters of water; applied 200 mL $plant^{-1}$ every 10 days periodically till final harvest period	- 100 mL PGPR, 2 kg Gliocompost, 0.5 kg humid acid; dissolved in 100 liters of water; applied 200 mL $plant^{-1}$ every 15 days till final harvest period. - 100 mL Bionutri-V; dissolved in 100 liters; applied 200 mL $plant^{-1}$ every 10 days till initial fruit formation. In the next step 100 mL Bionutri-V, 100 mL Bionutri-G; dissolved in 100 liters; applied 200 mL $plant^{-1}$ every 10 days till harvest period
6.	Plastic mulch	- Plastic mulch applied	- No plastic much utilized	- Plastic mulch applied
7.	Physical Border	- Physical border applied	- No physical border applied	- Physical border utilized

Taking and measuring all variables were carried out regularly following the growth and development of chilli plants. Furthermore, to determine farming efficiency derived from the effect of varieties and ACs in terms of revenue and production costs, return cost (R/C) ratio was calculated as described by Rambe et al. (2021). R/C ratio = Total Revenue (TR)/Total Cost (TC). If the R/C ratio is > 1, the farming system is said to be efficient; if the R/C ratio = 1, then the farm experiences is at the break-even point status; and if the R/C ratio < 1, then the farming system is inefficient. The production costs were calculated by compilating all factors affecting the costs such as seeds, fertilizers, pesticides, labor costs, etc.

Data analysis

Quantitative data in the experiment were analyzed by analysis of variance (ANOVA) using SAS Release Windows 9.1 (SAS Institute, Cary, NC). Significant differences between means were assessed by Tukey test, $P = 0.05$ (Mattjik & Sumertajaya, 2006).

RESULTS AND DISCUSSION

Based on periodical observations it was revealed that initial flowering was generally noted ± 22 DAP when the chilli plants had 24.9–34.9 cm in height and ± 28.2 cm in width of plant canopy. Initial fruits were recorded 42 DAP when the chilli plants had 61.6–66.2 cm in height and ± 32.5–38.9 cm in width of plant canopy. Pick flower number/plant was observed on 65–72 DAP and 79 DAP for fruit number produced plant^{-1}.

Utilization of the healthy seedlings derived from 'Kencana' and 'OR Twist 42', in fact, had significant improvement on growth-yield of chili. The significant improvement was noted based on number of flowers plant^{-1} (Fig. 1, A) and number of fruits plant^{-1} (Fig. 1, B) compared to farmer seedlings. The seedlings increased number of flowers per plant up to 24.9% and 14.2% for number of fruits plant^{-1}. Furthermore, utilization of 'OR Twist 42' variety had higher and better results compared to 'Kencana' variety with higher number of flowers plant^{-1} up to 23.6% (Fig. 1, C) and 19.5% number of fruits plant^{-1} (Fig. 1, D) than 'Kencana' variety. While IVegRI ACs increased 54.5% number of chilli flowers plant^{-1} (Fig. 1, E) and 25.5% for number of fruits plant^{-1} (Fig. 1, F). From the research it was determined that growth and yield performances of the two chilli varieties tested were significantly influenced by utilization of seedling types, varieties and ACs applied.

Optimal combination treatments give significant effect on growth-yield performances of chillies. 'OR Twist 42' planted using IVegRI ACs were the most appropriate combination in finding the optimal results. The combination was able to stimulate the growth of chilli plants up to 120 cm in height with 14.6 mm stem diameter; 88 cm plant canopy width; 400.8 number of flowers plant^{-1}; 3.98 g fruit weight fruit^{-1}; 279.2 number of fruits plant^{-1}; 1,111.4 g of fruit productivity per plant and 167 kg of chilli productivity plot^{-1} (Table 2; Fig. 2, B). The combination treatment was also able to increase number of fruits per plant up to 40.6%; 36.7% fruit productivity per plant and 26% chilli productivity plot^{-1} compared to others (Fig. 2, A). The second-best combination was shown by the 'OR Twist 42' cultivated using the farmer ACs (Fig. 2, D). Furthermore, the 'Kencana' variety, which was grown using the IVegRI

ACs, was not able to increase the productivity of chillies. In the 'Kencana' variety, the varieties planted with the farmer ACs gave the best results compared to other combinations (Fig. 4, A and 2, C).

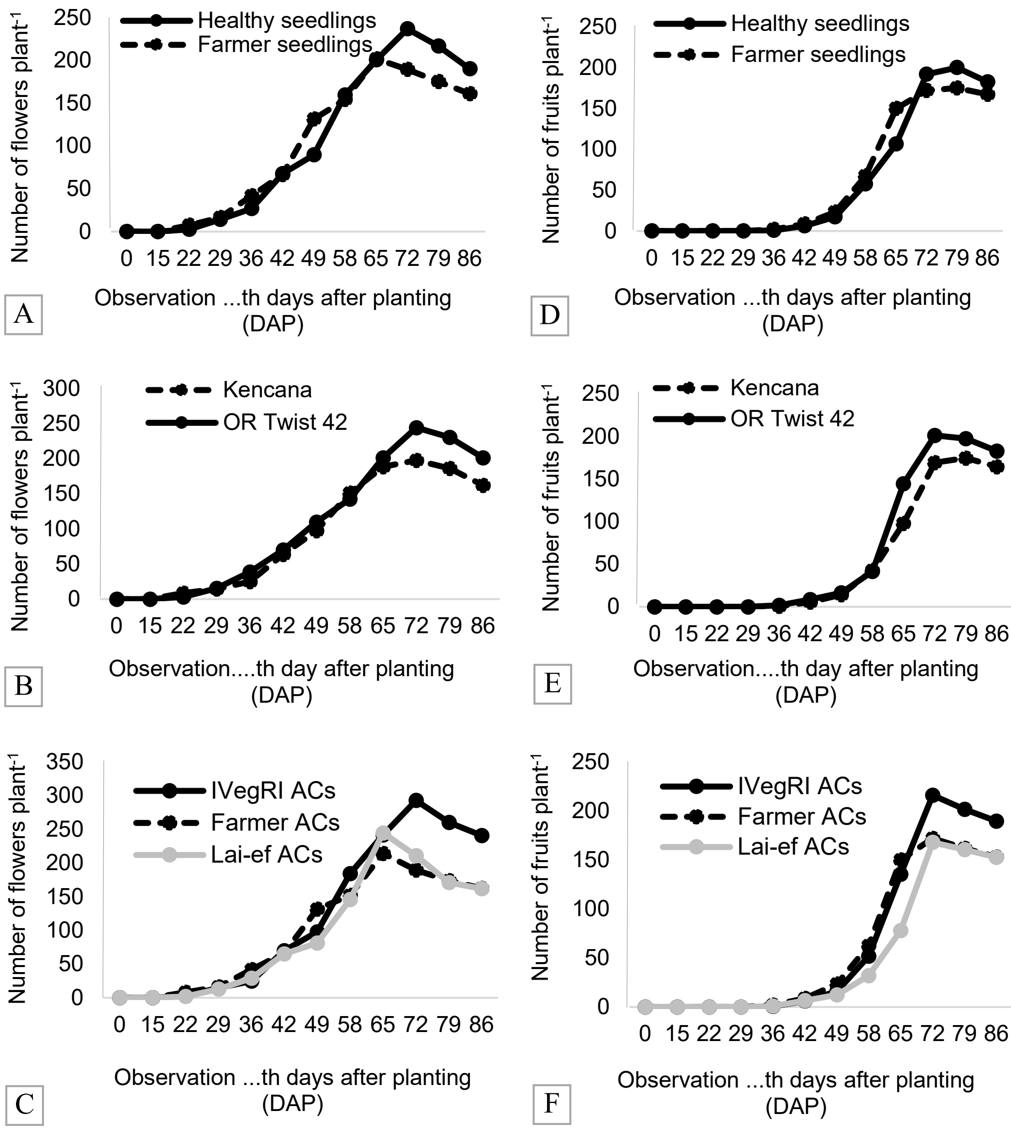

Figure 1. The effect of seedlings, varieties and ACs on growth-yield performances of curly chillies from initial planting till 86 DAP. Gradual alteration of flower number plant^{-1} affected by the seedlings (A), varieties (B) and ACs (C). Gradual changing fruit number plant^{-1} affected by the seedlings (D), varieties (E) and ACs (F).

Table 2. The effect of combination treatment of varieties (V) and ACs (T) on growth-yield performances of curly red chillies

Combination of Treatment	Plant height (cm)	Main branching height (cm)	Stem diameter (mm)	Plant canopy width (cm)	Number of flowers plant^{-1}	Weight of fruit per fruit (g)	Number of fruits plant^{-1}	Fruit productivity plant^{-1} (g)	Chilli productivity plot^{-1} (kg)
V1T1	87.9 bcd	2.9 c	12.9 ab	71.2 bcd	290.5 ab	3.01 b	200.4 b	610.4 bc	80.1 c
V1T2	73.9 d	34.2 b	12.3 b	62.2 d	287.6 ab	3.46 ab	185.8 b	640.6 bc	94.4 c
V1T3	85.8 cd	3.0 c	11.4 b	65.0 cd	224.3 b	2.95 b	158.8 b	468.2 c	56.2 d
V2T1	120.0 a	3,4 c	14.6 a	88.0 a	400.8 a	3.98 a	279.2 a	1,111.4 a	167.0 a
V2T2	95.5 bc	40.9 a	12.4 b	79.6 ab	279.7 ab	4.00 a	198.6 b	794.4 b	125.3 b
V2T3	104.6 ab	3.6 c	11.9 b	74.6 bc	230.1 b	3.76 a	170.4 b	642.0 bc	85.2 c
Coefficient of variation (*CV*, %)	6.92	11, 12	5.16	4.95	17.48	7.36	9.04	11.04	10.67

Means followed by the same letter in the same column are no significant difference based on Tukey's test, $P = 0.05$. V1T1, 'Kencana' planted using IVegRI ACs; V1T2, 'Kencana' cultivated using farmer ACs; V1T3, 'Kencana' grown under lai-ef ACs; V2T1, 'OR Twist 42' planted using IVegRI ACs; V2T2, 'OR Twist 42' cultivated using farmer ACs; V2T3, 'OR Twist 42' grown under lai-ef ACs.

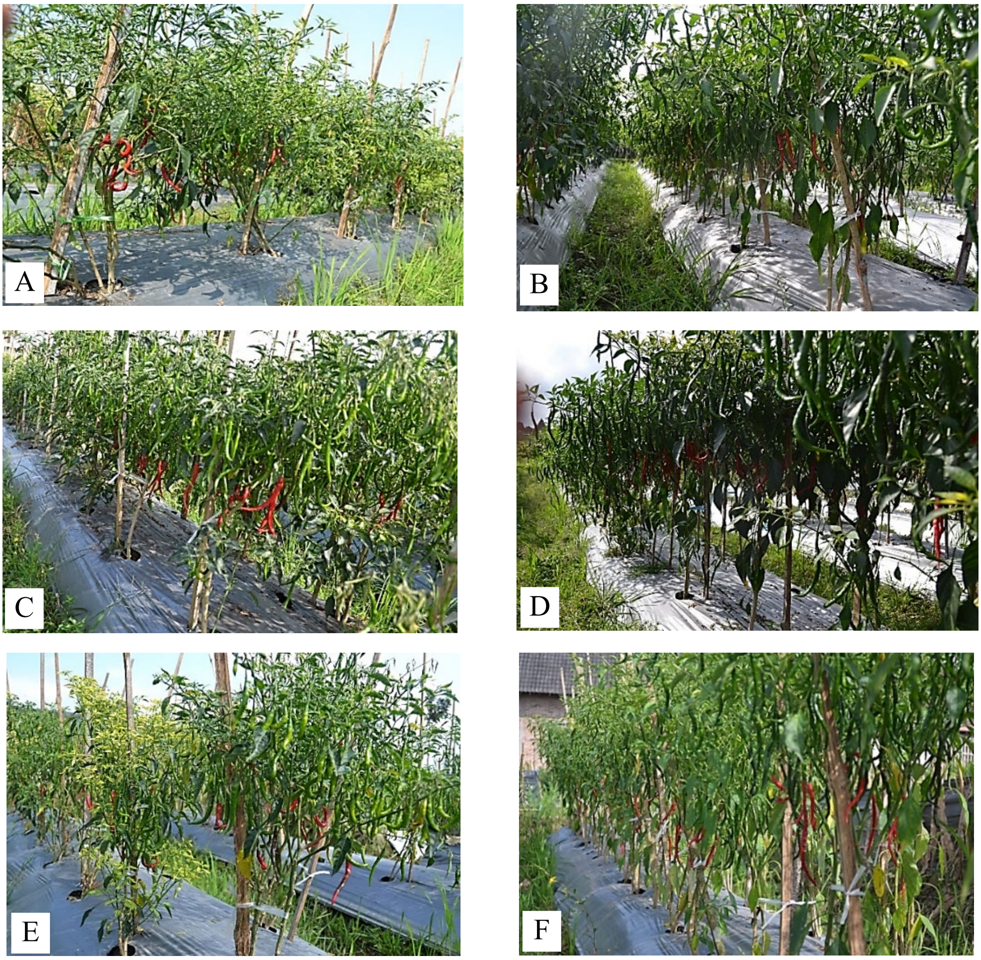

Figure 2. Performances of two curly chilli varieties of cultivated under different ACs. A. 'Kencana' planted using IVegRI ACs, B. 'OR Twist 42' planted using IVegRI ACs, C. 'Kencana' cultivated using farmer ACs, D. 'OR Twist 42' cultivated using farmer ACs, E. 'Kencana' grown under lai-ef ACs and F. 'OR Twist 42' grown under lai-ef ACs.

During harvest periods for 21 times, selling price of chillies kg^{-1} was fluctuated from Rp. 7,500 to 25,000 kg^{-1} with Rp. 15,700 kg^{-1} in average. Furthermore, under simple economic analysis to determine farming efficiency based on total revenue and production costs, most of combination treatments generally were in efficient status with the R/C ratios varied from 0.97 to 2.04 (Table 3). From the 2 varieties used, the 'OR Twist 42' variety showed higher R/C ratio than 'Kencana' in all combinations. From the 6 treatment combinations, 'OR Twist 42' planted under IVegRI ACs had the highest R/C ratio up to 2.04 with increasing percentage of farmer income up to 24.4%. The second-best result with 1.90 was actually exhibited by the 'OR Twist 42' cultivated using lai-ef ACs. These results provided evidence that though lai-ef ACs did not give significant effect on growth-yield performances of chilli, due to low agro-input cost, the ACs was able to provide better benefits than the farmer ACs with improving farmer income up to 15.9%.

Table 3. Simple economic analysis of the effect of 6 treatment combinations on the R/C ratio

Combination of treatment	Total production cost $plot^{-1*}$ (Rp)	Total chilli production $plot^{-1}$ (kg)	Total revenue $plot^{-1}$ (Rp)	R/C ratio
V1T1	1,286,511.3	80.1	1,256,000	0.97
V1T2	1,199,843.3	94.4	1,482,080	1.24
V1T3	726,549.2	56.2	882,340	1.21
V2T1	1,286,511.3	167.0	2,621,900	2.04
V2T2	1,199,843.3	125.3	1,967,210	1.64
V2T3	726,549.2	85.2	1,337,640	1.90

*Note: each plot had size 16 m in length and 8 m in width equal with 128 m^2.

Overall from the study, it was successfully explored that optimal growth-yield performances of chilli were significantly affected by utilization of healthy seedling, productive-adaptive variety, suitable cultivation ACs and their combinations. Under the observation, utilization of healthy seedlings of 'Kencana' and 'OR Twist 42' improved number of flowers $plant^{-1}$ up to 236.8 flowers (24.9%) (Fig. 1, A) and number of fruits $plant^{-1}$ up to 200.1 fruits (14.2%) (Fig. 1, B) compared to farmer seedlings. In other studies, the healthy seedlings of 'TM 999' variety stimulated 124.5 fruits per plant and increased number of fruits up to 21.8% compared to normal seedlings (Yuda et al., 2018), 228.5 flowers $plant^{-1}$ and 124.5 fruits $plant^{-1}$ of 'Kastilo' variety (Setiawati et al., 2018); and 101.8 fruits per plant with 18.6% improvement compared to farmer method (Atman et al., 2021). The above results confirmed that application of healthy seedlings improved chilli productivity significantly.

High adaptivity and productivity of chilli varieties are one of important factors usually applied in establishing optimal growth-yield performances of chilli. From the study it was clearly revealed that 'OR Twist 42' variety enhanced number of flowers $plant^{-1}$ up to 23.6% (244.3 flowers) with 19.5% fruits $plant^{-1}$ (201.2 fruits) than 'Kencana' with 197.7 flowers and 174.7 fruits, respectively. In other studies, 'Kajoli' variety was the most appropriate variety compared to 'Vadaria'>'Magura'>'Bogra Morich' variety with 265.5 fruits $plant^{-1}$ (Chowdhury et al., 2015), 327 fruits $plant^{-1}$ of 'Kajoli' than 'Akashi'>'Deshi Kacha Morich'>'Bogra Morich'>'Dongfou' variety with 8–200% improvement (Chowdhury et al., 2020). 'Lado' variety had high productivity up to 198 fruits per plant and 11.9% enhancing compared to 'Perintis' variety (Syafruddin et al., 2019). 'High Fly' variety produced high number fruits per plant up to 52.5 flowers and 184.6 g $plant^{-1}$ than 'Magma' variety with 10.7% increment (Jan et al., 2020). These results proved that utilization of suitable variety gave high effect on chilli productivity established.

Suitable cultivation ACs had closely relation to quality and productivity of chillies. IVegRI ACs with several components (Table 2) increased number of chilli flowers per plant up to 54.5% and 25.5% for number of fruits per plant with 292.7 flowers $plant^{-1}$ and 216 fruits $plant^{-1}$, respectively. In different studies, 25 farm yard manure in combination to 300 kg ha^{-1} urea, 60 kg ha^{-1} single super phosphate and 120 kg ha^{-1} muriate of potash induced high number of fruits $plant^{-1}$ up to 94 fruits and 600 g $plant^{-1}$ fresh fruit yield for 'Wgl Chapata' with 54.1% and 81% increasing, respectively (Srinivas et al., 2017). 10 ml L^{-1} biofertilizer, 1.5 kg ha^{-1} magnesium and 1.5 kg ha^{-1} boron improved productivity of chilli up to 54.5% compared to other treatments (Setiawati et al., 2020). 0.05% copper with standard N, P, K concentrations added treatment stimulated high number of pots per plant up to 47 pots, 109 g plant^{-1} yield with 25% yield increment compared to control treatment (Thennakoon et al., 2020). From these results, it was proved that suitable ACs applied in chilli cultivation leading to optimal plant productivity achieved.

Suitable combination treatments generally stimulate optimal growth and yield performances of plant. In the research, 'OR Twist 42' variety cultivated under IVegRI ACs induced number of fruits plant^{-1} as high as 279.2 fruits with 1,111.4 g of fruit productivity plant^{-1} and 167 kg of chilli productivity plot^{-1} with 40.6, 36.7 and 26% improvement, respectively. In different researches, combined application of 100 kg ha^{-1} of phosphorous and120 kg ha^{-1} of potassium for exotic land race of chilli was the optimal combination in producing 90.8 flowers plant^{-1}, 30.2 fruits per plant , and 465.5 g fruit plant^{-1} with 114.6%, 115.5% and 94.4% of improvement, respectively (Akram et al., 2017). Combined application of 135 kg ha^{-1} urea with 4.5 cow dung for 'California' variety showed significant increase in number of fruits plant^{-1} up to 16.6 fruits with 142.1 g fruit weight, and 38.5 kg replicate^{-1} with 88.6%, 22.2% and 80.8% of increasing percentage of them, respectively (Islam et al., 2017). Combination treatment of 'Lado F1' variety fertilized with urea 75 kg ha^{-1}, SP36 75 kg ha^{-1}, KCl 75 kg ha^{-1} with addition of 1.35 kg compost planting per hole resulted in high chilli productivity up to 253.35 g plant^{-1} (Anggraheni et al., 2019).

Under efficient farming, higher R/C ratio achieved, higher profit established. Combined application of organic and inorganic fertilizers (NPK, Urea, ZA, KCl) for hot chilli established R/C ratio up to 2.03 (Ardian et al., 2017). Application of organic chicken manure in combination with the inorganic fertilizers for 'Hot Beauty' variety resulted 3.05 R/C ratio (Iwan et al., 2017), 2.26 R/C ratio for 'Helix' variety (Sutardi & Wirasti, 2017). Combined utilization of basic fertilizers of 400 kg ha^{-1} of urea, 200 kg ha^{-1} of SP-36, 250 kg ha^{-1} of KCl applied to the soil and 0.5 kg ha^{-1} HF foliar fertilizer for 'TM999' variety had R/C ratio as high as 1.95 (Sugiyanta et al., 2018). Though utilization of local variety cultivated under the IVegRI ACs resulted in high R/C ratio up to 3.27 (Rambe et al., 2021). In the study, 'OR Twist 42' variety in combination with IVegRI ACs had R/C ratio up to 2.04 with 24.4% farmer profit improvement. These results clearly revealed that the all combination treatments induced efficient farming with high R/C ratio.

Interesting result was determined in the study due application of lai-ef ACs in combination with utilization of 'OR Twist 42' variety. Due to low agro input practiced, the combination stimulated higher R/C ratio as high as 1.90 than the farmer ACs combined with the 'OR Twist 42' variety that had R/C ratio as low as 1.64. High R/C ratio due to application of low agro input that generally used organic materials was also noted on curly red chilli with 2.19 (Qomariah & Pramudyani, 2014), hot chilli with 2.45 (Lestari et al., 2018), 'TM999' variety with 1.95 (Sugiyanto et al., 2018). From the information it was clear that though application of low agro input for chilli did not produce high productivity on chilli, the ACs generally regenerated higher R/C ratio compared to the farmer ACs with 15.9% farmer profit increasing.

CONCLUSIONS

The outstanding improvement of growth-yield of chilli was established derived from the study. Firstly, utilization of healthy seedlings actually improved growth-yield of chilli in all variables observed. The results confirmed that application of healthy seedlings significantly improved chilli growth and productivity. Secondly, high adaptivity and productivity of chilli varieties, high chilli growth-yield established. In the study 'OR Twist 42' was better than 'Kencana' varieties. These results proved that utilization of suitable variety gave high effect on chilli productivity. Thirdly, high suitability ACs in chilli cultivation led to optimal growth-yield of chilli that was also established with IVegRI ACs in the study. Suitable ACs applied in chilli cultivation led to optimal plant productivity achieved. Fourthly, the best combination treatment to stimulate optimal growth and yield performances of chilli was also established in the study by planting 'OR Twist 42' under IvegRI ACs. The evident proved that suitable combination treatment led to growth-yield improvement of chilli. Finally, high efficient farming under optimal combination treatment brought higher R/C ratio up to 2.04 due to maximal growth and high productivity compared to other combinations. While higher R/C ratio up to 1.90 derived from 'OR Twist 42' variety cultivated under lai-ef ACs due to low agro-input used in the combination than farmer ACs. The data determined that high R/C ratio was established due to maximal growth and productivity and low agro-input used. Summarizing, these data contribute to a better understanding the effect of the seedling types, chilli varieties, ACs and their suitable combination treatment utilized and applied for the optimal growth-yield of chilli was clearly established in the study by utilization of healthy seedlings of 'OR Twist 42' variety cultivated under IVegRI ACs. The suitable combination treatment led to high chilli productivity and profit.

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Location specific field performance of aman rice cultivars in tidal flood prone ecosystem of Bangladesh

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Abstract. Cultivation of modern high yielding rice varieties are ecologically limited in a tidal flood (TF) prone area of Bangladesh. Therefore, rice growers are cultivating local rice cultivars that resist tidal water pressure and survive under waterlogged condition in a tidal ecosystem. A farmers' participatory field experiment was conducted at Wazirpur, Bakergonj and Babugonj upazila of Barisal and Nalchity upazila under Jhalokati districts of Bangladesh to identify location specifies some aman rice cultivars that resist TF and give higher grain yield. There were fifty-sixty local rice cultivars along with five modern rice varieties were included in this trial. The experimental sites were extensively TF prone and 10 to 80 cm of flood water entered into the crop field during active tillering to flowering stage of rice. Data on plant height, yield and yield components were recorded. The results revealed that local rice cultivars performed better than modern one in the experimental sites. Taller plant, production of more panicles per unit area, higher number of grains panicle^{-1} and heavier grains were the most important traits associated with plant adaptation of aman rice cultivars in tidal areas. Collectively, this study suggested that Dudmona1 and Khoiyamota1 at Wazirpur (2.98 to 3.10 t ha^{-1}); Dishari1 and Sadamota2 at Bakergonj (2.92 to 2.98 t ha^{-1}); Shorna at Babugonj (3.56 t ha^{-1}); Moulata2, Achin and Sadamota2 at Nalchity (2.96 to 2.98 t ha^{-1}) were most promising rice cultivar in terms of adaptation and grain yield.

Key words: local rice cultivars, lodging, low yield, tidal flood, water pressure.

INTRODUCTION

Rice (*Oryza sativa* L.) is grown in a wide range of ecosystem and is considered one of the most important cereals for global food security, especially in Asia (GRiSP, 2013). Rice is staple food for about half of the world population (Worldmeters, 2020; UN, 2020).

Rice is cultivated throughout the year in Bangladesh and 80% of the total crop land of the country is occupied by rice. It is grown in three seasons namely aus (March to June), aman (July to November) and boro season (November to April). The total production of rice was 36.40 million tons grown in 2019 (BBS, 2019). However, the average yield of rice is 4.5 t ha^{-1}in Bangladesh, whereas it is 6.0 to 6.5 t ha^{-1} in China, Korea and Japan (BRRI, 2020). Drought, salinity, inset and pest, cold and submergence cause an enormous loss each year worldwide due to reduction in crop productivity and crop failure.

The seedlings of boro rice face cold injury during their early growing period during the month of December (BRRI, 2020). Therefore, growth of seedling reduces and becomes yellowing in color (Talukder et al., 2022). Aus season is the hottest time of the country and plants experiences high temperature during the growing period. Aman season is the rainy season and submergence causes' severe damage of the aman rice. The submergence may occur for a day to as long as few weeks (Mamun et al., 2021). The submergence problem is very common in aman season in norther region of Bangladesh. Bangladeshi scientists have developed some submergence tolerant rice varieties like BRRI dhan51 and BRRI dhan52 (BRRI, 2020). Islam et al. (2019) and Abedin et al. (2019) also reported that BRRI dhan33, BRRI dhan56 and BRRI dhan57 gave satisfactory yield after submergence.

The aman rice of southern region of Bangladesh experiences tidal flood (TF) submergence every year. About 2.0 m ha of land in Barishal, Patuakhali and Jhalokati districts of Bangladesh is TF prone where 10–80 cm of surge water enters twice a day in cropland from April to November every year (Roy et al., 2003; Brammer, 2014). Ecologically these areas are unfavorable and low productive because farmers can little scope of using modern improved rice varieties and management practices. Thus, the hydrology of these areas influences the cropping system, agricultural production, and farmer's socio-economic condition. Aman rice is the main crop in these areas and farmers cultivate local cultivars such as Sadamota, Lalmota, Rajashail, Kutiagni, Lalpayka, Mutha, Lothor, Lalchikon, Sadachikon, Moulata and Sadapajam, etc (Abedin et al., 2015). These cultivars are locally popular because they are tall, long duration, photoperiod sensitive and resistant to TF. Though the yield of these cultivars is low, but they perform better than modern rice varieties (Hamid et al., 2015). Usually, farmers transplant taller (60 to 70 cm) and older rice seedlings (50 to 60 days) maintaining wider spacing (40×40 cm) in TF-prone areas. These are not recommended practices for rice cultivation. On the other hand, nitrogen (N) fertilization is often impossible because of surface N losses to floodwater (Mamun et al., 2018). But the local cultivars were found responsive to the added N and have potentiality to give better yield under upland condition (Mamun et al., 2020).

The native N content of the soils of tidal floodplain soils are low (Mamun et al., 2016) and earlier studies concluded that the yield of local rice increases with urea deep placement due to absorption of higher amount of N by grains (Mamun et al., 2017a; Mamun et al., 2017b). Therefore, developing suitable high-yielding rice varieties for the TF ecosystem is essential but it requires much time through breeding process. Therefore, identification of location-specific suitable high-yielding rice cultivars might be an immediate solution for rice growers in TF-prone areas. The selection and adoption of the cultivars will improve farm productivity, farmer's income and ensure food security of tidal areas in Bangladesh.

MATERIAL AND METHODS

Experimental Site

The experimental site was located in the south coastal region of Bangladesh under Ganges Tidal Floodplain (Agro-Ecological Zone-13). The experiment was farmers' participatory and the sites included Wazirpur, Bakergonj and Babugonj of Barisal and Nalchity, Jhalokati districts. This region occupies an extensive area of tidal floodplain land in the south-west of the country. There is a general pattern of grey, slightly calcareous, heavy soils on river banks and grey to dark grey, noncalcareous, heavy silty clays in the extensive basins. Noncalcareous Grey Floodplain soil is the major component of General Soil Types.

In general, most of the top soils are acidic and subsoils are neutral to mildly alkaline. The experiment was conducted on July to December, 2021 called aman season in Bangladesh.

Planting Materials

Sixty-one (61) rice cultivars were included in this experiment (28 at Wazirpur, 11 from Nalchity, 11 Bakergonj and 11 from Babugonj). Among the cultivars 56 were local and 5 were modern. The cultivars at Wazirpur, Barisal were Dudmona1, Kutiagni1, Motadhan1, Lalpayka1, Jalkucha, Rajashail, Sadamota1, Lalmota1, Lalchikon, Lothor, Sada pajam, Sadachikon, Kalagura, Joina, BR25, Kajla, Balam1, Chikondhan, Haludmota1, Mothamota1, Moyna, Sonashail, Boleshormota, Kalizira, Kalomota, Moulata3, Khoiyamota1 and Kachamota. Similarly, the cultivars at Nalchity, Jhalokati were Khoiya, Moulata2, Aochin, Nakochimota2, Lalmota3, Dishari2, Dudkolom2, Sadamota2, BRRI dhan44, Haudmota2, Balam2. However, the cultivars at Bakergonj, Barisal were Dudkolom1, Motadhan2, Moulata1, Dishari1, Sadamota4, Nakochimota1, Lalmota4, Lalpayka2, Kutiagni2, Mothamota2 and Khoiyamota2. Furthermore, the cultivars at Babugonj, Barisal were Bhushiara, Chinigura, BRRI dhan76, Lalmota2, Dudmona2, BRRI dhan87, Shorna, Sadamota3, Shakhorkora, BRRI dhan41 and Moulata4.

Crop culture and management

The selected farmers were sown their own seeds in the seed bed. About 30-day-old seedlings were transplanted from first week of August to last week of September in 2021. About 3–4 seedlings hill^{-1} were transplanted maintaining 40 cm × 40 cm plant spacing. A randomized complete block design was followed for the experimentation. The size of each plot was 400 m^2. The number of farmers grown each cultivar is considered as replication and the number replications were 4. No fertilizer was applied to plots. One hand weeding was done at 20 days after transplanting to remove some aquatic weeds. The height of tidal water that entered into the crop field measured every day.

Data Collection

Water depth in the rice field, days to flowering (DF), days to maturity (DM), plant height (cm) (Ph), number of panicles m^{-2} (PPM), number of filled grains panicle^{-1} (GPP), 1,000-grain weight (g) (TGW) and grain yield (t ha^{-1}) (GY) were recorded. Yield of rice grain was determined by harvesting 5.0 m^2 areas of the middle of each plot. The grains were sun dried and winnowed before weighing and converted into t ha^{-1}. The grain yield was adjusted to 14% moisture content using the following formula:

$$\text{Adjusted weight} = \frac{W \cdot (100 - M_1)}{(100 - M_2)} \cdot 100$$

where, W, M_1 and M_2 were fresh weight, fresh and adjusted moisture percent of the grain, respectively.

Data Analysis

The data were analyzed using computer software CropStat 7.2 and the graphs were prepared using excel program. Descriptive statistics and multiple linear regression were performed for the collected data. Categorization of rice cultivars based on DM, Ph, TGW and GY. Correlation analysis, dendrogram, principal component analysis was done. Correlation, dendrogram, and principal component analysis were performed.

RESULTS AND DISCUSSION

Water depth in the crop field during crop growing period

Flood water entered into the crop field during the crop growing period. So that the crop faced challenges of tidal surge during their life cycle. At Wazirpur, the height of tidal water ranged from 0 to 68 cm. The highest water depth was measured during the month of September at Wazirpur. Similarly, the water depth in the rice field varied from 0 to 85 cm at Babugonj, 0 to 65 cm at Nalchity and 0 to 70 cm at Bakergonj upazila (Fig. 1). In all sites, the highest water depth was recorded during the month of 07 to 15 September. After September, the pressure of tidal water decreased gradually (Mamun et al., 2020). The experimental sites were near to sea and influenced by tides. These areas cover 32% of land area of Bangladesh with an average elevation between 3 and 6 m (MoWR, 2005). Lands were subjected to seasonal flooding and remain inundated July to October. High tide followed by low tide twice a day is the characteristics feature of the experimental sites (Roy et al., 2003; Brammer, 2004; Hamid et al., 2015).

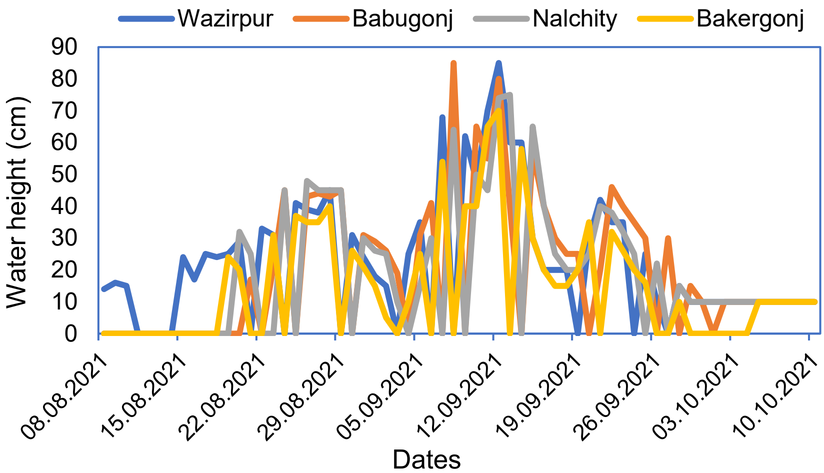


Figure 1. Water depth in the rice field during growing period.

Difference in morphology, phenology and yield

The rice plants needed 92 to 122 days for flowering, while 120 to 155 days for maturity (Table 1 and Fig. 2). The time for flowering and maturity were 108 and 141 days, respectively. The plant height of the rice cultivars varied from 120 to 178 cm with an average 143 cm. There was huge variation in panicle production in the testing rice material. The maximum number of panicles produced by the cultivars was 165 m^{-2}, while minimum was 75 m^{-2}. The number of grains ranged from 54 to 134 $panicle^{-1}$, while average was 90 (Table 1 and Fig. 2). The grain size (1,000-grain weight) of the testing cultivars ranged from 13.06 to 34.53 g (Table 1 and Fig. 2). The maximum grain yield was 3.56 t ha^{-1}, while minimum was 1.28 t ha^{-1}. The average grain yield was 2.51 t ha^{-1} (Table 1 and Fig. 2). Tiller production is an important trait for yield formation in rice. Large variation in tillering capacity among varieties has been reported (Mamun et al., 2020; Nuruzzaman et al., 2000). Indigenous variety Sadamota produced large number of tillers $hill^{-1}$ as reported by Hamid et al. (2015). Altering tiller production due to variation in agronomic practices has been reported earlier (Mamun et al., 2017a; Huang et al., 2011). Under field condition, the yield of local rice cultivar is relatively low (Senthilkumar et al., 2020). The genetic potentiality of cultivars may also have been deteriorated in the farmers' field for multiple reasons including out-crossing, admixtures of different cultivars and genetic erosion or mutation (Parlevliet, 2007). In addition, lack of proper management practices results in low yield of any cultivars (Liliane & Charles, 2020). The yield in local rice was mostly contributed by tiller $hill^{-1}$, filled grains and test weight. In many studies, similar plant characters including tillers production capacity, grains in a panicle and their weight were reported to be the major factors contributing to the yield (Sabri et al., 2020; Huang et al., 2020; Oladosu et al., 2018).

Table 1. Variations in the yield and yield contributing characters of locally popular landrace T. Aman rice cultivars in tidal flood prone region

Traits	Mean	Maximum	Minimum	*CV* (%)
Days to flowering	108.80	122.00	92.00	8.09
Days to maturity	141.72	155.00	120.00	8.28
Plant height (cm)	142.59	178.00	120.00	10.71
Panicle (no. m^{-2})	120.82	165.00	75.00	19.87
Grains (no. $panicle^{-1}$)	89.53	134.00	54.00	17.95
1,000-grain weight (g)	26.82	34.53	13.06	5.22
Grain yield (t ha^{-1})	2.51	3.56	1.28	0.44

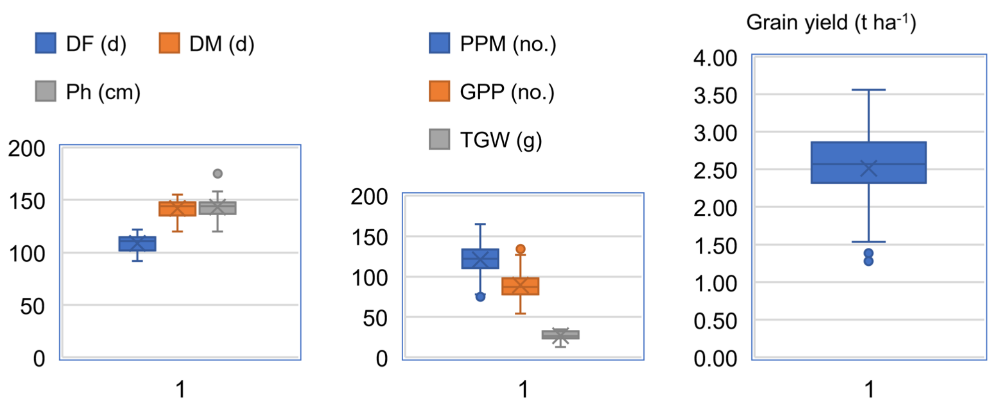


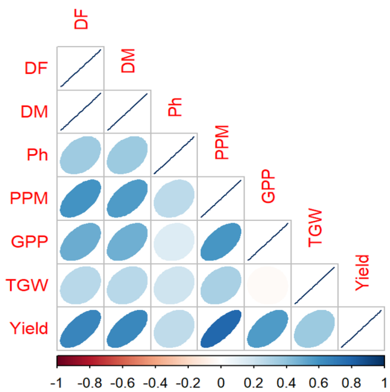
Figure 2. Phenology, plant height, panicle and grain production of T. aman rice cultivars.

Inter-trait associations among studied traits and contribution of yield attribute to yield of rice

Correlation analysis of studied parameters showed that grain yield had a strong positive association with days to flowering and maturity, plant height, panicle and grain production (Table 2 and Fig. 3). There was a positive but weak relation between grain yield and 1,000-grain weight. However, 1,000-grain weight also showed a weak but positive relationship with days to flowering and maturity, plant height, panicle and grain production. On the other hand, 1,000-grain weight had a negative but very weak relationship with grains panicl^{-1}. Similarly, number of grains panicle^{-1} had a strong association with days to flowering and maturity and panicle production. There was a weak but positive relationship between grain production and plant height. Plant height and panicle production had a strong association with days to flowering and maturity. The functional relationship between predicted yield and actual yield showed that $r = 83$ ($P < 0.01$) (Fig. 3). The number of panicle m^{-2}, grain production and 1,000-grain weight had great contribution in grain yield. Yield = - 1.360 – 0.0081 DF + 0.0213 DM – 0.0024 Ph + 0.0116** PPM + 0.0039* GPP + 0.0121* TGW. Higher yield in rice is associated with greater number of panicles per unit area, number of grains panicle^{-1} (spikelets), higher filled grains (Zhao et al., 2006; Hamid et al., 2015). Higher yield is often associated with high dry matter accumulation (Hu et al., 2015), but high translocation rate after heading directly contributes to grain formation that eventually results in higher yield in cereal crops.

Table 2. Inter-trait associations among studied traits of T. aman rice cultivars

	DF	DM	Ph	PPM	GPP	TGW	GY
DF	1.000						
DM	0.997**	1.000					
Ph	0.354**	0.363**	1.000				
PPM	0.586**	0.574**	0.261	1.000			
GPP	0.490**	0.485**	0.143^{ns}	0.582**	1.000		
TGW	0.275	0.270	0.199	0.317	-0.028^{ns}	1.000	
GY	0.651**	0.644**	0.644*	0.785**	0.571*	0.357	1.000



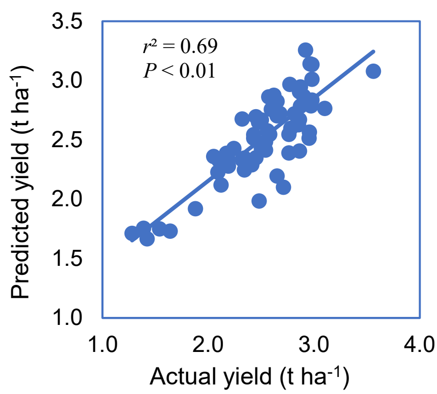


Figure 3. Inter-trait associations among studied traits and functional relationship between actual and predicted yield of rice cultivars of aman rice cultivars.
DF = days to flowering; DM = days to maturity; Ph = plant height; PPM = panicle m^{-2}; GPP = grains panicle^{-1}; TGW = 1,000-grain weight; GY = Grain yield.

Categorization of rice cultivars:

Based on growth duration, the rice cultivars were categorized into three classes (Fig. 4). The growth duration categories were short (< 120 days), medium (120–140 days) and long (> 140 days). The average yield of short duration cultivar was 2.1 t ha^{-1}, medium duration 2.34 t ha^{-1} and long duration 2.65 t ha^{-1}. It indicated that the grain yield increases with the increases of growth duration of the cultivars. However, only 2 rice cultivars were belonged to short duration, 21 from medium duration and rest 39 from long duration cultivars. The majority of rice cultivars in the TF prone areas were medium to long duration cultivars. Mamun et al. (2017b) reported that Rajashail and Kutiagni flowered mid and last week of October, respectively and they were short duration cultivars. However, rest of the cultivars flowered mid-November and they are long duration cultivar (Mamun et al., 2017b).

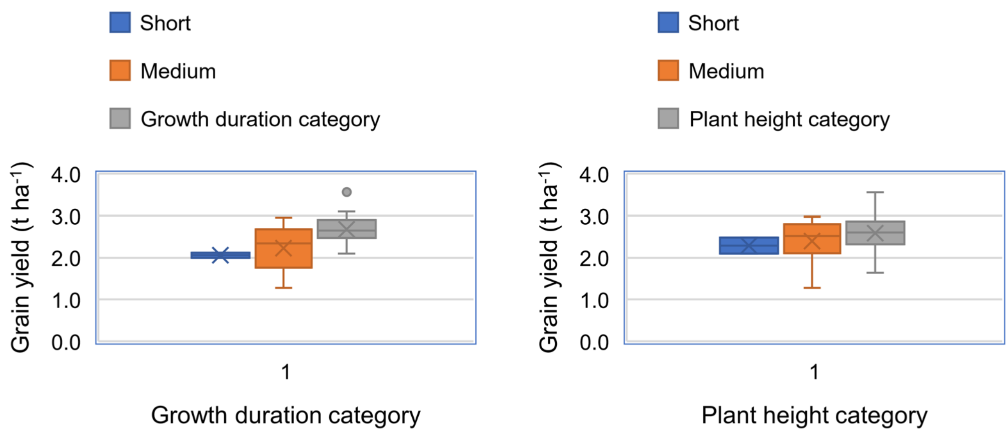


Figure 4. Categorization of rice cultivars based on growth duration and plant height.

Based on plant height, the rice cultivars were categorized into three classes (Fig. 4). The plant height categories were short (< 120 cm), medium (120–140 cm) and long (> 140 cm). The average yield of short stature cultivar was 2.3 t ha^{-1}, medium stature 2.52 t ha^{-1} and long stature 2.60 t ha^{-1}. It indicated that the grain yield increases with the increases of plant height of the cultivars. However, only 2 rice cultivars were belonged to short stature, 22 from medium stature and rest 38 from long stature cultivars. The majority of rice cultivars in the TF prone areas were medium to long stature cultivars. Hamid et al. (2015) reported the local rice cultivars are tall statured in nature.

Based on 1,000-grain weight, the rice cultivars were categorized into four classes (Fig. 5). The grain size categories were small (< 20.0 g), medium (20.0–25.0 g), large (20.0–25.0 g) and very large (> 30.0 g). The average yield of small grain sized cultivar was 1.6 t ha^{-1}, medium grain 2.68 t ha^{-1}, large grain 2.43 t ha^{-1} and very large grain 2.76 t ha^{-1}. It indicated that the grain yield increases with the increases of grain size of the cultivars. However, only 5 rice cultivars were belonged to small grain, 17 from medium grain, 19 from large grain and rest 20 from very large grain cultivars. The majority of rice cultivars in the TF prone areas were medium to very large grain sized cultivars. Previous study also confirmed that the local cultivars are having bold grained with grain size or 1,000-grain weight of 30.03 to 31.5 g (Hamid et al., 2015).

Based on grain yield, the rice cultivars were categorized into three classes. The grain yield categories were low (< 2.0 t ha^{-1}), medium (2.0–3.0 t ha^{-1}) and high (> 3.0 t ha^{-1}) (Fig. 5). The average yield of low yielded cultivar was 1.5 t ha^{-1}, medium 2.59 t ha^{-1} and high 3.33 t ha^{-1}. However, only 6 rice cultivars were belonged to low yielding, 53 from medium and rest 2 from high yielding cultivars. The majority of rice cultivars in the TF prone areas were medium yielding rice cultivars. Relatively lower yield in indigenous cultivars indicate the potential constraint of translocation of biomass to grains during grain filling stage (Hamid et al., 2015; Mamun et al., 2017a, Mamun et al., 2020). Grain yield of local aman rice cultivars were comparatively low (Mia et al., 2022; Senthilkumar et al., 2020). The low yield was due to low yield potential of local cultivars.

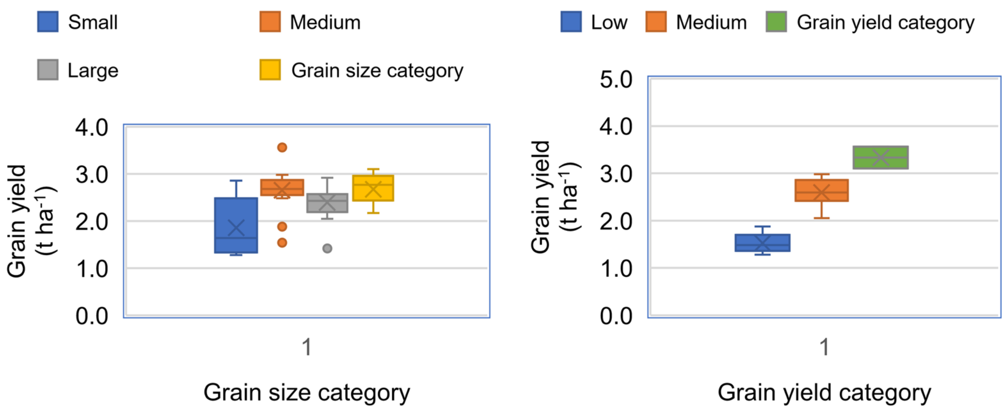


Figure 5. Categorization of rice cultivars based on grain size and grain yield.

When we categorize the landraces according to plant height, we observed that yield is significantly higher in the cultivars with longer plant height. This result contradicts with many previous findings where longer plant height is shown to be a negative character (Oladosu et al., 2018). In the context of coastal agro-ecosystems, it is, however, often a common phenomenon since dwarf plant cannot withstand tidal inundation/flooding. Therefore, longer plant of rice is a desired trait for cultivars to be grown in this ecosystem. Moreover, heavier grain is associated with higher grain yield, a phenomenon reported in the previous literature (Wenhui et al., 2019; Li et al., 2019). Considering all these factors, it can be suggested that longer plant with heavier grain are two important characters for local rice grown in coastal ecosystem.

Principal component and cluster analysis

Principal component analysis showed that different rice cultivars had association with different paraments (Fig. 6). Grain production show close association with BRRI dhan76, 1,000-grain weight with Sadamota 4, yield with Moulata. Dudmona1 and Khoiyamota1 at Wazirpur yielded 2.98 to 3.10 t ha^{-1}; Dishari1 and Sadamota2 at Bakergonj yielded 2.92 to 2.98 t ha^{-1}; Shorna at Babugonj gave 3.56 t ha^{-1}; Moulata2, Achin and Sadamota2 at Nalchity yielded 2.96 to 2.98 t ha^{-1}. These were most promising rice cultivar in terms of adaptation and grain yield in the study areas. In breeding program, these cultivars might be included.

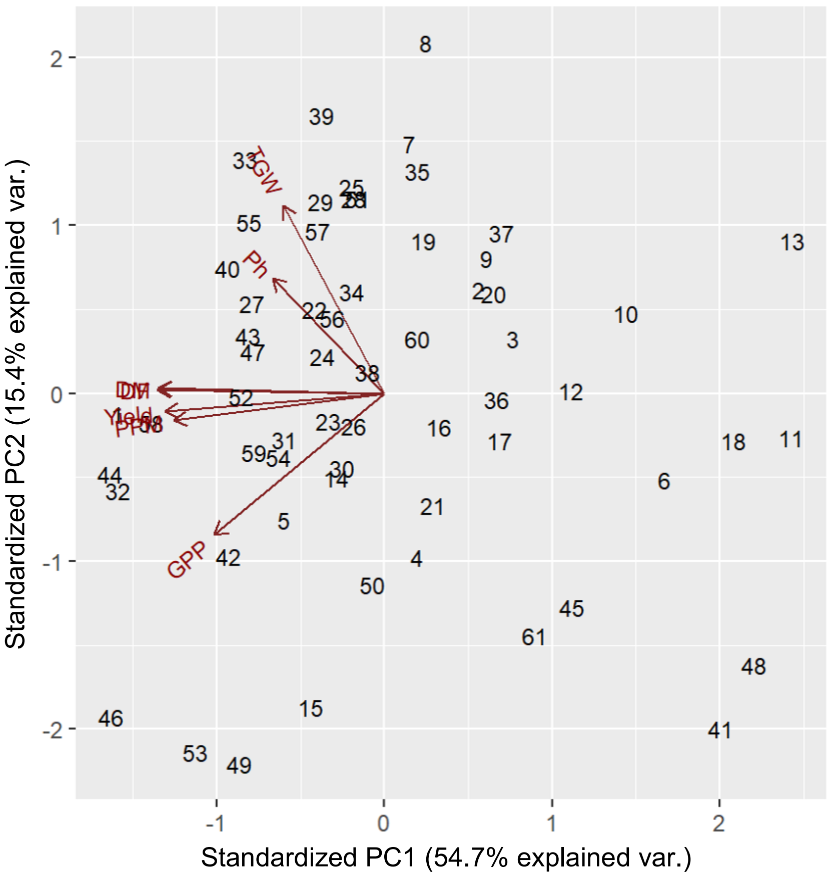


Figure 6. Inter relationship between studied parameters of aman rice obtained by principal component analysis.
DF = days to flowering; DM = days to maturity; Ph = plant height; PPM = panicles m^{-2}; GPP = grains panicle^{-1}; TGW = 1,000-grain weight; GY = Grain yield; 1 = Dudmona1; 27 = Khoiyamota1; 32 = Dishari1; 33 = Sadamota4 (TGW); 46 = Shorna; 52 = Moulata2; 53 = Achin; 58 = Sadamota2.

Based on the studied parameters, the cultivars were grouped into seven cluster (Fig. 7). Cluster 1 included 2 cultivars like Dudmona 1 and Dudmona 2; cluster 2 included 38 cultivars like Kutiagni, Mota dhan 1, Lalpayka, Jalkucha, Lalmota, Sadamota, Rajashail, Lalchikon, Sadachikon, Joina, Balam 1, BR25, Kajla, Haludmota 1, Mothamota 1, Moyna, Sonashail, Boleshormota, Kalizira, Kalomota, Moulata3, Khoiyamota1, Kachamota, Dudkalam, Moulata1, Sadamota 4, Nakochimota1, Lalmota 4, Lalpayka2, Kutiagni 2, Mothamota2, Khoiyamota3, Kiyamoat1, Lalmota3, Dishari2, Dudkolam2, BRRI dhan44 and Haludmota 2; cluster 3 included 3 cultivars like Lother, Sadapajam and Kalagura; cluster 4 included 5 cultivars like Motadhan2, Dishari1, Moulata2, Aochin and Nakochimota2; cluster 5 included 5 cultivars like Bhushiara, BRRI dhan76, Lalmota2, Sadamota3 and Moulata4; cluster 6 included 4 cultivars like Chinigura, BRRI dhan87, Shakhorkora and Balam2; and cluster 7 included 2 cultivars like Shorna and Sadamota2.

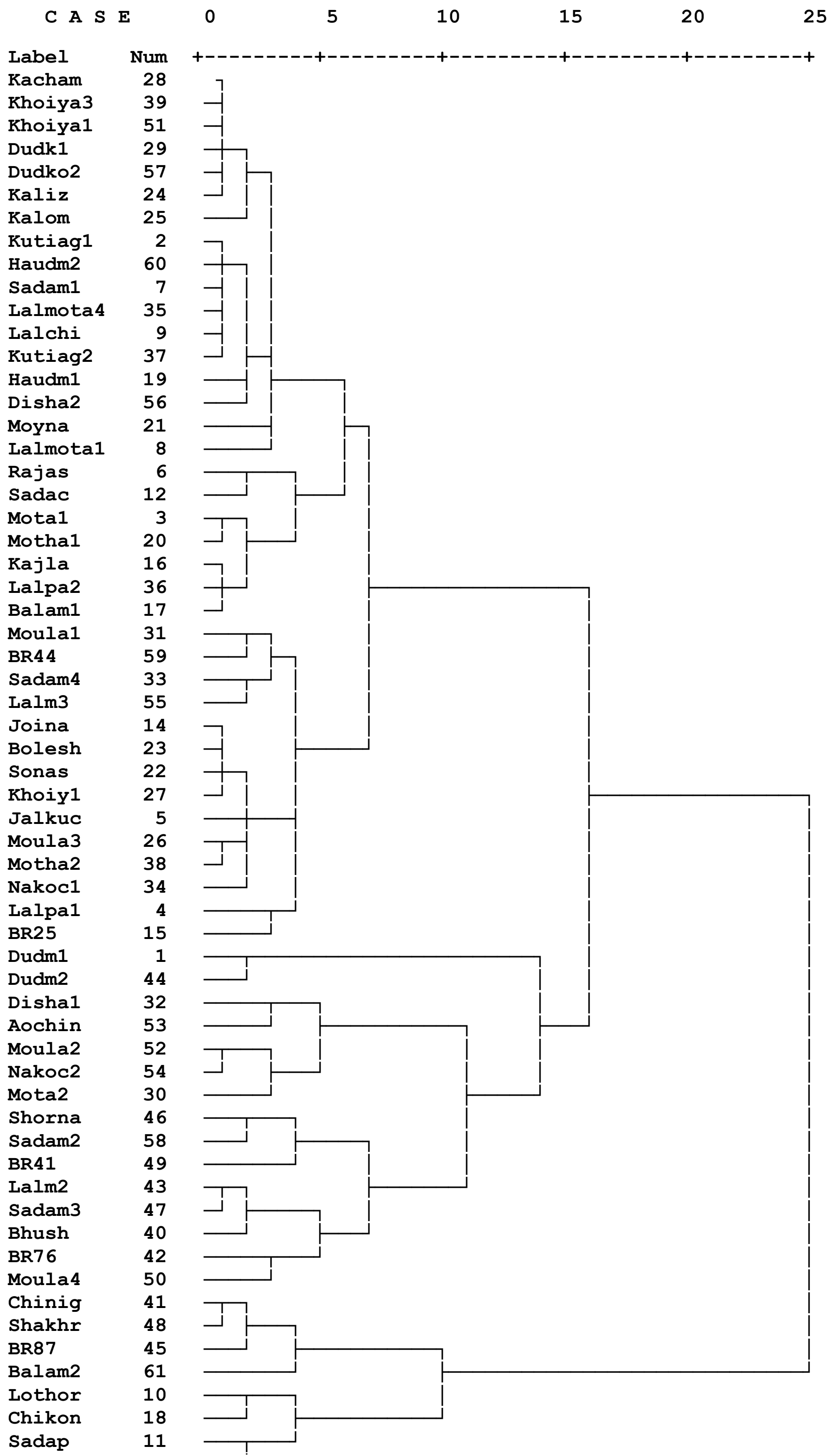


Figure 7. Dendrogram of studied T. Aman rice cultivars.

CONCLUSIONS

Local cultivars performed better than modern rice. Taller plant, production of more panicles per unit area, higher number of grains panicle^{-1} and heavier grains are the most important traits associated with plant adaptation of aman rice cultivars in tidal areas. Collectively, this study suggested that Dudmona1 and Khoiyamota1 at Wazirpur; Dishari1 and Sadamota2 at Bakergonj; Shorna at Babugonj; Moulata2, Achin and Sadamota2 at Nalchity were most promising rice cultivar in terms of adaptation and grain yield. In breeding program, these cultivars might be included. However, improved management practices should be developed from the research findings.

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Evaluating the drivers of environmental sustainability practices – mango farm managers' perspective

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Abstract. This study evaluated the drivers of environmental sustainability practices using mango farm managers in Thailand. The purpose was to ascertain farmers' ability to conserve natural resources and protect the ecosystem. This was necessitated to raise environmental awareness, promote sustainable human development, and increase economic growth and quality of life. The study adopted a quantitative survey design with primary data collected from mango farm managers in four provinces in Thailand. Data were collected from a total of 383 respondents. The data was analyzed using CFA and SEM techniques employing SPSS v26 and AMOS v26. The findings showed that Organizational Culture and Employee Training significantly and positively influence Environmental Sustainability Practices in managing mango as a natural resource. Organizational Culture was also found to positively mediate the effect of information system adoption. The study concluded that management and employees in mango farms should be trained about the importance of environmental sustainability and encouraged to cultivate a culture grounded in the sustainability practices of mango as a natural resource. They should be taught how advancing and adopting sustainable practices by subsistence and commercial mango farmers in Thailand can enhance sustainable development, economic growth, and regional cooperation by checking materials and methods.

Key words: natural resources, human development, organizational culture, information system adoption.

INTRODUCTION

Human existence and wellbeing are linked to the environment and natural resources available to them. Much of the problems faced by humans around the globe are linked to the health of the environment, and the scarcity or pollution of natural resources. Healthy living requires individuals to access fresh water and clean air to breathe and survive. The concept of environmental sustainability is thus the ability of people to behave in a manner that conserves natural resources and protects the ecosystems to ensure the promotion of health and wellbeing. Pata (2021) outlines that the concept of environmental sustainability is pegged on three pillars: renewable resources, pollution, and non-renewable resources. Under these pillars, environmental sustainability can be achieved by ensuring that the rate of using renewable resources does not exceed the rate of regeneration. Also, regarding pollution, the rate of waste generation should not exceed the assimilative capacity. Similarly, the use and depletion of non-renewable resources should be done with comparable renewable substitutes for the resources to enhance sustainability. In the age of Industry 4.0, more accurate information must be used as input data into virtual models for higher output (Herak et al., 2018; Wasserbauer et al., 2019; Wall et al., 2021).

Attaining environmental sustainability requires the adoption of practices that drive sustainable growth. Some of the key drivers in environmental sustainability include practices such as environmental management based on set policies and environmental legislation. These include antipollution and emission laws and policies (Risku-Norja & Mikkola, 2009; Naidoo, 2018; Havrysh et al., 2021). Greenhouse Gas Emissions (GHE) conservation is another practice that ensures sustainable environmental systems by passing and implementing laws and policies that limit GHE for the country. Also, climate change influences certain aspects of environmental sustainability (Amekawa, 2010; Singh, 2019; Rabbi et al., 2021). Reducing the levels of global climate change positively influences the environment towards sustainability. The effect of GHE has been felt in increasing global temperatures, which has affected agricultural yields, thus threatening the sustainability of humans (Amekawa, 2010). Measures like soil fertility conservation and soil degradation prevention play a vital role in ensuring sustainable farming practices and, consequently, a sustainable environment (Molnar et al., 2013; Turner et al., 2016; Escobar et al., 2019). Water quality is also vital by ensuring the non-contamination of groundwater sources and soil detoxification to remove harmful elements capable of reducing agricultural yield or crop failure. Various drivers of environmental sustainability are practiced worldwide, involving waste management, green labeling, and life cycle assessment.

Sustainability can be enhanced by also considerably reducing the carbon footprint in the distribution process by mango farmers. They can adopt measures such as ensuring the full loads of shipments to minimize emissions due to transportation. Mango fruits should be loaded onto trucks heading to the border, and firms should collaborate with other export sectors to ensure that other products are loaded onto trucks traveling back from the border. Farmers may also consider using independent trucking firms with the most fuel-efficient fleets to distribute mango produce and ensure they carry out routine vehicle maintenance. Mango farmers are encouraged to switch from flood irrigation to drip and dispersion irrigation to minimize the greenhouse gas emissions from pumping equipment. Mango farmers should employ photovoltaic cells as a renewable energy source to reduce the electricity used during production. They can further save on electricity by dividing up cool rooms in packing houses so that just the parts containing the mango produce are cooled, rather than refrigerating the entire room (National Mango Board, 2017). The novelty of the study is in the analysis and determination of drivers of environmental sustainability in the cultivation of mango as an export commodity to boost the trade economy of Thailand. Understanding the dynamics that make it a sustainable enterprise is critical when considering the Sustainability Development Goals (SDGs) and the growing influence of Thailand in the Southeast Asia region in promoting sustainability.

Environmental Sustainability Practices in Thailand

Thailand is one of the countries in Southeast Asia that has achieved increased social and economic growth in the past few decades. Despite the political uncertainty, the country recognizes environmental sustainability as one of the main objectives of economic and social development. According to the global stability index, Thailand is ranked 154th out of 195 countries based on its policies and actions toward environmental stability (Naidoo, 2018; Chmielarz et al., 2020). In a bid to achieve environmental sustainability, the country adopted measures to manage its natural resources sustainably. While the national strategy framework was put in place in 2017 as a 20-year strategy to 2036, the idea of sustainability is not new to the Thai people, who live under the 'Sufficient Economy Philosophy (SEP)'. The SEP principle that encourages people to live balanced lives based on moderation, reasonableness, and self-immunity. The concepts under the SEP philosophy align with the sustainable development goals.

Among the key factors promoting environmental sustainability in Thailand are environmental legislation, policies and sustainable farming practices. For instance, the Thai government has implemented the green integration policy and the national green directory, which aim to promote environmentally friendly products and services (Amekawa, 2010; Chaiyasoonthorn et al., 2019; Andersson et al., 2020; Csákay et al., 2021; Smirnova et al., 2021). The Thai government uses various agencies to promote its environmental policies and explain them to the public. For instance, the government facilitates environmental policies through institutions such as the national environment board and the ministry of natural resources. Non-governmental organizations also promote Thailand's environmental policies and philosophies to ensure social justice and sustainability (Chomchalow & Songkhla, 2008; Muangmee, 2021). For example, in many instances, many non-governmental organizations in Thailand argue against rapid capitalist expansion, terming it the basis for environmental degradation and economic inequality. Intertwining economic development with the environment plays a vital role in ensuring environmental sustainability in the country.

Sustainability in Thailand has also been largely linked to the sustainable agricultural practices in the country since the early 1900s. The Thai government has, over the years, encouraged sustainable farming practices through the extension of services such as incorporating green technologies into irrigation schemes as well as the provision of economic incentives to increase crop production. The other key factors in improving sustainable agriculture in Thailand involve integrating integrated farming systems where the farmers engage in two or more agricultural activities in the same field to lower costs and ensure the attainment of maximum potential from the farming lands (Chomchalow & Songkhla, 2008). The country also promotes organic farming, where alternative ways are utilized to limit the quantities of herbicides, pesticides, and fertilizers used in farming. Organic farming in Thailand is promoted by government research and non-governmental organizations (Panuwet et al., 2012; Larkin, 2017; Meemken & Qaim, 2018; Formoso, 2021). The other farming practices in the country aimed at promoting environmental sustainability include agroforestry, which encompasses farming of cash crops to coexist with cattle, and natural farming, which involves farming without applying chemicals to preserve the environment.

However, despite the environmental sustainability progress, Thailand continues to face various environmental problems related to pollution. Although the country has established good water treatment facilities, it lags in developing an enhanced piping system to deliver the water. Similarly, the retail and hotel industries continue to use plastic packaging, contributing to water and soil pollution. Environmental challenges in Thailand challenge economic growth through farming and social fulfilment.

Mango Farming in Thailand

Thailand is one of the largest mango producers globally after India and China, and in 2016, the country produced 3.4 million tons of mango. Mango farming in the country is mainly for commercial purposes, which involves processing and exporting fruit products. Much of the mango product produced in Thailand is exported to Japan, Malaysia, China and Singapore. However, despite being one of the world's highest mango farming countries, much of the mango fruit is sold in the domestic markets, with only 2% sold as exports. Sales from mango farming in Thailand are estimated to be over $50 million per year (Kumar et al., 2021).

Mango farming in Thailand takes various forms in different regions. For instance, farming of the mangoes is often done using dwarf trees under horticultural practices. This form of farming comes with the advantages of easy harvesting and reduced farming costs. It is also advantageous as it ensures minimal damage to the mangos. Similarly, the mango farming practices in Thailand also involve the use of tip pruning to reduce vegetative growth and ensure easy flowering and plant management. Other mango farming practices practiced in Thailand involve thinning, where the immature fruit is thinned to allow the remaining fruit to mature to its maximum size.

However, despite the high mango fruit production in Thailand, the farmers are increasingly faced with uncertainties due to the increasing scarcity of water resources for irrigation. Similarly, whether the anomalies pose a challenge to the off-season production of mango fruits in the country remains to be seen. To address these problems, farmers in Thailand adopted micro-irrigation systems that save water resources in the irrigation practices and are cost-effective as they are not labor-intensive (Kumar et al., 2021).

Mango farming in Thailand is based on the season. The rationale for the high season, which involves harvesting the fruits, is characterized by excess supply exceeding demand, resulting in low prices. However, farmers have in the past ignored the market-led production by developing an off-season production farming culture. The development of off-season farming methods through irrigation is a relatively high investment for the farmers (Chomchalow & Songkhla, 2008; Kumar et al., 2021). However, on the flip side, off-season farming also affects the environment through strains on the water resources for irrigation, and the use of herbicides and pesticides to control pests and diseases common during the off-season periods. Thus, to ensure environmental sustainability, mango farming in Thailand must address various aspects of farming practices, including the farming organizational culture, farming employee training, and adoption of technology that will positively affect environmental sustainability.

Information Technology Adoption and Its Effects on Environmental Sustainability

The effects of global warming and climate change on the environment have led to an increased focus on technologies that promote environmental sustainability around the globe. Various research indicates the success of the use of 'green technologies' in promoting environmental sustainability (Yumashev et al., 2020; Jayashree et al., 2021). Dezdar (2017) argues that adopting eco-technologies helps in developing practices that ensure non-toxic products are generated. The mango farming industry in Thailand takes environmental concerns seriously and, in turn, adopts various technologies aimed at promoting environmental sustainability. Some of the technological adoptions in Thailand's mango farming practices involve post-harvest technologies (Chomchalow & Songkhla, 2008). For instance, farmers in Thailand use specially made paper bags to cover the mango fruits to prevent them from being eaten by pests, especially the oriental fruit flies. The use of specially made paper bags benefits the fruit product by not allowing light to penetrate the fruit and providing a good environment to develop the perfect color for the ripe fruits for export. The paper bag and fruit wrapping technologies are environmentally sustainable. They prevent farmers from around the country from applying insecticides to prevent oriental fruit flies and consequently protect the environment (Karar, 2019). Similarly, the special wrapping papers are biodegradable, contributing positively to the environmental sustainability efforts in Thailand.

The use of information technology helps farmers learn about climate change and apply this information to farming methods to meet the conditions. Climate change has led to uncertainties in weather conditions that affect crop production and the quality of products. Due to the risks of the high cost of products, high labor costs, or labor shortages, not having the right information to tackle these factors in farming is often damaging, with adverse effects on the sustenance of the farm enterprise (Rolnick, 2019; Chaveesuk et al., 2020; Ndinojuo, 2020; Jędrzejczyk, 2021; Smimova et al., 2021). The use of information technologies helps search for information and share experiences related to weather and the environment. In the Thai farming industry, the farmers, with the help of the government, utilize the Near-Infrared Spectrometer (NIR) technology that provides information on the maturity of mangoes for harvesting (Chomchalow & Songkhla, 2008). Having information on whether the mangoes are ready for picking or not helps the farmers prepare other processes such as grade selecting and packaging for export, hence helping reduce costs during the selection process. The other aim of adopting information technologies in mango farming is to ensure an information connection between mango production and the market. Similarly, by maintaining a connection of information in the farming processes, the information is also used in determining climate change and ways to combat the severe effects of global warming. Adopting information technologies contributes positively to environmental sustainability efforts. From the above literature, the following Hypothesis has been developed:

Hypothesis (H1): The adoption of Information Technology significantly and positively influences environmental sustainability practices.

Organizational Culture and Its Effects on Environmental Sustainability

Organizational Culture involves the behavior based on shared common beliefs and values in particular organizations that leaders can communicate to influence employee perception and behavior. According to Chomchalow & Songkhla (2008), mango farmers in Thailand have formed coalitions that have turned into strong, productive organizations that work professionally to produce quality mangos to meet market demand. Fietz (2021) articulates that the concept of Organizational Culture plays a vital role in ensuring corporate environmental management and sustainability. The Thai authorities use the farmers' Organizational Culture to implement sustainability policies. For instance, the mango farmers adhere to the measures on agrochemicals put in place by the importing markets (Krause, 2016). Japan is one of the export markets for Thai mango fruits, and it has put in place measures on the level of chemicals to be used by farmers on the fruits. Such measures help build an Organizational Culture that positively affects environmental sustainability in Thailand.

Organizational Culture in Thailand: Mango farming affects sustainability by embedding environmentally sustainable farming practices among the farmers' behavior. Greater emphasis is being placed on the standardization of mango fruits to meet the export market's demands. In the research by Chomchalow & Songkhla (2008), various regulating bodies in Thailand, from the export regulating committees to agrochemical companies, have set sustainable standards that farmers should follow in producing mangoes. The sustainable market culture has led to the development of a sustainable mango farming culture among the farming cluster cooperatives in the country. Spreer (2013) states that establishing the one-stop service center, which facilitates the exporting process of the mango fruits in the country, involves all three parties in the mango farming industry, including farmers who cultivate the product; exporters who export the mango fruits to other countries from Thailand, and the government, which serves as the regulator in the entire process from production to export. The one-stop service center uses green labeling as the key driver toward implementing environmental sustainability practices. Under the labeling scheme, the regulators insist on the importance of farming management methods that adhere to the agrochemical regulations set by the exporter markets. Farmers in Thailand's mango industry thus must develop a sustainability culture in their farming practices to benefit from the export markets, thus positively impacting the country's environmental sustainability measures. From the above literature, the following Hypothesis has been developed:

Hypothesis (H2): Organization culture positively and significantly influences Environmental Sustainability Practices in Thailand.

Employee Training and Its Effects on Environmental Sustainability Practices

Attaining sustainability takes time, and training is one of the best ways to ensure continued defined behavior towards environmental sustainability. At the organizational level, employee training is one way to ensure sustainability discussions are held within the business and a way to begin the process of sustainable change in the future (Fernández et al., 2003). According to Ji (2012), employee training influences organizations' performance in sustainable development. Organizations adhere to various environmental regulations put in place by the government and other agencies in their line of business. Thus, training employees toward environmental sustainability helps organizations meet the regulatory measures while improving the firm's performance (Wagner & Schaltegger, 2003; Muangmee et al., 2021). The farmers under these clustered groups thus operate as the group employees who should moderate positively between the groups' environmental attitudes and performance towards sustainable development. Farmers operating in groups produce superior mango fruits that meet the market demand (Limniranku, 2010). Farmers working in groups gain a supply chain for the export market and support from the local governments to train themselves to adhere to the sustainability regulations demanded by the export markets (Haque & Yamoah, 2021). Consequently, these lead to a positive impact on the environmental sustainability efforts in the country.

The organization's employees are the first point of contact between the organization and the customers; hence, training the employees on environmental sustainability helps organizations meet the customer's desire for eco-friendly options while maintaining improved performance. (Jędrzejczyk, 2021). In the Thai mango industry, the farmers operate as business groups, and the members are considered employees (Vasudha & Agarwal, 2019). Thus, to ensure the mango fruits meet the consumer demands of going green, the community business groups conduct farmer training to adhere to sustainability measures. Chomchalow & Songkhla (2008) and Pachura (2019) remind us that the community business groups formed by the farmers invite agricultural experts to train the members on sustainable farming methods, including fruit wrapping, tree pruning, harvesting, and chemical spraying, among other methods that reduce environmental degradation while improving their production performance. By training the farmers on sustainable farming methods, the Thai farming industry contributes positively to the environmental sustainability efforts in the country. From the above literature, the following Hypothesis has been developed:

Hypothesis (H3): Employees Training positively and significantly influences Environmental Sustainability Practices in Thailand.

Conceptual Framework

Based on the evaluation of the literature review in the previous section, the conceptual framework bearing the study model was developed. The model comprises four variables: Environmental Sustainability Practices (which is the dependent variable) and three independent variables, which include Information Systems Adoption (ISA), Organizational Culture (OC), and Employee Training (ET). The relationship between these variables is presented in Fig. 1.

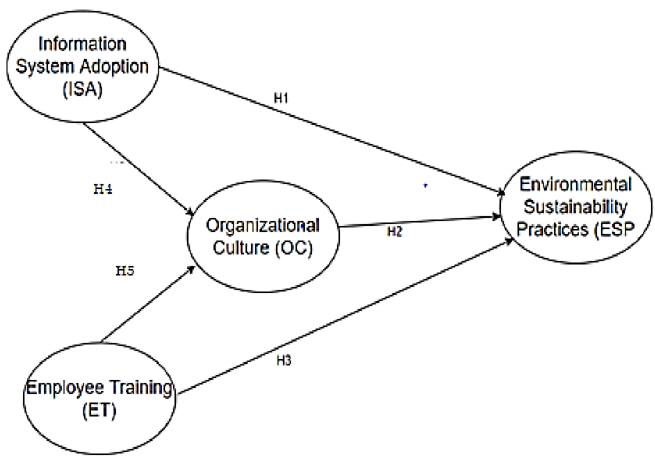

Figure 1. Conceptual model.

Hypothesis 1 (H1): Information system adoption (ISA) positively influences Environmental Sustainability Practices in Thailand.

Hypothesis 2 (H2): Organizational culture positively influences Environmental Sustainability Practices in Thailand.

Hypothesis 3 (H3): Employees Training positively influences Environmental Sustainability Practices in Thailand.

Hypothesis 4 (H4): Organizational culture mediates the relationship between Information system adoption and environmental sustainability in Thailand

Hypothesis 5 (H5): Organizational culture mediates the relationship between Employees Training and environmental sustainability in Thailand.

MATERIALS AND METHODS

This study adopted the quantitative method, where primary data was collected from the mango farmers in Thailand. The population of the study comprised mango farmers in five provinces in Thailand: Suphan Buri, Prachinburi, Phitsanulok, and Chainat. These provinces were considered suitable because they are regarded as the highest producers of mangoes. The data was collected using a questionnaire from a total of 383 mango farmers through personal interviews with the aid of the Association of Mango Producers in those regions. Regarding the data collection, the four variables of the study, Environmental Sustainability Practices (ESP), Organizational Culture (OC), Information System Adoption (ISA), and Employee Training (ET), were measured by adapting existing scales that have been validated in previous studies. All the measures were undertaken using 5-point Likert Scales (1 = strongly disagree to 5 = strongly agree) as follows: Environmental Sustainability Practices (ESP) with six indicators adopted from Pachura (2019) and Silvestre & Tîrca (2019); Organizational Culture (OC) with six indicators from (Baird et al., 2017); Information System Adoption (ISA) with six indicators from (Wagner & Schaltegger, 2003; Pérez-Méndez & Machado-Cabezas, 2015); Employee Training (ET) with seven indicators (Consoli et al., 2016; Rojo-Ramirez et al., 2019). The analysis of the data was conducted using various techniques. The first analysis was descriptive statistics of the data, followed by an evaluation of the suitability of the model using confirmatory factor analysis (CFA). Reliability and validity analysis were analyzed using Cronbach's Alpha, and average variance was extracted (AVE). The hypotheses were evaluated using structural equation modeling (SEM). The analysis was carried out using SPSS (version 26) and AMOS (version 26).

RESULTS AND DISCUSSION

Characteristics of the Respondents

The evaluation of the respondent's characteristics was carried out using the data collected. The results indicated that most respondents were female, 243 (63%), with males being at least 140 (37%). The largest age group was 31–40 years old (201, 52.48%), followed by 41–50 years old (64, 16.71%). The highest education level of the respondents was college (226, 59%), followed by high school and below (79, 21%). The study also evaluated the income of the respondents from the mango farming activities. The highest income level was 35,000–45,000 Baht, followed by 5,000–15,0000 Baht per season of mango harvesting. A summary of the demographic data is presented in Table 1.

Several techniques were adopted to evaluate the fitness of the model adopted in the study. The techniques applied include the reliability analysis, which was statistically conducted using Cronbach's Alpha and Convergent Reliability (CR). The validity of the constructs used in the study was evaluated using average variance extracted (AVE). The model used for the study was evaluated using confirmatory factor analysis (CFA). The results of the CFA revealed that the chi-square statistic for the model was significant ($\chi2$ [458] = 936.716, p <0.01) to the check of (CMIN/df = 2.045), which was considered acceptable since it was below the threshold of 3.0 (Hu & Bentler, 1999; Schumacker & Lomax, 2004). Additional statistics included IFI = 0.939, TLI = 0.933, CFI = 0.938, NFI = 0.886, and RMSEA = 0.052. RMSEA was below the threshold of 0.80 and verified the suitability of the model (Hu & Bentler, 1999), while the variables for Convergent Reliability and Cronbach Alpha were above the 0.9 thresholds as recommended by (Thompson, 2004; Kline, 2005).

Table 1. Demographic data of respondents

Demographic features	Possible options	Frequency (n)	Percent (%0)
Gender	Male	140	36.6
	Female	243	63.4
Age	18–30 years	82	21.41
	31–40 years	201	52.48
	41–50 years	64	16.71
	above 51 years	36	9.40
Education	High school and below	79	20.6
	College	226	59
	Bachelor	76	19.8
	Master	2	0.5
Income (Baht)	Below 15,000	135	35.2
	15,001–25,000	40	10.4
	25,001–35,000	29	7.6
	35,000–45,000	141	36.8
	above 45,000	38	9.9

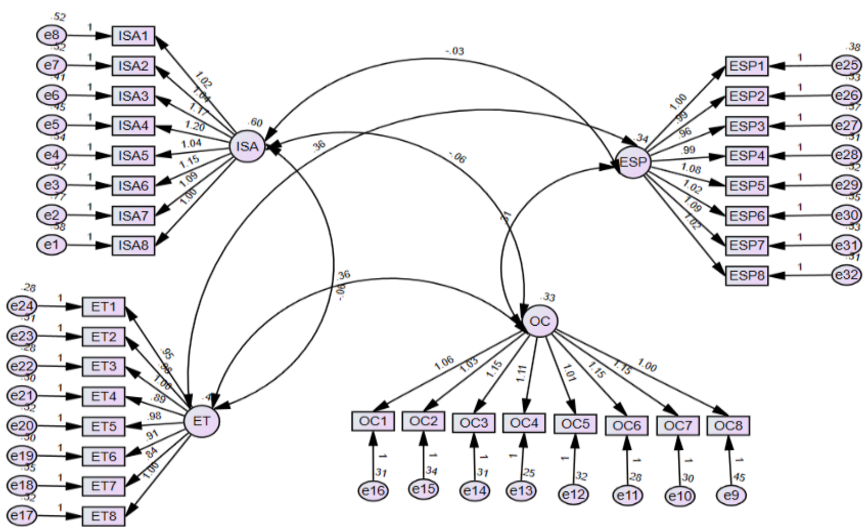

Figure 2. Model Fitness.

The suitability of the observant variables and latent variables was evaluated using factor loadings, the Convergent Reliability (CR), Average Variance Extracted (AVE), and Cronbach's Alpha as presented in Fig. 2. From the results, the CR ranged from 0.894 to 0.918. These values were above the required threshold of 0.7 according to Fornell &

Larcker (1981). Considering the AVE, the values ranged between 0.512 and 0.583 (see Table 2), which were above the required threshold of 0.5 according to (Segars, 1997). The satisfaction of all the required thresholds proved that the study model was suitable for conducting the study.

Table 2. Validity and reliability analysis

Constructs	Items	Factor Loadings	CR	AVE	Cronbach's Alpha
	Information System Adoption (ISA)		**0.918**	**0.583**	**0.917**
ISA1	The use of the internet is a good source to obtain information to innovate in the company environmental sustainability practices	0.74			
ISA2	The advances of science presented at fairs and other events favour environmental sustainability practices	0.746			
ISA3	The different levels of government share their skills and information with companies environmental sustainability practices	0.819			
ISA4	Development of a web page is needed for the company to inform customers and suppliers regarding environmental sustainability practices	0.812			
ISA5	The information generated in technological R&D centres contribute to the innovative production processes of the firm environmental sustainability practices	0.741			
ISA6	The information about the ecological situation, forests, soil resources, and available water is grounded in managing the company	0.827			
ISA7	Information Technology encourages improved environmental sustainability practices	0.696			
ISA8	Internet and web services are vital in spreading the word regarding environmental sustainability practices	0.715			
	Organizational Culture (OC)		**0.907**	**0.550**	**0.906**
OC1	Not being constrained by many rules encourages environmental sustainability practices	0.738			
OC2	Being quick to take advantages of opportunities boosts environmental sustainability practices	0.711			
OC3	Being Innovative is an aspect of environmental sustainability practices	0.765			
OC4	Being achievement-oriented is vital to environmental sustainability practices	0.788			
OC5	Having high expectation for performance encourages environmental sustainability practices	0.715			
OC6	Being results-oriented is important to environmental sustainability practices	0.781			
OC7	Paying attention to detail is sometimes not suitable for environmental sustainability practices	0.772			
OC8	Being precise is critical for environmental sustainability practices	0.65			

Table 2 (continued)

	Employee Training (ET)		**0.914**	**0.570**	**0.913**
ET1	Training is a key to the development of the environmental sustainability practices	0.773			
ET2	Learning is a key to improving production processes in the company's environmental sustainability practices	0.765			
ET3	Knowledge management is a key in the company's environmental sustainability practices	0.791			
ET4	Attending events fairs is a key to getting more knowledge for the staff that works in the company's environmental sustainability practices	0.738			
ET5	The professionalization of workers and managers is a key in the activities of environmental sustainability practices	0.761			
ET6	Self-training is a key to personal and professional development of workers and managers in the firm for environmental sustainability practices	0.749			
ET7	The level of education is a key to the relationship between workers and employers in the company's environmental sustainability practices	0.692			
ET8	Managerial encouragement is critical for encouraging employees best practices	0.765			
	Environmental Sustainability Practices (ESP)		**0.894**	**0.512**	**0.893**
ESP1	The organizations carry out fertility tests and soil moisture	0.69			
ESP2	The irrigation systems used in the company are of the latest technology	0.71			
ESP3	The cultivation (conventional/ecological) is related to productivity and financial performance	0.674			
ESP4	Natural resources are used rationally in the company	0.724			
ESP5	The companies have environmental certifications	0.744			
ESP6	In the organization, they care about the good management of toxic inputs	0.711			
ESP7	Climate change issues are part of organizational policies	0.739			
ESP8	We are always aware of environmental sustainability practices	0.731			

Structural Equation Modelling

Structural Equation Modeling (SEM) was used to evaluate the five hypotheses of the study. The result of SEM is presented in Fig. 3 and Table 3.

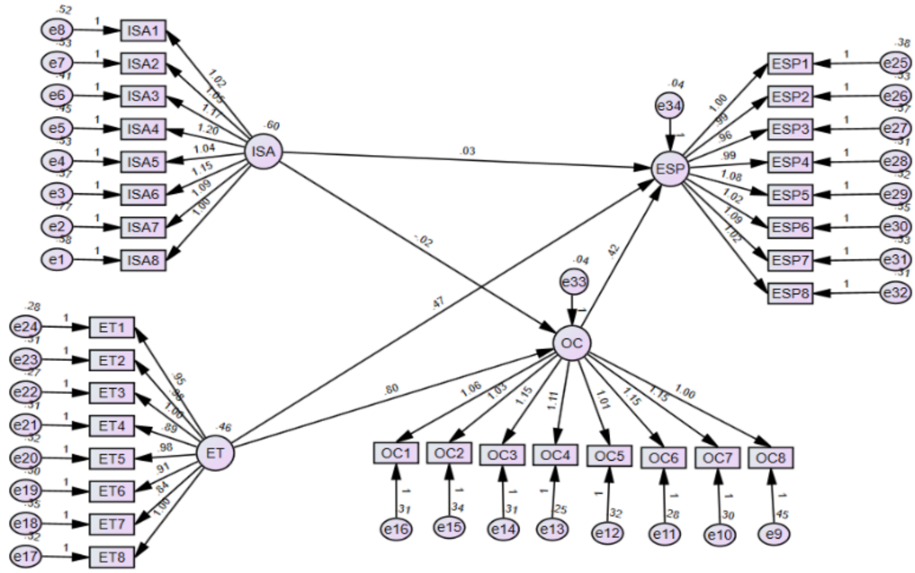

Figure 3. SEM analysis.

The SEM model was evaluated for suitability before the actual analysis of the hypothesis. The data revealed that the chi-square statistic for the model was significant (χ2 [458] = 936.716, $p < 0.01$) the check of (CMIN/df = 2.045), which was considered acceptable since it was below the threshold of 3.0 (Pérez-Méndez & Machado-Cabezas, 2015; Consoli et al., 2016). Additional statistics included IFI = 0.939, TLI = 0.933, CFI = 0.938, NFI = 0.886, and RMSEA = 0.052. RMSEA was below the threshold of 0.80 and showed the suitability of the model (Schumaker & Lomax, 2004), while the variables (IFI, TLI, and CFI) were above the 0.9 thresholds as recommended by Kline (2005) and Thompson (2004).

Table 3. SEM analysis for Thailand mango farmers

Hypo-thesis	Relationship	β	Accept?
	ISA ----> OC ---> ESP	0.415***	
H1	ISA ----> ESP	0.027	No
H2	OC ----> ESP	0.415***	Yes
H3	ET ----> ESP	0.470***	Yes
H4		-0.007***	Yes
H5	ET ----> OC ---> ESP	0.331	No

*H2, H3 and H4 were accepted while H1 and H5 were rejected from the SEM analysis.

From the SEM, the five hypotheses of the study were evaluated. The results showed that the path coefficients between Information System Adoption (ISA) and Environmental Sustainability Practices (ESP) (H1) (β = 0.027, $p > 0.00$) indicated that Information System Adoption (ISA) positively but insignificantly influences Environmental Sustainability Practices in Thailand. This led to the rejection of Hypothesis 1. The path coefficients between Organizational Culture (OC) and Information System Adoption (ISA) (H2) (β = 0.415, $p < 0.00$), indicated that Organizational Culture positively and significantly influences Environmental Sustainability Practices in Thailand. This led to the acceptance of the second

Hypothesis of the study. The results also showed that the path coefficients between Employee Training (ET) and Environmental Sustainability Practices (ESP) (H3) ($\beta = 0.470$, $p < 0.00$), indicate that Employee Training positively and significantly influences Environmental Sustainability Practices in Thailand. This led to the acceptance of the third hypothesis. The effect of Information System Adoption (ISA) on Environmental Sustainability Practices (ESP) through Organizational Culture (OC) (H4) ($\beta = -0.007$, $p < 0.00$) indicated that Organizational Culture mediates the relationship between Information System Adoption and environmental sustainability in Thailand. This led to the acceptance of the fourth Hypothesis of the study.

The effect of Employee Training (ET) on Environmental Sustainability Practices (ESP) through Organizational Culture (OC) (H5) ($\beta = 0.331$, $p > 0.00$) indicated that Organizational Culture does not mediate the relationship between Information Systems Adoption and environmental sustainability in Thailand. This led to the rejection of the fifth Hypothesis of the study.

Effects of Information system adoption on environmental sustainability practices

This section is discussed with reference to the first Hypothesis of the study, which investigated how information system adoption influences environmental sustainability practices. According to the findings of the study, it was found that the information system does not significantly influence the environmental sustainability practice among Thai mango farmers. This implies that the aspects of information system adoption, such as the use of the internet as a good source to obtain information to innovate in the company's environmental sustainability practices, the advances of science presented at fairs and other events that favor environmental sustainability practices, and the different levels of government sharing their skills and information with companies' environmental sustainability practices do not influence the environmental sustainability practices. The findings of this Hypothesis do not agree with the literature by Dezdar (2017), who opined that eco-technologies play an important role in the development of practices that ensure environmentally friendly products. Mango farmers in Thailand have not keyed into this important resource about environmental sustainability, which is available via the adoption of information systems. Similarly, Rolnick (2019) also found that Information Technology is important in informing people about the benefits of environmentally friendly production practices. The non-adoption of information systems will hamper the inclusion of sustainable environmental practices in mango farming. The reason for this non-adoption could be that in mango farming in Thailand, the use of high technology has not been widely adopted and implemented in the production practices of mango, considered in many aspects a natural resource.

Effects of Organizational Culture on environmental sustainability practices

In this study, Organizational Culture was considered a critical aspect of the Environmental Sustainability Practices of Thailand's mango farmers. It incorporated the behaviors and common beliefs and values shared among people, a community, or an organization engaged in mango farming in Thailand. The results of the study indicated that Organizational Culture has a significant and positive influence on Environmental Sustainability Practices in Thailand. These results were in agreement with that of Fietz

(2021), who indicated that the concept of Organizational Culture plays a vital role in ensuring corporate environmental management and sustainability. The reason is because the Organizational Culture makes it easy for the management to implement environmental practices and guidelines to ensure sustainability. As Turner et al. (2016) point out, problems associated with contemporary agriculture and natural resources are on the rise globally and have affected social order and the ability to tackle issues associated with climate change. From the perspective of this research, the specific Organizational Culture aspects that influence Environmental Sustainability Practices include the cultivated organizational innovativeness, achievement-oriented, high expectations from the members, and being results-oriented in mango farming.

Effects of Employee Training on Environmental Sustainability Practices

Employee Training was considered development of farm managers' continued commitment to adopting and implementing Environmental Sustainability Practices for natural resources. The findings of this study indicated that Employee Training contributes positively and significantly toward the Environmental Sustainability Practices in Thailand. These findings agreed with that of Ji (2012), who indicated that Employee Training influences organizations' performance in sustainable development. This aspect is grounded on the argument that Employee Training at the organizational level is one of the ways to ensure sustainability discussions are held within the business and a way to begin the process of sustainable change in the future and the management of natural resources. These findings were further supported by Fernández et al. (2003), who indicated that training employees toward environmental sustainability helps organizations meet the regulatory measures while improving the firm's performance. From the perspective of this study, Employee Training aspects that contribute to Environmental Sustainability Practices include considering training as a key employee development strategy; learning is a key to improving production processes, and investing in knowledge management to ensure sustainable harnessing of natural resources associated with an area. Thailand, as one of the top global mango producers (Chomchalow & Songkhla, 2008), should incorporate sustainable practices into its operations toward the sustainable development of its mango farms.

Mediating Role of Organizational Culture

In this study, organizational culture was evaluated as a mediator of the effect of employee training and information system adoption on environmental sustainability practices. The findings of the study indicated that organizational culture mediated the effect of information system adoption on environmental sustainability practices but did not significantly mediate the effect of employee training on environmental sustainability practices. The interesting finding from the study is that when the organizational culture was included as a mediator, the information system adoption changed from having an insignificant influence to having a significant influence on environmental sustainability practices. This paper proposed that to be effective, the information system should be incorporated by the people, community, and organizational culture, or rather, considered as an aspect of the inherent values to effectively influence the Environmental Sustainability Practices in mango farming in Thailand.

CONCLUSIONS

From the findings of this study, several policy recommendations were made both to the mango farmers in Thailand as well as the concerned stakeholders in the sector, especially about harnessing mango as a natural resource for Thailand. The first recommendation is that in this study and from the literature, employee training is an important variable for enhancing environmental sustainability practices. Therefore, management and employees on the mango farms should be trained regarding the importance and aspects of environmental sustainability. The second recommendation is that organizational culture could play a critical role as far as environmental sustainability practices are concerned. Cultivating an organizational culture grounded in environmental sustainability practices and aspects could play a great role in advancing it, both for mango farmers in Thailand. Another policy recommendation is that information system adoption by itself may not influence environmental sustainability practices. It should be incorporated into the organization's culture so that it can have a significant influence on environmental sustainability practices.

The first limitation of the study is that it was specific to Thailand. This is because it particularly focused on mango farmers in Thailand, and data was collected from five provinces. The limitation is that mango is cultivated in many areas of Thailand. As a result, the application of the results and generalization of the findings should be applied in recognition of the study's specificity. The next limitation is that the study variables and sample size were quite small. This study recommends that future studies should consider increasing the sample size and the study variables, as well as covering a larger study area for comparisons. The last limitation of the research is the non-inclusion of seasonal production as a variable and how it affects mango production in Thailand among farmers. Future studies should include this as a variable in studies about mango farming and other crops with seasonal variants.

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The response by selected rice genotypes to organic ameliorants in tidal swampland which is affected by Fe toxicity

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Abstract. Ferrous Fe toxicity in rice has been reported to be one of several major limitations in terms of wetland rice production. Previous studies have reported a decrease in paddy rice yields of between 12–100% due to this problem. A study was conducted in order to determine the growth and yield factors for selected rice genotypes as a response to the presence of organic ameliorants, and their interaction in controlling ferrous Fe toxicity levels in rice which is grown in tidal swampland. Experiments formed part of this study, with these being conducted in tidal swampland around Danda Jaya village and Belandean village in Barito Kuala Regency, South Kalimantan. The experiment was arranged in a split-plot design to test the organic ameliorant treatments (the control was fresh *Salvinia sp*, with the compost being formed of *Salvinia sp*, plus rice straw, and cow manure as the main plots, and sub-plots being formed of rice genotypes (TOX-4136, Inpara-1, Inpara-2, Inpara-4, and IR-64). Results from the experiments revealed the fact that organic ameliorants could reduce ferrous Fe toxicity levels, as well as Fe content in plant tissues, while plant height and the number of tillers also decreased. Rice genotypes which are medium tolerant or fully tolerant to ferrous Fe toxicity when organic amelioration treatments are added can serve to decrease ferrous Fe toxicity and increase the number of filled grains and yield. Applications of fresh S*alvinia* and S*alvinia* compost were as effective as an application of rice straw and cow manures when it came to successfully increasing the yield of rice grown in tidal swampland. Ferrous iron toxicity in rice which has been produced in tidal swampland can be overcome by using tolerant genotypes (Inpara-1 and Inpara-4), or organic ameliorants (*Salvinia* sp).

Key words: land amelioration, Fe toxicity, rice, *Salvinia* sp, tidal swampland.

INTRODUCTION

There exists approximately 8.92 million hectares of tidal swampland in Indonesia. All of i t has the potential of being converted into agricultural land (BBSDLP, 2015). Productivity levels of rice which is grown in tidal swampland areas is generally low due to high acidity levels and poor soil nutrient conditions, plus Al, Fe, and H_2S toxicities (Sarwani et al., 1994).

Ferrous Fe (Fe^{+2}) toxicity levels in rice have been reported to be one of the single major limitations of wetland rice production, resulting in a decrease in paddy rice yields by between 12–100% (Sahrawat, 2010). Rice plants which experience high ferrous Fe toxicity levels suffer from poor growth and low tillering, resulting in low-to-zero production (Audebert & Sahrawat, 2000). Ferrous Fe toxicity in rice may vary with genotypes, nutrient status, land and water management regimes, temperature, and light intensity levels (Sahrawat, 2010; Audebert & Sahrawat, 2000).

Symptoms of ferrous iron toxicity in rice only occur under specific conditions, where the rice has been subjected to flooding. The occurrence of 'reduction conditions' in flooded lowlands has resulted in the dissolution of all forms of soil Fe into dissolved forms of Fe^{+2} (Beckers & Ash, 2005; Audebert, 2006). The occurrence of ferrous Fe toxicity in plants is not only caused by high concentrations of Fe^{+2} in the soil solution which itself is the result of reduction conditions, it is also caused by low pH levels, low nutrient status, and imbalances in the soil (Dobermann & Fairhurst, 2000).

Ferrous iron toxicity can adversely affect morpho-physiological characteristics, resulting in a disruption to rice plant growth (Nugraha et al., 2016; Turhadi et al., 2018; Onyango et al., 2019; Novianti et al., 2020). Several genotypes which are tolerant to ferrous iron toxicity can grow and produce normally, such as Inpara-2, Inpara-6, Siam Saba, Mashuri, Hawara Bunar, and Pokkali (Nugraha et al., 2016; Turhadi et al., 2018). The mechanism which involves the transition between rice tolerance to iron toxicity is related to the genetic characteristics of the specific varieties of rice which are involved in the process. Ensuring the use of tolerant genotypes is the most economical way for farmers to be able to grow rice in tidal swampland areas. However, levels of tolerance may vary with different rice genotypes and soil conditions.

The use of organic fertiliser for soil amelioration can reduce the need for chemical fertilisers while also providing support for sustainable agriculture (Ningsih et al., 2017). Organic-based agriculture which limits the use of chemical fertilisers and pesticides can reduce environmental burdens, ensure food sustainability, and provide a stable income (Hokazano et al., 2009). Using organic fertiliser can help to improve land quality while also decreasing toxic elements in the soil and increasing plant yield. Results from the use of organic fertiliser decomposition has contributed to macro and micronutrient status, while also having formed complex compounds which contain toxic metal ions (Al, Fe, and Mn), and increasing soil exchange capacity, which itself leads to high levels of soil nutrient retention (Juarsah, 2014). Applications of organic materials such as rice straw and cow manure have been reported to improve the physical properties of soil, but these are not always available on site. Another organic material which has the potential to be able to ameliorate soil conditions is *Salvinia* sp, which is widely grown in tidal swampland areas.

Salvinia sp is a water fern which has widely been used as a phytoremediator in order to clean-up water which has been polluted by heavy metals. *Salvinia* is an effective bio sorbent when it comes to removing heavy metals from waste water (Olguín et al., 2005; Dhir & Kumar, 2010). Plants which can be used for phytoremediation must possess characteristics such as being native, and having a rapid growth rate, a high biomass yield, the ability to absorb large levels of heavy metals, the ability to transport metals to those parts of plants which are above ground, and the ability to tolerate the metal toxicity mechanism (tolerant) (Ali et al., 2013; Burges et al., 2018).

The objectives behind these experiments involved the study of growth and yield levels in selected rice genotypes, and the response to organic ameliorants and their interaction in controlling iron toxicity levels in rice which is grown in tidal swamplands.

MATERIAL AND METHODS

Implementation procedure

A field experiment was conducted in the tidal swampland at Barito Kuala, South Kalimantan, on acidic sulphate soil - overflow type B - at two different locations which had differing ferrous Fe toxicity levels: ie. Belandean (with heavy toxicity levels) and Danda Jaya (with medium toxicity levels). The experiment was conducted between February and August 2011.

The experiment was arranged in a split-plot design employing land amelioration as the main plots, and with rice genotypes as the sub-plots. The main plots consists of the following: 1) control plots; 2) the application of one month-old *Salvinia sp* plants, buried under the soil prior to planting; 3) an application of *Salvinia sp* compost at 2.0 t ha^{-1}; 4) an application of rice straw compost at 2 t ha^{-1}; 5) an application of cow manure compost at 2 t ha^{-1}. The subplot (rice genotypes) consist of prospective genotypes TOX4136-5-1-1-KY-3, Inpara-1, Inpara-2, Inpara-4, and IR-64 (an Fe-sensitive genotype which was set as the control).

The treatments were assigned to plots of 4 m × 5 m, with three replications for each treatment. The *Salvinia sp* (treatment 2) source was collected from the River Kambat in Barito Kuala, South Kalimantan. *Salvinia sp* seeds were broadcasted at 100 g m^{-2} (fresh).

Composting was conducted for four weeks by adding decomposing bacteria to speed up the decomposition process. Other compost forms - rice straw and cow manure - were also composted and buried under the soil one week prior to planting. Organic matter with a water content of 35% was applied at a dose of 2 t ha^{-1}. Three week-old rice seedlings were planted 20 cm × 25 cm apart, with two seedlings per plot. Fertiliser was used of N, P, and K types, being applied at 75 kg N ha^{-1}, 37.5kg P_2O ha^{-1}, and 37.5 kg K_2O ha^{-1}. A half dose of N, and full doses of P and K, were applied seven days after planting; the remaining N was applied four weeks later. Weeding, plant protection, and maintenance were conducted according to recommended rice culture practices.

Soil and plant sampling

Measurements were carried out on soil Fe levels and the pH of the main plots before planting took place. This was seven days after the application of organic ameliorant, at the end of the vegetative growth stage, and after the harvest had been completed. Soil samples were taken from each plot using a soil drill at a depth of 20 cm, covering as many as six points. Soil from these six points was then composited, and about 500 g was taken for analysis in the laboratory.

The yield of agronomical characters and other yield components were recorded. The height and number of tillers were observed at the end of the vegetative phase for the plant, covering ten plants (or clumps) in each treatment plot. Observations were carried out for the rice yield component (involving the number of panicle tillers^{-1}, the number of filled grain panicles^{-1}, and the panicle length) by taking samples of six clumps in each treatment plot a t harvest. Grain yield was measured by taking a sample of 2.5 m × 2.5 m per plot.

Readings were taken twice for levels of ferrous Fe toxicity in rice, namely at the age of four weeks after planting and again at eight weeks (at the end of the plant's vegetative phase). Ferrous Fe toxicity was scored according to those methods which had been developed by IRRI-INGER (1996): a score of 1 means highly tolerant; 2–3 is tolerant; 5 is medium tolerant; 7 is sensitive; and 9 is highly sensitive. Plant samples were collected at the end of the vegetative stage for the purpose of tissue Fe analysis.

Soil and plant analysis

A measurement took place of soil pH levels using a digital pH meter, at a ratio of 1:2.5 soil and water. Iron (Fe) was extracted with one mol L^{-1} NH4 OAc (ammonium acetate), and then Fe levels in solution were determined through Atomic Absorption Spectrophotometry (AAS).

Nutrient contents (P, Ca, Mg, K, and Fe) in organic matter (ameliorant) as used in this study were extracted with a mixture of strong acid HNO_3+$HClO_4$. Phosphorus was measured by means of a staining system which used a spectrophotometer. Calcium (Ca), Mg, K, and Fe were measured using Atomic Absorption Spectrophotometry (AAS). Nitrogen (N) contents were determined by means of extraction using H_2SO_4+H_2O_2 (N-kjeldahl), and N levels were measured by means of distillation. The analysis of Fe in paddy plant tissue was the same as for the analysis of nutrient contents in organic matter (ameliorant).

Statistical analysis

The results which were obtained were subjected to an 'Analysis of Variance' (ANOVA), and further means of treatment effects were compared with testing results by using a Duncan Multiple Range Test (DMRT) at a confidence level of 95%. Data analysis was carried out using the SAS V.9 version program.

RESULTS AND DISCUSSION

Soils characteristic

The results of soil analysis at research locations revealed the acidity levels of the soil (pH): this was very acid. Meanwhile, soil pH levels at the Belandean site were lower (3.80) than they were at Danda Jaya (4.10). Toxic elements such as Al-exchangeable (9.70 me per 100 g^{-1}) and Fe which were extracted with Ammonium Acetate pH 4.8 (631 ppm) at the Belandean location were higher than they were at the Danda Jaya Al-exchangeable 6.37 me 100 g^{-1} and 425 ppm Fe. The depth of the pyrites layer ($FeS_2 \geq 2\%$) at Belandean was more shallow (≥ 40 cm) than at Danda Jaya (≥ 54 cm), while level of pyrites at Belandean was also higher (4.37%) than at Danda Jaya (2.48%) (Lubis et al., 2016).

Nutrient content of organic ameliorants

The nutrient content of the organic ameliorants can be seen in Table 1. Levels of organic-C ranged between 15.71–24.92%, while Nitrogen was between 0.87–1.08%, P was 0.09–0.32%, K was 1.35–1.85%, Ca was 0.24–0.35%, Mg was 0.22–0.28%, and C/N was 16–26 (Table 1).

Composts which included *Salvinia sp* had the lowest carbon-nitrogen ratio, whereas variants of rice straw had the highest Potassium levels (1.86%) when compared to the other ameliorants. Variants of cow manure had the highest P level (0.32%). Fresh *Salvinia* had the highest Fe levels (5.23%), followed by *Salvinia* composts, whereas rice straw and cow manure had the lowest levels of Fe (0.25%).

Table 1. The nutrient content of those ameliorants which were used in the research

Nutrient	Rice Straw Compost	Cow Manure Compost	*Salvinia sp.* Compost	Fresh *Salvinia sp.*
C (%)	24.92	16.6	15.71	24.83
N (%)	0.96	0.87	0.98	1.06
C/N	26.0	19.08	16.0	23.4
P (%)	0.19	0.32	0.09	0.11
K (%)	1.86	0.95	1.35	1.75
Ca (%)	0.35	0.18	0.24	0.31
Mg (%)	0.22	0.39	0.28	0.26
Fe (%)	0.62	0.25	1.62	5.23

Changes in soil Fe and pH

Soil analysis which was conducted prior to planting (seven days after soil ameliorant treatmen t had been conducted, at the end of the vegetative stage, and after harvest), demonstrated that soil pH and Fe levels at both experimental sites had changed (Figs 1 and 2). Soluble Fe and soil pH decreased with time; being at their lowest after harvest. Soils at Danda Jaya were more acidic than were those at Belandean in all treatments, whereas soil Fe levels at Danda Jaya were lower than were those at Belandean.

Organic ameliorant treatments did not always change soil pH levels. Soil pH at Belandean before planting was 3.65–3.90 when compared to a reading of 3.50 in the control soil, whereas at Danda Jaya it was between 3.90–4.10 when compared to a reading of 3.90 in the control soil (Fig. 1).

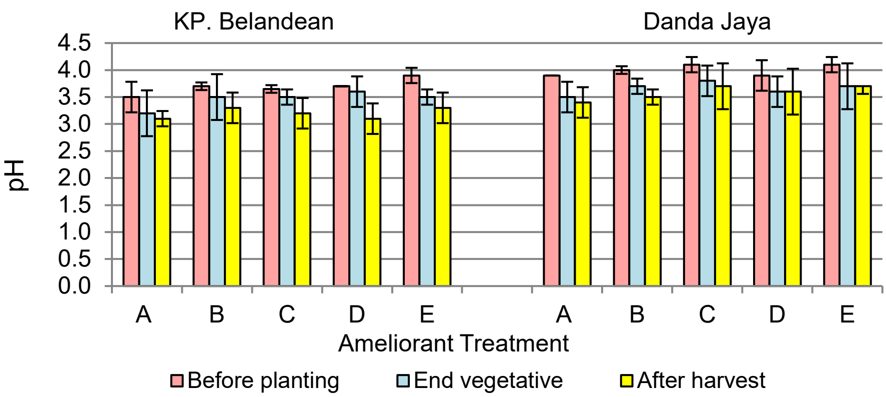

Figure 1. Changes in soil pH content prior to planting, at the end of the vegetative stage, and after harvest, as affected by ameliorant treatment in the tidal swampland of Belandean and Danda Jaya, South Kalimantan, with: a) control; b) *Salvinia sp*, grown; c) *Salvinia sp* compost; d) compost rice straw; and e) compost farmyard manure.

The decrease in soil Fe levels following amelioration treatment was at a figure of 11%, or between 761 ppm (for the control) down to between 525–681 ppm at Belandean. For the Danda Jaya location, the figure was between 9–19%, or from 528 ppm to 428–480 ppm respectively. The decrease in soil Fe levels a t harvest could be related to the waterlogging in this lowland area. Water levels decreased as the plants matured during the start of the dry season; oxidation processes in the soil reduced the levels of soil Fe^{+2} (Fig. 2).

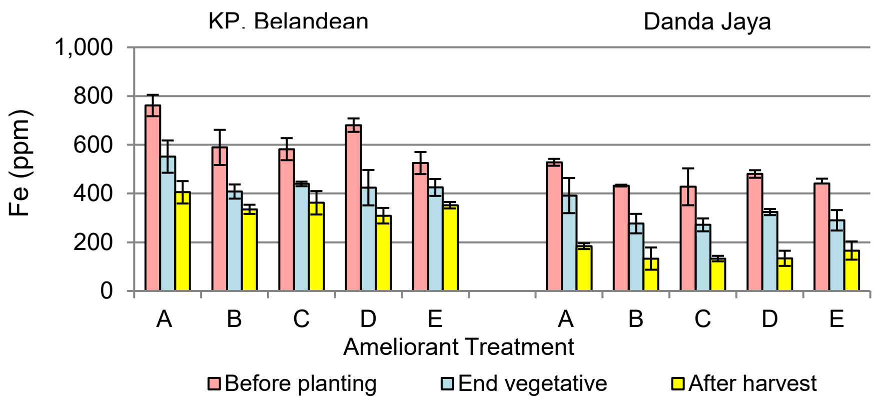

Figure 2. Changes in soil Fe levels prior to planting, at the end of the vegetative stage, and after harvest as affected by ameliorant treatment in the tidal swampland at Belandean and Danda Jaya, South Kalimantan: a) the control; b) *Salvinia sp*, grown; c) *Salvinia sp* compost; d) rice straw compost; and e) farmyard manure compost.

The results revealed that soil iron content in both locations was quite high, with a very low pH level. Ferrous iron toxicity in rice plants was due to the high Fe^{+2} content in the soil, something which occurs in flooded conditions (being known as reduction), where ferric iron (Fe^{+3}) is converted into ferrous iron (Fe^{+2}). The reaction which involved the reduction of ferric iron to ferrous iron occurs in flooded conditions (known as reduction), which involves anaerobic microbes which utilise electrons from NO^{-3}, MnO^{+4}, Fe^{+3}, and $SO4^{-2}$ (Emerson & Moyer, 1997). The Fe oxidation-reduction reaction can be described as follows (Dent, 1986):

$$Fe(OH)_3 + 3H^+ + e- \Leftrightarrow Fe^{2+} + 3H_2O$$

Acidity (soil pH) triggers the dissolution of ferric iron (Fe^{+3}) into a ferrous form (Fe^{+2}), which can be absorbed by plants and can subsequently result in toxicity. The results have shown that low soil pH levels could encourage iron toxicity in rice plants (Dobermann & Fairhurst, 2000; Sahrawat & Diatta, 1995). Fe levels of 100 ppm at pH 3.7 and 300 ppm Fe at pH 5.0 causes iron toxicity in rice (Sahrawat et al., 1996), while Fe levels of 250–500 ppm with a pH of between 4.5–6.0 causes iron toxicity in rice (Majerus et al., 2007; Mehraban et al., 2008).

The ameliorant application of organic matter in soil can decrease the Fe content in that soil (Fig. 2), and decrease the Fe content in the same soil due to the act of chelating organic matter with Fe. The decrease of Fe content in the soil is also a result of the reduction of Fe content which is absorbed by the plant due to the ameliorant application of organic matter in that soil. According to Bernal et al. (2007), organic matter has to control the mobility of heavy metals in soils by decreasing available concentrations through precipitation, adsorption, or complexion processes.

The results from research which was undertaken by Mardalena et al. (2018), revealed that aquatic plants such as water hyacinth (*Eichornia crassive*), water lettuce (*Distia stratiotes*), and floating fern (*Salvinia natans*) can be used as materials for phytoremediation in terms of water which has been contaminated by coal waste due to the ability of these plants to be able to absorb heavy metals such as Fe and Mn.

Fe toxicity levels in plants

The symptoms of iron toxicity vary with rice genotypes. The most common visual symptoms are bronzing or yellowing in rice, tiny brown spots on the lower leaves of plants, spreading towards the leaf base, or the entire leaf colour turning an orange-brown and drying out (Peng & Yamauchi, 1993).

Organic amelioration treatments and rice genotypes significantly affected the levels of ferrous Fe toxicity in rice at four and eight weeks after planting. There was no significant interaction between organic ameliorants and rice genotypes. Organic amelioration treatments could be seen to reduce ferrous Fe toxicity levels in rice (see Table 2).

Table 2. The effect of land amelioration and rice genotypes on ferrous Fe toxicity levels in rice grown in the tidal swampland at Belandean and Danda Jaya in South Kalimantan

Treatment	Belandean		Danda Jaya	
	Fe toxicity Scores (4 weeks)	Fe toxicity Score (8 weeks)	Fe toxicity Scores (4 weeks)	Fe toxicity Scores (8 weeks)
	Soil Ameliorant			
Control	3.93 a	4.32 a	3.33 a	3.42 a
Fresh *Salvinia sp*.	2.53 c	3.06 b	3.00 ab	2.68 b
Salvinia sp. compost	3.00 b	3.28 b	2.73 b	2.58 b
Rice straw compost	3.13 b	3.40 b	3.20 ab	2.88 b
Cow manure compost	3.00 b	3.14 b	3.07 ab	2.72 b
	Rice Genotypes			
TOX4136	2.87 b	3.34 b	2.80 b	3.02 b
Inpara-1	2.33 c	2.74 bc	2.00 c	1.80 c
Inpara-2	3.20 b	3.32 b	3.20 b	3.14 b
Inpara-4	2.20 c	2.26 c	2.07 c	1.60 c
IR 64	5.00 a	5.54 a	5.27 a	4.72 a

Note: Values which are followed by letters within a column are significantly different according to DMRT at $\alpha = 5\%$.

The iron toxicity levels in four-week-old plants which had been grown in ameliorant-treated plots in Belandean was lower - at between 2.53–3.13 - than in the control plots (where the reading was 3.93). Similar results were obtained at Danda Jaya, with readings of between 2.73–3.20 in ameliorant-treated plots and 3.33 in the control plots. Plants which had been treated with *Salvinia sp* compost showed the lowest toxicity symptoms.

The results which are shown in Table 2 revealed the fact that TOX 4136, Inpara-1, Inpara-2, and Inpara-4 varieties are more tolerant when it comes to iron toxicity than is IR-64. Inpara-1 and Inpara-4 plants revealed the lowest symptoms of toxicity in both experimental sites, and at four and eight weeks. The ratings for Danda Jaya were at 1.80 and 1.60 for Inpara-1 and Inpara-4 respectively, in contrast to a reading of 4.72 for IR-64. Similarly, ratings at Belandean were at 2.74 and 2.26 for Inpara-1 and Inpara-4 respectively, in contrast to the figure of 5.54 for IR-64 (Table 4).

Inpara-1 and Inpara-4 show low iron toxicity symptoms both with and without ameliorant applications, indicating the fact that both of these varieties are tolerant of iron toxicity in tidal swampland. According to Becker & Ash (2005), rice plants have a mechanism of avoidance and/or morphological and physiological tolerance when it comes to being able to overcome and survive adverse conditions in soils which contain toxic Fe elements and large amounts of Fe in plants. This mechanism is important in the selection of adaptive or tolerant rice genotypes.

The tolerance of rice genotypes to ferrous Fe toxicity is related to the biosynthesis and lignification processes in root cell walls. Increased lignin concentration in root cell walls and changes of Fe permeability increase the capacity to avoid excessive Fe uptake (Stein et al., 2019). According to Saikia & Baruah (2012), iron toxicity tolerance in rice is related to anti-oxidative enzyme activity. There was an exclusion mechanism here which was determined by its root architecture to be conducive to air transport giving it, therefore, the ability to oxidise Fe^{2+} in the rhizosphere, while there was also a tolerance mechanism which was mainly related to shoot-based mechanisms (a tolerance-inclusion mechanism) (Wu et al., 2014).

Iron levels in plants

Ameliorant treatments significantly reduced Fe levels in rice plant tissues (Fig. 3). The Fe levels were found to be at a figure of 1,299 ppm in the control, and between 759–1,096 ppm in ameliorant-treated plants which were being grown at Belandean. Similar results were obtained from plants which had been grown at Danda Jaya, with a figure of 939 ppm in the control and figures of between 622–703 ppm in ameliorant-treated plants (Fig. 3).

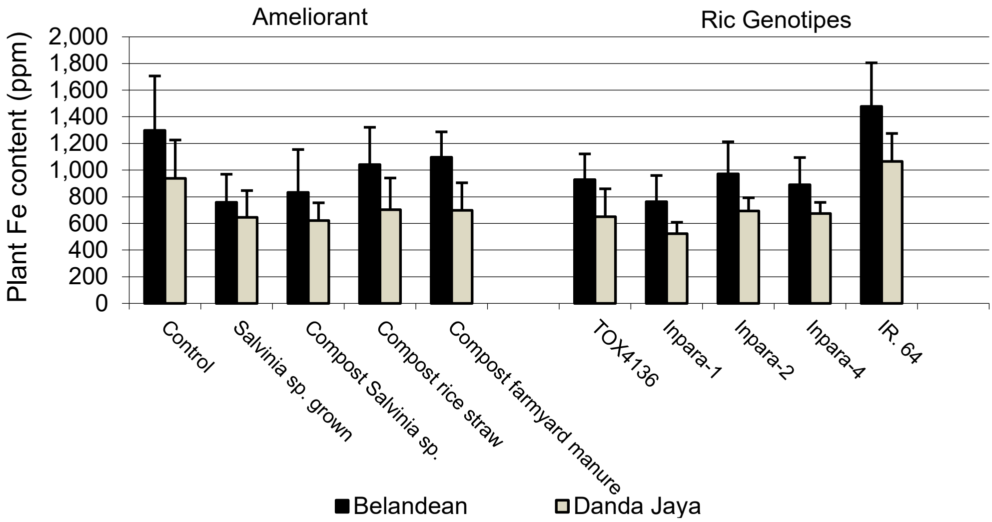

Figure 3. Average iron content in rice under land ameliorant and rice genotype treatments in tidal swampland: Belandean and Danda Jaya, South Kalimantan.

Rice genotype IR-64 is susceptible to iron toxicity, so this genotype could be seen to possess higher iron levels in the plant tissues. Iron levels in IR-64 which was rown at Belandean was at 1,477 ppm, which was higher than the comparative readings for other genotypes (TOX, Inpara-1, Inpara-2, and Inpara-4), which ranged between 762–971 ppm. Similar results were obtained from Danda Jaya, with a reading of 1,066 ppm Fe in IR-64, and only 524–694 ppm Fe in other genotypes.

Previous studies reported that levels of iron toxicity tend to vary with plant varieties and susceptibility. Iron toxicity occurs if the Fe intake by plants is more than 300 ppm (Yamauchi & Peng, 1995), whereas Sulaiman et al. (1997) reported that the figure was 200 ppm Fe for IR-64 which had been grown in tidal swampland areas. The soil Fe content which was used in this particular experiment was somewhat high (at 1,626 ppm Fe), in soil with a high Fe content which can cause iron toxicity in plants. The research results by Noor et al. (2012) showed that FE concentrations in a solution which was set at ≥ 325 ppm Fe caused symptoms of severe iron toxicity in IR-64 rice varieties, which resulted in the presence of a level of inhibition of rice plant growth and which decreased the plant's dry weight to 85.5%.

The level of Fe in rice tissue (Fig. 2) was associated with the iron toxicity figures (Table 2), and Fe levels in the soil (Fig. 2). The result of research by Turhadi et al. (2018), showed that leaf bronzing figures correlated with Fe content levels in the shoots themselves. The research results also showed that IR-64 (sensitive) absorbs high levels of Fe in plant tissues when compared to Hawara Bunar and Pokkali (tolerant), and Indragiri, Inpara-2, and Inpara-6 (moderately tolerant).

Plant growth

Ameliorant treatments significantly affected plant growth heights at the Balandean site, whereas the effects of rice genotypes were significant both at Balandean and Danda Jaya. There was no interaction between amelioration and rice genotypes in terms of affecting plant growth at the two sites (Table 3).

Table 3. The effects of land ameliorants and rice genotypes on plant height and the number of tillers in tidal swampland at Belandean and Danda Jaya in South Kalimantan

Treatment	Belandean		Danda Jaya	
	Plant Height (cm)	Number of tillers	Plant Height (cm)	Number of tillers
	Soil Ameliorant			
Control	76.6 b	12.9 b	76.9 a	14.8 b
Fresh *Salvinia sp.*	85.4 a	15.0 a	79.5 a	16.1 ab
*Salvinia*sp. compost	82.4 a	14.8 a	79.4 a	17.1 a
Rice straw compost	82.3 a	13.9 ab	78.6 a	15.8 ab
Cow manure compost	80.9 ab	13.5 b	81.6 a	15.5 b
	Rice Genotypes			
TOX4136	91.6 a	11.9 c	84.9 b	14.2 c
Inpara-1	89.0 a	14.3 b	89.9 a	16.3 b
Inpara-2	92.3 a	13.5 b	94.0 a	14.3 c
Inpara-4	65.6 b	17.0 a	64.8 c	20.6 a
IR 64	69.2 b	13.4 b	62.3 c	13.8 c

Note: Values which are followed by letters within a column are significantly different according to DMRT at $\alpha = 5\%$.

An application of fresh *Salvinia*, plus *Salvinia* compost, and various types of rice straw all served to increase plant height; this was noted as being between 82.3–85.4 cm in treated plants, compared to a figure of 76.6cm in the control. At both experimental sites, TOX-4136, Inpara-1, and Inpara-2 plants were taller than were those of Inpara-4 and IR-64 (Table 3).

Land ameliorants significantly affected the number of tiller clumps^{-1} at both sites. Belandean's plants, when treated with fresh *Salvinia* and *Salvinia* compost, had the greatest number of tillers, reaching figures of 15.0 and 14.8 respectively. Similar results were obtained at Danda Jaya, with readings of 17.1 for plants which had been treated with fresh *Salvinia*. The number of tillers between genotypes was variable, being at its highest in Inpara-4, with a reading of 17.0 at Belandean and 20.6 at Danda Jaya.

TOX-4136 had the lowest number of tillers at both sites, providing a reading of 11.9 at Belandean and 14.2 at Danda Jaya. Treatment with fresh *Salvinia* and *Salvinia* compost significantly increased the number of tillers, whereas treatment with various forms of rice straw and cow manure did not (Table 3).

Yield and the yield component of rice

Land ameliorants significantly affected grain yields at both sites, along with the number of panicles per tiller, and the number of filled grains per panicle. Similarly, plant genotypes significantly affected yield and yield components, and there were no interactions between land ameliorant treatments and plant genotypes when it came to affecting yield and yield components (Tables 4 and 5).

Table 4. The effects of land ameliorants and rice genotypes on dry grain yield (t ha^{-1}), the number of panicles per tiller, the number of filled grains per panicle, and panicle length (cm) in tidal swampland in Belandean, South Kalimantan

Treatment	Belandean			
	Grain (t ha^{-1})	Number of panicle tiller^{-1}	Number of filled grain panicle^{-1}	Panicle length (cm)
	Land Ameliorant			
Control	3.15 b	9.5 c	112.0 b	21.6 a
Fresh *Salvinia* sp.	3.81 a	12.3 a	127.5 a	21.8 a
Salvinia sp. compost	3.54 a	11.2 ab	131.5 a	22.5 a
Rice straw compost	3.73 a	11.0 b	126.4 a	22.3 a
Cow manure compost	3.60 a	10.8 b	136.6 a	21.9 a
	Rice Genotypes			
TOX4136	2.97 b	9.0 c	125.1 c	22.9 a
Inpara-1	4.08 a	11.0 b	140.6 b	23.3 a
Inpara-2	3.91 a	9.8 b	125.8 c	21.8 b
Inpara-4	4.13 a	12.5 a	170.7 a	21.6 b
IR 64	2.67 b	12.4 a	71.6 d	20.5 c

Note: Values which are followed by letters within a column are significantly different according to DMRT at $\alpha = 5\%$.

Organic ameliorant treatments increased dry grain yields, from 3.15 t ha^{-1} in the control to between 3.54–3.81 t ha^{-1} at Belandean. Fresh *Salvinia*, plus *Salvinia* compost, various forms of rice straw, and cow manure all increased yields by 21%, 12%, 18%, and 14% respectively (Table 4). Dry grain yields at Danda Jaya increased from

3.94 t ha^{-1} in the control to between 4.50–4.73 t ha^{-1}. Fresh *Salvinia*, plus *Salvinia* compost, various forms of rice straw, and cow manure all served to increase yields by 15%, 20%, 14%, and 17% respectively (Table 5).

Table 5. The effects of land ameliorants and rice genotypes on dry grain yield (t ha^{-1}), the number of panicles per tiller, the number of filled grains panicles^{-1}, and panicle length (cm) in tidal swampland at Danda Jaya, South Kalimantan

Treatment	Danda Jaya			
	Grain (t ha^{-1})	Number of panicle tiller^{-1}	Number of filled grain panicle^{-1}	Panicle length (cm)
	Land Ameliorant			
Control	3.94 b	12.8 b	115.3 b	23.0 a
Fresh *Salvinia* sp.	4.55 a	14.5 a	125.6 ab	22.7 a
Salvinia sp. compost	4.73 a	15.2 a	135.5 a	22.8 a
Rice straw compost	4.50 a	13.9 ab	131.4 a	23.0 a
Cow manure compost	4.63 a	14.0 ab	124.6 ab	23.2 a
	Rice Genotypes			
TOX4136	3.70 c	11.6 d	120.8 b	24.1 ab
Inpara-1	5.59 a	15.4 b	152.0 a	24.4 a
Inpara-2	4.50 b	12.5 cd	127.1 b	23.5 b
Inpara-4	5.61 a	17.8 a	158.9 a	21.6 c
IR 64	2.95 d	13.5 c	73.6 c	21.2 c

Note: Values which are followed by letters within a column are significantly different according to DMRT at $\alpha = 5\%$.

Organic ameliorant treatment increased the number of panicle tillers^{-1}, from 9.5 in the control to between 11.0–12.3 at Belandean, and from 12.8 for the control to between 13.9–15.2 at Danda Jaya. Inpara-1 and Inpara-4 at Danda Jaya had results of 14.8 and 17.8, which was significantly more than for IR-64 (which produced a reading of 13.5). *Salvinia*-treated plants, both fresh and composted, had more panicles than did the control plants, whereas those which had been treated with various forms of rice straw and cow manure were similar to the control treatment at Danda Jaya (Tables 4 and 5).

Organic ameliorant treatment increased the number of filled grain panicles^{-1} from 112.0 for the control to between 126.4–136.6 at Belandean, and from 1,115.3 for the control to between 124.6–135.5 at Danda Jaya. Inpara-1, Inpara-2, Inpara-4, and TOX-4136 all produced significantly higher readings than did IR-64 at both locations (Tables 4 and 5).

Organic ameliorant treatment at both locations did not affect panicle length. The average panicle length at Belandean was between 21.6–22.5 cm, while at Danda Jaya it was between 22.7–23.0 cm. At both locations, the genotype which had the longest panicle was Inpara-1 (which produced readings of 23.3 cm at Belandean and 24.4 cm at Danda Jaya), and the shortest was IR-64 (at 20.5 cm for Belandean and 21.2 cm for Danda Jaya).

Fresh *Salvinia* increased grain yields by 21% at Belandean, whereas *Salvinia* compost increased yields by 20% when compared to results for other ameliorant treatments. These results demonstrated that, just like various forms of rice straw and cow manures, *Salvinia*, both fresh and as compost, can be used as organic ameliorants in tidal swampland areas. Aquatic plants have the potential to be used for the phytoremediation of water which has been contaminated with inorganic (such as nutrients or heavy metals), and also organic pollutants due to their ability to absorb and bind elements which solute in the water (Wani et al., 2017; Mardalena et al. 2018; Ali et al., 2020; Ansari et al., 2020). The technology which was used in the phytoremediation of metal which had contaminated environments should be effective, cost-effective, and environmentally friendly (Sharma et al., 2015; Ashraf et al., 2018).

Organic ameliorant treatment and the use of tolerant genotypes increased rice yields through their roles in preventing or reducing ferrous Fe toxicity in rice (Tables 4 and 5). The use of tolerant genotypes at Belandean increased grain yields to 4.13 t ha^{-1} at Belandean, whereas the yield of the susceptible IR-64 was only 2.67 t ha^{-1}. Similarly, the use of tolerant genotypes at Belandean increased grain yields to 5.61 t ha^{-1}, whereas the yield of the susceptible IR-64 was only 2.95 t ha^{-1}. The yields of tolerant-genotypes (TOX-4136, Inpara-1, Inpara-2, and Inpara-4) at Belandean were between 11.20–54.70% higher than the figures for IR-64. Similarly, the yields were between 25.4–90.2% higher than those for IR-64 at Danda Jaya. Inpara-1 and Inpara-4 had grain yields of 4.08 and 4.13 t ha^{-1} at Belandean respectively, whereas at Danda Jaya the results were 5.59 t ha^{-1} and 5.61 t ha^{-1}.

The results which have been gathered together under this study have revealed that levels of iron toxicity tended to vary with soil characteristics and rice genotypes. The toxicity levels at Belandean were higher than those at Danda Jaya, something which may be related to differences in soil characteristics between the two sites (Table 2). The soil at Belandean was more acidic and had higher levels of exchangeable Al and Fe.

Ferrous Fe toxicity in plants is caused by high levels of soil-soluble Fe. Most mineral soils are rich in Fe, and ferrous Fe toxicity in plants generally occurred in flooded areas where a reduction process involving microbes converted insoluble Fe^{3+} to soluble Fe^{+2} (Beckers & Ash, 2005).The critical concentration, according to Sulaiman et al. (1997), using extraction methods with 1 N NH_4OAC in tidal swampland areas was 260 ppm. The toxicity level in IR-64 plant tissue was 200 ppm.

Research results from Lubis et al. (2016) regarding tidal swampland in South Kalimantan with a soil Fe content of 631 ppm, involved paddy IR-64 (sensitive varieties) which showed severe symptoms of iron toxicity (the ferrous Fe toxicity score was 7.0), but Inpara-4 (tolerant varieties) showed only light symptoms of iron toxicity (the ferrous Fe toxicity score was 3.0). In conditions of elevated iron toxicity, the individual rice plant experienced a decrease in morpho-physiological performance as indicated by a decrease in plant height, length, and width, the number of leaves, and the contents of chlorophyll and carotenoids (Novianti et al., 2020). According to Onyango et al. (2019), iron toxicity also affects several characteristics of rice, such as the length of the roots, increased bronzing of leaves, photosynthesis, transpiration, soluble sugars, proteins, and starch.

Organic ameliorant treatments at both sites served to reduce ferrous Fe toxicity and increased rice yields. The application of organic matter will improve soil quality and reduce phytotoxicity. Some organic acids - both naturally and as a result of the decomposition of organic matter - can bind to metal ions and reduce their solubility in the soil. Organic acids are abundantly released by the plants, and can be used as the natural chelators of metal ions (Montiel-Rozas et al., 2016), Research results from

Herviyanti et al. (2011) revealed that the application of humic matter from rice straw compost with a dose of 600 kg ha^{-1} on rice fields which have always flooded can reduce the concentration of Fe^{2+} in the soil, from 694 ppm to 476 ppm, and increase grain yield by 51.4% when compared to soil which does not contain humic materials. Research results from Mowidu et al. (2019) revealed that the application of straw compost at 5 t ha^{-1} increased the grain yield of Inpari-1 genotypes by 20.83% when compared to using no straw compost at all.

An application of fresh or composted *Salvinia sp* was comparably effective to cow manure or various forms of rice straw. This result demonstrated the potential use of *Salvinia sp* as organic matter, and this species is widely available in the tidal swampland. Four week-old *Salvinia* produced 225 kg of fresh weight 100 $(m^2)^{-1}$, or about 22.5 t ha^{-1} at Belandean, and a 186 kg $plot^{-1}$ or about 18.6 t ha^{-1} at Danda Jaya. The time taken to multiply *Salvinia sp* at Belandean and Danda Jaya was 5.1 and 5.9 days respectively.

There are several mechanisms in environmental phytoremediation which can be useful in remediating heavy metals from contaminated soils, with phytoextraction and phytostabilisation being used as the most promising and alternative methods (Mahar et al., 2016; Sarwar et al., 2017). Several environmental factors, such as pH, solar radiation, nutrient availability, and salinity, all greatly serve to influence the phytoremediation potential and growth of the plant (Reeves et al., 2018; Tewes et al., 2018).

Salvinia sp plants have been reported to contain major nutrients (N, P, K, Ca, and Mg), along with growth regulators, particularly cytokine and IAA (Arthur, 2007). In addition, *Salvinia sp* biomass has the ability to bind heavy metals. Begum & Hari Krishna (2010) reported that *Salvinia sp* bound 88.8% Fe from a 5 ppm solution after ten days. Sanchez-Galvan et al. (2008) reported that *Salvinia sp* has a wide specific surface ($264 m^2 g^{-1}$), one which is rich in carbohydrates (48.5%) and carboxyl (0.95 mmol g^{-1}).

CONCLUSION

1. Organic ameliorant treatments reduced soil Fe, ferrous Fe toxicity scores, and Fe content in plant tissues, and increased plant height and the number of tillers in selected rice grown under medium and high levels of ferrous Fe toxicities in tidal swampland.

2. Rice genotypes which have medium levels of tolerance or which are fully tolerant to iron toxicity and organic ameliorants treatments tend to decrease iron toxicities and increase the number of filled grains and yields of rice grown in tidal swampland. An application of fresh *Salvinia sp* and *Salvinia sp* compost were as effective as an application of various forms of rice straw and cow manure compost when it came to increasing yields.

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Selection of resistant upland cotton genotypes challenged with aggressive isolates of *Meloidogyne incognita* race 3

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Abstract. This study aimed to select populations of *M. incognita* race 3 for screening cotton genotypes as part of a breeding program for the development of resistant cotton cultivars. Five isolates of *M. incognita* race 3, collected in Western Paraná, Brazil, were tested for virulence and aggressiveness against the cotton cultivars FM966 (susceptible), IAC 24 (resistant), CD 409, and FMT 701 (moderately resistants) under greenhouse conditions, and following a factorial design with five replicates. Thirty-one cotton genotypes were screened against the three most aggressive isolates of *M. incognita* race 3 tested before and kept under greenhouse conditions following a factorial design with five replicates. Experiments run under greenhouse conditions had single cotton plants inoculated with 5,000 eggs/J2 of *M. incognita* and were assessed at 120 days after inoculation considering the variables gall index, egg mass index, total eggs, and reproduction factor. The same genotypes tested under greenhouse conditions were also grown in a field infested with *M. incognita* race 3 in a randomized block design with 10 replicates. In the field, the *M. incognita* population was monitored by the quantification of J2 forms in soil samples collected before sowing, 60 days after sowing (DAS), and 120 DAS. A gall index score was used to evaluate the roots of cotton genotypes at 120 DAS. The isolate from Umuarama was the most aggressive, followed by Moreira Sales and Iporã. The genotypes CD 05-419, CD 05-945, CD 05-1087, and CD 05-1170 showed resistance against *M. incognita* race 3 under greenhouse and field conditions.

Key words: aggressiveness, genetic resistance, *Gossypium hirsutum*, root-knot nematode, virulence.

INTRODUCTION

Herbaceous cotton (*Gossypium hirsutum* L.) is one of the most important annual crops in Brazil, due to its excellent economic return and market competitiveness (Dohlman et al., 2019). In the 2020/2021 harvest, Brazil was ranked as the second largest exporter and fourth largest cotton producer. Cotton growing areas in Brazil are spread mainly in the states of Mato Grosso and Bahia, which together respond for nearly 90 percent of all the Brazilian cotton production (Coêlho, 2021; Meyer & Dew, 2022). The high yield obtained in the cotton crop in Brazil has been affected by plant-parasitic nematodes with no cost estimation (Pires et al., 2008; Machado, 2014). In the USA, yield losses caused by *M. incognita* in cotton were estimated at 2,3% in season 2021 (Langstone, 2022).

One of the major nematode species of the cotton crop is the southern root-knot nematode *Meloidogyne incognita* (Lu et al., 2014). This nematode is of great importance in cotton-growing areas worldwide due to its aggressiveness and large host range. The main symptoms caused by this nematode in cotton plants are the presence of galls on the roots and plant growth suppression, which lead to a decrease in crop yield. Cotton losses are mainly caused by the population density of the nematode in the soil and its distribution in the growing area (Starr & Page, 1990).

M. incognita complex presents four host races, but only races 3 and 4 are known as parasites of cotton worldwide (Starr & Page, 1990). However, race 3 is prevalent in cotton-growing fields in Brazil (Kirkpatrick & Sasser, 1983; Pires et al., 2008).

The difficulties found when strategies are applied for the control of *M. incognita* in infested fields lie in the large land extension and improper management. The latter factor is the most significant as farmers fail to rotate crops, seeking an immediate return from the land. This leads to a sharp increase in the density of the nematodes, which soon become epidemic (Starr et al., 2007).

In cotton growing areas, chemical control of *M. incognita* may be performed through the use of nematicides. Nonetheless, these molecules are highly toxic and harmful to the environment, and also increase the costs of cotton production (Overstreet et al., 2014). Furthermore, prolonged use of chemical molecules can foster the selection of resistant variants in the nematode population. In addition, the large range of host plants makes crop rotation a difficult task when *M. incognita* is the target.

The use of resistant cotton cultivars is the most desirable method for controlling *M. incognita* for being safe and without additional cost for farmers. Moreover, when growing resistant cotton, the density of *M. incognita* in the soil is decreased (Wheeler et al., 2020), which prevents the appearance of new pathogen biotypes (Starr & Roberts, 2004). The genetic variability present in plant germplasm banks has been investigated by breeders worldwide for incorporating resistance genes in major crops against plant-parasitic nematodes and other plant pathogens (Razukas et al., 2009; Alves et al., 2017).

Different strategies have been used for assessing resistance to *M. incognita* in cotton germplasm collections. Davis & May (2003) and Carneiro et al. (2005) reported the use of single *M. incognita* isolates to assess cotton genotypes without a previous test for aggressiveness. Ogallo et al. (1997) screened cotton germplasm for resistance against virulent isolates of *M. incognita* with increased reproduction on resistant cotton. However, this procedure may complicate the selection of plant genotypes expressing oligogenic resistance (Zhou et al., 2000) and the selection of other plant genotypes with a different resistant gene (Phillips & Blok, 2008).

In this study, three isolates of *M. incognita* race 3 were selected and used to test the reaction of cotton genotypes, searching for sources of resistance to this nematode.

MATERIALS AND METHODS

Collection, establishment, and identification of *M. incognita* isolates

The five isolates of *Meloidogyne incognita* (EST I1, race 3) used in this study were previously collected from commercial cotton planting areas in the state of Paraná, Brazil, and kept in a glass-enclosed greenhouse at 25 °C. *Meloidogyne incognita* isolates were identified by the esterase phenotype (Esbenshade & Triantaphyllou, 1990) and host race was determined (Pires et al., 2008), according to Hartman & Sasser (1985).

Single egg masses were extracted from cotton roots and inoculated on tomato plants cultivar Rutgers for nematode reproduction. Tomato plants were grown in pots with 1.5 kg of a sterile substrate (1:1 v/v soil and sand), watered daily, and kept in a greenhouse under temperatures ranging from 25 to 28 °C.

Extraction of eggs and J2 of *M. incognita* from tomato roots

Sixty days after inoculation (DAI), tomato roots were separated from the shoot, washed with tap water, sectioned into small pieces, and crushed in a blender with sodium hypochlorite (NaOCl) at 0.5% for 60 seconds and under low rotation (Hussey & Barker, 1973). The suspension was passed through a sieve with a pore size of 230 μm stacked over a sieve with a pore size of 25 μm. Eggs and J2 (second-stage juveniles) retained on the sieve with a pore space of 25 μm were transferred to a beaker and quantified on a Peters' slide before inoculation.

Selection of *M. incognita* isolates

The experiment was carried out in a 5×4 factorial design consisting of the 5 isolates of *M. incognita* race 3 and four cotton varieties: resistant - IAC 24 (Cia et al., 2002), susceptible - FM966 (Galbieri et al., 2009), and moderately resistant - CD 409 and FMT 701 (Galbieri et al., 2009). Cotton plants were inoculated with 5,000 eggs of *M. incognita*, kept in a glasshouse at 25–28 °C, and assessed at 120 DAI based on the following variables: gall index, the total number of eggs, and reproduction factor. This experiment was performed twice in time.

Cotton genotypes tested in greenhouse and field experiments

Cotton genotypes, developed by the Central Cooperative of Agricultural Research (Coodetec) in collaboration with CIRAD-France, were tested against three populations of *M. incognita* race 3 (Table 1). The cultivars IAC 24 (Cia et al., 2002) and FM966 (Galbieri et al., 2009) were used as resistant and susceptible cotton varieties, respectively. The cultivars FMT 701, and CD 409, which are moderately resistant to *M. incognita* (Galbieri et al., 2009), were also included in this work.

Table 1. Genealogy of cotton genotypes and cultivars challenged with *Meloidogyne incognita* race 3

Genotype/Cultivar	Crossings	Genotype	Crossings
CD 406	[1]OC165 x Sicala V1	CD05-206	[12]CD98-39 x CD98-378
CD 408	[1]OC165 x Sicala V1	CD05-243	[12]CD98-39 x CD98-378
CD 409	[1]OC92-165 x Sicala 3-2	CD05-419	[13]CD991 x CD97-545
CD 410	[2]DPAc90 x P288	CD05-485	[14]CD97-122 x CD96-252
CD02-621	[3]OC92-165 x SP 8324	CD05-700	N320-2-9 x CD405
CD02-1637	[1]OC92-165 x Sicala 3-2	CD05-865	*N419-1-191 x CD401
CD03-5198	[4]SP8334 x Ston BR110	CD05-945	N315 RNR x CD401
CD04-4939	[5]CD98-218 x OC94-434	CD05-1039	[15]M315 RNK x OC96-276(CD404)
CD04-3361	[6]CD98-213 x CD98-420	CD05-1087	[16]M155 RKN x CD 401
CD04-3040	[7]CD98-578 x CD98-378	CD05-1170	[16]M155 RKN x CD 401
CD04-3278	[8]CD98-39 x CD98-578	CD05-1222	[17]M155 RKN x CD 405
CD04-3816	[9]CD98-991 x CD97-122	CD05-1323	[18]M155 RKN x OC94-434
CD04-4721	[10]CD98-218 x CD405	CD04-5281	[19]CD98-450 x OC94-434
CD04-5081	[11]CD98-361 x CD 401	CD04-2990	[7]CD98-578 x CD98-378

[1](P288/DP41)/Unknown; [2]Unknown/(Allen x HAR); [3](P288/DP41)/Fundo US; [4]Fundo Argentino; [5]92-165x SicalaV1/AllenxHAR/IAC20; [6]92-165xSicala32/HAR/IAC20; [7](Yuc/TniHoa/Au56)/HR102/DPAc90/Auburn56; [8]P288/DP41/(Yuc/TniHoa/Au56)/HR102; [9]SP8334 x DPAc90/IRCT223 x P288; [10]92-165 x SicalaV1/(CNPA86-387xP288)xPR3060/87; [11]Sealand542xIAC20Reba/CD401; [12]92-165xSicala32/DPAc90/Auburn56; [13]SP8334x DPAc90/Sealand542xIAC20; [14]IRCT223xP288;[15]Auburn634/DeltaPine61/SP8334/DPAc90; [16]Auburn634/Coker310/CD401; [17]Auburn634/Coker310/N'Kourala/(Allen x HAR)/Auburn56; [18]Auburn634/Coker310/HAR/IAC20; [19]Sealand542 X IAC20Reba/HAR/IAC20.

Inoculation of *M. incognita* race 3 on cotton genotypes

Cotton genotypes were kept in a greenhouse at a temperature of 27 °C and 60% relative humidity. Single cotton plants were grown in plastic tubes of 7×18 cm containing sterile substrate composed of soil and sand in the ratio of 1.5:1 and fertilized with 2 grams of N-P-K 8-20-20.

Plants with two true leaves were inoculated with 3 mL of a suspension containing 5,000 eggs and juveniles of *M. incognita* race 3. Cotton plants were evaluated at 120 DAI based on the following variables: Number of galls (GA), gall index (GI) (Taylor & Sasser, 1978), total eggs (Coolen & D'Herde, 1972), and reproduction factor (RF) (Oostenbrink, 1966). For egg extraction, cotton roots were ground in a blender in a solution of 0.5% sodium hypochlorite.

The quantification of eggs and juveniles was determined on a Peters' slide using a light microscope. The cotton plants were watered twice a day during the experiment. The experimental design was completely randomized with 31 treatments (cotton genotypes/varieties) and ten replicates.

Data obtained for eggs were transformed to log X + 1. The RF was calculated using the equation RF = Fp/Ip, where Fp means final population and Ip means initial population. Cotton genotypes with RF values < 1.0 were classified as resistant (R), moderately resistant (MR) when 1 ≥ RF < 2, moderately susceptible (MS) when 2 ≥ FR < 3, and susceptible (S) when RF >3. This classification followed Khan et al. (2016).

Field experiment

Cotton genotypes were grown in a commercial area of 120.75 m² infested with *M. incognita* race 3 where cotton had been cultivated for three years without rotation. The area is located in the municipality of Moreira Sales, northwest of Paraná state, Brazil.

A randomized block experimental design was adopted with 31 treatments (cotton genotypes/cultivars) and 10 replicates. Seeds of each treatment (Table 3) were sown with 50 cm plant spacing and row spacing. The cultivars IAC-24 and FM 966 were used as resistant and susceptible checks, respectively.

The population of *M. incognita* found in Moreira Sales was also collected and tested in the greenhouse experiments. Analysis of variance was used on the data, and the means of the treatments were compared by the Scott-Knott clustering algorithm at a 5% significance level (Scott & Knott, 1974). All analyses were performed using the SISVAR software (Ferreira, 2011).

Quantification of J2 in soil samples and physical and chemical soil analyses

Soil samples were collected for chemical, physical, and nematological analyses. A composite sample of ten cores and 1 kg of soil was selected at random in the experimental area. The grid had one composite sample composed of ten cores. Soil chemical contents were used for fertilizer recommendation. For quantification of J2, soil samples were collected at 0–20 cm depth before sowing and after sowing at 60 days and 120 days.

Extraction of nematodes

Nematodes were extracted from 100 cm^3 of soil following Jenkins (1964), and eggs were extracted from cotton roots according to Coolen & D'Herde (1972). A sieve with a pore size of 300 µm was stacked over a sieve with a pore size of 37 µm for the separation of nematodes. Eggs and J2 of *M. incognita* were quantified under a light microscope using a Peters' slide.

Evaluation of the field experiment

For the field trial, an assessment was conducted 126 days after sowing. Cotton plants were uprooted and the root system was evaluated for root gall rating on a scale of 0 (none) to 5 (severe), following Colyer et al. (2000). Each interval in the rating scale was related to its correspondent resistance level, according to Khan et al. (2016) (Table 2).

Table 2. Rating-scale for galls to assess the resistance of cotton genotypes to *M. incognita* race 3 in the field experiment (Khan et al., 2016)

Score	Galls	Ranking
0	0	HR
1	0–3	R
2	4–10	MR
3	11–30	MS
4	31–100	S
5	> 100	HS

RESULTS AND DISCUSSION

The isolate from Umuarama (UM) was the most virulent and aggressive for most cotton varieties, considering the variables number of galls (GA), Gall index (GI), total eggs, and reproduction factor, followed by the isolatess from Moreira Sales (MS), Iporã (IP), Pérola (PL) and Santa Lúcia (SL). Regarding the variables GA and GI, the UM isolate was more aggressive than PL and SL isolates but it did not differ from the isolates MS and IP. MS and IP isolates were more aggressive than PL and SL isolates for most variables, except for the variable GI (Table 3). Based on the results, UM, MS, and IP were selected for screening the cotton genotypes used in this study.

Table 3. Virulence and aggressiveness of *Meloidogyne incognita* race 3 isolates on cotton cultivars for germplasm assessment

Cultivar	Variable																							
	Ga (values x 10)						Gi						Eggs (valuesx10.000)						Rf					
Isolate	Um	Ms	Ip	Pl	Sl	V	Um	Ms	Ip	Pl	Sl	V	Um	Ms	Ip	Pl	Sl	V	Um	Ms	Ip	Pl	Sl	V
FM 966[1]	27	14	13	7.3	6.2	135 C	5	5	5	3.8	4	4.6 C	5	3.2	2.1	1.1	0.8	2.4	1	6.4	4.7	1.8	1.7	6.1 A
IAC-24[2]	2.9	1.9	1.5	1.1	1	17 A	2.8	2.6	2.4	2.0	1.8	2.3 A	1.3	0.8	0.5	0.5	0.3	0.6	2.5	1.6	0.9	0.7	0.5	1.5 C
CD 409[3]	16	12	8.4	4.1	6	92 B	5	4.6	4	3.2	3.8	4.1 C	2.1	1.4	1.7	0.7	0.6	1.3	4.3	3.5	2.7	1.3	1.1	3.2 B
FMT 701[3]	16	7.5	7.6	3.1	3.1	74 B	5	3.8	3	3.5	3.4	3.7 B	2.9	1.6	0.9	0.5	0.5	1.3	5.9	3.2	1.8	1	1.2	3.3 B
Isolate	15 a	8.7 b	12 B	3.9 c	4.1 c		4.4 a	4 ab	2.9 Ab	3.1 bc	3.2 bc		0.3 a	0.2 b	1.3 c	0.7 d	0.5 d		5.6 a	3.7 b	2.5 c	1.2 d	1.1 d	
CV%	18.27						6.12					15.92						12.60						

Data transformed to square root of X + 1 with original data kept; mean values represent five plants/treatment and a total of 99 degrees of freedom; averages followed by the same letter in the column (varieties) or row (isolates) did not differ by Tukey' test at 5% probability; Ga = number of galls; Gi = Gall index (Taylor & Sasser, 1978): 0 = no galls or egg masses; 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100, and 5 >100; Rf = Reproduction factor; Pl = Population from Pérola, PR; Ip = Population from Iporã, PR; Um = Population from Umuarama, PR; Ms = Population from Moreira Sales, PR; Sl = population from Santa Lúcia, PR;

[1]susceptible cultivar - Fibermax 966; [2]resistant cultivar; [3]moderately resistant cultivars. V = varieties. *CV* = Coefficient of variation; Combined analysis of greenhouse experiments 1 and 2.

According to Castagnone-Sereno (2002), the genetic variability of plant-parasitic nematodes should be taken into account when evaluating plant resistance and its durability. Additionally, previous tests with a large collection of nematode isolates should reduce the risks of a rapid breakdown of plant resistance genes.

Differences in the reproduction of *M. incognita* isolates in cotton genotypes were also reported by Silva et al. (2014) and for other root-knot nematodes by Van der Beek et al. (1998) on potato cultivars. This latter, reports an isolate-by-cultivar interaction between *M. hapla* and potato cultivars.

The gall and RF values obtained for the cotton genotype treatments after 120 DAI with the three isolates allowed the genotypes to be ranked into different categories based on their reaction to *M. incognita* (Table 4).

Table 4. Mean of galls and reproduction factor of three isolates of *Meloidogyne incognita* race 3 inoculated on cotton genotypes and cultivars under greenhouse experiments

Treatment	Galls	Reproduction factor
FMT 701	217.40 e	2.08 c
CD 406	225.20 e	2.13 c
CD 408	295.53 g	3.00 d
CD 409	220.33 e	2.04 c
CD 410	272.06 f	2.58 d
CD02-621	157.13 d	1.42 b
CD02-1637	190.86 d	1.73 c
CD03-5198	349.53 g	4.21 f
CD04-4939	326.13 g	3.54 e
CD04-3361	326.60 g	3.79 e
CD04-3040	236.06 e	2.39 c
CD04-3278	263.06 f	2.83 d
CD04-3816	150.26 d	1.27 b
CD04-4721	189.00 d	2.27 c
CD04-5081	173.86 d	1.41 b
CD04-5281	225.53 e	1.74 c
CD05-206	310.40 g	2.67 d
CD05-243	514.60 h	4.60 f
CD05-419	113.26 c	0.81 a
CD05-485	109.40 c	0.81 a
CD05-700	207.73 d	1.18 b
CD05-865	350.86 g	1.89 c
CD05-945	74.60 b	0.56 a
CD05-1039	261.80 f	2.14 c
CD05-1087	46.70 a	0.37 a
CD05-1170	59.00 a	0.47 a
CD05-1222	56.40 a	0.48 a
CD05-1323	61.13 a	0.54 a
CD04-2990	165.13 d	2.00 c
FM 966	615.73 i	16.53 g
IAC 24	84.00 b	1.03 b
CV (%)	14.37	13.37

Also, the isolate UM was more aggressive than MS and IP, while MS was more aggressive than IP isolate in the greenhouse experiments, as shown in Table 5.

Table 5. Overall mean of galls and reproduction factor of three isolates of Meloidogyne incognita race 3 on cotton cultivars in greenhouse experiments

Isolate	Galls	Reproduction Factor
IP	147.17 a	1.49 a
MS	195.70 b	1.69 b
UM	319.96 c	2.02 c

*Means followed by the same letter do not differ by Tukey's test at 5% probability. Data transformed by the *square root of X + 1* with original data kept; combined analysis of greenhouse experiments 1 and 2. IP = Iporã, MS = Moreira Sales, UM = Umuarama.

In both greenhouse experiments, the genotypes CD05-1087, CD05-1170, CD05-1222, and CD05-1323 produced the lowest values for GA (averages ranging from 47 to 61) and RF (averages ranging from 0.4 to 0.5). CD05-945, CD05-419, and CD05-485 produced more galls on the roots (averages ranging from 109 to 113) than the previously cited genotypes, but their RFs did not differ statistically (average of 0.81).

The cultivar IAC 24, used as a resistance check, had a GA mean of 84.0 and RF mean of 1.0. The RF of IAC-24 did not differ statistically from the RFs of CD05-700 (mean of 1.18), CD04-5081 (mean of 1.41), CD04-3816 (mean of 1.27), and CD02-621 (mean of 1.42), but these genotypes had a larger number of galls on the roots, which ranged from 150 to 173.

The genotypes FMT 701, CD 406, CD 409, CD04-3040, and CD04-5281 produced more galls (217 to 225) on the roots than CD02-1637 and CD04-4721 (189 to 191), but these genotypes did not differ statistically from each other based on the RFs, which ranged from 1.7 to 2.4.

Genotypes with means of the RF ranging from 2.6 to 3.0 are statistically similar, but for the GA variable, CD04-3278 (263 galls) and CD 410 (272 galls) are statistically different from CD 408 (295 galls) and CD 05-206 (310 galls).

Other genotypes, such as CD04-4939 (mean RF of 3.5, 326 galls) and CD04-3361 (RF of 3.8, 327 galls) are statistically different from the genotypes CD03-5198 (RF of 4.2, 349 galls), and CD05-243 (RF 4.6, 515 galls). The susceptible check, cultivar FM966, had the highest values for means of RF (16.5) and galls (616).

The UM isolate was the most aggressive, inducing an overall average of 320 galls per cotton plant and RF mean of 2.0, followed by MS with 196 galls and RF of 1.7, and IP with 147 galls and RF of 1.5 (Table 4). There was a positive correlation between the galls produced in different cotton genotypes and the RF of the three isolates tested ($r = 0.86$; $R^2 = 0.97$; $P < 0.05$).

Soil samples analysis revealed that silt, sand, and clay contents were at 4%, 88%, and 8%, respectively. The analysis revealed the presence of J2 of *M. incognita* in the soil of the experimental area before the beginning of the field trial, and an increase in the J2 density during cotton cultivation (Fig. 1).

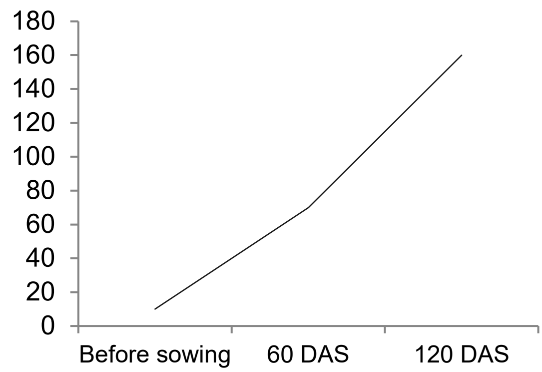

Figure 1. Dynamics of *M. incognita* J2 in the soil of a field experiment assessed before sowing and at 60 and 120 days after sowing (DAS).

The results obtained in the field showed that the evaluation by rating scale allowed the separation of the genotypes into 4 groups according to their resistance level (Table 6). According to the evaluation criterion adopted (rating scale), CD05-419 (GI = 1.90), CD05-1087 (GI = 1.70), CD05-1170 (GI = 1.70), and CD05-945 (GI = 1.60) were classified as resistant due to GIs less than 2.0. The other genotypes were classified as moderately resistant, moderately susceptible, and susceptible. The susceptible check, FM 966, had a GI of 4.40 and was classified as susceptible. The resistant check, IAC-24, had a GI of 2.20 and was classified as moderate resistant (Table 6).

Several works addressing the resistance level of cotton genotypes from germplasm collections were previously reported by Shepherd (1974, 1982), Shepherd et al. (1996), and Starr & Smith (1999). Other studies involving the search for new sources of resistance to root-knot nematodes were reported by Sheperd (1983), Mota et al. (2013), and inheritance of resistance in cotton accessions (Faske & Starr, 2009; Alves et al., 2017).

Table 6. Host status of cotton genotypes and cultivars inoculated with *M. incognita* race 3 and assessed under greenhouse and field conditions

Genotype	Host Status*	Field Score	Host Status**	Genotype	Host Status*	Field Score	Host Status**
CD 05-945	R	1.60 a#	R	CD 04-5281	MR	2.40 b	MR
CD 05-1087	R	1.70 a	R	CD 409	S	2.60 b	MR
CD 05-1170	R	1.70 a	R	CD 02-621	MR	2.60 b	MR
CD 05-419	R	1.90 a	R	CD 05-700	MS	2.70 b	MR
CD 05-1222	MR	2.00 a	MR	CD 02-1637	MS	2.80 c	MR
CD 05-1323	MR	2.00 a	MR	CD 410	S	2.90 c	MR
CD 05-485	R	2.00 a	MR	FMT 701	MR	3.00 c	MS
CD 04-3816	MR	2.10 a	MR	CD 05-1039	MS	3.10 c	MS
CD 04-4939	MR	2.20 b	MR	CD 408	S	3.20 c	MS
IAC - 24	MR	2.20 b	MR	CD 406	MS	3.60 d	MS
CD 04-3361	MR	2.20 b	MR	CD 03-5198	S	3.70 d	MS
CD 04-3278	MR	2.20 b	MR	CD 05-865	MS	3.70 d	MS
CD 04-3040	MR	2.30 b	MR	CD 05-243	S	3.80 d	MS
CD 04-4721	MR	2.40 b	MR	CD 05- 206	MS	3.90 d	MS
CD 04-5081	MR	2.40 b	MR	Fiber Max 966	S	4.40 e	S

*Column showing host status of cotton genotypes for *M. incognita* in the greenhouse experiments; **Column containing the ranking of cotton genotypes for *M. incognita* in the field experiment; # Means followed by the same letter do not differ by the Scott-Knott test at 5% probability; ***Ranking adapted from Khan et al. (2016).

The status of some cotton genotypes was different when comparing data from field and greenhouse. CD05-1222 (RF = 0.48) and CD05-1323 (RF = 0.54) were classified as resistant in the greenhouse experiments and moderate resistant in the field (mean of GI = 2.0). In this case, these genotypes were classified as presenting moderate resistance.

Differences in the response of cotton genotypes to the nematode *M. incognita* were also reported by Ogallo et al. (1997) and Galbieri et al. (2009), for tests carried out under greenhouse and field conditions.

Field experiments are challenging because plants are exposed to different climate and soil conditions and also to biotic stresses caused by organisms other than nematodes. All this contributes to a better understanding of the cotton response to plant parasites such as the root-knot nematode *M. incognita*. In this study, the genotypes CD05-419, CD05-1170, CD05-1087, and CD05-945, were classified as resistant to *M. incognita* due to the performance obtained under greenhouse and field conditions. These resistant cotton genotypes will be targeted for further studies about their agronomic performance aiming at the development of new cultivars.

CONCLUSIONS

The results obtained in this study allowed the selection of four cotton genotypes (CD05-945, CD05-1170, CD05-1087, and CD05-419), through greenhouse and field experiments, for which three *M. incognita* isolates had a low reproductive rate. Additionally, the selection of virulent and aggressive isolates of *M. incognita* before screening genotypes for resistance was very important and helpful in the selection of resistant cotton genotypes.

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Geogenic and anthropogenic contamination of groundwater in a fragile eco-friendly region of southern Kerala, India

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Abstract. In environmentally fragile regions that rely solely on groundwater resources, the hazards to the environment and human health are amplified by geogenic and anthropogenic pollution through the supply and use of groundwater for drinking and irrigation use. Groundwater from borewells in the study area was evaluated through hydrogeochemical analysis of 17 parameters in 2018 and 2019 across three seasons: pre-monsoon, monsoon, and post-monsoon. The study area, Kainakary, a fragile eco-friendly area in South India, was specifically chosen, as agriculture is the predominant anthropogenic activity in the region and other anthropogenic activities with known negative effects are negligible compared to other parts of India. Despite diligent attention paid to sustainable practices in Kainakary, iron, fluoride, and ammonia components in groundwater exceeded the permissible limits stipulated by the World Health Organization and Indian drinking water standards. Significant need for water resources due to below sea level farming practices of rice cultivation and potable water requirements result in over-extraction of groundwater, an inevitable cause of geogenic pollution. Anthropogenic pollution of groundwater sources was evidenced by the presence of coliform bacteria in samples. Determining the origins of major geogenic and anthropogenic pollutants, as well as understanding irrigation use patterns, play a key role in mitigating the overuse of groundwater sources. This study contributes to evolving strategies for reducing geogenic and anthropogenic pollution and for groundwater management in ecologically fragile areas toward achieving Sustainable Development Goal 12, which focuses on responsible consumption and production.

Key words: fluoride, groundwater, geogenic pollution, Kainakary, SDG 12.

INTRODUCTION

Many countries worldwide are facing acute water scarcity, due to increasing demand for, and diminishing availability of this resource. Rapid depletion of groundwater resources has resulted in distress for nearly 30% of the world's largest groundwater systems. Unavailability of accurate knowledge of the quantity of groundwater sources and inadequate monitoring and quality of these sources are hampering the ability to make informed decisions. Water pollution, population density, and economic growth is now viewed as a threat to sustainable development (Boretti & Rosa, 2019).

Groundwater is considered to be safer than surface water in terms of quality and mineral composition, and to a certain extent, to be beneficial for human health (Rohde et al., 2017). Therefore, most of the effort currently revolves around groundwater availability to fulfill the water demands of an increasing population, rather than its quality for drinking purposes. Groundwater quality is impacted by natural hydrochemical and geochemical processes, increased groundwater abstraction, and potential contaminants from agriculture, human excreta and sanitation, industry, mining, solid waste disposal, and landfill leachate. To safeguard groundwater quality and quantity, in 2006, the Ground Water Rule was implemented in the USA and the Groundwater Directive in Europe.

India is among the largest groundwater users (Chinnasamy & Agoramoorthy, 2015; Mukherjee et al., 2015; Kulkarni et al., 2015; Patel et al., 2016; Arun, 2017). As of April 2015, the water resource potential of India was 1,869 billion cubic meter (BCM) per year in terms of natural runoff (water flow) in rivers. However only ~60% of it was usable due to its erratic and variable distribution and topographical constraints. The usable 1,123 BCM per year is comprised of 690 BCM per year of surface water and 433 BCM per year of groundwater (Central Water Commission, 2015). A report by NITI Aayog, the Indian government's think tank, has forecasted that by 2030, India's water demand will be twice the available supply, resulting in nearly 6% loss in GDP (NITI Aayog, 2018).

Hard-rock aquifers with poor permeability to recharge through rainfall are a predominant characteristic of nearly 65% of peninsular India. Over extraction of groundwater results in deterioration of groundwater quality (Mukherjee et al., 2015). Discharge from industrial waste, agricultural activities, urban wastewater, seawater intrusion in the coastal tract, and landfill leachate are common groundwater contaminants (Banerjee et al., 2012). Harmful substances in the groundwater in many parts of India are directly related to anthropogenic factors, specifically agricultural water use and landscape changes (Burow et al., 2017; Verma et al., 2017; Jia et al., 2018; Prasad et al., 2020). Fluoride and arsenic are the major geogenic contaminants of groundwater in India, and a major health concern, stemming from weathering processes (Banerjee et al., 2012). Increased incidence of chronic kidney disease associated with uranium has also been reported in India (Rajapurkar et al., 2012). Fluorosis associated with fluoride intake is common in Rajasthan (Agrawal et al., 1997; Choubisa, 2001).

According to a 2011 Indian census, groundwater through dug wells and borewells/tube wells is the source of potable water for nearly 65% of the population in the State of Kerala (Nair et al., 2014). Over the last 100 years, the per capita water availability in Kerala has decreased dramatically, while water demand has markedly increased. Kerala receives 2.5 times more rainfall than the national average in India, but it supports an ~4-fold population than the national average, as well as water utilization by a rich and diverse vegetation (Varma, 2017). Groundwater in Kerala is vulnerable to both geogenic and anthropogenic pollution. The Critical Zone, which extends from the treetops to the aquifer bottom, has exhibited drastic changes as a result of environmental degradation, poor water management, and urbanization. Moreover, changes in groundwater quality are evident in the pre-monsoon (PrM), monsoon (Mon), and post-monsoon (PoM) seasons. Manjula & Warrier (2019) opined that the groundwater and river water chemistry in regional studies in Kerala is influenced by existing rock chemistry rather than by precipitation and evaporation. Hydrochemistry analyses revealed that Na^+ with Ca^{2+}, Cl^- and K^+ are dominant during the PrM season, whereas Ca^{2+} and mg^{2+} are dominant during the Mon season and HCO_3^- during the PoM season, possibly resulting from hydrochemical reactions in the aquifer, as posited by Vutla & Ravichandran (2011). Reza & Singh (2019) found a higher concentration of dissolved metals during the PrM vs. PoM season, probably due to more dissolution of metals during rock–water interactions in the relatively stagnant and low groundwater flow in the former season. Jasmin & Mallikarjuna (2014) showed that concentrations of eight parameters (total dissolved solids [TDS], nitrate [NO_3^-], N, Na^+, Cl^-, K^+, F^- and hardness) exceed their permissible limits during PrM and PoM seasons, with quality in the latter being worse than in the former season in the state of Tamil Nadu in Southern India, probably due to increased influx of contaminants from industries, mining areas, waste-disposal sites and agricultural fields during the monsoon season. Taken together, there is a pressing need for a better understanding of the quality of groundwater sources, its changing pattern before, during, and after monsoons, and the related anthropogenic factors, to ensure the sustainability of water resources and sustainable agriculture.

This study was carried out in a region where agriculture is the major anthropogenic activity, whereas other potentially negative anthropogenic activities are minimal. The goal of this study was to (i) compare groundwater quality parameters before, during, and after the monsoons, (ii) determine the presence of geogenic and anthropogenic pollution and identify the predominant pollutants (iii), determine the Water Quality Index (WQI) of groundwater sources for 2018 and 2019 in Kainakary, Kerala, and provide suitable management strategies for the region. The insights gained from this study will aid policymakers to implement appropriate management strategies for managing geogenic and anthropogenic pollution towards achieving sustainable development.

MATERIALS AND METHODS

Assessment of the geogenic and anthropogenic contaminants and the extent of pollution was done using previous and current analytical data, aggregated observations, and historical government data, due to the limited accessibility to region-specific data on the availability, water quality, and recharge practices.

Study Area

Alappuzha, the smallest coastal district in southern Kerala, is situated between latitudes 9°51' N and 9°45' N, and longitudes 76°45' E and 76°1' E (Prasad & Ramesh, 2019). The study area, Kainakary Panchayat (aka Grama Panchayat - village council in India), is an ecologically sensitive area of the Kuttanad wetland ecosystem and is an important tourist destination in India, attracting millions of people from within and outside the country. Apart from tourism, the study area is globally recognized as a Ramsar site- a wetland site listed under UNESCO's Ramsar Convention to promote sustainable use of its natural resources. The mission of the Ramsar Convention is 'the conservation and wise use of all wetlands through local and national actions and international cooperation, as a contribution towards achieving sustainable development throughout the world' (Gardner & Davidson, 2011). According to a 2011 Indian census (Census, 2011), the estimated population of Kainakary was 23,696 residents in 5,689 households. The practice of paddy field farming below sea level is a distinctive feature of agriculture in this area. Battered by heavy rainfall, the area is prone to severe floods, disrupting the ecosystem's natural balance with devastating environmental consequences, as evidenced by the flooding havoc in 2018. The study area is a peninsula, and the landmass is interspersed with canals, ponds, and paddy fields.

Warkalli beds of Tertiary Age (Upper Miocene to Pliocene), the dominant geological feature of this region, are comprised of alternating beds of clayey sandstone, white and variegated clay, and carbonaceous clay. Notable lithological features in the study area include ferruginous laterite at depths of 0–6 m and 36–61 m. Data from government sources indicate that the phreatic aquifer is polluted in the study area. The geographical features, such as annual rainfall, temperature, geology, geomorphology, land-use pattern, soil, and detailed specifications of the study area have been elaborated by Prasad & Ramesh (2019).

Sampling

Three borewells, located at the outskirts of Kainakary Panchayat (approximately 6.5 km away from Alappuzha's town center), are the sole source of potable water for the entire population. Samples were collected during three seasons of the year in 2018–2019 from Kainakary Panchayat: (i) pre-monsoon (PrM), from February to May, (ii) monsoon (Mon), from June to September, and (iii) post-monsoon (PoM), from October to January. The sample nomenclature and locations are listed in Table 1. The collection of water samples conformed to APHA standard methods (Baird, 2017), and analyses were performed within 48 h of collection.

Table 1. Sample nomenclature and location

Sample name	Source	Depth	Location coordinates
S1	Borewell	120 m	9°47'17.4"N, 76°36'21.7"E
S2	Borewell	108 m	9°46'34.1"N, 76°36'44.6"E
S3	Borewell	80 m	9°46'52.1"N, 76°36'40.6"E

Analytical methods

The water samples collected were analyzed in the laboratory for physical, chemical, and biological parameters employing the standard methods (American Public Health Association; Baird 2017). Sample pH was measured using a portable pH meter (Eutech PCS Testr 35), color, turbidity, and hardness were determined by a Systronics Digital Nephelo Turbidity Meter, which was set to 100 with 40 NTU standards, electrical conductivity (EC) was measured by a portable conductivity meter (Elico) and TDS was computed by the EC meter. Analyses of the physicochemical and bacteriological parameters followed standard methods (Baird, 2017). A flame photometer (Systronics India Ltd.) was used to estimate the Na^+ and K^+ contents in the water. To estimate total hardness (TH) and calcium Ca^{2+} contents, the EDTA titrimetric method was used, whereas mg^{2+} levels were computed. Total alkalinity (TA) and bicarbonate (HCO_3^-) were determined by acid titration, and sulfide (S^{2-}) and Cl^- were determined by argentometric titration. A UV–VIS spectrometer (Thermo Scientific Evolution 201) was used to quantify Fe, sulfate (SO_4^{2-}), NO_3^-N, total ammoniacal nitrogen (TAN), and F^- levels. Phenolic compounds were estimated by spectrophotometry. Total coliform and Escherichia coli were determined by the most probable number (MPN) method. A Piper trilinear diagram (Piper, 1944), illustrating the predominant hydrochemical facies, was plotted with Aquachem Scientific software version 4.0 (AqQA).

RESULTS

Physicochemical Parameters

Physicochemical characteristics can provide insights into the nature of contaminants present in the groundwater sources of Kainakary Panchayat during the PrM, Mon, and PoM seasons. The range, average, and standard deviation of the measured physicochemical parameters of these samples obtained during 2018 and 2019 are summarized in Table 2.

Color of the samples ranged from 1–9 Hazen units (HU). In 2018, more than 50% of the PrM samples exhibited color above the acceptable limit of 5 HU (WHO, 2011); overall these samples showed more color in both years. Turbidity was above acceptable limits in the PrM samples from both years and the PoM samples. The pH of the groundwater samples in the study area varied from 6.90 to 8.00, indicating a slightly alkaline nature and conforming to World Health Organization (WHO) standards and Bureau of Indian Standards (BIS). EC is a good indicator of groundwater water quality, as groundwater will have more dissolved ions than surface water (RamyaPriya & Elango, 2018). The EC values of the samples ranged from 460 to 1,381 µS cm^{-1}. The estimated TDS values calculated from the EC values varied between 290 and 870 mg L^{-1}. According to the WHO (2011), TDS level in potable water should be less than 500 mg L^{-1}. Apart from S2 (for all seasons) in 2019, TDS values for all samples exceeded permissible limits, indicating that these sources are less suitable as drinking water and require treatment to meet potability standards. Cl^-, a major inorganic anion in groundwater, ranged between 56.4 and 280 mg L^{-1} in 2018 and 2019. Cl^- levels in the S1- PrM and S1- Mon samples were above the WHO limit. Fig. 1 shows the average Fe concentration in PrM, Mon, and PoM borewell samples. The limit specified by the WHO and BIS for Fe is 0.3 mg L^{-1}. In 2018 (except for one value in the Mon season), all samples showed high Fe concentrations; in 2019, all Mon values showed a decrease in concentration compared to the preceding year (Fig. 1).

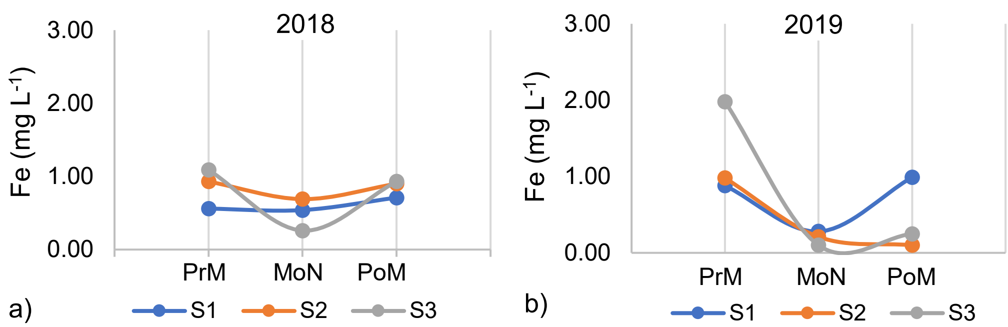

Figure 1. Fe concentration in borewell samples in 2018 (a) and 2019 (b).

Fig. 2 shows the average F concentrations in the PrM, Mon, and PoM groundwater samples. F^- levels in the samples from the study area were between 0.75 and 1.8 mg L^{-1}. The acceptable and permissible limits for F^- in groundwater are 1.0 mg L^{-1} and 1.5 mg L^{-1}, respectively (BIS, 2012). F^- was above safe limits in all borewells in the Mon seasons of both years.

Table 2. Physicochemical analyses and corresponding drinking water standards of the World Health Organization (WHO, 2011) and Bureau of Indian Standards (BIS, 2012)

Parameter	2018 PrM	2018 Mon	2018 PoM	2019 PrM	2019 Mon	2019 PoM	WHO 2011	BIS 2012 AL	BIS 2012 PL
Color (HU)	5–9 (7.0 ± 2.0)	2–4 (3.0 ± 1.0)	5–6 (5.33 ± 0.58)	2–4 (3.0 ± 1.0)	1–1 (1 ± 0)	1–2 (1.3 ± 0.6)	5	5	15
Turbidity	2.2– 4.3 (2.97 ± 1.16)	0.8–1.8 (1.3 ± 0.5)	1.5–2.1 (1.83 ± 0.31)	2.1–2.3 (2.17 ± 0.12)	0.4–1.0 (0.7 ± 0.30)	1.0–2.8 (1.67 ± 0.99)	1.5	1	5
pH	6.90–7.20 (7.05 ± 0.15)	6.75–7.11 (6.92 ± 0.18)	7.20–7.43 (7.28 ± 0.13)	6.93–8.00 (7.3 ± 0.61)	6.98–7.16 (7.05 ± 0.1)	7.30–7.43 (7.36 ± 0.07)	6.5–8.5	6.5–8.5	NR
EC ($\mu S\ cm^{-1}$)	463–1249 (749, ± 434.88)	460–998 (687 ± 303.06)	619–1380 (947, ± 391.82)	655–1278 (920, ± 321.62)	655–1373 (962 ± 367.30)	703–1281 (949 ± 298.05)	1,500	–	–
TDS ($mg\ L^{-1}$)	336–787 (471.67 ± 273.97)	290–629 (433 ± 175.6)	390–870 (596.67 ± 246.85)	413–805 (579.33 ± 202.62)	419–865 (606.33 ± 231.4)	443–807 (598.33 ± 187.77)	500	500	2,000
Na^+ ($mg\ L^{-1}$)	22.30– 142.10 (67.5 ± 65.09)	24.50–84.70 (53.83 ± 30.13)	28.20–92.80 (60.2 ± 32.3)	26.80–108.20 (61.23 ± 42.12)	39.40–113.80 (72.43 ± 37.89)	31.40–82.80 (58.53 ± 25.82)	200	–	–
K^+ ($mg\ L^{-1}$)	0.7–1.2 (0.97 ± 0.25)	1.0–3.4 (2.1 ± 1.21)	0.9–2.6 (1.63 ± 0.87)	0.5–0.9 (0.67 ± 0.21)	0.8–2.2 (1.37 ± 0.74)	0.7–1.9 (1.17 ± 0.64)	12	–	–
TH ($mg\ L^{-1}$)	78.3–186.2 (117.73 ± 59.52)	85.6–168.6 (121.03 ± 42.81)	104.0–220.0 (155.37 ± 59.13)	87.1–198.0 (125.75 ± 62.62)	112.0–220 (158.87 ± 55.4)	116–200 (155.33 ± 42.25)	–	200	600
Ca^{2+} ($mg\ L^{-1}$)	19.6–53.6 (31.5 ± 19.16)	19.7–43.9 (29.47 ± 12.76)	27.2–50.16 (36.25 ± 12.23)	19.17–46.1 (29.16 ± 14.75)	28–44.1 (34.9 ± 8.29)	28.0–38.0 (32 ± 5.29)	75	75	200
Mg^{2+} ($mg\ L^{-1}$)	7.52–21.8 (12.8 ± 7.34)	10.2–17.36 (13.72 ± 3.58)	12.89–24.88 (18.56 ± 6.02)	9.2–20.2 (13.73 ± 5.75)	12.1–23.7 (17.72 ± 5.81)	14.0–27.0 (18.67 ± 7.23)	50	30	100
Cl^- ($mg\ L^{-1}$)	56.8–280.0 (141.8 ± 120.74)	57.4–245.8 (135.73 ± 98.13)	60–190 (120.67 ± 65.43)	56.4–270.8 (144.33 ± 112.27)	83.4–156.8 (172.73 ± 98.27)	68.0–219.0 (138.33 ± 76.03)	250	250	1,000
TA ($mg\ L^{-1}$)	148.36–208.20 (157.41 ± 46.92)	131.15–155.74 (144.75 ± 12.5)	163.28–205.74 (179.68 ± 22.82)	113.90–179.51 (148.27 ± 32.91)	152.61–207.38 (182.23 ± 27.66)	170.49–195.00 (180.31 ± 13.02)	500	200	600
Fe^{2+} ($mg\ L^{-1}$)	0.56–1.09 (0.86 ± 0.27)	0.26–0.69 (0.5 ± 0.22)	0.71–0.93 (0.85 ± 0.12)	0.88–1.98 (1.28 ± 0.61)	0.1–0.28 (0.2 ± 0.09)	0.1–0.99 (0.45 ± 0.48)	0.3	0.3	NR
SO_4^- ($mg\ L^{-1}$)	3–5.2 (4.20 ± 1.11)	1.02–2 (1.37 ± 0.54)	2.28–4.2 (2.98 ± 1.06)	3.32–5.03 (3.90 ± 0.98)	2.22–4.07 (3.13 ± 0.93)	4.96–6 (5.39 ± 0.54)	250	200	400
NO_3^-N ($mg\ L^{-1}$)	1.06–1.45 (1.21 ± 0.21)	1.72–3.77 (2.49 ± 1.11)	3.72–5.91 (4.93 ± 1.11)	1.6–2.70 (2.32 ± 0.62)	1.25–3.30 (1.98 ± 1.14)	2.89–4.40 (3.83 ± 0.82)	45	45	NR
F^- ($mg\ L^{-1}$)	1.02–1.13 (1.07 ± 0.06)	1.08–1.46 (1.32 ± 0.21)	0.98–1.8 (1.5 ± 0.45)	0.75–1.43 (1.08 ± 0.34)	0.98–1.22 (1.12 ± 0.12)	0.89–1.49 (1.29 ± 0.34)	–	1	1.5
TAN ($mg\ L^{-1}$)	3.82–4.51 (4.51 ± 0.7)	3.18–4.1 (3.59 ± 0.47)	3.34–4.85 (4.23 ± 0.79)	3.04–5.6 (3.94 ± 1.44)	6.69–8.96 (7.15 ± 1.63)	3.98–5.96 (4.72 ± 1.08)		0.5	NR

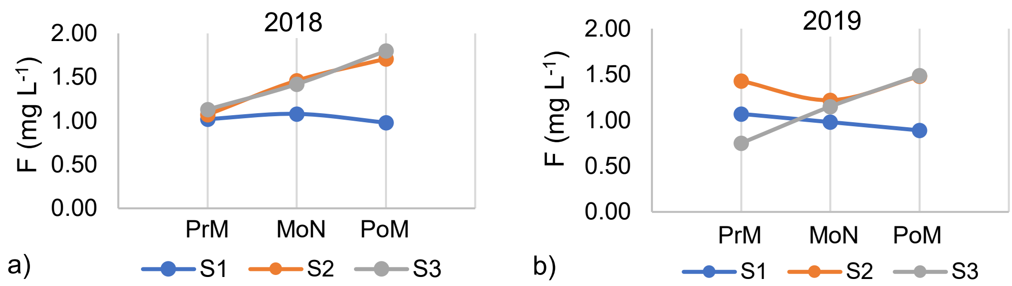

Figure 2. F concentration (mg L^{-1}) in borewell samples in 2018 (a) and 2019 (b).

TAN is the total amount of nitrogen as ammonia (NH_3) and dissolved ammonium ions (NH_4^+). Fig. 3 shows the average TAN concentrations in the borewell samples for the PrM, Mon, and PoM seasons. Total NH_3 was much above the permissible level of 0.5 mg L^{-1} in all groundwater sources in all seasons.

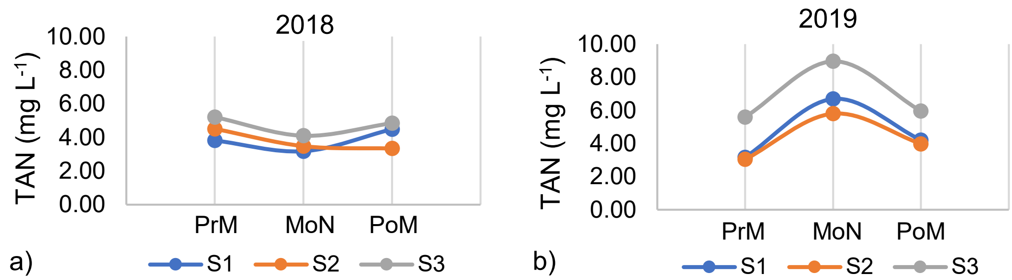

Figure 3. TAN level (mg L^{-1}) in borewell samples in 2018 (a) and 2019 (b).

TA, SO_4^{2-} and NO_3^- were found to be within the permissible limits in all samples across all seasons. Values of Al, phenolic compounds, and S^{2-} in the water samples were below detection limits across all seasons in 2018 and 2019.

DISCUSSION

The color of the samples is possibly due to reduced groundwater volumes during the PrM season. Turbidity in groundwater is caused mainly by clay, silt, reduced Fe precipitates, and other oxides originated from the erosion of rocks in aquifers, vegetative debris, and microorganisms present in water bodies (Baluch & Hashmi, 2019; Cheremisinoff, 2019). We have done a survey in the study area and the participants indicated that the water supply through faucets was visibly muddy quite often. The particulate matter responsible for turbidity settles at the bottom of the storage tanks, making the water dirtier, even during the supply of clear water, and coalescing of particulate matter can potentially clog water piping (Cheremisinoff, 2019), which is commonly seen in the study area. High turbidity in potable water can be associated with a higher incidence of infections of the human gastrointestinal tract (WHO, 2011).

Significantly increased rainfall, a departure from normal rainfall pattern, during the months of July and August in both the years (IMD Annual Report 2018 and IMD Annual

Report 2019), did significantly influence the pH of groundwater. Bicarbonates, originating from carbonate minerals and dissolved soil gases, are the major contributors to the natural alkalinity of groundwater. Point source pollution from rainwater and floodwater runoff, wastewater discharge, or anthropogenic activities also contribute to the alkalinity of surface water (Mattson, 2009; Prasad et al., 2021).

Higher values of EC can reduce the aesthetic appearance of water and are also insensitive to infants and heart patients (WHO, 2003). Variations in TDS are probably a result of geogenic pollution, such as weathering of sedimentary rocks and erosion of the earth's surface resulting in the formation of salts as NaCl, Ca^{2+}, Mg^{2+} and K^{+}, SO_4^{-}, and HCO_3. These salts can accumulate through the continuous process of precipitation and evaporation. Higher levels of TDS in groundwater are usually a result of its contact time with underlying rocks and sediments (Gjessing, 1976). Excessive solids impart an undesirable taste, emanating primarily as Cl^{-} content in the water, as well as gastrointestinal irritation.

Chloride in groundwater is contributed by both natural and anthropogenic sources, as runoff containing fertilizers, landfill leachates, industrial and sewage effluents, irrigation drainage, and seawater intrusion in coastal areas (WHO, 2003). Cl^{-} as an anthropogenic pollutant can be attributed to agricultural activities, particularly paddy cultivation as frequent washing of the soil with burnt lime is undertaken to neutralize alkalinity (FAO, 2016). The presence of Cl^{-} rich minerals or rocks are the sources of geogenic contamination. Cl^{-} exists as NaCl in groundwater; however, the Cl^{-} content may exceed that of Na due to the phenomenon of base exchange. CaCl and MgCl are rare in groundwater (Nair et al., 2018).

Fe is present naturally in soils, rocks, and minerals, and is a constituent in oxides, hydroxides, sulfides, sulfates, arsenates, and carbonates. Fe can be present in groundwater due to weathering of Fe-bearing minerals in rocks. When the concentration of Fe approaches 0.3 mg L^{-1}, the water has a metallic taste and becomes less potable. Natural mobilization of Fe from alluvial deposition and natural recycling are the sources of Fe in the Alappuzha region, in which the study area belongs. Increased mobilization of Fe is seen when the increased speed of water flow occurs, especially during monsoon season and flooding instances (Sivanandan & Ambili, 2018), which is not a rare incident in this region. Groundwater that is acidic or low in oxygen may have higher dissolved Fe concentrations (Zucker et al., 2015). Increased well water volumes during the Mon season probably resulted in lower Fe concentration. Another peculiarity of this region is its effluent seepage from septic tanks, silage clamps from paddy fields, slurry pits, landfills, or other sources of anthropogenic pollution which can contribute Fe to the groundwater over long periods of time. Anaerobic groundwater can contain increased levels of Fe^{2+} without displaying any discoloration or turbidity when pumped directly from the well. However, oxidation of this Fe^{2+} to Fe^{3+} occurs upon exposure to the atmosphere, turning the water a reddish-brown color. Fe can also increase the growth of unwanted bacteria, resulting in biofouling in the well, pump, and water pipes (Bachmann & Edyvean, 2005). From the personnel communication, several villagers complained that in some parts of the study area they feel stringent odor in water. They often get muddy or reddish-brown colored water, which causes stains in the clothes, while washing. Fe deposits and biofouling can clog the intake of a well or affect the pump, making the well less efficient. At levels above 0.3 mg L^{-1}, Fe stains laundry and plumbing fixtures. Fe values of samples showed an increasing trend in the PrM period (Fig. 1).

F is a naturally occurring time-variant geogenic pollutant that is commonly found in bedrock as F minerals, such as F^-, hornblende, biotite, and fluorapatite (Nair et al., 2018). Managing F^- pollution is tricky because the source of the pollution is unknown. The tertiary sediments at Alappuzha- where the study area locates - shows the presence of a common mineral, fluorapatite, which is the source of fluoride in groundwater. The key factors accountable for the high fluoride content in the groundwater are sediment–groundwater interaction, prolonged residence time, and facies changes (Ca-HCO_3 to Na-HCO3) (Raj & Shaji., 2016). F^- is essential for healthy bones and teeth, and consumption levels below 0.5 ppm can lead to dental caries and weak bones in children (WHO, 2011). On the other hand, consumption of F^- in excess of 1.0 ppm can result in fluorosis of the bones and/or teeth (Susheela, 1991).

Intense agricultural activities during the monsoon season of 2019 resulted in a dramatic increase in TAN levels. The N cycle signifies the exchange and movement of N between plants, animals, the atmosphere, soil, microorganisms, surface water, and groundwater. N is found in nature as NH_3 as a result of the decomposition processes of organic material (Mahler & Garner, 2009; Prasad et al., 2020). Fertilizers, such as ammonium nitrate, manure used in agriculture in the study area, and occasionally, sewage contamination, are common reasons for anthropogenic pollution by NO_3^- and NH_3 (Zhang et al., 2018). NO_3^- is considered a reliable indicator of contamination resulting from agricultural activity. The causes for such high TAN levels in groundwater sources in Kainakary is a research focus for future work.

Hydrogeochemical Facies

The Piper (1944) trilinear diagram can be used to elucidate, compare and contrast the characteristics of groundwater (Todd, 1980). Plots of cations and anions and their concentrations using Aquachem Scientific software version 4.0 are shown in Fig. 4. The common water types in the study area were $CaHCO_3$ type, NaCl type, and mixed CaMgCl type. The predominance of Cl and HCO_3 types indicates sample salinity.

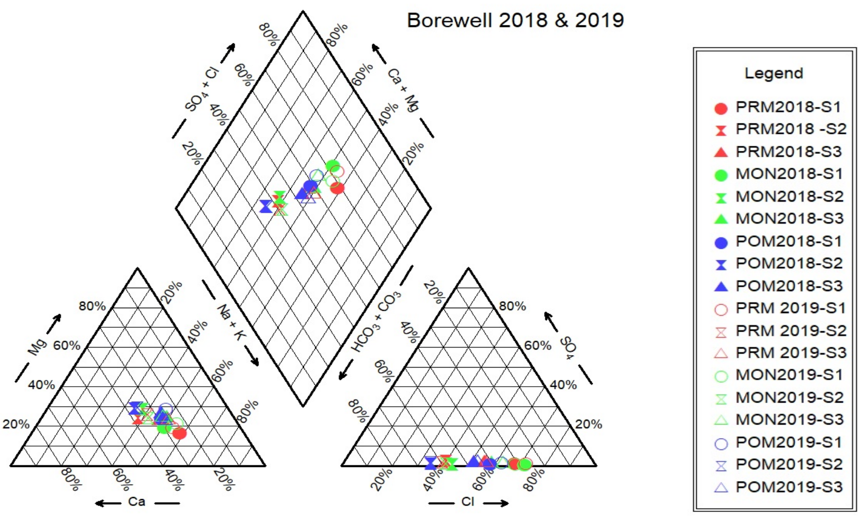

Figure 4. Piper diagram illustrating the relationship between dissolved ions in groundwater samples in PrM, Mon, and PoM seasons for 2018 and 2019.

Ionic Ratios

Ionic ratios of groundwater samples taken during the PrM, Mon, and PoM seasons for 2018 and 2019 are shown in Table 3, Fig. 5, and Fig. 6. The mean Ca^{2+}+ + Mg^{2+}/ Na^{+}+ K^{+} ratios during PrM, Mon, and PoM for each year (Table 3) indicated that the presence of Ca^{2+} and Mg^{2+} ions was due to silicate minerals (Katz et al., 1997; Nair et al., 2018). The mean HCO_3^-/ Ca^{2+} + Mg^{2+} ratios for the three seasons in 2018 and 2019 (Table 3) reflected a higher value during the PrM season. Higher HCO_3^-/ Ca^{2+} ratios were noted in the PoM season, an indication of increased dissipation of atmospheric CO_2 during rainfall. Minor fluctuations of ionic components were seen across the 2018 and 2019 seasons, indicating the absence of any drastic compositional change.

Table 3. Ionic ratios of groundwater samples during PrM, Mon, and PoM seasons for 2018 and 2019

Ionic Ratio	2018								
	PrM			Mon			PoM		
	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max
Ca^{2+} + mg^{2+}/Na^{+} + K^{+}	1.14	0.71	1.60	1.21	0.96	1.63	1.40	1.11	1.91
HCO_3^-/Ca^{2+} + mg^{2+}	1.65	1.15	2.27	1.46	1.05	1.97	1.38	1.10	1.72
HCO_3^-/Ca^{2+}	2.75	1.90	3.69	2.65	1.73	3.65	2.53	2.00	3.05
Ca^{2+}/Na^{+}	0.70	0.43	1.01	0.69	0.55	0.92	0.78	0.61	1.11
Mg^{2+}/Na^{+}	0.47	0.28	0.64	0.55	0.39	0.78	0.64	0.51	0.86
Ca^{2+}/Mg^{2+}	1.49	1.34	1.59	1.28	1.11	1.54	1.20	1.07	1.29
Ca^{2+}/ SO_4^{2-}	17.50	10.72	24.74	58.68	29.76	103.29	32.46	17.94	52.80
Mg^{2+}/Ca^{2+}	0.67	0.63	0.75	0.80	0.65	0.90	0.84	0.78	0.93
HCO_3^-/HCO_3^- + SO_4^{2-}	0.98	0.98	0.98	0.99	0.99	0.99	0.99	0.98	0.99
Ionic Ratio	2019								
	PrM			Mon			PoM		
	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max
Ca^{2+} + mg^{2+}/Na^{+} + K^{+}	1.09	0.84	1.45	1.10	0.83	1.37	1.33	1.02	1.84
HCO_3^-/Ca^{2+} + mg^{2+}	1.53	1.11	2.16	1.45	1.22	1.90	1.45	1.16	1.68
HCO_3^-/Ca^{2+}	2.75	1.90	3.85	2.61	2.28	3.25	2.78	2.51	3.06
Ca^{2+} /Na^{+}	0.61	0.49	0.82	0.62	0.45	0.82	0.71	0.53	1.03
Mg^{2+} /Na^{+}	0.49	0.35	0.65	0.49	0.39	0.58	0.64	0.46	0.84
Ca^{2+} /Mg^{2+}	1.27	1.15	1.39	1.23	1.14	1.41	1.10	0.86	1.22
Ca^{2+} / SO_4^{2-}	17.25	13.86	22.00	27.88	19.22	34.14	14.20	13.55	15.20
Mg^{2+} /Ca^{2+}	0.79	0.72	0.87	0.82	0.71	0.88	0.93	0.82	1.16
HCO_3^- /HCO_3^- + SO_4^{2-}	0.98	0.98	0.98	0.99	0.98	0.99	0.98	0.97	0.98

Bivariate diagrams (mixing diagrams) of Ca^{2+}/ Na^{+} vs. Mg^{2+}/ Na^{+} and HCO_3^-/ Na^{+} vs. Ca^{2+}/ Na^{+} for all seasons and years are given in Fig. 5 and 6, respectively, indicating that the groundwater's geochemical character is due to weathering of aluminosilicate minerals and atmospheric dissolution (Gaillardet et al., 1999).

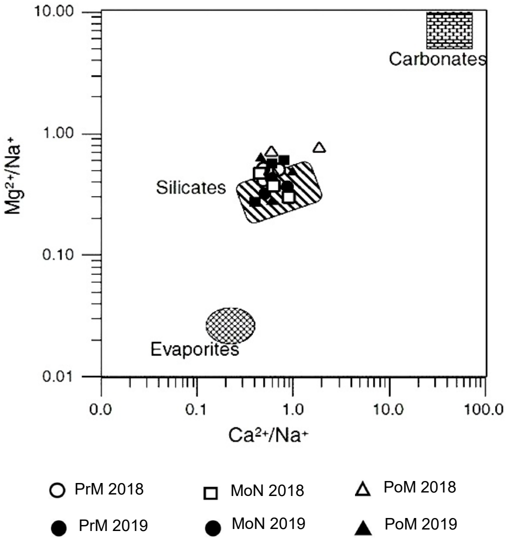

Figure 5. Scatter plot of mg^{2+}/Na^{+} vs. Ca^{2+}/Na^{+} relating carbonate and silicate members in the study area.

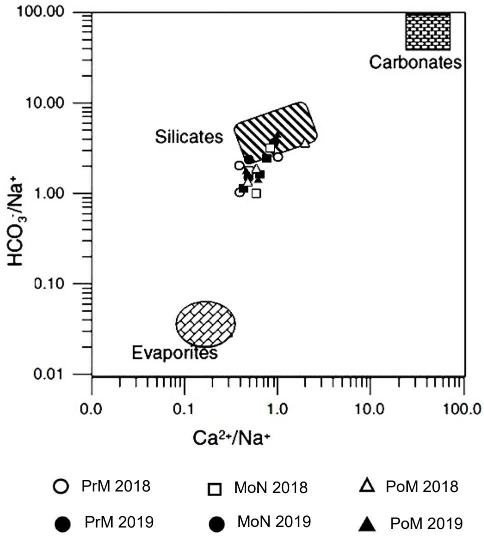

Figure 6. Scatter plot of HCO_3^{-}/ Na^{+} vs. Ca^{2+}/ Na^{+} relating carbonate and silicate members in the study area.

Ion Exchange

Ion-exchange processes can represent the variations in geochemical processes that lead to changes in potable water quality. The variation in the chemical composition of groundwater along its flow path is understood by studying the Chloro Alkaline Indices (CAI). Schoeller (1977) has recommended two Chloro-Alkaline Indices CAI-I and CAI-II for the interpretation of ion exchange between groundwater and the host environment. The Chloro-Alkaline Indices is expressed as:

$$\begin{aligned} CAI\text{–}I &= Cl^{-} – (Na^{+}+ K^{+})/ Cl^{-} \\ CAI\text{–}II &= Cl^{-} – (Na^{+}+ K^{+})/ (SO_4^{2-} + HCO_3^{-} + NO_3^{-}) \end{aligned} \quad (1)$$

where, CAI–I and CAI–II are Chloro-Alkaline Indices.

The positive value of CAI in all samples indicates that the predominant base exchange is between Na^{+} and K^{+} ions in the water source and Ca^{2+} and mg^{2+} ions in the rocks, as shown in Table 4. It can be inferred that the water samples in this region are exposed to similar geochemical processes and the fate of the environmental contaminants is better predicted.

The tendency of Ca^{2+} and mg^{2+} to engage in reactions that leave insoluble mineral deposits rendering groundwater hard varies from region to region. Increasing hardness requires water treatment processes to be in place before delivery for potable and irrigation use. It is more difficult for plants to absorb and break down hard water when compared to soft water leading to an increased rate of irrigation to compensate for poor utilization. This in turn leads to additional strain on the groundwater resources.

Table 4. Total ion concentration and chloro-alkali index (CAI) of groundwater samples

Year	Season	Sample no.	TZ-	TZ+	CAI–I (meq L^{-1})	CAI–II (meq L^{-1})
2018	PrM	S1	13.16	14.35	0.33	1.18
		S2	5.42	4.16	0.39	0.2
		S3	5.46	5.31	0.33	0.35
	Mon	S1	10.89	10.76	0.39	0.73
		S2	5.51	4.65	0.34	0.17
		S3	6.29	6.83	0.29	0.33
	PoM	S1	10.69	13.05	0.24	0.3
		S2	6.53	5.76	0.27	0.11
		S3	7.59	8.49	0.24	0.22
2019	PrM	S1	12.31	12.65	0.38	0.74
		S2	5.47	4.64	0.26	0.13
		S3	6.02	6.05	0.29	0.34
	Mon	S1	13.19	13.55	0.37	0.62
		S2	7.05	6.37	0.27	0.15
		S3	8.31	8.75	0.37	0.49
	PoM	S1	11.26	11.76	0.38	0.49
		S2	6.60	6.26	0.31	0.13
		S3	8.19	8.42	0.34	0.29

TZ-, total anions; TZ+, total cations.

Coliform Counts

Studies have shown that nearly 70% of open wells in Kerala are rife with bacterial contamination (Harikumar & Chandran, 2013; Jaya Divakaran et al., 2019). Groundwater samples were analyzed to determine the presence of coliform bacteria, specifically E. coli, to assess source contamination due to anthropogenic activities. The presence of bacteria was confirmed in all borewells during the PrM and Mon seasons of 2018. PoM samples in 2018 were devoid of bacteria, which can be attributed to the extensive and elaborate chlorination measures following the great flood of August 2018. The presence of bacteria was noted in two of the three borewells during the Mon season of 2019, although at a lower concentration than in 2018, as shown in Fig. 7. One of the primary causes of water pollution is untreated wastewater, often discharged into streams, and affecting downstream water quality as well as lakes, open wells, and even deeper groundwater sources as seen during 2018 as a result of flooding (Jaya Divakaran et al., 2019; Amritanand et al., 2020), which is very familiar in the study area too.

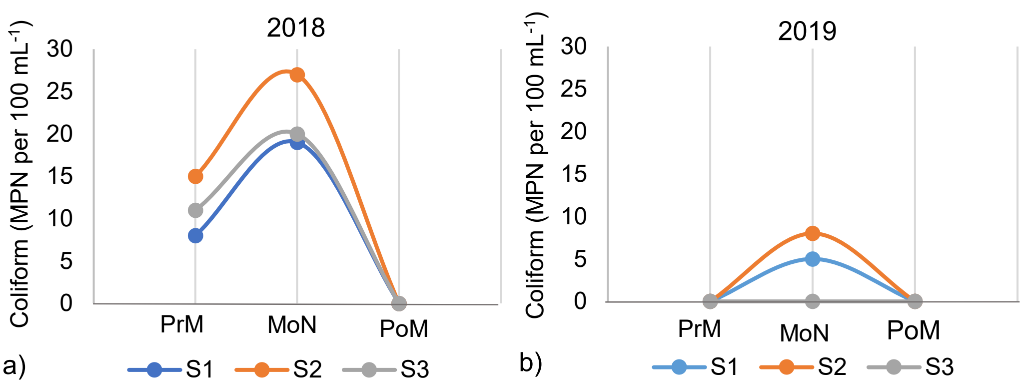

Figure 7. Bacteriological parameters of groundwater samples from the study area collected in the PrM, Mon, and PoM seasons of 2018 (a) and 2019 (b).

Drinking-Water Suitability

The WQI is the most common parameter for assessing the quality of drinking water. Various researchers have adopted different methodologies to calculate the WQI (Tiwari & Mishra, 1985; Mishra & Patel, 2003; Gebrehiwot et al., 2011; Nair et al., 2018). A WQI < 50 is indicative of excellent water quality and a WQI between 50 and 100 denotes good water quality. A WQI of 100–200 denotes poor water quality, and a WQI between 200 and 300 is indicative of very poor water quality. Finally, a WQI > 300 signifies a non-potable water source. The weightages (*wi*) accorded to the hydrochemical parameters on the basis of their impact on potable water quality and the computed relative weight (*Wi*) of the chemical parameter are highlighted in Table 5. Based on the concentration of the chemical parameters in the samples and the guideline values as defined by the BIS and the WHO, a quality rating scale was constructed.

Table 5. Weightage accorded to physicochemical parameters based on their impact on the quality of potable water (Gebrehiwot et al., 2011; Nair et al., 2018)

Chemical parameter	BIS (2012)	WHO (2011)	Weight (wi)	Relative weight (Wi)
pH	6.5–8.5	8.5	4	0.09
EC (µS cm^{-1})	500	500	4	0.09
TDS (mg L^{-1})	500	500	5	0.11
Na^+ (mg L^{-1})	–	200	2	0.04
K^+ (mg L^{-1})	–	12	2	0.04
TH (mg L^{-1})	200–600	–	3	0.06
Ca^{2+} (mg L^{-1})	75–200	75	2	0.04
Mg^{2+} (mg L^{-1})	30–100	50	2	0.04
TA (mg L^{-1})	–	500	3	0.06
Cl^- (mg L^{-1})	250–1,000	250	3	0.06
SO_4^- (mg L^{-1})	200–400	250	4	0.09
NO_3^-N (mg L^{-1})	45	45	5	0.11
Fe^{2+} (mg L^{-1})	0.3	0.3	4	0.09
F^- (mg L^{-1}L)	1	1	4	0.09

Fig. 8 illustrates the decline in most of the WQI in the Mon and PoM seasons of 2018 and 2019. An improvement in the quality was noted in 2019, although it remained in the 'Good' category. The water must be treated to remove Fe, F^- and ammoniacal N components before its distribution through potable water systems.

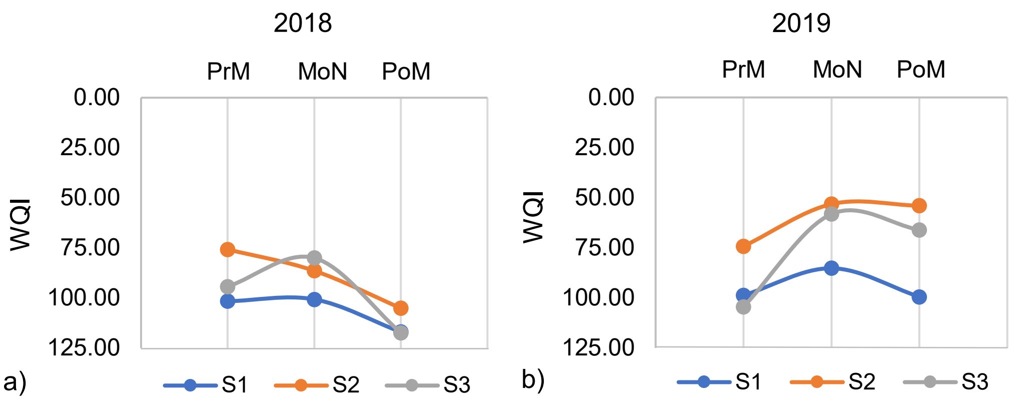

Figure 8. Water quality index of groundwater samples in the study area collected during the PrM, Mon, and PoM seasons of 2018 (a) and 2019 (b).

In August 2018, the state of Kerala witnessed a massive flood, which affected millions of people. The groundwater levels monitored by the Central Ground Water Board (CGWB) at the end of August 2018 in Kerala showed that the water level varied from less than 1 m to 20 m below ground level (Central Ground Water Board, 2020). A year-over-year comparison indicated a fall in water levels in most of the dug wells - 57.88% of the total wells monitored. There was a rise in water level in 41.45% of the wells, and only a few wells showed no change in water level, indicating that groundwater level did not increase considerably during Mon 2018. During periods of heavy rainfall, nearly 95% of rainwater ends up as surface runoff. However, in November 2018, 81% of the dug wells showed a rise in water level and only 19% showed a falling trend (CGWB, 2020), suggesting more groundwater recharge from the normal rainfall occurring in the PoM season than from the heavy spells, and this increased groundwater recharge resulted in improved groundwater quality.

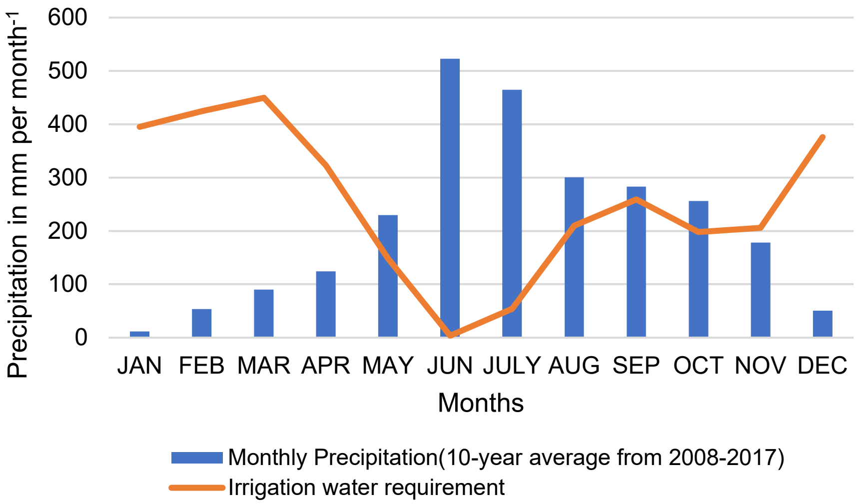

Figure 9. Ten year (2008–2017) average monthly rainfall vs monthly irrigation water need.

Understanding irrigation water requirements in the study area from the perspective of replenishment of water resources can promote both agriculture and sustainability. Fig. 9 illustrates the mismatch between the 10-year average (2008–2017) monthly irrigation needs versus the 10-year average monthly rainfall. Surplus rainfall during the monsoon season corresponds to minimal irrigation needs, while during the season of rice cultivation, the quantum of rainfall cannot minimize the extraction of groundwater to meet the irrigation water needs. Infrastructure to save surplus rainfall for groundwater recharge or to be utilized for conjunctive use in agriculture during non-rainy seasons can minimize the stress on groundwater resources in the study region.

CONCLUSIONS

Given that the region of study is an agricultural and eco-friendly zone, anthropogenic activities with deleterious effects were minimal relative to other regions in India. It is well known that point source pollution from wastewater and agricultural runoff leads to polluted surface waters, however, the direct and indirect effects of surface water pollution on groundwater sources are yet to be elucidated. Anthropogenic contamination of the groundwater in the study area was evidenced by the presence of bacteria and NH_3 components and indicates interconnectivities between surface water and groundwater sources. Geogenic contamination of groundwater sources in the study region was evidenced by the presence of Fe and F- levels above permissible limits and are the result of natural geogenic processes exacerbated by over-extraction of groundwater for potable and irrigation use during dry seasons. Appropriate water treatment measures including defluoridation may be required to prevent health hazards. The observed improvement in water quality during PoM season was associated with groundwater recharge, reiterating that artificial recharge of groundwater could significantly remediate geogenic pollution and promote sustainable use of groundwater resources in the study region and other ecologically fragile areas. The need for responsible consumption of natural resources in the context of agriculture, the fabric of culture, and economic activity in Kainakary. contribute to the achievement of Sustainable Development Goal (SDG) 12 and other SDGs directly or indirectly, as they are all integrated. In addition, activities such as awareness campaigns, mitigation of flood risks and enforcement of rehabilitation measures, monitoring of groundwater quality periodically, and widening the observation areas, can supplement the natural replenishment of groundwater and reverse geogenic pollution, and achieve sustainable consumption and production patterns.

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Maize Growth and Yield characteristics with application of mushroom waste substrate vermicompost in Ultisol

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Abstract. Improve maize production can be done by expanding the planting area in Ultisol. The main problems with planting maize in Ultisol are high soil acidity (pH < 4.5), high Al saturation (> 65%), and low P availability (± 1.7 ppm). The objective of this study was to know the effect of the application of mushroom waste substrate vermicompost on the characteristics of maize growth and yield. in Ultisol. Randomized Complete Block Design with the treatments of vermicompost at a dose of 0 t ha^{-1}, 15 t ha^{-1}, 30 t ha^{-1}, and 45 t ha^{-1} with six replications was tested. The observed variables were Ultisol's chemical and biological properties, maize growth, and yield. The soil characteristics of the pH, C-organic, P-available, and phosphate solubilizing microbes, and maize growth and yield characters viz. the number of leaves, shoot dry weight, flower emergence, leaf chlorophyll content, the efficiency of P use, the protein content of seeds, number and weight of maize seeds improved with the application of 45 t ha^{-1} of vermicompost. From the results it could be concluded that applying the vermicompost would enable farmers increase yield of maize in Ultisol soil.

Key words: maize growth performace, marginal land, organic compost, soil characters.

INTRODUCTION

Maize production in Indonesia increased by about 19 million tons in 2014 and 28.9 million tons in 2017 under 3.8 million hectares of harvested area. Improving maize production in Indonesia can be expanded to Ultisol as a potential area of 45.7 million hectares (Fitriatin et al., 2014).

Soils of Ultisol are not fit for crop production due to high soil acidity (pH < 4.5), high aluminum (Al) saturation (> 65%), and deficient availability of P (± 1.7 ppm) (Taisa et al., 2019), and deficiency of Ca, Mg, and K (Widiatmaka et al., 2016; Purwanto et al., 2021; Utami et al., 2021). In addition, Ultisol has low levels of C-organic, N-total, Cation Exchange Capacity (CEC), and Base Saturation (Syahputra et al., 2015).

Application of vermicompost improves soil conditions such as N-total, soil pH, and C-organic (Rakhmalia et al., 2015, Setiawan et al., 2015). Some researchers reported that the effect of vermicompost increases total N levels, soil pH, P-available, and C-organic

(Aryani et al., 2019, Fitria et al., 2018, Sianturi et al., 2019, Yuka et al., 2017), P content, cation exchange capacities (CEC) of K, Ca, Mg, soil fungi, and bacteria (Emalinda et al., 2005). According to Adhikary (2012) and Bellitürk et al.(2022), vermicompost increases 2.2% N, 2.2% P, 1.5% K, and some micronutrients such as Na, Ca, Zn, S, Mg, and Fe. Vermicompost can also reduce soil acidity and increase the number of microorganisms (Fernández-Gómez et al., 2010).

Some studies have done that the application of vermicompost improves the soil properties of Ultisols. However, a study of the application of pure mushroom waste substrate vermicompost without additional ingredients has not been revealed yet. Therefore, it is critical to investigate the impact of applying mushroom waste vermicompost to Ultisol on morpho-physiological characters, and yield components of maize.

MATERIALS AND METHODS

The study was done at the Experimental Garden of the Faculty of Agriculture, the Universitas Muhammadiyah Purwokerto, located in Karangsari Village, Kembaran District, Banyumas Regency from April - to November 2019. The vermicompost was made with the activity of *Eudrilus eugeniae* worms on the mushroom waste substrate. Ultisol was taken from Gorowong Village, Parungpanjang District, Bogor Regency, West Java. Soil samples were taken randomly at a depth of 0–20 cm with conditions of pH, C-organic, total N, total P_2O_5 and Al saturation of 4.21, 0.77%, 0.04%, 1,200 ppm and 66.07%, repectively. Then, the soil was air-dried and sieved with a 2 mm sieve. The application of vermicompost was by doses viz. 0 t ha^{-1}, 15 t ha^{-1}, 30 t ha^{-1}, and 45 t ha^{-1}. The chemical and biological compositions of vermicompost were 1.44% total N, 2.03% total P_2O_5, 3.19% total K_2O, 31.0×10^5 cfu ga^{-1} N-fixing microbes and 39.0×105 cfu ga^{-1} phosphate solubilizing microbes.

20 kg of sieved soil (30% moisture content) was mixed with vermicompost (17% moisture content) and then put into a 45 cm × 50 cm polybag (± 30 cm diameter). The soil and vermicompost mixture was watered until the field capacity was reached. Subsequent watering was applied according to conditions in the polybag. Sukmaraga maize seeds were planted in polybags at a depth of about 2–3 cm, with two seeds in each polybag and arranged distance among seed at a spacing of 20 cm. Nitrogen fertilizer with a dose of 92 kg ha^{-1} was given at seven days after planting (DAP) and 30 days after planting, in half dose each. Potassium (K_2O) fertilizer with a dose of 60 kg ha^{-1} was given seven days after planting (Wahyudi, 2009). Calculation of the fertilizer dose per polybag was the volume of soil in polybags divided by the volume of soil per hectare, multiplied by the dose of fertilizer for one hectare. Harvesting was done when the plant reached physiological maturity, with the criteria that the husks were dry and hard. Variables observed were chemical and biological properties of Ultisol, and morphological, physiological, and yield components of maize.

Soil chemical and biological analysis

Soil pH was analyzed by using a glass electrode, while C-organic analysis using the Walkley and Black method, the total P-level was extracted with HNO_3 and analyzed by Olsen and Bray method. The available P was analyzed by the Bray-II method (Sulaeman et al., 2005). Total Phosphate Solubilizing Microbial (PSM) was calculated using the Most Probability Number method (Saraswati et al., 2007).

Plant morphological observations

Plant height was measured when male flowers appeared by measuring plant height from the base of the stem to the base of the last flag leaf (Peiffer et al., 2014). Stem diameter was measured at the time of the end vegetative stage by measuring the diameter of the stem at the lowest internode at a distance of 15 cm from the base of the stem (Bintoro, 1989). The number of leaves, counting all the leaves that formed and have fully opened was done 56 days after sowing time (Sitompul & Guritno, 1995). Leaf Area Index was calculated by the formula:

$$Leaf\ Area\ Index = \frac{Leaf\ Area}{Ground\ Area} \quad (1)$$

The calculate of leaf area is leaf length x maximum leaf width × 0.75×9.39 (Bintoro, 1989). The ground area is the spacing used for maize plants with plant distance of 20 cm × 75 cm (Sitompul & Guritno, 1995). Dry shoot weight was weighed at the end of the vegetative stage. The weighing was carried out after the shoots were dried in the oven at a temperature of 60 °C until the weight was constant (Bintoro, 1989).

Plant physiological observations

Physiological analysis of the amount of chlorophyll was measured in the third leaf using the Arnon method (1949). The concentrations of proline were determined in the ear-leaf during the vegetative phase (35 days after planting) and the seed filling phase (60 DAP) using the Bates method (1973). The protein content of seeds was measured at harvest time using the Kjeldahl method (AOAC, 1990), absorption efficiency and efficiency of P use were measured at harvest time (Fageria & Baligar, 1997) with the formula as follows:

$$Uptake\ Efficiency\ P = \frac{Dry\ Seed\ Weight\ (g)}{Total\ P\ uptake\ of\ plant\ (g)} \quad (2)$$

$$Efficiency\ of\ using\ P = \frac{Dry\ plant\ weight\ (g)}{Total\ P\ uptake\ of\ plant\ (g)} \quad (3)$$

Observation of maize yield and yield components

The yield and yield components of maize were observed at the end of the study, which consisted of cob length, cob diameter, grain weight per cob, weight of 100 dry seeds, number of seeds per cob (Subaedah et al., 2021). Harvest index, calculated at harvest time with the formula:

$$Harvest\ Index = \frac{Oven\ Dry\ Seed\ Weight\ (ODSW)\ (g)}{ODSW + Oven\ Dry\ Canopy\ Weight\ (g)}\ x\ 100\% \quad (4)$$

Statistic analysis

The effect of vermicompost application on Ultisol's chemical and biological properties, morpho-physiological characters, yield, and maize yield components was analyzed using variance analysis ($\alpha = 0.05$) with the SPSS 25. Multiple comparisons for treatments with a significant effect were using *Duncan's Multiple Range Test* (DMRT) with $\alpha = 0.05$ (Gomez & Gomez, 1984).

RESULTS AND DISCUSSION

Chemical and biological properties of Ultisol

Vermicompost had a significant effect on Ultisol pH. Vermicompost was able to increase the pH by 67.5%. The application of vermicompost in soil about 45 t ha^{-1} showed the highest pH, 6.92. There was a significant difference in the C-organic content of the soil due to the addition of vermicompost along with the addition of 45 t ha^{-1} P_2O_5 (Table 1).

Table 1. Effect of dose of vermicompost from the mushroom waste substrate on pH, C-organic and P-total of Ultisol

Vermicompost dosage	pH	C-organic (%)	P-total (ppm)
0 t ha^{-1}	4.30 ± 0.10a	0.83 ± 0.017a	0.148 ± 0.013a
15 t ha^{-1}	6.70 ± 0.03b	0.89 ± 0.016b	0.210 ± 0.022b
30 t ha^{-1}	6.79 ± 0.04b	0.90 ± 0.020b	0.250 ± 0.018c
45 t ha^{-1}	6.92 ± 0.08c	0.96 ± 0.031c	0.264 ± 0.042c

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with $\alpha = 0.05$.

The high total P-total was shown in the treatment with vermicompost of 45 t ha^{-1}, but it was not significantly different with 30 t ha^{-1}. However, the available P was highest with the application of vermicompost of 45 t ha^{-1}. Meanwhile, dose of 45 t ha^{-1} vermicompost raised the number of phosphate solubilizing microbes from 4.6×10^8 cfu g^{-1} to 189.9×10^8 cfu g^{-1} and reduced Al saturation from 60.96% to 17.39% (Table 2).

Table 2. Effect of dose of vermicompost from the mushroom waste substrate on P-available, phosphate solubilizing microbes and Al saturation of Ultisol

Vermicompost dosage	P-available (ppm)	Phosphate solubilizing microbes (x10^8 cfu g^{-1})	Al saturation (%)
0 t ha^{-1}	5.42 ± 0.181a	4.6 ± 1.0a	60.96 ± 1.76d
15 t ha^{-1}	6.03 ± 0.627b	39.7 ± 14.8b	34.78 ± 6.77c
30 t ha^{-1}	6.08 ± 0.706c	70.8 ± 8.03c	24.76 ± 2.49b
45 t ha^{-1}	6.40 ± 0.782d	189.9 ± 90.2d	17.39 ± 1.06a

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with $\alpha = 0.05$.

The increase in soil pH due to the application of vermicompost was in line with Mahmud et al. (2018), which stated that the application of vermicompost can increase soil pH. According to Butterly et al. (2010), the application of organic matter to acid soils (with high Al saturation) can raise soil pH because Al is bound by organic acids resulting from decomposition and forms complex compounds (chelates) so that Al is not hydrolyzed anymore.

Some studies mentioned that the addition of organic matter increases the C-organic content of the soil and improves the physical, chemical and biological properties of soil (Mekki et al., 2017). Soil C-organic can be improved by application of organic materials such as manure, compost, agro-industrial waste and urban waste (Vicente-vicente et al., 2016). The amount of P-total and P-available increased with increasing vermicompost.

The application of organic matter has been reported to increase P availability in the soil (Sharif et al., 2014). According to Mekki et al. (2017) , the increasing amount of P-total and P-available is related to the amount of carbon as a food source for soil microorganisms. An increase in the amount of carbon can stimulate the activity of microorganisms for the process of decomposition, P dissolution, and N fixation. According to Opala et al.(2012), the increase in soil P availability is due to the presence of soil microorganisms that can mediate the mineralization of organic P into inorganic P.

The increase in available P is also caused by the decomposition of organic matter, which produces organic acids that act as chelators (Siregar et al., 2017). According to Gusnidar et al. (2010), during the decomposition process, organic acids are produced, which then form Al complex compounds, reducing the Al-exch content and reducing P adsorption by Al while increasing P availability. Organic acids formed during organic material degradation can form chelate bonds with Al and Fe ions, lowering their solubility and increasing P availability. Organic acids produced by the breakdown of organic materials can also liberate adsorbed phosphorus and increase P availability (Bloom & Skyllberg, 2012). Ayodele & Shittu (2014) and Balík et al. (2019) reported that soil Al-exch decreased due to the application of, manure, compost and vermicompost.

Brady & Weil (2014) stated that P fertilization on acid soils needs to be accompanied by providing ameliorant materials, including organic matter. Previous research has shown that phosphate fertilizer combined with green manure (Rick et al., 2011), organic waste (Korzeniowska et al., 2013), or compost can increase available P. (Chien et al., 2011). Phosphorus solubility increases through microbial metabolism during the decomposition of organic materials, which release functional phosphate molecules for plants (Galvez-sola et al., 2010, Korzeniowska et al., 2013, Lu et al., 2014).

Adding organic matter increases microorganisms' activity and density, including phosphate solubilizing microbes (Agbede et al., 2017, Frimpong et al., 2017, Seshachala & Tallapragada, 2012). The relationship between the amount of Phosphate-solubilizing microbes and organic matter in the soil is that carbon compounds become the primary energy source for phosphate-solubilizing microbes (Alori et al., 2017). Various carbon sources influence the growth and activity of phosphate solu bilizing microbes. This carbon source has been reported to affect the production of enzymes to release organic P (Qureshi et al., 2010). Thus, Nur et al. (2019) have proven that giving compost about 20 t ha^{-1} increases the activity of phosphate solubilizing microbes.

Maize plant morpho-physiological character

The application of vermicompost significantly affected plant height, stem diameter, leaf area, leaf area index, and shoot dry weight of maize. The application of vermicompost up to a dose of 45 t ha^{-1} showed an increase in plant height, stem diameter, number of leaves, leaf area index, and shoot dry weight. 219%, 157%, 117%, 645%, and 1,263%, respectively. Vermicompost application of 45 t ha^{-1} resulted in a shorter appearance on male and female flowers of 30 dan 38 days earlier, respectively, compared to without vermicompost application (Table 3 and 4).

The efficiency of P uptake and P utilization increased by 100% and 106% compared to the dose of 15 t ha^{-1} with the application of vermicompost of 45 t ha^{-1}. The addition of vermicompost significantly increased the protein content of maize seeds. When vermicompost was applied at a rate of 45 t ha^{-1}, protein content of 9.1 percent was obtained, whereas a compost addition of 15 t ha^{-1} resulted in protein content of 6.0 percent (Table 6).

Table 3. The effect of different doses of vermicompost from the mushroom waste substrate on plant height, stem diameter, number of leaves per plant and leaf area index of maize

Vermicompost dosage	Plant height (cm)	Stem diameter (cm)	Number of leaves per plant	Leaf area index
0 t ha^{-1}	45.9 ± 3.3a	0.7 ± 0.05a	5.7 ± 0.6a	0.40 ± 0.16a
15 t ha^{-1}	107.6 ± 9.3b	1.2 ± 0.05b	11.0 ± 0.4b	2.03 ± 0.35b
30 t ha^{-1}	115.4 ± 6.5b	1.5 ± 0.03c	12.2 ± 0.1c	2.34 ± 0.20b
45 t ha^{-1}	146.5 ± 5.4c	1.8 ± 0.06d	12.4 ± 0.4c	2.98 ± 0.31c

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with α = 0.05. DAP = Day After Planting.

Table 4. The effect of different doses of vermicompost from the mushroom waste substrate on shoot dry weight, time for male flowers to appear and time for female flowers to appear of maize

Vermicompost dosage	Shoot dry weight (g tan^{-1})	Time for male flowers to appear (DAP)	Time for female flowers to appear (DAP)
0 t ha^{-1}	7.3 ± 2.5a	83.3 ± 1.6c	92.9 ± 0.6d
15 t ha^{-1}	42.7 ± 2.7b	53.9 ± 0.7b	60.3 ± 0.6c
30 t ha^{-1}	59.6 ± 4.5c	51.1 ± 0.3a	56.3 ± 0.6b
45 t ha^{-1}	99.5 ± 11.d	50.3 ± 0.1a	54.7 ± 0.4a

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with α = 0.05. DAP = Day After Planting.

According to Abdou et al. (2016), the improved growth of maize after receiving vermicompost is due to a mineralization process that occurs when compost is applied to the soil. The addition of compost in soil could increase the N, P, K and micronutrients content (Khan et al., 2017), increase the amount of soil organic carbon and soil pH (Agegnehu et al., 2016, Anhar et al., 2018, Karimuna et al., 2016, Zaman et al., 2010) and improve activity of microorganisms (Agbede et al., 2017, Frimpong et al., 2017). Other studies showed that the application of organic fertilizers on maize crop could increase plant height, the number of leaves, stem diameter and fresh weight at harvest (Okoroafor et al., 2013, Soro et al., 2015). Plant height also increased due to pig and chicken manure (Coulibaly et al., 2019). Meanwhile, Mahmood et al. (2017) stated that maize crop with applications of chemical fertilizers and poultry manure in dose of 7 t ha^{-1} resulted in the maximum Leaf Area Index.

When compared to unfertilized plots, the use of vermicompost increased plant height, which could be attributed to vermicompost's ability to improve photosynthesis rate, free radical scavenging, and soil enzymatic activity (Laugale et al., 2020). According to Kareem et al. (2017) an increase in plant height was caused by an increase in apical meristem activity, while an increase in stem diameter was caused by an increase in mitotic cell division and cell enlargement in the stem, as well as an increase in leaf area caused by an increase in cell division in the leaves.

Some previous studies have reported that an increase in plant dry weight was due to composts of cow dung, waste (Naderi & Ghadiri, 2010), cassava (Adejumo & Togun, 2014), and livestock manure with addition of phosphorus (Sharif et al., 2011). The increase in plant dry weight caused by composting is intimately connected to compost's function in improving soil fertility, both chemically and biologically, so that plants can grow better. According to Huang et al. (2010) and Zhang et al. (2016), composting increases soil fertility and nutrient uptake by plants, resulting in increased plant growth, yield, and yield components. Organic fertilizers improve soil fertility and microbial activity and increase the efficiency of chemical fertilizer use to improve crop production (Mahmood et al., 2017, Schoebitz & Vidal, 2016). The quickest time for male and female flowers to emerge was achieved using up to 45 t ha^{-1} of vermicompost. Imran & Khan (2015) believe that increasing the compost dosage can hasten the release of male and female maize flowers.

The appearance of flowers is a sign that the plant has entered the generative phase. In general, to enter the generative phase, plants need sufficient phosphorus. Phosphorus deficiency can cause delays in plants entering the generative phase. In this study, adding more vermicompost can significantly increase the availability of phosphorus (Table 2), so corn plants can enter the generative phase more quickly than those without vermicompost. According to Sabastian et al. (2018), phosphorus plays an important role in energy storage and transfer. An adequate supply of phosphorus in the early stages of plant life is critical for the reproductive phase of the plants. The optimal application of phosphatic fertilizers is required for rapid growth, early maturity, and improvement in the quality of vegetative growth, whereas its deficiency will slow overall growth (Ahmad et al., 2013).

Application of vermicompost increased the chlorophyll and proline content of maize leaves (Table 5). Naeem et al. (2017) reported that applying artificial fertilizers along with compost increased the chlorophyll content of leaves compared to applying synthetic fertilizers only. Chlorophyll content also increased in plants fed with organic fertilizers (Amujoyegbe et al., 2010), sawdust (Adekayode & Olojugba, 2010), urban waste compost (Pirdashti et al., 2010), and cow dung, poultry and rice straw (Siavoshi & Laware, 2013). According to Udom & Kamalu (2019), an increase in proline levels is caused by the rise in the three components of maize: leaf area index, stem diameter, and plant dry crown weight. As a result, with the same watering volume, the vermicompost-treated soil will quickly experience water shortages because water needs will increase with a larger canopy.

Table 5. The effect of different doses of vermicompost from the mushroom waste substrate on chlorophyll, proline at the end vegetative phase and proline in the seed filling phase of maize plants

Vermicompost dosage	Chlorophyll (mg g^{-1})	Proline at the end vegetative phase (μ mol g^{-1})	Proline in the seed filling phase (μ mol g^{-1})
0 t ha^{-1}	15.3 ± 2.0a	1.3 ± 0.1a	1.3 ± 0.1a
15 t ha^{-1}	24.7 ± 1.9b	2.7 ± 0.4b	2.8 ± 0.2b
30 t ha^{-1}	26.3 ± 1.9b	3.8 ± 0.4c	3.5 ± 0.4c
45 t ha^{-1}	29.1 ± 1.5c	6.0 ± 1.2d	4.6 ± 0.4d

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with $a = 0.05$.

Humic acid in vermicompost helps to increase the efficiency of P use. According to El-Etr et al. (2011), the presence of humic acid increases P use efficiency in maize sesame plants. The addition of compost (Majeed et al., 2018), manure (Ademba et al., 2015, Andriamananjara et al., 2019, El-Eyuoon & Amin, 2018), and Tithonia biomass was also reported to improve P uptake or use efficiency (Endris, 2019). Because the addition of vermicompost affects soil phosphorus levels, the protein content of maize seeds increases; according to Shahbazi & Nematollahi (2019), increasing the phosphorus application from 0 to 150 kg ha^{-1} increased the protein content significantly from 13.56

percent to 15.53 percent. The addition of phosphorus to the fertilization process can increase the protein content of maize kernels (Radulov et al., 2010), wheat (Rahim et al., 2010).

Table 6. The effect of different doses of vermicompost from the mushroom waste substrate on P absorption efficiency, efficiency of using P and seed protein of maize plants

Vermicompost Dosage	P absorption efficiency (dry seed weight / total P uptake) ($g\ g^{-1}$)	Efficiency of using P (dry plant weight / total P uptake) ($g\ g^{-1}$)	Seed protein (%)
0 t ha^{-1}	NA	NA	NA
15 t ha^{-1}	5.2 ± 0.4a	23.0 ± 1.3a	6.0 ± 1.4a
30 t ha^{-1}	7.7 ± 0.9b	30.6 ± 2.2b	6.8 ± 1.5b
45 t ha^{-1}	10.4 ± 1.2c	47.4 ± 4.6c	9.1 ± 1.1c

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with α = 0.05. NA = Not Available.

Yield and yield components of maize

The application of vermicompost increased the length and diameter of the cobs significantly. With the addition of 15 t ha^{-1} of vermicompost, the size and diameter of the cobs increased by 137 percent and 517 percent, respectively. In comparison, a dose of 45 t ha^{-1} of vermicompost increased by 205 and 683 percent, respectively. The maximum yield index, seed weight per ear, number of seeds per ear, the weight of 100 grains, and highest yield index were all achieved with vermicompost up to 45 t ha^{-1} (Table 7 and 8).

Table 7. The effect of different doses of vermicompost from the mushroom waste substrate on cob lenght, cob diameter and seed weight per cob of maize

Vermicompost dosage	Cob length (cm)	Cob diameter (cm)	Seed weight per cob (g)
0 t ha^{-1}	5.7 ± 0.1a	0.6±0.05a	NA
15 t ha^{-1}	13.5 ± 0.7b	4.0 ± 0.08b	61.9 ± 7.2a
30 t ha^{-1}	15.5 ± 0.2c	4.3 ± 0.12c	94.0 ± 5.8 b
45 t ha^{-1}	17.4 ± 0.4d	4.7 ± 0.07d	130.1 ± 4.6 c

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with α = 0.05. NA = Not Available.

Table 8. The effect of different doses of vermicompost from the mushroom waste substrate on weight 100 seeds, number of seeds per cob and harvest index of maize

Vermicompost dosage	Weight 100 seeds (g)	Number of seeds per cob	Harvest Index
0 t ha^{-1}	NA	NA	NA
15 t ha^{-1}	25.5 ± 1.9a	246.9 ± 24.3a	0.51 ± 0.02a
30 t ha^{-1}	28.3 ± 1.0b	333.2 ± 22.9b	0.59 ± 0.01b
45 t ha^{-1}	31.7 ± 1.4c	425.3 ± 8.55c	0.64 ± 0.01b

Note: Value followed by the same letter in the same column showed no significant difference based on the Duncan Multiple Range Test with α = 0.05. NA = Not Available.

The increased yield and yield components of maize due to vermicompost treatment were linked to increased soil fertility and plant development. Growing plants can supply photosynthate to storage organs or plant yields. Previous research found that using vermicompost (Jjagwe et al., 2019) or compost (Amanullah et al., 2015) increased the number of cobs per plant and seeds per row, the number of rows per cob, and the number of seeds per cob of maize. Ganjali et al. (2013), Glaser et al. (2014), and Doan et al. (2015) all reported increased maize yield and yield components with compost application. When organic fertilizers were used, the number of seeds per cob, the weight of 1,000 grains, and the weight of seeds increased (Achieng et al., 2010). Iqbal et al., 2015) reported increased yields and yield components of maize with organic matter. Bekele et al. (2019) found that combining nitrogen, phosphorus, and vermicompost application resulted in a 21.5 percent increase in groundnut pod harvest index over the control.

CONCLUSIONS

Vermicompost application of up to 45 t ha^{-1} can increase pH, C-organic, P-total, P-available, and phosphate solubilizing microbes while decreasing Al saturation. Plant height, stem diameter, the number of leaves, leaf area index, and dry crown weight increased by up to 45 t ha^{-1} with vermicompost. Male maize flowers appeared 30 days earlier when Ultisol was combined with 45 t ha^{-1} of vermicompost. The addition of 45 t ha^{-1} of vermicompost increased the levels of chlorophyll and proline. In the application of vermicompost up to 45 t ha^{-1}, the highest seed protein content, P uptake efficiency, and P use efficiency were achieved. The length of the ear, the diameter of the ear, weight of seeds per ear, the weight of 100 seeds, and the highest number of seeds were also achieved with vermicompost applications of up to 45 t ha^{-1}.

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Genetic diversity analysis of Indonesian rice germplasm (*Oryza sativa* L.) with simple sequence repeat markers

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Abstract. The characterization of germplasm provides information on the regional rice genetic diversity and variety kinship classification. This study aimed to provide information on the agro-morphological traits and genetic diversity of fifty local rice varieties from Java and Borneo Island in Indonesia. The variability of thirteen agronomic traits showed the differentiation among the accessions, while the phenotypic traits were grouped into six clusters. The genotyping characterization was conducted using SSR (Simple Sequence Repeats) markers (22 microsatellites), and continued with genetic diversity and Polymorphism Information Content (PIC) analysis. The agro-morphological clustering based on Ward's Hierarchical constructed six sub-clusters. The PC1 and PC2 had 86.3% of the total percentage. The UPGMA method was used to construct six different groupings, as the correlation between each group and its collecting source was significant. Furthermore, the UPGMA dendrogram clustered the 50 accessions into six main clusters, while the PIC showed a polymorphism value range of 0.41–0.74. RM162 located on chromosome 5, which was considered as the best marker for fifty-one genotypes. At the same time, the lowest PIC value of 0.41 was observed in RM431 located in chromosome 1. This classification can be helpful as a detailed information for plant breeders to characterize and select the germplasm, while conducting backcrosses between rice accessions.

Key words: agro-morphological, diversity, germplasm, rice, SSR.

INTRODUCTION

Over half of the world's population consumes rice as a primary food (Garris et al., 2005; Ghneima et al., 2008; Hokazono et al., 2009; Sabu et al., 2009; Singh et al., 2016). Rice is classified into other two large groups, namely *indica* and *japonica* subspecies

(Zaidi et al., 2006; Agrama et al., 2010). Rice plant is a mostly-cultivated cereal crop in Asia, especially in Indonesia, because of its wide range of ecological, geographical and climate aspects (Ni et al., 2002). The country has several diversities, including landraces, cultivar, and wild accession. This condition occurs as Indonesia is a tropical country that has a strategic geographical position located on the equator. Indonesia is known as the largest archipelagic country in the world with more than seventeen islands, as each of which has its own uniqueness and characteristics according to the landscape. Furthermore, there are over seventeen thousand rice genetic resources served as a potential natural source.

The climatic condition of Indonesia helps the production of several local rice diversities, including the ones resistant to biotic stress (brown planthopper, blast, and blight disease) (Park et al., 2022). The utilization of local germplasm is relatively low, as more than thirty-five thousand accessions have been characterized through continuous natural selection, resulting in gradual changes among the crosses of genetically related accessions during the evolutionary time (Caicedo et al., 2007; Pachauri et al., 2013). As a consequence, the adaptation process produced new rice varieties with diverse morphology, physiology, genetic, and habitat (Vaughan et al., 2008; Jasim Aljumaili et al., 2018).

Before the green revolution policy, farmers in each region have been planting and cultivating the germplasm rice for generations and centuries. This policy has a substantial negative effect due to rapid replacement of local varieties with the modern ones. However, some of them are still planted by farmers because of their adaptation and competition potential in sub-optimal natural conditions (Pandey et al., 2015).

Genetic diversity provides an opportunity to obtain high benefits through rice breeding program opportunity to improve the yield and characterization quality and quantity (Chakravarthi & Naravaneni, 2006; Varshney et al., 2008; Huang et al., 2010; Yadav et al., 2013; Roy et al., 2016; Skipars et al., 2021). The characterization of local germplasm is extremely important to determine the superior genes potential from local germplasm, while genetic diversity helps ensure the efficiency level (Prysiazhniuk et al., 2020; Karimah et al., 2021; Shanina & Likhodeyevsky, 2021). Several studies have been conducted on these concepts in many countries of the world. However, there is a lack of information sources to describe the genetic diversity of rice germplasm on a local scale (Thomson et al., 2009). In addition, this information is also required to provide a detailed picture of the rice genetic diversity in a country, besides its complex interactions with the germplasm potential (Barry et al., 2007).

Therefore, this study used 22 SSR markers distributed from chromosome number 1 until 12 to examine the genetic diversity of 50 genotypes, and their efficacy was compared to the utilized rice germplasm.

MATERIALS AND METHODS

Plant material

In this study, we collected fifty sources of rice germplasm from Java and the Borneo Islands of Indonesia. In Java Island, the samples were obtained from East Java, Central Java, Banten, and West Java Provinces. Meanwhile on Borneo Island, the samples were obtained from Central Borneo and East Borneo Provinces, as shown in Table 1. The control varieties included the *Japonica* (*Nagdong*, *Black Madras*, and *Bekinju*) and *Indica* (*Tetep* and *TN1*) subspecies. The experiment was designed to focus on the insect standard handling, plant geometry, and fertilizer application. All of these activities were conducted under rainy lowland conditions.

Table 1. List of varieties

No	Accession name	Origin	No	Accession name	Origin
1	*Tetep*	Vietnam	26	*Hoing*	Banten
2	*Madras*	Korea	27	*Ketan Hitam 1*	West Java
3	*Bekinju*	Korea	28	*Ketan Keuyeup*	West Java
4	*Nampyeong*	Korea	29	*Genjah Batu*	West Java
5	*TN1*	China	30	*Si Rendet*	West Java
6	*Kali Culuk*	East Java	31	*Botel*	West Java
7	*Merah SP*	East Java	32	*Pulut Rigoti*	West Java
8	*Bondowoso 1*	East Java	33	*Ketan Putri*	West Java
9	*Inpago 8IPB*	East Java	34	*Aek Sibundong*	West Java
10	*Gogo Niti 2*	East Java	35	*Batang Paiman*	West Java
11	*Mansur*	East Java	36	*Bondoyudo*	West Java
12	*Merah Wangi*	East Java	37	*Cigeulis*	West Java
13	*MS Pendek*	East Java	38	*Cilamaya Muncul*	West Java
14	*Genjah Nganjuk*	East Java	39	*Ciliwung*	West Java
15	*Super Manggis*	East Java	40	*Cisantana*	West Java
16	*Kropak*	East Java	41	*Cisokan*	West Java
17	*Inpago*	East Java	42	*Cempo Selamet*	Middle Java
18	*Sereh*	Banten	43	*Cempo Telouluk*	Middle Java
19	*Waren*	Banten	44	*Mamas*	Middle Java
20	*Pare Jaketra*	Banten	45	*Cempo Wulut*	Middle Java
21	*Pare Caok*	Banten	46	*Hawara Batu*	Middle Java
22	*Bulu Putih*	Banten	47	*Kero*	Middle Java
23	*Pandan Ungu*	Banten	48	*Ketan Nangka*	Middle Java
24	*Roti*	Banten	49	*Ketan Benjar*	Middle Borneo
25	*Amas*	Banten	50	*Pulut Hitam*	East Bormeo

Morphology clustering and characterization

After 20 days of rice seedling (DAS), we transplanted the rice germplasm to the experimenting field at Jember University, located on 100–200 masl with rainfall level of 2.396 mm year^{-1} and humidity of 84%–95%. Fifty accessions were cultivated during the rainy season from October, 2017 to April, 2018 in 1×1 m plots (5 lines/accession) with a 0.25 m spacing and 0.5 m spacing between the rows using a block design. We used the Standard Evaluation System to describe the detailed morphology of rice after the standard agronomy (Allhgholipour et al., 2014). The phenotypic data included traits, such as the plant height (Ht), culm length (CL), culm's internode length (CmIL), panicle length (PL), number of panicles per plant (PnP), leaf length (LL), leaf width (LW), flag leaf length (FLL), flag leaf width (FLW), and ligule length (LgL). Other traits include grain length (GrL), grain width (GW), and grain length to width (GrLW). The total phenotypic data from these samples were analyzed further using a Principal Component Analysis (PCA) to reveal the critical parameter correlation matrix data (Roy et al., 2016). Then, the Ward's Hierarchical Clustering was used to group the agro-morphological characteristics. We also used PAST 3 software to examine the parameter clustering and characterization.

Genotyping characterization

The fifty leaf samples were collected for DNA isolation from young leaf tissue using the SDS method (Thomson et al., 2007). Using a UV Reader for 30 minutes, we examined the DNA quality at 100 V in a 1.7% agarose gel containing 1 x TAE buffer (Tris-acetate-EDTA). The DNA concentration was determined using a NanoDrop Spectrophotometer (Nanovue Plus-UK) and the purified DNA was diluted to 100 ng using TE buffer to preserve its quality, when stored at –20 °C. The total PCR reaction obtained a volume of 10 μl, which included 5 μl PCR Nexpro master mix reaction, 3 μl DDH2O water, 1 μl for forward and reverse primer, and 1 μl DNA template. The 22 SSR markers mapped by McCouch et al. (2002) were used to distribute the 12 chromosomes (Table 2) and cover the genome for each sample.

Table 2. List of 22 SSR markers

Primer No	Locus	Chr.	Repeat motif	PCR primer (5' to 3')	
1	RM431	1	(AG)16	F	TCCTGCGAACTGAAGAGTTG
				R	AGAGCAAAACCCTGGTTCAC
2	RM259	1	(CT)17	F	TGGAGTTTGAGAGGAGGG
				R	CTTGTTGCATGGTGCCATGT
3	RM154	2	(GA)21	F	ACCCTCTCCGCCTCGCCTCCTC
				R	CTCCTCCTCCTGCGACCGCTCC
4	RM452	2	(GTC)9	F	CTGATCGAGAGCGTTAAGGG
				R	GGGATCAAACCACGTTTCTG
5	RM489	3	(ATA)8	F	ACTTGAGACGATCGGACACC
				R	TCACCCATGGATGTTGTCAG
6	RM55	3	(GA)17	F	CCGTCGCCGTAGTAGAGAAG
				R	TCCCGGTTATTTTAAGGCG
7	RM307	4	(AT)14(GT)21	F	GTACTACCGACCTACCGTTCAC
				R	CTGCTATGCATGAACTGCTC
8	RM124	4	(TC)10	F	ATCGTCTGCGTTGCGGCTGCTG
				R	CATGGATCACCGAGCTCCCCCC
9	RM334	5	(CTT)20	F	GTTCAGTGTTCAGTGCCACC
				R	GACTTTGATCTTTGGTGGACG
10	RM161	5	(AG)20	F	TGCAGATGAGAAGCGGCGCCTC
				R	TGTGTCATCAGACGGCGCTCCG
11	RM162	6	(AC)20	F	GCCAGCAAAACCAGGGATCCGG
				R	CAAGGTCTTGTGCGGCTTGCGG
12	RM11	7	(GA)17	F	TCTCCTCTTCCCCCGATC
				R	ATAGCGGGCGAGGCTTAG
13	RM118	7	(GA)8	F	CCAATCGGAGCCACCGGAGAGC
				R	CACATCCTCCAGCGACGCCGAG
14	RM408	8	(CT)13	F	CAACGAGCTAACTTCCGTCC
				R	ACTGCTACTTGGGTAGCTGACC
15	RM284	8	(GA)8	F	ATCTCTGATACTCCATCCATCC
				R	CCTGTACGTTGATCCGAAGC
16	RM404	8	(GA)33	F	CCAATCATTAACCCCTGAGC
				R	GCCTTCATGCTTCAGAAGAC
17	RM215	9	(CT)16	F	CAAAATGGAGCAGCAAGAGC
				R	TGAGCACCTCCTTCTCTGTAG

Table 2 (continued)

18	RM171	10	(GATG)5	F	AACGCGAGGACACGTACTTAC
				R	ACGAGATACGTACGCCTTTG
19	RM552	11	(TAT)13	F	CGCAGTTGTGGATTTCAGTG
				R	TGCTCAACGTTTGACTGTCC
20	RM536	11	(CT)16	F	TCTCTCCTCTTGTTTGGCTC
				R	ACACACCAACACGACCACAC
21	RM19	12	(ATC)10	F	CAAAAACAGAGCAGATGAC
				R	CTCAAGATGGACGCCAAGA
22	RM277	12	(GA)11	F	CGGTCAAATCATCACCTGAC
				R	CAAGGCTTGCAAGGGAAG

Moreover, the DNA samples were amplified using the T100 Thermal Cycler (Bio-Rad-US). The PCR program was performed in 5 minutes of predenaturation at 95 °C, 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 55–60 °C, a minute of extension at 72 °C, and 5 minutes of final extension at 72 °C. The samples were conducted on a 1.7% agarose gel at 100V for 40 minutes using electrophoresis.

Statistical and genetic diversity analysis

The visible DNA bands were appropriated to the molecular weight and scored using the Allele's format, which represented the band presence as number one and band absence as zero number, until the DNA tape had the lowest size (Sajib et al., 2012). The genetic diversity, allele frequency, and PIC value (Polymorphic Information Content) were analyzed using the PowerMarker V3.25 (Liu & Muse, 2005). The PIC value was used to represent the polymorphism value in a variety of ways. The allele score matrixes were used to develop a phylogeny tree by applying the UPGMA method. However, the tree was constructed using the NTSYPC program and Jaccard's coefficient to determine the relationship among the varieties (Pandey et al., 2015).

RESULTS AND DISCUSSION

Morphological characterization

This study was conducted to characterize the agronomic trait variability of the samples collected from different provinces in Indonesia (Banten, Borneo, East Java, Central Java, and West Java). The variety evaluation in 13 morphological characters revealed the highest value of plant height trait (187.40 cm) in *Cempo Selamet* variety and the lowest value in Ciliwung variety (53.60 cm). This condition can be attributed to the number of panicles per plant, ranging from 5 to 48 per plant. Based on the grain characteristics, GrL was found among 5.09–7.53 cm and GrW was among 1.56–3.41 cm. As shown in Table 3, the average of grain size was discovered at 3.06, which indicates that the grain is on the medium side.

The Principal Component Analysis (PCA) of 13 traits is presented from PC1 to PC5 (Eigenvalue > 1). The analysis was conducted to identify the most important variables contributing to the total agro-morphological variation of the samples. At the same time, the multivariate method visualizes the accessions clustering that depends on the component loading with the multivariate method (Singh et al., 2016). The detailed information about these values is enlisted in Table 4. The PC1 and PC2 represent the distribution of the 50 accessions, besides the number and names of the accessions listed in Table 1. However, the results showed that the PC1 and PC2 only obtained 86.3% of the total percentage. The first principal value was 71.5% of the percentage variance. This condition was characterized by high positive loading, containing Ht, CL, FLL and LL. The second principle was 14.8%, with high positive loadings of FLL and LL. Another trait, namely PnP, obtained a high positive loading of 0.86, as listed in PC3 (Table 4).

Table 3. Agro-morphological characters of 50 accessions

Agronomic traits	Mean ± SD	Min	Max	% CV
Ht	114.47 ± 13.17	53.60	187.40	12.32
CL	86.83 ± 12.71	28.00	163.40	15.87
CmIL	9.52 ± 0.51	1.60	51.00	5.03
LL	39.56 ± 5.51	23.90	87.90	14.17
LW	1.40 ± 0.06	0.70	2.56	4.93
FLL	30.61 ± 3.60	12.10	87.60	13.29
FLW	1.26 ± 0.08	0.90	2.00	6.32
PL	25.24 ± 2.55	13.10	39.80	9.78
PnP	20.65 ± 2.03	5.00	48.00	11.82
GrL	6.42 ± 0.11	5.09	7.53	1.79
GrW	2.15 ± 0.06	1.56	3.41	2.84
GrLW	3.06 ± 0.11	1.65	4.40	3.64
LgL	1.44 ± 0.07	0.40	2.60	5.87

Note: Ht = plant height; CL = culm length; cmIL = culm internode length; LL = leaf length; LW = leaf width; FLL = flag leaf length; FLW = flag leaf width; PL = panicle length; PnP = panicle per plant; GrL = grain length; GrW = grain width; GrLW = grain length to width; LgL = ligule length.

Table 4. Principal component analysis

Characteristics	PC1	PC2	PC3	PC4	PC5
Ht	0.755	-0.071	-0.321	0.518	0.108
CL	0.605	-0.279	0.251	-0.653	-0.172
CmIL	0.079	0.101	0.163	0.172	0.554
LL	0.145	0.529	0.233	-0.293	0.578
LW	0.003	-0.005	-0.007	-0.014	0.002
FLL	0.184	0.774	0.063	0.076	-0.547
FLW	0.002	0.002	-0.005	-0.004	0.009
PL	0.032	0.054	-0.093	0.027	0.036
PnP	0.041	-0.158	0.860	0.428	-0.126
GrL	-0.002	0.002	-0.021	-0.009	0.024
GrW	0.001	0.002	-0.012	-0.014	0.008
GrLW	-0.003	-0.003	0.011	0.017	0.000
LgL	0.001	0.003	-0.008	0.000	-0.011

Note: Ht = plant height; CL = culm length; cmIL = culm internode length; LL = leaf length; LW = leaf width; FLL = flag leaf length; FLW = flag leaf width; PL = panicle length; PnP = panicle per plant; GrL = grain length; GrW = grain width; GrLW = grain length to width; LgL = ligule length.

The PCA visualization could assist in suggesting the most appropriate genotype selection to improve the yield based on the component loading value. The distribution of the separated accessions is presented in scare plots of PC1 and PC2 (Fig. 1).

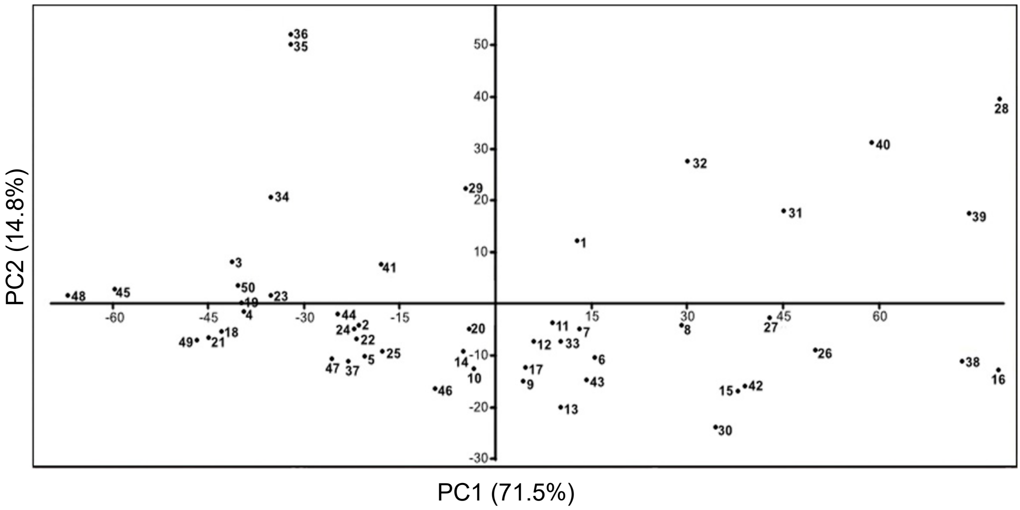

Figure 1. Principal Component Analysis with PC1 and PC2 value: (1) *Tetep*; (2) *Madras*; (3) *Bekinju*; (4) *Nampyeong*; (5) *TN1*; (6) *Kali Culuk*; (7) *Merah SP*; (8) *Bondowoso* 1; (9) *Inpago 8IPB*; (10) *GogoNiti 2*; (11) *Mansur*; (12) *Merah Wangi*; (13) *MS Pendek*; (14) *Genjah Nganjuk*; (15) *Super Manggis*; (16) *Kropak*; (17) *Sereh*; (18) *Waren*; (19) *Pare Jaketra*; (20) *Pare Caok*; (21) *Bulu Putih*; (22) *Pandan Ungu*; (23) *Roti*; (24) *Amas*; (25) *Inpago*; (26) *Ketan Hitam 1*; (27) *Ketan Keuyeup*; (28) *Genjah Batu*; (29) *Si Rendet*; (30) *Ketan Benjar*; (31) *Cempo Salamet*; (32) *Cempo Telouluk*; (33) *Mamas*; (34) *Cempo Wulut*; (35) *Hawara Batu*; (36) *Kero*; (37) *Ketan Nangka*; (38) *Pulut Hitam*; (39) *Hoing*; (40) *Botel*; (41) *Pulut Rigoti*; (42) *Ketan Putri*; (43) *Aek Sibundong*; (44) *Batang Paiman*; (45) *Bondoyudo*; (46) *Cigeulis*; (47) *Cilamaya Muncul*; (48) *Ciliwung*; (49) *Cisantana*; (50) *Cisokan*.

The plots with high-value loading were presented on the right, namely *Mamas*, *Mansur*, *Merah Wangi*, *Merah SP*, *Sereh, Inpago 8IPB*, *Aek Sibundong*, and *Kali Culuk*. Meanwhile, plots with low positive loading were presented on the left, namely *Cilamaya Muncul*, *Ketan Nangkam Inpago*, *Amas*, *Pandan Ungu*, and *Hawara Batu*. The agro-morphological clustering based on Ward's Hierarchical Cluster is shown in Fig. 2.

Two larger groups were constructed, containing two sub-clusters tagged as IA, IB, IIA, and IIB. Two sub-clusters of IB were created, using *Black Madras* and *Bekinju* varieties, while *Nampyeong* and *TN1* varieties were used as controls. However, *Pandan Ungu* and *Amas* were very closed to *Black Madras*. At the same time, *Cisokan* was very closed to *Bekinju* as *Japonica* subspecies control. The other sub-cluster, i.e. IIA, was also divided into two smaller sub-clusters. *Cempo Telouluk* variety was very closed to *Tetep* as a variety control, and other varieties, such as *Bondowoso 1, Ketan Keuyeup, Ketan Putri, Super Manggis*, and *Ketan Benjar*. Furthermore, the IA and IIB sub-clusters only contain one smaller sub-cluster.

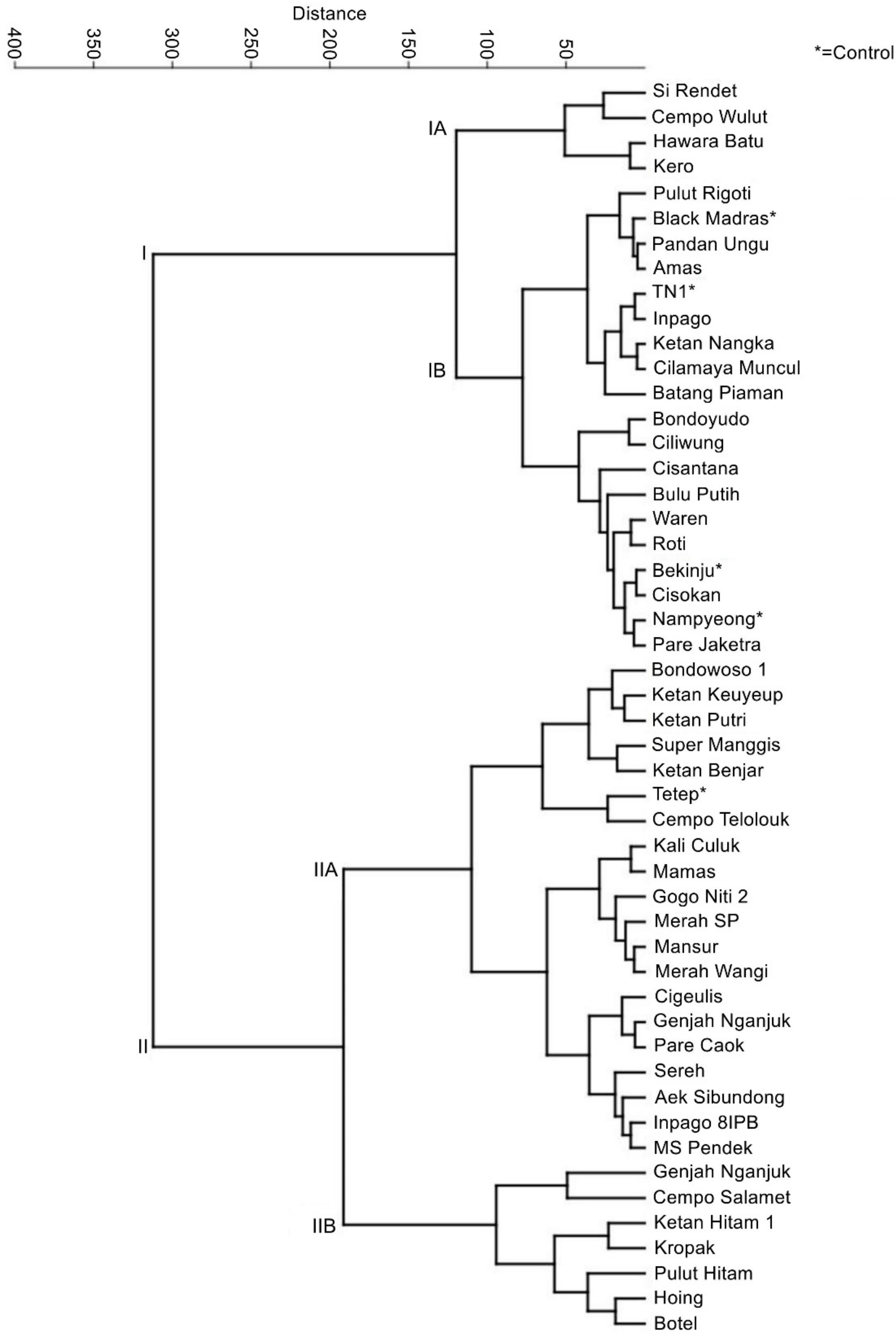

Figure 2. The 50 accessions grouping based on the agro-morphological characters with Ward's hierarchical clustering.

The SSR markers and genetic diversity analysis

The 22 markers used in the 50 accessions mostly showed polymorphic characteristics. The total amplified fragments (alleles) was 1074 of 22 primers with a mean of 48.8 alleles for each locus. The detailed information is presented in Table 5.

The Pi value showed the average frequency of alleles in each locus at 0.23–0.49. Therefore, PIC can indicate the polymorphism value of a molecular marker. The highest value of 0.74 was found in RM162, followed by RM408 with 0.72 and RM171 with 0.69. Therefore, RM162, located on the chromosome 5, is considered as the best marker due to high level of polymorphism compared to others. However, the lowest PIC value of 0.41 was observed in RM431 located in the chromosome 1. This level was determined using a mean value of 0.56, whereas a value of > 0.5 showed high polymorphism and a value of < 0.5 showed low polymorphism. Other studies also obtained similar results at 0.23–0.84, with an average of 0.61 (Brondani et al., 2006), or varied among 0.45–0.65, with an average of 0.57 (Roy et al., 2016), and among 0.28–0.98, with an average of 0.63 (Jasim Aljumaili et al., 2018). These values are markedly higher than the values obtained in this study.

Table 5. Summary of SSR analysis

No	Marker	Amplified Fragment (alleles)	Pi (frequency alleles)	PIC (polymorphic information content)
1	RM431	52	0.34	0.41
2	RM259	48	0.48	0.52
3	RM154	67	0.44	0.42
4	RM452	43	0.42	0.64
5	RM489	51	0.25	0.65
6	RM55	47	0.46	0.52
7	RM307	55	0.27	0.62
8	RM124	39	0.38	0.66
9	RM334	62	0.30	0.50
10	RM161	51	0.25	0.59
11	RM162	44	0.29	0.74
12	RM11	48	0.47	0.55
13	RM118	39	0.39	0.64
14	RM408	45	0.30	0.72
15	RM284	49	0.49	0.52
16	RM404	54	0.35	0.62
17	RM215	43	0.42	0.63
18	RM171	40	0.39	0.69
19	RM552	70	0.23	0.44
20	RM536	46	0.45	0.59
21	RM19	38	0.38	0.71
22	RM277	43	0.43	0.62

Genetic diversity depends on the gene recombination, mutation, and selection due to capable of creating new varieties. Furthermore, genetic diversity is explored to identify the potential genes that can be used to repair germplasm (Allhgholipour et al., 2014). Every crop improvement program is the primary source of variability, which acts as a reservoir for identifying superior alleles controlling the agronomic and qualitative key parameters by mapping the allele mining associations (Nachimuthu et al., 2015). The cluster analysis results of fifty genotypes based on Jaccard's Coefficient successfully classified the genotypes into two main clusters. In contrast, the genetic similarity among the genotypes was 0.2–1.0. Group I consisted of 5 genotypes, while Group II consisted of with two sub-clusters (IIA and IIB). Group IIA consisted of A (5 genotypes) and B (16 genotypes). In addition, Group IIB consisted of C (5 genotypes), D (11 genotypes), and E (8 genotypes). The detailed UPGMA dendrogram with the genetic relationship among accessions is presented in Fig. 3.

Five control varieties, namely *Tetep, Bekinju, Nampyeong, Black Madras*, and *TN1*, were distributed in group II. The *TN1* and *Bekinju* varieties were closed to *Bondowoso 1,* *Merah SP, Merah Wangi*, and *MS Pendek*. Other varieties, including *Black Madras, Tetep,* and *Nampyeong*, were closed to *Bulu Putih* and *Pare Caok*. The method divided them into six genetic clusters confirmed to correspond with the *Japonica* and *Indica* subspecies.

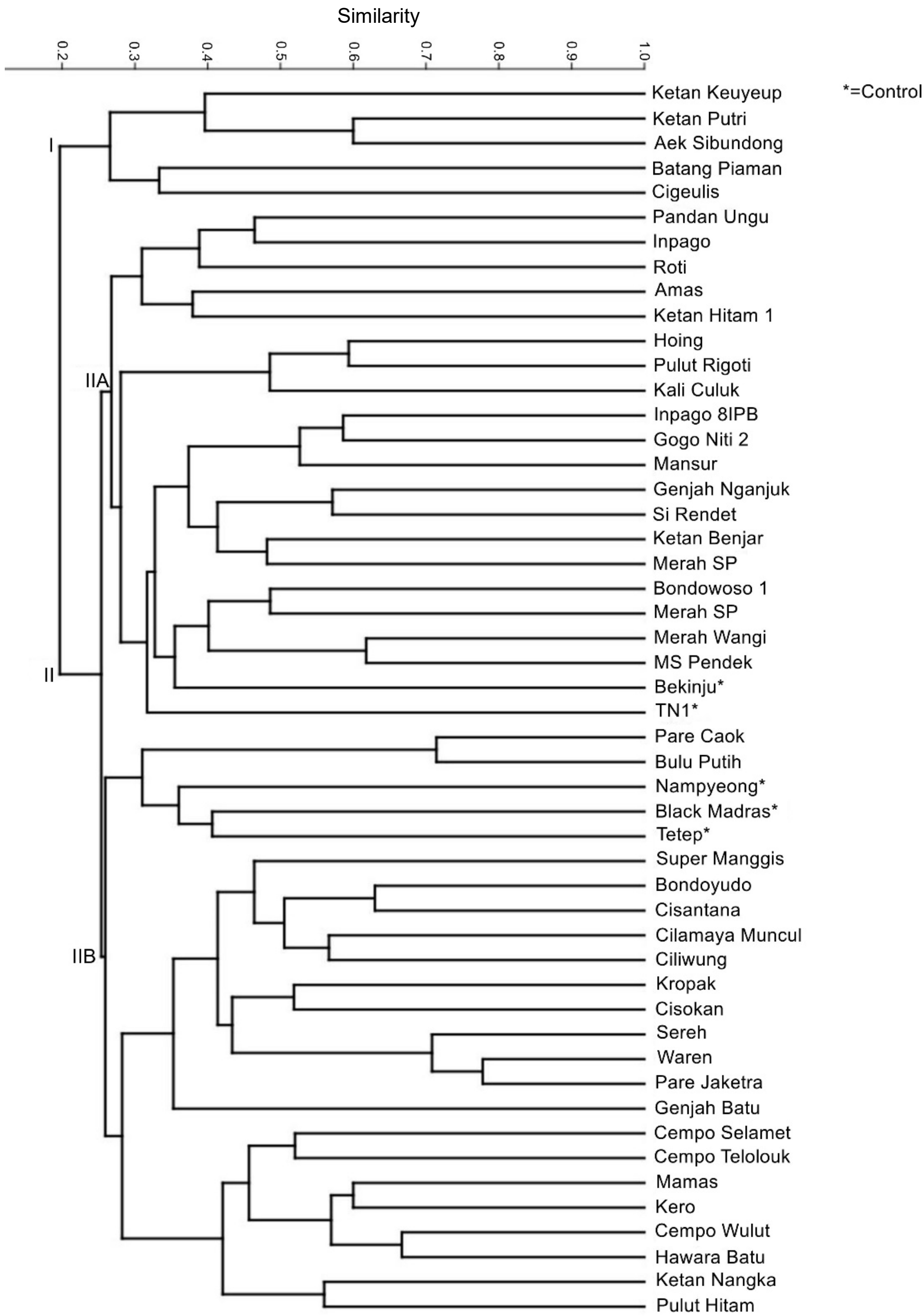

Figure 3. The 50 genotypes grouping based on the 22 SSR markers with UPGMA method.

Therefore, it can be deduced that the Principal Coordinate Analysis (Fig. 4) indicates the separated fifty accessions based on their genetic relationship with the majority group closed to the *Indica* subspecies, rather than the *Japonica* subspecies.

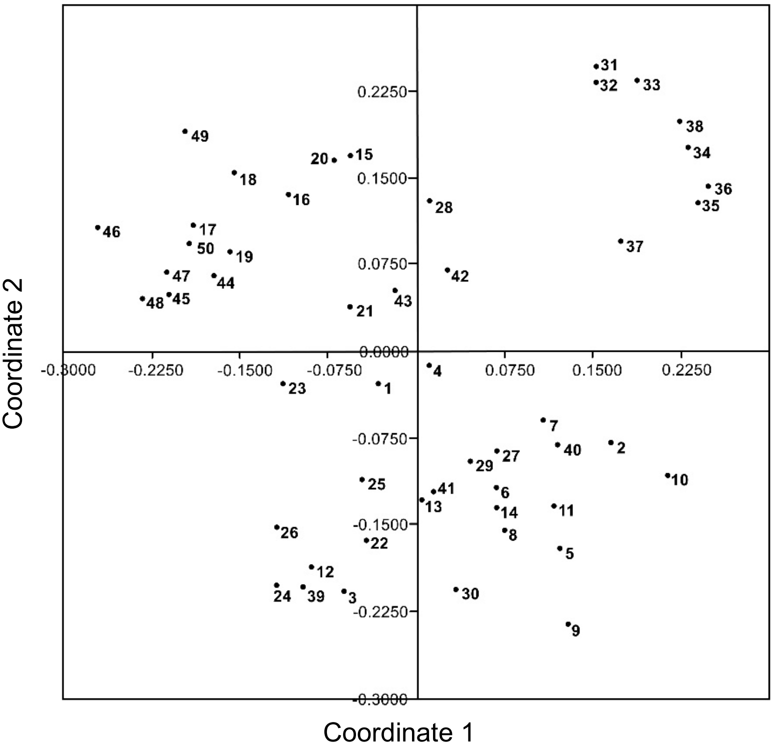

Figure 4. Principal Coordinate Analysis: (1) *Tetep*; (2) *Madras*; (3) *Bekinju*; (4) *Nampyeong*; (5) *TN1*; (6) *Kali Culuk*; (7) *Merah SP*; (8) *Bondowoso 1*; (9) *Inpago 8IPB*; (10) *GogoNiti 2*; (11) *Mansur*; (12) *Merah Wangi*; (13) *MS Pendek*; (14) *Genjah Nganjuk*; (15) *Super Manggis*; (16) *Kropak*; (17) *Sereh*; (18) *Waren*; (19) *Pare Jaketra*; (20) *Pare Caok*; (21) *Bulu Putih*; (22) *Pandan Ungu*; (23) *Roti*; (24) *Amas*; (25) *Inpago*; (26) *Ketan Hitam 1*; (27) *Ketan Keuyeup*; (28) *Genjah Batu*; (29) *Si Rendet*; (30) *Ketan Benjar*; (31) *Cempo Salamet*; (32) *Cempo Telouluk*; (33) *Mamas*; (34) *Cempo Wulut*; (35) *Hawara Batu*; (36) *Kero*; (37) *Ketan Nangka*; (38) *Pulut Hitam*; (39) *Hoing*; (40) *Botel*; (41) *Pulut Rigoti*; (42) *Ketan Putri*; (43) *Aek Sibundong*; (44) *Batang Paiman*; (45) *Bondoyudo*; (46) *Cigeulis*; (47) *Cilamaya Muncul*; (48) *Ciliwung*; (49) *Cisantana*; (50) *Cisokan*.

CONCLUSIONS

The thirteen agronomic trait variabilities showed the differentiation among the accessions, while phenotypic traits found were grouped into six clusters. The genotyping characterization was conducted using SSR (Simple Sequence Repeats) markers (22 microsatellites), continued with the genetic diversity and Polymorphism Information Content (PIC) analyses. The agro-morphological clustering based on Ward's Hierarchical constructed six subclusters with the PC1 and PC2 obtained 86.3% of the total percentage.

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Prediction of fruit rot disease incidence in Arecanut based on weather parameters

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Abstract. The occurrence of pests and diseases in arecanut crops has always been an important factor affecting the total production of arecanut. Arecanut is always dependent on environmental factors during its growth. Thus monitoring and early prediction of the occurrence of the disease would be very helpful for prevention and therefore more crop production. Here, we propose artificial intelligence-based deep learning models for fruit rot disease prediction. Historical data on fruit rot incidence in representative areas of arecanut production in Udupi along with historical weather data are the parameters used to develop region-specific models for the Udupi district. The fruit rot disease incidence score value is predicted using recurrent neural network variants (i.e., Vanilla LSTM, Vanilla GRU, stacked LSTM, and Bidirectional LSTM) for the first time. The predictive performance of the proposed models is evaluated by mean square error (MSE) along with the 5-fold cross-validation technique. Further, compared to other deep learning and machine learning models, the Vanilla LSTM model gives 1.5 MSE, while the Vanilla GRU model gives 1.3 MSE making it the best prediction model for arecanut fruit rot disease.

Key words: crop disease, deep learning, prediction, cross-validation, fruit rot disease.

INTRODUCTION

The different technologies like robotics platforms, the Internet of things, and remote sensing belonging to the industry are now applied to solve agriculture challenges, leading to intelligent farming. Smart farming is essential to meet consumer demands, as well as for sustainability and productivity. According to the annual report of 2018–19 from the Department of Cooperation, Agriculture, and Farmers welfare, 54.6% of the entire workforce is affianced in allied and agricultural sector activities. Farming the arecanut is a financially rewarding endeavor for farmers. Over 90% of all arecanuts produced in India are produced in Karnataka, Assam, Kerala, Mizoram, Meghalaya, West Bengal, and Nagaland. Arecanut is exported worldwide and 700 million people depend on it for many reasons (Balanagouda et al., 2021).

Crop disease always depends on three factors: environmental fluctuation, host, and pathogen (Fenu & Malloci, 2021). Arecanut is exceptionally vulnerable to several pathogens like bacteria, fungi, and viruses. Heavy rainfall, humidity, and high-intensity rains reduce pollination and increase the disease rate reducing yield (Sujatha et al., 2018). Arecanut in India is especially threatened by fruit rot disease which causes a 10–70% loss in crop yield and sometimes leads to the tree collapsing in itself. The fruit rot disease (FRD) problem is escalating yearly because of the lack of human resources available to spray fungicides. This increases the disease severity and the ailment spreads to nearby regions. Hence, the scattering of diseases was assessed across many locations and the risk correlated with FRD (Balanagouda et al., 2021), which will help the extension officers and farmers to take control actions and prevent the further expropriation of FRD into adjacent places. Farmers rely more on forewarning systems at an initial stage as they can avoid the roll-out of disease and also minimize chemical usage. Literature shows that arecanut is comprehensively used in medicinal practice in India, China, and other countries in Asia (Peng et al., 2015). It is used as traditional medicine for abdominal health issues and for treating parasitic diseases, gastrointestinal diseases, and edematous diseases.

Deep learning is a modern tool that was later integrated with the agriculture domain, which has already succeeded in other disciplines. The applications of deep learning in agriculture are surveyed by a few authors (Kamilaris & Prenafeta-Boldú, 2018; Santos et al., 2019; Ren et al., 2020). They summarized that deep learning models give superior performance in terms of accuracy when compared with machine learning models. Image-based disease detection, weed detection, land cover classification, and yield prediction are some of the more common uses of DL. Even then, it is rarely used for early disease detection in crops based on weather. Recent research states that long short-term memory (LSTM) performs better in sequential data processing when compared with deep feedforward neural networks (Kim et al., 2017).

Researchers have developed a variety of weather-based crop disease forecasting models using deep learning techniques. Along with that, many crop disease detection models are developed using images. Analysis of agriculture big data through machine learning and deep learning has emerged lately. The analysis and classification of different algorithms used recently for plant and crop disease forecasting are explored (Fenu & Malloci, 2021). The study categorizes the forecasting models into image-based data, weather-based information, and data from heterogeneous sources, in which weather-based forecasting is more examined. Support vector machine, artificial neural network (Malicdem & Fernandez, 2015; Fenu et al., 2019), LSTM (Kim et al., 2017), extreme learning machine(ELM) (Anshul Bhatia et al., 2020), random forest (Jawade et al., 2020), and multi-layer perceptron (de Oliveira Aparecido et al., 2020) techniques are used to predict crop and plant disease based on weather parameters. Similarly, CNN, MLP, RF, ELM, and support vector regression techniques are frequently used to forecast crop and plant disease based on images (Zhu et al., 2017; Duarte-Carvajalinoet al., 2018). Along with images and weather data, data from different sources - i.e. remotely sensed variables like vegetation index, water stress index, land surface temperature, leaf area index, and soil temperature - are used to predict crop and plant diseases in their early stages.

The enhanced LSTM model is used in the rice crops' pest attack forecasting with the help of weather data (Wahyono et al., 2020). Daily temperature, humidity, and rainfall data are the inputs given for the model over 16 years. Vanilla LSTM, stacked LSTM, and bidirectional LSTM models were compared with the sliding window concept. They found that bidirectional LSTM performed better due to the forward and backward processing of the data.

Rice blast disease (RBD) is forecasted before occurrence using LSTM RNN (Recurrent Neural Network) (Kim et al., 2017). To test the model, they considered 17 different rice varieties from 12 regions in South Korea. The proposed LSTM model is evaluated by varying the input parameters relative humidity, air temperature, sunshine hours, and rice blast disease score. Among several LSTM models, the BlastTHS LSTM model gives more accuracy because it contains all the input parameters to predict the disease in the Cheolwon region.

The LSTM model is used to predict the pest and disease occurrence in the cotton crop based on weather factors (Xiao et al., 2018; Xiao et al., 2019). The Apriori algorithm is used to find the association rules between the weather parameters and disease data. The advantages of the LSTM model are shown by comparing it with the support vector method (SVM) and random forest method. Relative humidity, rainfall, minimum and maximum temperature, wind speed, and sunshine hours are the parameters used for the model development along with 63 datasets of cotton pests and diseases.

A new deep LSTM method is proposed to predict the rice crop pest with the help of anomaly climate data (Wahyono et al., 2021). In this study, two prediction models are developed and prediction results are compared. Among the two models, the first model uses climate data with anomaly as input, and the second model uses data without anomaly. Good accuracy was seen in the first model when experimented with 100 epochs. An attempt was made to predict the rice crop disease using an artificial intelligence-based hybrid CNN (Convolution Neural Network)-LSTM model (Jain & Ramesh, 2021). The study presents both region-specific prediction and classification models, where SVM is used to classify yellow stem border disease in rice crops. The model takes relative humidity, temperature, rainfall, evaporation, sunshine hours, and pest value captured from the light trap as validation parameters.

Recently, ANN and SVM learning models were developed to forecast the incidence and severity of RBD to prevent or mitigate the escalating of the disease (Malicdem & Fernandez, 2015). The PCA algorithm is used to select the features contributing more to disease occurrence. SVM gives a more accurate prediction when the result is compared with the ANN model. A system was proposed to predict the RBD based on weather parameters like rainfall, temperature, wind speed, wind gust, sun hours, visibility, humidity, and UV index (Sriwanna, 2022). The agriculture office has documented the RBD occurrence between 2013 and 2019. The class 1 label is given to the recorded disease data elements but the class 0 label is given to ‘no disease’ data elements. After combining the blast data and weather data, they used ensemble feature rankings to select the weather features that have a meaningful effect on disease occurrence. The classification performance was measured using the F1 score, ROC AUC score, balanced accuracy, and geometric mean over DT, KNN, SVM, NB, and MLP classification algorithms using the top 10 features.

LSTM and multilayer perceptron algorithms are used to develop a predictive model for RBD in the Davangere region (Varsha et al., 2021). The data for blast disease is considered based on the literature study and climate data collected from the Karnataka state natural disaster monitoring station. Hyperparameters are adjusted and based on the readings shown by the authors, the final results have outstanding accuracy. Image-based deep convolution encoder network model is proposed to predict and classify the diseases in different seasonal crops (Khamparia et al., 2020). The crop leaf images from the Plant Village dataset are considered for the experiment for five various diseases. Due to the combination of autoencoders and CNNs, the model is in hybrid mode. This network performed better than the conventional techniques.

A CNN model was developed to identify the tomato crop disease using leaf images. In comparison to different machine learning models like Naive Bayes, kNN, and decision trees, the proposed model has 98.4% accuracy, which is relatively high (Agarwal et al., 2020). The proposed model is tested using a different dataset from other domains, and additional model variants are used in the experiments. Accuracy, F1 score, and AUC-ROC metrics are used to measure the model's achievement. Rice diseases are identified through leaf images using the deep CNN model (Lu et al., 2017). The proposed model achieves 95.48% accuracy under a 10-fold cross-validation strategy, which is relatively high compared to conventional machine learning models such as SVM and particle swarm optimization. 10 different rice diseases are identified from the model. Apple scab disease was detected at its early stage using CNN and transfer learning methods (Kodors et al., 2021). The imbalanced dataset containing images of apple fruit and apple leaves is considered for the experiment.

A summary was made on detecting and categorizing various arecanut diseases using image processing and machine learning methods (Puneeth et al., 2021). The results of the study indicate that not much work is being done on the early identification of arecanut disease. The authors found no weather-based prediction model for arecanut disease as part of their review process. Authors have also detected and classified wheat disease using improved deep convolution architecture (Goyal et al., 2021). The spike and leaf parts are the most affected in the wheat plant. The proposed method detects nine diseases and one healthy class from the Large wheat disease classification dataset 2020. The proposed CNN model is compared with VGG16 and RESNET50 CNN models and found high testing accuracy of 97.88%.

Current state-of-the-art ML models are applied to predict the fruit rot disease in arecanut crops after data collection (Krishna et al., 2022). The dataset is created by integrating disease data and weather data. The decision tree regression (DTR), multilayer perceptron regression (MLPR), random forest regression (RFR), and support vector regression (SVR) models are used to predict the disease. RFR model gives the best performance with 0.9 as Mean Absolute Error and 1.9 as Mean Square Error.

The development of image-based arecanut crop disease identification models has recently gained much traction. For instance, there was an identification of different arecanut diseases using a multi-gradient direction-based deep learning model (Mallikarjuna et al., 2021; Mallikarjuna et al., 2022). In the proposed method, a multi-Sobel directional kernel is applied to each input image to generate multi-gradient directional images. These images are given as input to the ResNet CNN architecture for disease identification. The author has generated the dataset with a total of 281 images including healthy, rot, split, and rot-split images. Precision, F-measure, and recall performance metrics are used to evaluate the generated results.

The literature shows that disease identification and prediction models are developed for different plants and crops. To list citrus, cucumber (Liu et al., 2022), potato, olive, apple, tomato, tobacco, mango, barley, coffee, cotton, grape, rice, wheat, orange, strawberry, oil palm, ginger, and sugar beet can be considered. The main contribution of this research work is as follows:

- Even though many prediction models exist in the crop disease management domain for various crops, there is no deep learning-based prediction model for the arecanut crop. Hence the present study is the first effort to develop weather-based arecanut crop disease forecasting using deep learning models.
- A novel aspect of this study is integrating historical weather data with arecanut crop disease data to create a unique dataset.
- The performance of four deep learning methods is compared and analyzed.

Consequently, sincere effort and research are required to develop novel solutions to prevent or mitigate the effect of crop disease on yield loss at an early stage.

MATERIALS AND METHODS

The flow diagram for the proposed work is shown in Fig. 1. First, the disease and weather data are collected from different sources and integrated. Second, the data is pre-processed to make it fit for further processing. After this, different deep-learning models are applied to predict the disease score value. Finally, based on the validation loss and training loss, all the models are compared and the results are analyzed.

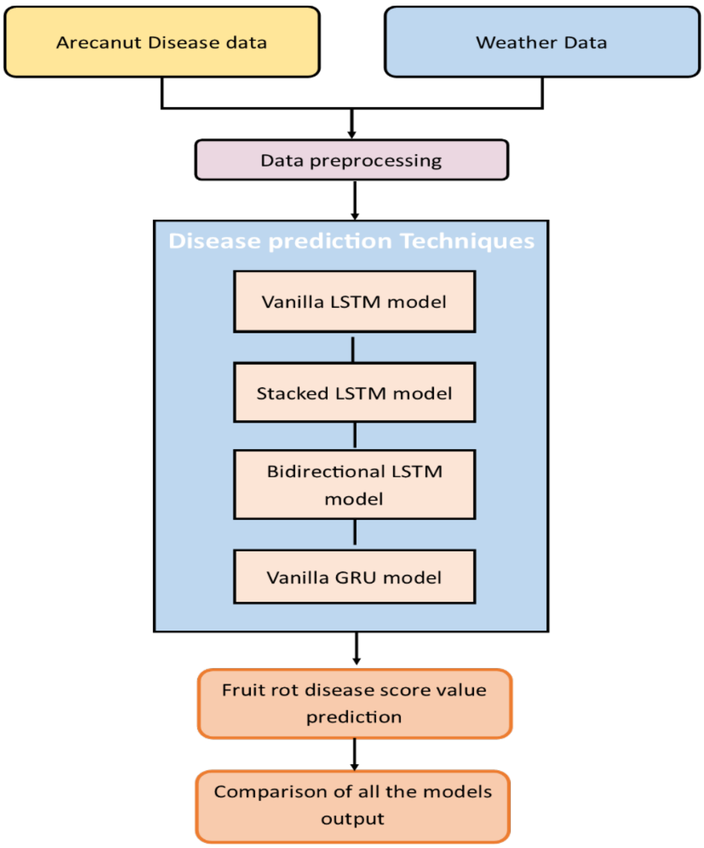

Figure 1. Flow diagram of the proposed work.

Sources of Data

In this research work, arecanut fruit rot disease data and historical climate data are used to generate an FRD forewarning model. The arecanut disease data is collected from literature resources, arecanut disease recommendations, and field surveys with nearly 60 farmers (Krishna et al., 2022) from the Udupi region in Karnataka state, India. Zone Agricultural and Horticultural Research Station, Brahmavar, Udupi has provided 21 years of weather data from 2000 to 2020. Only temperature, rainfall, humidity, sunshine hours, and cloud direction parameters are considered in the experiment. The data before pre-processing/integration is shown in Table 1 for September 2000.

Table 1. The historical weather data sample for September 2000 before preprocessing. The parameters are: Rainfall (RF), Maximum temperature (Max Temp), Minimum Temperature (Min. Temp), Relative Humidity in the morning (RH - I), Relative Humidity in the evening (RH - II), Cloud measure (I & II in oktas)

Date	RF	Max. Temp	Min. Temp	RH-I	RH-II	Cloud I	Cloud II	Sunshine Hours
01.09.2000	0	30	22	97	97	6	6	0
02.09.2000	0	30	22	98	95	8	7	0
03.09.2000	0	30	23	93	91	8	6	0
04.09.2000	0	30	22	100	97	8	6	0
05.09.2000	0	30	23	95	91	8	8	0
06.09.2000	0	31	22	92	90	8	8	8
07.09.2000	6	31	22	96	91	8	8	9
08.09.2000	12	31	23	94	91	8	6	9
...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...
29.09.2000	0	31	23	94	94	8	6	4
30.09.2000	0	30	25	92	95	8	8	9

Data Pre-processing

The FRD occurs due to heavy rainfall, high humidity, and low temperature. It is around May through October that the climate will be conducive to disease activity. Hence from 21 years of collected data, only these months are considered. Arecanut diseases usually directly depend on the weather pattern. Therefore, the disease and weather data are integrated as shown in Algorithm 1 to create the final dataset, which is used to develop the prediction model. This study represents the relationship between weather parameters and FRD in arecanut crops by predicting the score value. The score value varies from 0 to 35 based on the weather pattern. As the score value increases, there is a greater chance of disease outbreaks and spreading. The following rules are used to calculate the disease score value.

Algorithm 1: Rules used to calculate disease score value

If Rainfall > 15 mm and Temperature < 24 °C and Humidity > 90,
then Score value increments
if Rainfall > 5 mm and Sunshine > 5 hrs,
then Score value increments
if Rainfall < 10 mm and Temperature > 24 °C,
then Score value decreases.
In all other cases score value remains same.

After integrating/preprocessing the fruit rot disease data and weather data, the final data can be used to predict the fruit rot disease score value. It is possible to forecast disease severity for farmers based on the range of score values. The range from 0 to 15 indicates no disease, 15–25 indicates a medium risk of getting a fungal infection, 25–34 shows an occurrence of disease and a score value above 35 indicates a high severity of the infection. Once the infection starts it will spread to the entire farm with the help of wind. Table 2 shows the final integrated data after preprocessing for September 2000.

Table 2. The historical weather data sample for September 2000 and score value after integration/preprocessing through algorithm 1

Date	RF	Max. Temp	Min. Temp	RH-I	RH-II	Cloud I	Cloud II	Sunshine Hours	Score value
01.09.2000	0	30	22	97	97	6	6	0	16
02.09.2000	0	30	22	98	95	8	7	0	15
03.09.2000	0	30	23	93	91	8	6	0	14
04.09.2000	0	30	22	100	97	8	6	0	13
05.09.2000	0	30	23	95	91	8	8	0	12
06.09.2000	0	31	22	92	90	8	8	8	11
07.09.2000	6	31	22	96	91	8	8	9	12
08.09.2000	12	31	23	94	91	8	6	9	13
...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...
29.09.2000	0	31	23	94	94	8	6	4	3
30.09.2000	0	30	25	92	95	8	8	9	2

Disease prediction Techniques

A recurrent neural network is the best technology to work with sequential time series data (Samarawickrama & Fernando, 2017; Zhang & Dong, 2020). Hence the present study has experimented with different variants of RNN, that include LSTM and GRU. A detailed explanation of the models is given in the next section.

LSTM model

Recently LSTM has become a state-of-the-art model for different time series prediction problems. It is a part of an RNN capable of learning long-term data dependencies, hence it is more useful in time series sequential analysis. The LSTM cell is shown in Fig. 2. It is also called a memory cell because it stores some information that is essential for the next decision. This LSTM cell contains three gates; each has its functionality; which provides read, write, and reset operations for the cell.

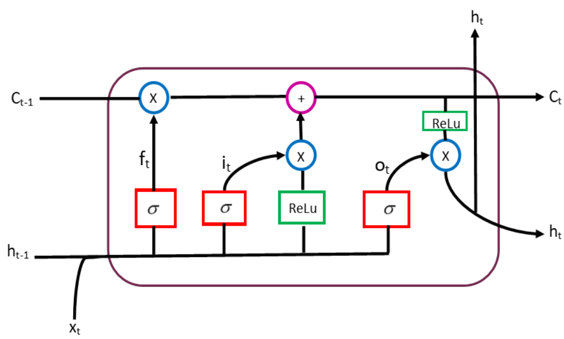

Figure 2. LSTM cell with three gates: input, output and forget.

Forget Gate: Decides what information has to be discarded from the cell.

$$f_t = \sigma(W_f . [h_{t-1}, x_t] + b_f \quad (1)$$

Input Gate: Decides the values from the inputs to update the cell.

$$i_t = \sigma(W_i.[h_{t-1}, x_t] + b_i \quad (2)$$

Output Gate: Decides what to output based on the input and content of the cell.

$$o_t = \sigma(W_o.[h_{t-1}, x_t] + b_o \quad (3)$$

In the equation, W is the weight given to the neuron, h is the output from the previous cell, b is the bias, C_t is the cell state and x is the input at time step t.

Vanilla LSTM model: As shown in Fig. 3(a), the model with a single hidden layer is called the vanilla LSTM model (Wu et al. 2018) used the vanilla LSTM model to estimate the remaining useful life of engineered systems.

Stacked LSTM model: In this model, multiple LSTM layers are stacked one after the other as shown in Fig. 3(b). According to the literature, stacked LSTM performs better than vanilla LSTM.

Bidirectional LSTM model: As shown in Fig. 3(c), in this model, instead of training a single model, two models are trained with forwarding LSTM using input sequence and backward LSTM using reversed input sequence. The output from the forward and backward LSTM is concatenated to feed to the dense layer.

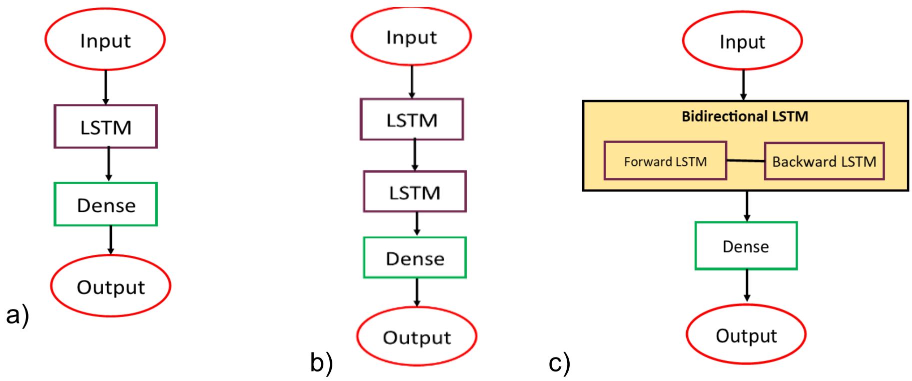

Figure 3. Variants of RNN (a) Vanilla LSTM (b) Stacked LSTM (c) Bidirectional LSTM.

GRU Model

It is similar to LSTM but a new variant of RNN. The GRU has not maintained the cell state like LSTM. It controls the flow of information through the reset gate and the update gate. The GRU unit is shown in Fig. 4.

Update Gate: It combines the input and the forget gates into a single update gate.

$$z_t = \sigma(w_z.[h_{t-1}, x_t]) \quad (4)$$

Reset Gate: It stores the short-term memory of the network that is the hidden state.

$$r_t = \sigma(w_r.[h_{t-1}, x_t]) \quad (5)$$

In the equation, W is the weight given to the neuron, h is the output from the previous cell, b is the bias, and x is the input at time step t.

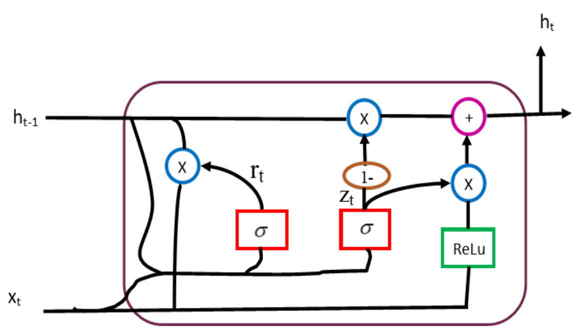

Figure 4. GRU cell with two gates.

Both LSTM and GRU performance varies from case to case, but GRU has only two gates, so it processes faster than LSTM. K-fold cross-validation is used to validate the model. It is a statistical method that estimates the performance of the learning models on new data samples. Here the parameter K refers to the number of splits in the dataset. K value 5 is taken in the present experiment. In this method, the dataset is divided into 5 subsets; from the subset, one set is used for testing, and the remaining four sets are used for training. By using this technique, we ensure that the model's score does not depend on the selected subset for training and testing.

A diagram of the neural network layers for the different deep learning models used in the study is presented in Fig. 3. A detailed parameter list for the models used in the experiment are shown in Table 3. ReLu activation function is used in all four models, along with a 0.001 learning rate. A dataset with step size 15 is used to train the model. A Python environment and the TensorFlow library are used for the experiment. TensorFlow supports deep learning and machine learning applications.

Table 3. Parameter list for the different DL models

Units	First layer		Dense layer	Dropout layer
Vanilla LSTM	64		32	0.2
Stacked LSTM	64 (Lstm 1)	32 (Lstm 2)	32	0.2
Vanilla GRU	64		32	0.2
Bidirectional LSTM	64 (Lstm 1)	64 (Lstm 2)	32	0.2

RESULTS AND DISCUSSION

The present study compares the DL models to predict the arecanut crop disease score value. According to the current literature, deep learning models were not applied to predict the arecanut FRD. Hence for the first time, vanilla LSTM, vanilla GRU, stacked LSTM, and bidirectional LSTM are used in this present experiment. Models are measured through validation loss and training loss with the help of mean square error (MSE) as shown in Eq. 6.

$$MSE = \frac{\sum(actual\ value - predicted\ value)^2}{total\ number\ of\ test\ samples} \quad (6)$$

Table 4 shows the validation loss of different DL algorithms with the help of the 5-fold cross-validation technique. Similarly, Table 5 shows the training loss of different DL models. In training, the loss represents how well the model fits the training samples, while in the validation, the loss represents how well the model fits newly arrived samples.

Table 4. Comparison of validation loss with different learning models and training sets

	Validation Loss				
	Fold 1	Fold 2	Fold 3	Fold 4	Fold 5
Vanilla LSTM	2.9	1.5	2.2	2.2	2.9
Stacked LSTM	3.7	1.8	3.0	2.2	2.4
Vanilla GRU	2.0	1.5	2.1	1.3	1.6
Bidirectional LSTM	2.2	5.5	2.5	1.9	1.8

Similarly, Table 4 shows the training loss of different DL models. Observation from Tables 4 and 5 shows that the loss can vary depending on the subset of the sample.

Vanilla LSTM and stacked LSTM give better accuracy (lower validation loss) in their second fold, whereas vanilla GRU performed best in its fourth fold and bidirectional LSTM gives the best performance in the fifth fold. The measured loss

MSE is the mean data of the squared differences between actual and predicted values. MSE performance metric is always used in regression problems. The training loss and validation loss of Vanilla LSTM for 5-fold cross- validation are shown in Fig. 5.

Table 5. Comparison of training loss with different learning models and training sets

	Training Loss				
	Fold 1	Fold 2	Fold 3	Fold 4	Fold 5
Vanilla LSTM	9.6	7.2	6.3	5.8	4.7
Stacked LSTM	7.1	6.6	5.4	5.1	4.3
Vanilla GRU	8.3	7.1	6.3	5.5	4.3
Bidirectional LSTM	6.7	1.7	4.6	3.5	3.0

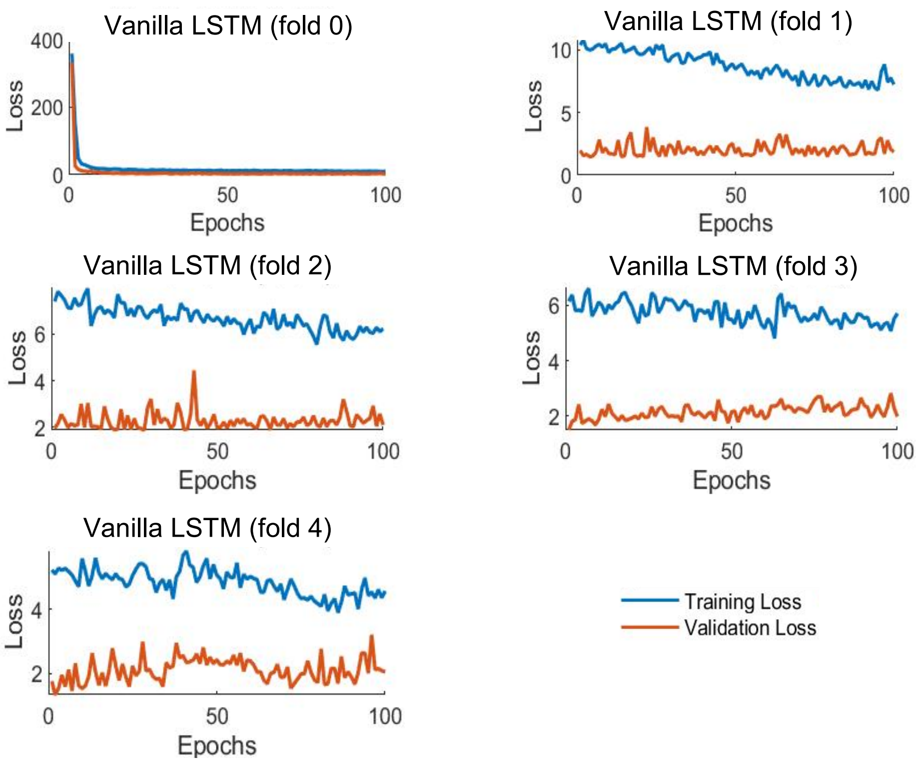

Figure 5. Training loss and validation loss for Vanilla LSTM at different fold cross-validation stage.

The training loss is slightly higher than the validation loss in all the validation folds when 100 epochs are executed. As shown in the figure, it is observed that as the epochs increase, the training loss is decreasing. When 1,000 epochs are executed, the training loss is almost equal to the validation loss, which is the best fit. The training loss and validation loss of Vanilla GRU for 5-fold cross-validation are shown in Fig. 6. In the K-fold cross-validation method each data sample is used at least once in testing, and (K-1) times in training. Hence the result generated from cross-validation reduces bias and variance. The training loss and validation loss of stacked LSTM for 5-fold cross-validation are shown in Fig. 7. The difference between training loss and validation loss for a bidirectional model is lower than in other models. The bidirectional model performed well when the K-fold cross-validation technique was applied. Fig. 8 shows the loss obtained during the experimentation with a bidirectional model.

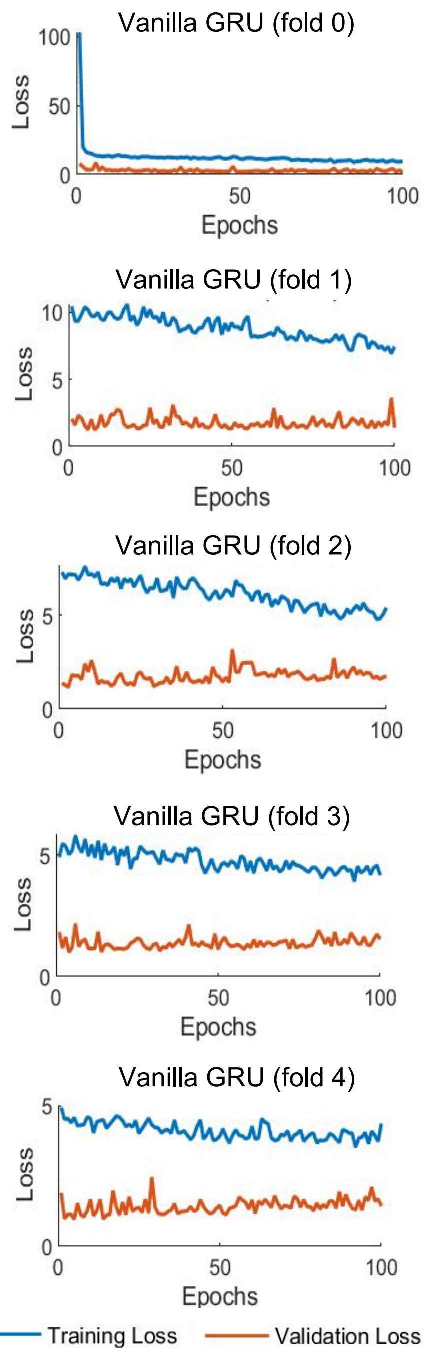

Figure 6. Training loss and validation loss for Vanilla GRU at different fold cross-validation stage.

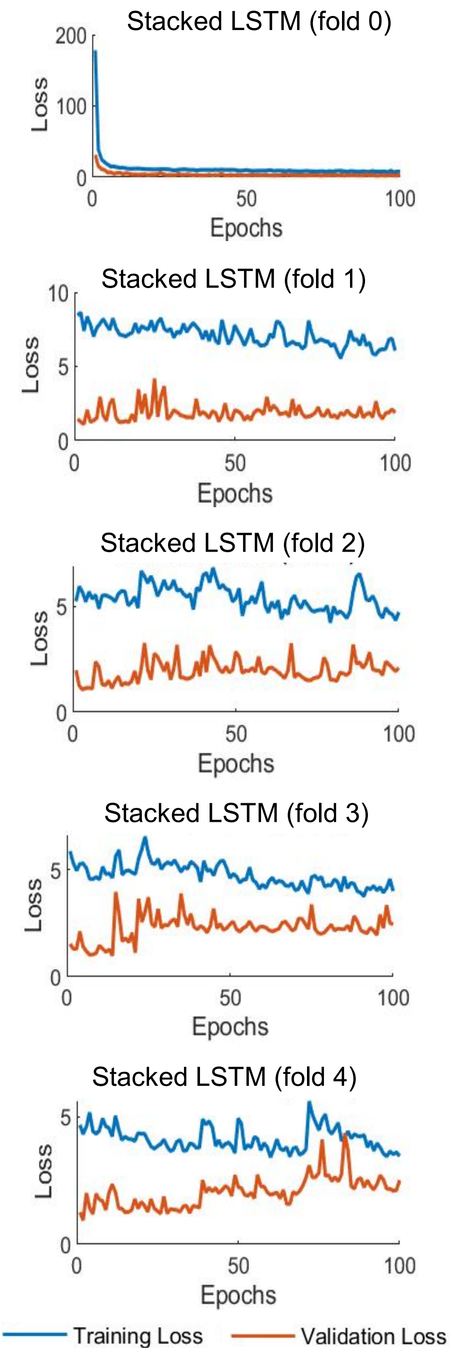

Figure 7. Training loss and validation loss for stacked LSTM at different fold cross-validation stage.

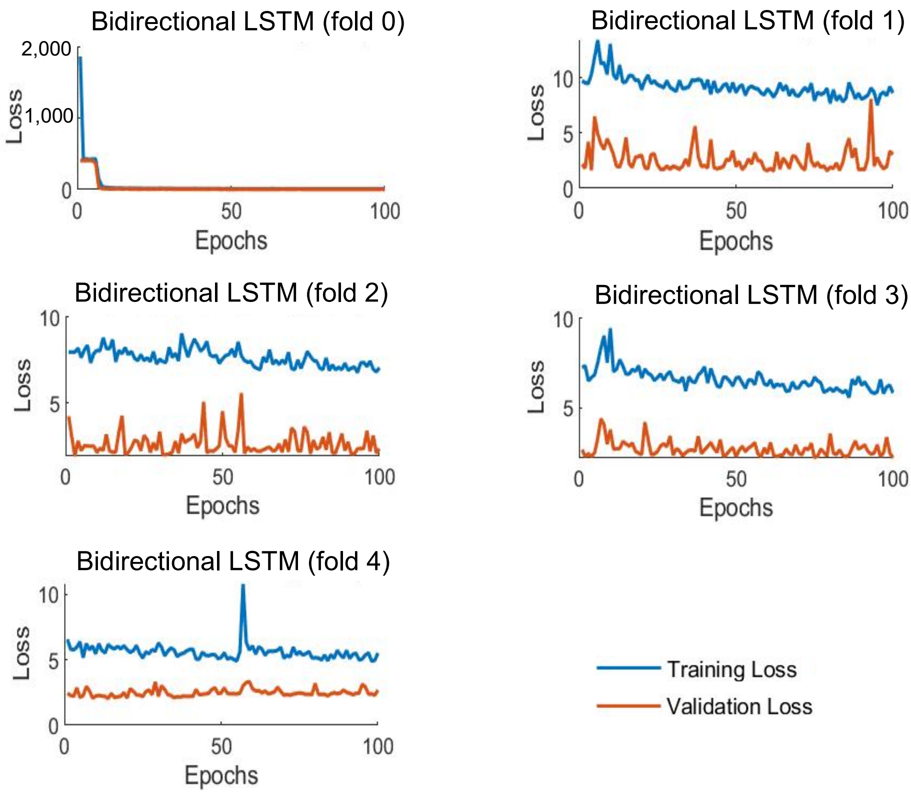

Figure 8. Training loss and validation loss for bidirectional LSTM at different fold cross-validation stage.

From Figs 5, 6, 7, and 8, it can be observed that as the number of epochs increases the training loss decreases. The training is done with 1000 epochs and it is observed that the training (TL) and validation loss (VL) is almost equal as shown in Fig. 9. In the conducted experiment, the predicted value is slightly different from the actual value. The graph for prediction values versus actual values is shown in Fig. 10.

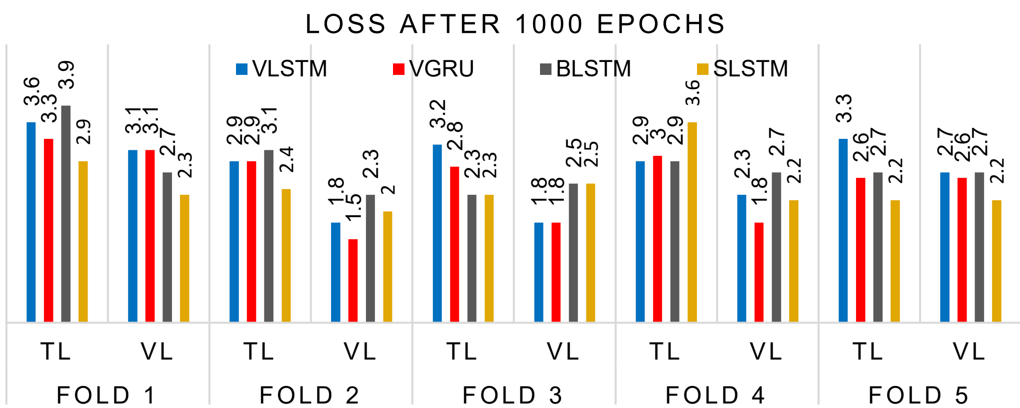

Figure 9. Loss rate after 1000 epochs training with 5-fold cross-validation.

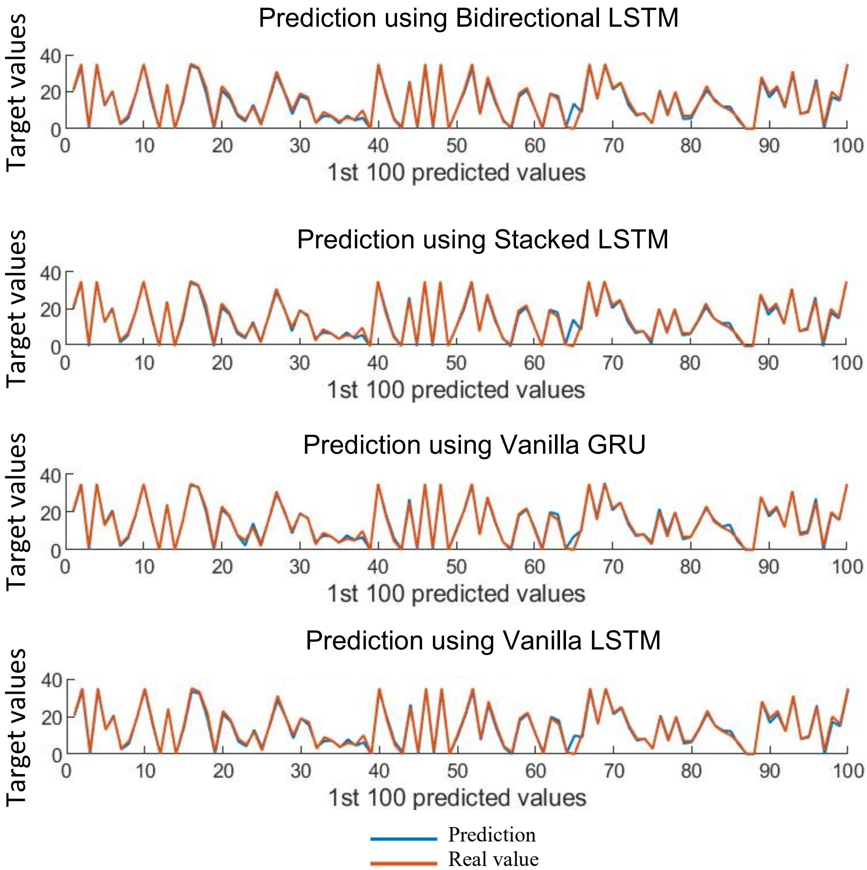

Figure 10. Plotting of first 100 actual values vs prediction value: Bidirectional LSTM, Stacked LSTM, Vanilla GRU, and Vanilla LSTM.

There are 3,152 samples in the dataset, 80% of samples are used for training, and 20% are used for testing purposes. From the 630 testing samples, only 100 predicted values are shown in the graph. The disease score value is predicted from the model and using this score value, disease incidence severity classification can be done. The proposed study and the current study were compared in Table 6 based on different crops and methods used to make crop disease predictions. It is difficult to compare the results with other related works since the use of deep learning models in arecanut crop disease forecasting is a novel approach. In contrast, LSTM models are used to predict disease in crops such as rice and cotton.

Rice blast disease is forecasted at an early stage based on RH, sunshine, temperature, and disease score data, with 67.4% prediction accuracy (Kim et al., 2017). The model is developed using only the LSTM technique. Similarly, the present study experimented with a different crop, and the rainfall parameter plays an important role here. Along with LSTM, the GRU model is also considered for the study, which gives a 1.3 MSE value. Arecanut disease classification is done with the help of CNN architecture (Mallikarjuna et al., 2022). The model takes only images as input and produces an 86.8% accuracy rate on average. However, in the present study, weather data is incorporated as input, allowing for an earlier disease diagnosis. Arecanut disease data and weather data are integrated as novel data sets, which are used as input to the different machine learning models (Krishna et al., 2022). This article considers the same dataset but experiments on different deep-learning models.

Table 6. The proposed study and the existing study are compared concerning crops and methods

Reference	Crop type	Input parameters	Techniques used	Prediction
(Kim et al., 2017)	Rice	Relative humidity, sunshine, temperature, disease score data	LSTM	Rice blast disease
(Xiao et al., 2019; Xiao et al., 2018)	Cotton	Rainfall, wind speed, humidity, sunshine, temperature	SVM, RF, LSTM	Pest and disease
(Wahyono et al., 2021)	Rice	Temperature, rainfall, humidity, climate anomaly	Deep LSTM	Pest attack
(Mallikarjuna et al., 2022; Mallikarjuna et al., 2021)	Arecanut	Images	ResNet CNN	Rot, split, rot split, and healthy
(Krishna et al., 2022)	Arecanut	Rainfall, Relative humidity, sunshine, temperature, disease score data	DTR, SVR, RFR, MLPR	FRD score value
Proposed study	Arecanut	Rainfall, Relative humidity, sunshine, temperature, disease score data	LSTM, GRU, Bidirectional LSTM	FRD score value

The DL algorithm results are compared with the ML algorithm results for the arecanut disease prediction. Table 7 shows the MSE values of ML and DL algorithms applied to the arecanut dataset to predict fruit rot disease score values.

Table 7. Compares MSE loss with machine learning and deep learning models

Model	Algorithm	MSE value
ML (Krishna et al., 2022)	Support Vector Regression	6.1
	Random Forest Regression	1.9
	Decision Tree Regression	3.4
	Multi-layer Perceptron Regression	3.3
DL	Vanilla LSTM	1.5
	Stacked LSTM	1.8
	Vanilla GRU	1.3
	Bidirectional LSTM	1.8

DL models give better accuracy in prediction when compared to ML models. Vanilla GRU is the best model compared with all other models, with a low error rate of 1.3 and less processing time due to its fewer gates.

CONCLUSIONS

In the present study, deep learning approaches are first used to predict the fruit rot disease in arecanut based on weather parameters. This novel approach primarily focused on the relationship between weather parameters and fruit rot disease in arecanut crops.

Historical weather data is taken from the agriculture research station in Brahmavar, India, and disease data is generated by referring to the arecanut disease recommendations. Vanilla GRU gives a lower error value of 1.3 MSE compared to different LSTM models. Bidirectional LSTM does not show promising results with the current dataset since its MSE value is 1.8. The stacked LSTM model also shows the same MSE value. K-fold cross-validation gives a less biased model than a single training and testing data set.

Since the validation loss is not very low, it can be surmised that the accuracy of the model is not very high. Nevertheless, it is a meaningful starting point, being the first attempt to predict arecanut crop disease using deep learning techniques based on weather parameters. This effort will help farmers take precautionary measures and prevent the spread of crop disease. In future work, disease and weather data from different regions can be considered to develop the model. Different optimization techniques can be used to increase the accuracy of the model.

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Possibility of using nutmeg flesh (M*yristica fragrans* houtt) extract in broiler diet to improve intestinal morphology, bacterial population, blood profile and antioxidant status of broilers under high-density condition

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Abstract. This study investigated the impact of nutmeg flesh extract on intestinal morphology, bacterial population, blood profile and antioxidant status of broiler chickens stocked at high density pens. After 15 days of rearing, 370 Lohmann broiler chicks (unsexed) were assigned to five treatment groups based on a completely randomized design, including T0 (chicks were raised at a density of 10 birds m^{-2} and received no nutmeg flesh extract), T1 (chicks were raised at a density of 16 birds m^{-2} and received no nutmeg flesh extract), T2 (chicks were raised at a density of 16 birds m^{-2} and received 0.5 mL kg^{-1} nutmeg flesh extract), T3 (chicks were raised at a density of 16 birds m^{-2} and received 1.0 mL kg^{-1} nutmeg flesh extract), and T4 (chicks were raised at a density of 16 birds m^{-2} and received 1.5 mL kg^{-1} nutmeg flesh extract). Sampling was conducted on day 35 of age. The results showed that the administration of nutmeg flesh extract (regardless of its levels) to broilers diets at high density (16 birds m^{-2}) significantly ($p \leq 0.05$) increased the performance, villi height in the duodenum, jejunum and ileum, decreased pH in the ileum and cecum, increased lactic acid bacteria and decreased coliform bacteria in the ileum and cecum, decreased malondialdehyde (MDA) and increased superoxide dismutase (SOD) and had no significant effect ($p > 0.05$) on the blood profile of broiler chickens. The findings demonstrated that nutmeg flesh extract improved performance, intestinal bacterial population and morphology and antioxidative status of broilers raised under high density condition.

Key words: antioxidant, broilers, intestinal bacterial population, intestinal morphology, nutmeg flesh extract.

INTRODUCTION

Raising of broilers at high densities causes broilers to experience pressure and stress during their growth period, although rearing at high densities is a strategy to gain profits by increasing meat production per square meter. Apart from the production efficiency, increasing the density of the cage has a negative influence on the intestinal microbial balance, reduces beneficial bacteria, and increases pathogenic bacteria, resulting in decreased growth performance of broilers (Astaneh et al., 2018). Stress causes adverse effects on the physiology, immunology, and microbiology of broiler chickens which in turn can impair chicken performance (Sugiharto et al., 2017a). In particular, stress due to high density decreases body weight gain and feed consumption, decreases the quality of poultry products, and in severe cases increases mortality (Silas et al., 2014; Agusetyaningsih et al., 2022; Sugiharto & Turrini 2022). Stress due to high density has been reported to impair intestinal function associated with impaired nutrient absorption, causing an increase in the heterophile to lymphocyte ratio (H/L ratio) as well as involution of lymphoid organs (Astaneh et al., 2018). Furthermore, Sugiharto & Turrini (2022) reported that stress due to high density causes a decrease in antioxidant enzymes activities.

Molecular changes due to stress are reported to increase the production of free radicals or reactive oxygen species (ROS) and trigger oxidative stress (Sugiharto et al., 2019). To reduce the negative impact of oxidative stress, feed supplementation using synthetic antioxidants is common in broiler rearing practices. However, the use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in the long term and in excess doses can leave residues in the meat that endanger the health of broiler consumers. Owing to these facts, natural sources of antioxidants are needed so that the use of synthetic antioxidants in broiler chickens can be reduced. Recent knowledge shows that plant extracts contain many phenolic compounds that can function as antioxidants. Herbal extracts have been reported to improve intestinal morphology (Liu et al., 2021), increasing the growth of beneficial bacteria in the digestive tract (Martínez et al., 2021), and improve broiler immunity (Agusetyaningsih et al., 2022). The application of herbal extracts in poultry feed has been reported to increase body weight gain, improve the rate of nutrient metabolism, and improve meat quality by lowering cholesterol levels and inhibiting lipid peroxidation (Oloruntola et al., 2020).

Nutmeg (*Myristica fragrans* Houtt) is a native Indonesian plant that is widely used as a spice. Nutmeg is an aromatic tropical plant (with a distinctive aroma) and has bioactive substances and can function as antioxidant, antimicrobial, painkiller, antiobesity, and hepatoprotective (Periasamy et al., 2016; Vangoori et al., 2019). Nutmeg consists of flesh, seed, and mace. Seed and mace are the main product of nutmeg which is used as spice, while nutmeg flesh is a waste that has no economic value. So far, studies regarding the use of nutmeg flesh extract on broilers to reduce the negative impact of stress due to high density (16 birds m^{-2}) have never been reported. Taken the antioxidative and antimicrobial properties of nutmeg flesh into consideration, administration of nutmeg flesh extract was expected to improve intestinal morphology, bacterial population, blood profile and antioxidant status of broilers reared under high density condition. Currently nutmeg flesh has not been used, and as waste nutmeg flesh can have a negative impact on environmental health if not treated properly. Therefore, the application of nutmeg flesh in broiler chicken production not only has a positive impact on chicken health and productivity, but also has a beneficial impact on the environment. Overall, the present study aimed to investigate the impact of nutmeg flesh extract on intestinal morphology, bacterial population, blood profile and antioxidant status of broilers reared under high density conditions. It was hypothesized that nutmeg flesh extract improved intestinal morphology and bacterial population, blood profile and antioxidant status of broilers stocked in high density pens.

MATERIALS AND METHODS

Preparation of nutmeg flesh extract

Nutmeg flesh discarded by farmers after harvesting was obtained from the nutmeg plantation in Ternate City, North Maluku Province, Indonesia. Before use, the nutmeg flesh was peeled and thinly sliced, then air-dried and ground into flour before use. A 1 kg of nutmeg flesh flour was extracted based on a maceration technique by soaking in 4 litre of 96% ethanol solution for 3×24 hours. During the maceration process, stirring was carried out twice, i.e., in the morning and evening. The results of maceration in the form of filtrate were then filtered and evaporated using a rotary evaporator to produce a solution of nutmeg flesh extract (Sapsuha et al., 2021).

Antioxidant activity and phytochemical composition of nutmeg flesh extract

The AOAC method (AOAC, 2007) was used to determine the proximate content of nutmeg flesh extract. Total phenol content was determined using the Folin-Ciocalteu method (Sahreen et al., 2010), and total flavonoid content was determined using the spectrophotometric method (Mayur et al., 2010). The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assay was used to determine antioxidant activity (Sochor et al., 2010). The disc diffusion method (Kirby-bauer) was used to conduct the antibacterial inhibition test against *Escherichia coli*.

In vivo Experiment

The Animal Ethical Committee of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro, Semarang approved the *in vivo* study with the approval number 57-01/A-7/KEP-FPP. A total of 370 Lohmann broiler chickens (unsexed) were reared together from 0 to 14 days of age. From day 15 onward, the chickens (average body weight of 420 ± 2.75 g) were allocated into five treatment groups (based on a completely randomized arrangement), including T0, T1, T2, T3, and T4, with five replicate pens in each group. The birds in T0 were reared at a density of 10 birds m^{-2} (i.e., normal stocking density), while the birds in T1, T2, T3, and T4 were reared at a density of 16 birds m^{-2} (i.e., high stocking density). Overall, the treatment group T0 consisted of 50 birds and for T1, T2, T3 and T4 each consisted of 80 birds. Chicks in groups T0 and T1 were not provided with nutmeg flesh extract. Chicks in groups T2, T3, and T4 were fed with diets containing nutmeg flesh extract at doses of 0.5, 1.0, and 1.5 g kg^{-1}, respectively. Because research using nutmeg flesh extract as an additive for broiler feed is still rare, the determination of the level of use of nutmeg flesh extract in feed was based on the fact that in general the use of feed additives for broiler chickens ranges from 0.05 to 0.2% of the total feed. The nutmeg flesh extract was added to the feed ('on top') after all the ingredients for the broiler ration were mixed. The chicks were distributed into treatment groups starting on the 15^{th} day because on that day the body weight of the chickens had reached more than 400 g $bird^{-1}$, allowing stress due to high density starting to occur in the chickens. In addition, the digestive organs in broilers have developed optimally in broilers aged 15 days so that the utilization of nutmeg flesh extract can be optimal. The feed given was in the form of mash and formulated (Table 1) as starter feed (days 1–21) and finisher feed (days 22–35). The entire formulated feed did not contain antioxidants, enzymes, antibacterial, antifungal, and antiprotozoa. Feed and drinking water were provided *ad libitum* using manual feeder and drinker until day 35. Feeding was done little by little periodically to prevent spilled feed. On day 4, all chicks were vaccinated with Newcastle disease (ND)-infectious bronchitis disease (IBD) vaccines (Caprivac ND-R®, PT. Caprifarmindo Laboratories, Indonesia) through eye drop and ND-avian influenza (AI) vaccines (Caprivac ND-AI K®, PT, Caprifarmindo Laboratories) through subcutaneous injection at a dose of 0.15 mL bird^{-1}. Gumboro vaccine (Cevac Transmune IBD®, Ceva Animal Health, Indonesia) was also given on day 14 through the drinking water. During the rearing period, the chicks were raised in an open-sided broiler house with beds made of rice husks (thickness about 12 cm). A continuous lighting schedule was applied throughout the study period. During the study there was no blower fan provided in the broiler house, so air circulation occurred naturally. No mortality was observed during the *in vivo* experiment.

Table 1. Feed compositions of broilers as starter (days 1–21) and finisher (days 22–35)

Feed ingredients:	Starter (%)	Finisher (%)
Yellow corn	56.10	63.55
Soybean meal	36.64	29.83
Palm oil	2.40	2.40
DL-methionine	0.30	0.30
Bentonite	1.10	0.46
Limestone	1.42	1.42
Monocalcium phosphate	1.45	1.45
Premix[1]	0.20	0.20
Chlorine chlorite	0.08	0.08
NaCl	0.31	0.31
Nutrient contents:		
Metabolizable energy (kcal kg^{-1})[2]	2,924	3,040
Crude protein (%)	21.75	19.25
Crude fiber (%)	3.31	3.21
Crude fat (%)	4.43	4.82

[1]Premix contained (per kg of diet) of vitamin A 7,750 IU, vitamiin D3 1,550 IU, vitamin E 1.88 mg, vitamin B1 1.25 mg, vitamin B2 3.13 mg, vitamiin B6 1.88 mg, vitamiin B12 0.01 mg, vitamin C 25 mg, folic acid 1.50 mg, Ca-d-pantothenate 7.5 mg, niacin 1.88 mg, biotin 0.13 mg, Co 0.20 mg, Cu 4.35 mg, Fe 54 mg, I 0.45 mg, Mn 130 mg, Zn 86.5 mg, Se 0.25 mg, L-lysine 80 mg, Choline chloride 500 mg, DL-methionine 900 mg, CaCO3 641.5 mg, Dicalcium phosphate 1,500 mg.

Chicken body weight, feed consumption, and feed efficiency were measured at days 21 and 35. Daily weight gain, daily feed consumption and feed efficiency was then determined as described by Agusetyaningsih et al. (2022) as follows:

$$\text{Daily weight gain (g/bird/day)} = \frac{\text{Final body weight} - \text{initial body weight}}{\text{Days of rearing}}$$

$$\text{Daily feed consumption (g/bird/day)} = \frac{\text{Total feed consumption during rearing}}{\text{Days of rearing}}$$

$$\text{Feed efficiency (\%)} = \frac{\text{Daily weight gain}}{\text{Daily feed consumption}} \times 100\%.$$

On day 35, one male chick with a body weight close to the average body weight of each pen was taken and blood was taken from the wing veins and then put into a tube with anticoagulant (ethylenediaminetetraacetic acid/EDTA) for complete blood profile assessment. The remaining blood was stored in another tube (without coagulant) to produce blood serum after coagulation at room temperature for about 2 hours. Male chicks were selected for blood sampling to avoid physiological errors due to sex variations. For practical reasons, the chicken from which the blood sample was previously taken was then slaughtered, and the internal organs were removed and weighed (empty condition). To measure the population of intestinal bacteria, digesta was taken from the ileum and cecum and put into a sterile sample container. Digesta was also collected from the duodenum, jejunum, ileum, and cecum for measurement of pH values (using an electronic pH meter, Thermo Fisher Scientific Inc.). For the assessment of the small intestine morphology, the segment of intestine was taken approximately 2 cm from each part of the small intestine and put into a sample tube containing 10% neutral formalin buffer solution. Samples were taken from the mid-point of the duodenum, from the mid-point between the point of entry of the bile duct and Meckel's diverticulum (for jejunum) and from the mid-point of the ileum.

A complete blood profile was determined using the Prima Fully-Auto Hematology Analyzer (PT. Prima Alkesindo Nusantara, Jakarta, Indonesia) following the manufacturer's protocol. To perform histological analysis, duodenal, jejunal, or ileal slices (5 μm each) were stained with hematoxylin and eosin. The villous height and crypt depth were measured using an optical microscope with a camera. Total coliform bacteria were counted on MacConkey agar (Merck KGaA, Darmstadt, Germany) medium and incubated under aerobic conditions for 24 hours at 38 °C. Coliform bacteria that grow on agar media turn red, and bacterial colonies were counted. Total lactic acid bacteria (LAB) were counted on de Man, Rogosa, and Sharpe agar (MRS; Merck KGaA) medium, then incubated anaerobically at 38 °C for 48 hours (Sugiharto et al., 2017b).

The thiobarbituric acid reactive substance (TBARs) method as described by Yuanita et al. (2019) was followed to determine malondialdehyde (MDA) levels in serum. The method was based on the ability to form a pink complex between MDA and thiobarbituric acid (TBA). A mixture of 0.5 mL of serum and 4.5 mL of saline phosphate buffer (PBS) was mixed and centrifuged for 15 minutes before removing 4 mL of the supernatant. The supernatant was combined with 1 mL of 15% trichloroacetic acid (TCA) and 1 mL of TBA and heated in an 80 °C water bath for 15 minutes before being cooled at room temperature for 60 minutes. After centrifugation for 15 minutes, the absorbance was measured with a spectrophotometer at a wavelength of 532 nm. The MDA concentration (nmol mL^{-1}) was calculated using the 1,1,3,3-tetramethoxypropane standard curve.

The measurement of superoxide dismutase (SOD) activity in the sample was carried out according to the method described by Peters (Peters et al., 2014). A total of 0.06 mL of the supernatant was reacted with a mixture of 2.70 mL of 50 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10), 0.06 mL of xanthine 10 mM, 0.03 mL of 0.5% bovine serum albumin (BSA) and 0.03 mL of 2.5 mM NBT. The xanthine oxidase (0.04) unit was then added. After 30 minutes, the absorbance was measured at a wavelength of 560 nm. The PBS containing 11.5 g L^{-1} KCl was used as a control solution. Enzyme activity was measured in units per milliliter of sample (U mL^{-1}).

Statistical analysis

The study was conducted based on a completely randomized design, and the data obtained were treated using analysis of variance according to the statistical model below:

$$Yij = \mu + \tau i + \varepsilon ij \quad (1)$$

where Yij is the observation value, μ is the average value of treatment, τi is the effect of treatment and εij is the treatment error.

RESULTS AND DISCUSSION

Antioxidant and antibacterial activity of nutmeg flesh extract

The chemical and functional compositions (antioxidant activity) as well as antibacterial activity of nutmeg flesh extract are shown in Table 2. The nutmeg flesh extract showed a good potential for antioxidant source as indicated by its high DPPH radical scavenging activity (3.45 µg mL^{-1}, based on IC_{50}) and the content of total phenolics (1.62 g per 100 g) and flavonoids (1.45 g per 100 g). The nutmeg flesh extract also exhibited antibacterial activity against *E. coli* (diameter of inhibition zone 13.7 mm).

Table 2. Chemical and functional compositions and antibacterial activity of nutmeg flesh extract

Items	Values
Moisture (%)	51.66
Ash (%)	0.15
Fat (%)	13.79
Proteins (%)	0.99
Carbohydrates (%)	33.42
Total phenolics (g per 100 g)	1.62
Flavonoids (g per 100 g)	1.45
DPPH radical scavenging activity (IC_{50} [µg mL^{-1}])	3.45
Antibacterial activity against *E. coli* (mm)	13.7

Phytobiotics (herbs and spices) are important sources of phytochemicals with strong antioxidant activity against free radicals, and they are commonly used in poultry and other livestock feeds to obtain various biological activities such as antibacterial, antifungal, antiviral, antiparasitic, and antioxidants (Sugiharto, 2020). The results of this study indicated that the nutmeg flesh extract showed strong antioxidant activity (based on the value of IC_{50} of DPPH radical scavenging activity) and contained the substantial amounts of phenols and flavonoids. These findings were consistent with previous studies showing the antimicrobial and antioxidant activities of the aromatic nutmeg plant (*Myristica fragrans* Houtt) (Gupta et al., 2013; Adu et al., 2020). The antibacterial activity test of nutmeg flesh extract against *E. coli* using the disc diffusion (Kirby-bauer) method were 13.7 mm, indicating that the nutmeg flesh extract had moderate inhibition against *E. coli* growth (Morales et al., 2003). Our present finding was similar to that of reported by Atmaja (2017) showing that nutmeg flesh extract had an inhibitory zone of 11.37 mm against the growth of *E. coli* at the concentration of 10×10^5 ppm.

Productive performance of broiler chickens

The data on performance of broilers are presented in Table 3. It was shown that for the entire study period (days 15–35) daily weight gain, daily feed consumption and feed efficiency were higher ($p < 0.01$) in T0 than that in T1 chicks. Moreover, the T4 group showed higher ($p < 0.01$) daily weigh gain as compared to T0, T1 and T2, but did not differ from that of T3 group. The T4 had the highest ($p < 0.05$) daily feed consumption as compared to other treatment groups. Throughout the study period, feed efficiency was better (p = 0.02) in T0, T3 and T4 as compared to T1 group. In general, the treatment effects on daily weight gain, daily feed intake and feed efficiency were more pronounced during days 22–35 than days 15–21. It was most likely that the chicks weigh more during the finisher period, causing them to experience more stress from the high stocking density than they did during the days 15–21. With regard particularly to the performance of chicks, the results of this study showed that the daily weight gain of broiler chickens age 15–35 days ranged from 56.04–64.07 g/bird/day, which indicates a normal body weight gain range. Indeed, Dei & Bumbie (2011) showed that the daily weight gain of broiler chickens at 35 days ranged from 58.0 to 64.3 g/bird/day.

Table 3. Performance of broilers chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Daily weight gain (g/bird/day)							
15–21 day	23.28	23.78	24.21	25,32	25.77	3.27	0.32
22–35 day	38.71^{b}	32.26^{a}	37.48^{b}	38.31^{b}	38.92^{b}	2.45	< 0.01
15–35 day	61.98^{b}	56.04^{a}	61.70^{b}	63.62^{cb}	64.67^{c}	1.28	< 0.01
Daily feed intake (g/bird/day)							
15–21 day	30.21	30.65	31.11	32.08	32.80	3.65	0.13
22–35 day	69.71^{b}	67.24^{a}	72.08^{c}	72,98^{c}	73,82^{d}	1.89	< 0.01
15–35 day	100.94^{b}	97.07^{a}	103.19^{c}	105.42^{d}	107.60^{e}	1.09	< 0.01
Feed efficiency (%)							
15–21 day	77.06	77.51	77.82	78.93	78.57	5.21	0.62
22–35 day	55.53^{b}	47.98^{a}	52.02^{b}	52.49^{b}	52.72^{b}	2.11	< 0.01
15–35 day	61.41^{b}	57.73^{a}	59.79^{ab}	60.35^{b}	60.10^{b}	1.73	0.02

^{a,b,c,d,e} On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; *SE*: standard error.

The data in this study clearly showed that rearing broilers in high density pens particularly during the finisher period had negative impacts on broiler production performance. Daily body weight gain, daily feed consumption and feed efficiency were lower in the chickens at high density pens, when compared to the chickens at normal density pens. In line with this, several studies have shown that high density condition is associated with impaired chicken growth and low feed efficiency (Kryeziu et al., 2018). The decrease in growth performance of broiler chickens at high density pens is often associated with several factors, including an increase in temperature and a decrease in air circulation in the pens, causing uncomfortable conditions (stress) for chickens, and limited space for movement and access to feed which can cause nutritional deficiencies in chickens (Yin et al., 2017; Ahmed et al., 2018).

The data in this study indicated that the extract of nutmeg flesh can reduce the negative impact of rearing broilers with high density (16 birds m^{-2}) on the growth performance of broilers. There are currently no studies in the literature that explain the effect of nutmeg flesh extract on increasing body weight gain in broiler chickens. The positive effect of nutmeg flesh extract could most likely be attributed to the synergistic action of various phytochemicals present in the nutmeg flesh which in turn improve physiological conditions in chickens. The latter condition has an effect on increasing feed utilization and efficiency in order to improve broiler growth performance. Due to their ability to scavenge/neutralize free radicals and maintain intestinal mucosal integrity, phytochemicals such as flavonoids, phenols, and saponins have been shown to promote higher growth rates and better feed efficiency in broilers (Astaneh et al., 2018). In addition, previous studies have shown that nutmeg flesh has antibacterial, antiparasitic, antifungal, anti-coccidiotic, and hepatoprotective properties (Panggabean et al., 2019) which is able to stimulate the growth of beneficial bacteria, inactivate pathogenic bacteria, as well as facilitate the metabolism of nutrients and absorption in the digestive tract which in turn can improve the growth performance of broiler chickens.

In term of feed consumption, administration of nutmeg flesh extract resulted in higher feed consumption during the rearing period. Study showed that phytochemicals especially essential oil may improve feed taste and delicacy, thus increasing feed consumption and weight gain of animals (Sugiharto, 2016). Indeed, nutmeg flesh contains about 10% essential oil, which primarily consists of terpene hydrocarbons (sabinene and pinene), myrcene, phellandrene, camphene, limonene, terpinene, myrcene, p-cymene, and other terpene derivatives (Nagja et al., 2016). In this study, the increased feed efficiency was seen in broiler fed nutmeg flesh extract, indicating better feed utilization. In accordance with this, other study found that using plants as phytogenic agent in broiler feed can improve intestinal digestibility, which in turn increases broiler chicken growth (Amad et al., 2011). In addition, previous studies have shown that the introduction humic acids of natural origin in drinking water to the diet of broiler chickens has a positive effect on the body's natural resistance and feed digestibility (Korsakov et al., 2019).

Intestinal morphology of broiler chickens

Data on the intestinal morphology of broiler chickens are presented in Table 4. Villi height of duodenum and jejunum was lower ($p < 0.01$) in T1 than that in other treatment groups. Treatment with nutmeg flesh extract increased ($p < 0.01$) the villi height of duodenum, jejunum and ileum of broilers stocked at high density pens. The dietary treatment also increased villi height to crypt depth ratio of jejunum ($p = 0.04$) and ileum ($p = 0.02$) of broilers raised under high density pens. In this study, the treatment had no significant effect on crypt depth in the duodenum ($p = 0.31$), jejunum ($p = 0.99$) and ileum ($p = 0.51$) of broiler chickens. The current findings showed that the height of the villi of the duodenum, jejunum and ileum ranged from 933.55–1,250.03 µm, 984.58–1194.48 µm and 625.02–890.40 µm, where these respective values were within the normal conditions. According to Jazi et al. (2017), normally the villi height in the duodenum ranges from 1,058–1,318 µm, whereas Fard et al. (2014) mentioned that the normal height of the villi in the jejunum and ileum ranges from 1,010–1,239 µm and 508–635 µm.

Broilers raised at normal densities had higher villi in the duodenum, jejunum, and ileum than chickens raised at high density pens. Normal density-chickens also had a higher villi height to crypt depth ratio in the jejunum and ileum than the high density-broilers. These findings were consistent with the study of Tabeekh et al. (2017) who discovered that rearing chickens at normal density (12 birds m^{-2}) increased villi height and crypt depth compared to high density (18 birds m^{-2}). Sugiharto et al. (2017a) reported that stress conditions due to high density have a negative impact on gut morphology.

Table 4. Intestinal morphology of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Duodenum							
Villi height (μm)	998.18^{b}	933.55^{a}	1,097.74^{c}	1,138.66^{c}	1,250.03^{d}	120.57	< 0.01
Crypt depth (μm)	98.93	94.17	94.10	102.92	110.50	13.84	0.31
VH/CD	10.14	9.99	9.52	11.38	11.53	1.55	0.21
Jejunum							
Villi height (μm)	1,145.53^{b}	984.58^{a}	1,155.84^{b}	1,186.02^{b}	1,194.48^{b}	99.22	< 0.01
Crypt depth (μm)	72.46	71.24	71.77	72.47	72.68	4.85	0.99
VH/CD	15.95^{b}	13.88^{a}	16.15^{b}	16.41^{b}	16.44^{b}	1.59	0.04
Ileum							
Villi height (μm)	682.36^{a}	625.02^{a}	749.94^{a}	886.15^{b}	890.40^{b}	138.90	< 0.01
Crypt depth (μm)	67.57	65.72	68.38	70.24	70.47	4.71	0.51
VH/CD	10.19^{a}	9.59^{a}	11.00^{ab}	12.62^{b}	12.64^{b}	1.94	0.02

^{a,b,c,d} On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract, VH/CD: villi height to crypt depth ratio; *SE*: standard error.

Moreover, the corticosterone hormone seemed to be responsible for intestinal mucosa damage because it can delay the proliferation of intestinal epithelial cells, resulting in a decrease in the height of the intestinal villi and the depth of the crypts in high temperature-stressed birds (Sugiharto et al., 2017a). With regard to the broilers raised at high density pens, administration of nutmeg flesh extract was able to increase the hight of the villi in the duodenum, jejunum, and ileum, as well as the villi hight to crypt depth ratio in the jejunum and ileum. The findings of this study were consistent with the findings of Saragih et al. (2019), who discovered that administration of the *Spirogyra jaoensis* plant increased villi hight and crypt depth in the duodenum, jejunum, and ileum. Pathogenic bacteria and stress, according to Sugiharto (2016), have a negative impact on intestinal microflora or intestinal epithelium, resulting in changes in cell permeability as the body's natural resistance, making it easier for harmful components and pathogenic bacteria to penetrate small intestinal cells, interfering with metabolism, digestion, and nutrient absorption. These conditions can cause chronic inflammation of the intestinal mucosa, which ultimately leads to decreased villi height, impaired digestion and absorption in chickens. The positive effect of nutmeg flesh extract on intestinal morphology has been attributed to its bioactive compounds that can stimulate the proliferation and growth of cells in the digestive tract, resulting in higher villi hight to crypt depth ratio. The mechanism by which nutmeg flesh extract provides benefits in improving intestinal morphology could be that the herbal plants can protect intestinal tissue from the microbial attack (Sugiharto, 2016).

pH values and selected bacteria population in intestine of broiler chickens

The pH values and numbers of selected bacteria in the intestine of broilers are presented in Table 5. While had no effect on the pH values of duodenum (p = 0.59), jejunum (p = 0.71) and ileum (p = 0.02), stocking the chicks at high density pens increased the pH values of cecum (p < 0.01). The nutmeg flesh extract considerably decreased pH in the ileum (p = 0.02) and cecum (p < 0.01) of broilers reared at high density, but had no effect on the pH of the duodenum (p = 0.59) and jejunum (p = 0.71). In this study, the pH values of the duodenum, jejunum, ileum and cecum of broiler chickens ranged from 6.23–6.56, 5.50–5.90, 4.73–6.10, and 6.28–7.73, respectively. These values were still within the normal ranges of pH of the digestive tract of broiler chickens. This was in accordance with Mabelebele et al. (2014) reporting that the normal pH values of the digestive tract of broiler chickens was between 6.62 and 6.43.

Table 5. pH and selected bacteria population in the intestine of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
pH							
Duodenum	6.35	6.56	6.42	6.23	6.26	0.34	0.59
Jejunum	5.89	5.90	5.87	5.64	5.50	0.53	0.71
Ileum	6.03^{b}	6.10^{b}	5.52^{ab}	5.24^{ab}	4.73^{a}	0.79	0.02
Cecum	7.10^{b}	7.73^{c}	6.58^{ab}	6.37^{a}	6.28^{a}	0.68	< 0.01
Lactic Acid Bacteria (log cfu g^{-1})							
Ileum	10.30^{b}	9.48^{a}	11.11^{bc}	11.00^{bc}	11.22^{c}	0.87	p < 0.01
Cecum	10.76^{b}	9.74^{a}	11.43^{c}	11.45^{c}	11.68^{c}	0.79	p < 0.01
Coliform (log cfu g^{-1})							
Ileum	6.20^{a}	7.95^{b}	5.85^{a}	5.76^{a}	5.61^{a}	1.37	< 0.01
Cecum	7.97^{b}	8.69^{b}	7.52^{ab}	7.52^{ab}	6.24^{a}	1.33	0.04

^{a,b,c} On the same row, different superscripts indicated a significant variation (p < 0.05); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract, VH/CD: villi height to crypt depth ratio; *SE*: standard error.

Our current study showed that the pH of cecum increased with raising broilers at high density condition. Similarly, Tsiouris et al. (2015) reported an increase in pH of the cecum of broilers reared at high density. The rise in cecum pH was most likely caused by a decrease in litter quality caused by an increase in humidity and temperature in the broiler house, which could affect the microbiota activity of intestinal commensal bacteria. The main factors that determine pH in the intestine are gastrointestinal secretions and volatile fatty acids produced by the gut microbiota. Giving nutmeg flesh extract to high-density broilers could lower pH in the ileum and cecum in the current experiment. This study supports the findings of Sunu et al. (2021) who discovered that supplementing plant extracts as phytobiotics can lower pH in the ileum and cecum. Carbohydrate content, particularly oligosaccharides found in almost all plants, can be a good substrate for fermentation, which promotes the growth of beneficial microbes. Lactic acid bacteria-fermentation produces a high concentration of lactic acid, which affects pH decrease and reduces the growth of harmful bacteria (Nkukwana et al., 2015). The decrease in the intestinal pH caused by the administration of nutmeg flesh extract in this study was similar to the findings of Ferdous et al. (2016), who found that the administration of plant extracts in feed significantly reduced the intestinal pH of broiler chickens. The decrease in pH in the ileum and cecum due to the administration of nutmeg flesh extract was associated with an increase in the population of lactic acid bacteria in the ileum and cecum. Lactic acid is a metabolite of lactic acid bacteria and one of the organic acid components that contribute to pH reduction. The decrease in pH in the ileum and cecum caused by nutmeg flesh extract administration reduced the number of pathogenic bacteria in the intestines while increasing nutrient digestibility. Note that high acidity inhibits the growth of pathogenic bacteria (Song et al., 2014), resulting in healthier intestines.

Rearing broiler at high density pens increased ($p < 0.01$) the number of coliforms, while decreasing ($p < 0.01$) lactic acid bacteria numbers (Table 5). Moreover, the administration of nutmeg flesh extract significantly ($p < 0.01$) increased lactic acid bacteria and decreased ($p < 0.01$) coliform bacteria in the ileum and cecum of broilers raised at high density pens. Khosravifar et al. (2014) stated that the number of coliform bacteria in the intestine broilers of the finisher period was 6.04 log cfu g^{-1}. Previous study by Yeh et al. (2018) reported that the normal range of lactic acid bacteria in the digestive tract of broiler chickens ranged from 8.09 to 8.11 log cfu g^{-1}. It was very likely that differences in environmental conditions, hygiene and nutritional content of feeds led to variations in the population of lactic acid bacteria in the digestive tract between this study and other studies.

The data from this study showed that raising broilers at high density could reduce the population of lactic acid bacteria and increase coliform bacteria in the ileum and cecum of broilers. The effect of raising broilers at high density can cause stress, which is characterized by behavioural, biochemical, and physiological changes subjected to re-establishing homeostasis, which can alter the composition of gut microbes (Sugiharto et al., 2017b). Wang et al. (2021) reported the results of their study showing the abundance of lactic acid bacteria in broilers reared at normal densities compared to high densities. Our further research data confirmed that administration of nutmeg flesh extract increased the population of lactic acid bacteria and decreased coliform bacteria in the ileum and cecum of broiler chickens. These findings are consistent with the findings of Ferdous et al. (2016), who found that supplementing plant extracts as phytobiotics increased the population of lactic acid bacteria while decreasing *E. coli* and total coliform in the intestine. Acidic conditions in the digestive tract, particularly the ileum and cecum, aid in the balance of the digestive tract microflora, increasing the population of lactic acid bacteria while decreasing the population of pathogenic bacteria (Yadav & Jha, 2019). Most pathogenic bacteria grow in pH close to 7 or tend to be neutral. On the other hand, beneficial microorganisms live in acidic pH (5.8–6.2) and compete with pathogenic bacteria, which allows competitive exclusion (Rahmani et al., 2005). Lactic acid bacteria produce lactic acid which is able to maintain the pH of the digestive tract of broiler chickens to be acidic. Furthermore, acidic conditions in the digestive tract can improve the efficiency of the nutrient absorption by the digestive tract, ensuring that nutritional needs are satisfied (Mabelebele et al., 2014; Sugiharto, 2016).

Blood profiles of broiler chickens

The data on blood profile of broilers reared at high density are presented in Table 6. In general, blood profile of broilers was not affected ($p > 0.05$) by the treatments applied during the study. Broilers reared at high densities had blood profiles that are not substantially different from those reared at normal densities. Although the specific reasons for this condition were not known with certainty, broilers reared at high density pens from days 15 to 35 were very likely to be able to adapt to high density conditions so that the chickens can compensate and adjust physiological processes in their bodies to stress. Apart from the treatment effect, the levels of erythrocytes and leukocytes of broilers in this study were within normal haematological range. Scanes & Christensen (2014) reported that five-week-old broilers had erythrocyte values of $2.56–3.2\times10^{12}$ L^{-1}. In line with this, Ning et al. (2014) pointed out that the normal values of broiler red blood cells are around $2.0–3.2\times10^{12}$ L^{-1}. Likewise, the normal leukocyte counts in broiler chickens are in the range of $12–30\times10^{9}$ L^{-1} (Ullah et al., 2018)

Table 6. Blood profile of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
Erythrocytes (10^{12} L^{-1})	2.96	3.38	3.17	2.69	2.92	0.48	0.19
Leukocytes (10^{9} L^{-})	14.38	20.48	18.30	17.58	15.84	4.24	0.19
Haemoglobin (g dL^{-1})	10.30	12.20	10.40	9.70	10.20	1.71	0.18
Haematocrits (%)	36.40	41.20	35.90	32.60	35.20	5.80	0.22
Thrombocytes (10^{9} L^{-1})	13.80	23.20	28.80	16.20	22.80	13.84	0.47
Lymphocytes (10^{9} L^{-1})	137.00	198.10	176.40	169.10	151.50	41.43	0.17
Heterophils (10^{9} L^{-1})	7.40	6.70	13.20	4.99	6.90	7.20	0.46
MCV (10^{-15} L)	123.50	123.40	121.50	120.72	121.30	2.34	0.2
MCH (10^{-12} g)	34.74	36.00	35.06	35.52	34.96	1.44	0.69
MCHC (g L^{-1})	28.26	29.36	29.08	29.60	28.96	1.33	0.62
RDW-SD (10^{-15} L)	45.72	47.92	46.08	46.82	45.34	3.46	0.81
RDW-CV (%)	9.76	9.62	10.02	10.26	9.90	0.90	0.85
MPV (10^{-15} L)	9.20	7.90	7.36	8.72	8.54	1.41	0.74

T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; RDW-SD: red blood cell distribution width-standard deviation; RDW-CV: red blood cell distribution width-coefficient variation; MPV: mean platelet volume, *SE*: standard error.

Antioxidant activity of broiler chickens

Table 7 shows the MDA and SOD levels in the serum of broiler. MDA levels were lower ($p < 0.05$) in the T4 group compared to the T0, T1, T2, and T3 groups. There was no significant difference ($p > 0.05$) in MDA levels between the T0 and T1 groups. The SOD levels were lower ($p < 0.01$) in the T1 group than in the T0, T2, T3, and T4 treatments. Broiler chickens in the T4 treatment had the highest ($p < 0.01$) serum SOD level when compared to other treatments. The results of this study showed that the values of MDA and SOD ranged from 0.85 to 1.16 nanomol mL^{-1} and 37.58 to 51.84 U mL^{-1}, respectively. According to Sunu et al. (2019), SOD levels of chicken ranged from 44.79 to 66.49 U mL^{-1}, whereas according to Fathi et al. (2016) MDA levels of chicken range from 2.30 to 3.17 nanomol mL^{-1}. The inconsistent levels of SOD and MDA in broilers across studies appeared to be associated with the various environmental factors and stress levels in chickens.

Table 7. Serum levels of superoxide dismutase and malondialdehyde of broiler chickens

Items	Treatment groups					*SE*	*p* value
	T0	T1	T2	T3	T4		
MDA (nanomol mL^{-1})	1.09^{bc}	1.16^{c}	1.04^{bc}	0.99^{b}	0.85^{a}	0.14	< 0.01
SOD (U mL^{-1})	45.69^{b}	37.58^{a}	50.80^{bc}	49.44^{bc}	51.84^{c}	6.34	< 0.01

^{a,b,c} On the same row, different superscripts indicated a significant variation ($p < 0.05$); T0: chicks raised at normal density of 10 chicks m^{-2}; T1: chicks raised at high density of 16 chicks m^{-2}; T2: chicks raised at high density and fed with 0.5 mL kg^{-1} nutmeg flesh extract; T3: chicks raised at high density and fed with 1.0 mL kg^{-1} nutmeg flesh extract; T4: chicks raised at high density and fed with 1.5 mL kg^{-1} nutmeg flesh extract; *SE*: standard error.

Under most conditions, stress is associated with increased MDA and decreased SOD levels, which is a protective response of broilers against excessive free radical production (Akbarian et al., 2016). A recent study found that raising broilers at high densities increased MDA levels while decreasing SOD levels in serum (Nasr et al., 2021). A high MDA concentration indicates an oxidation process in the cell membrane. Aside from the effect of pen density, the use of nutmeg flesh extract in this study may have reduced MDA due to the antioxidant role of nutmeg flesh, which can inhibit lipid peroxidation. According to Sugiharto & Turrini (2022), MDA in the body is formed as a result of oxidative stress conditions, specifically an imbalance between the formation of reactive oxygen species (ROS) and the presence of antioxidants, where free radicals outnumber antioxidants. According to Zhao et al. (2014), a decrease in blood MDA levels indicates free radical inhibition by antioxidants, and thus a high antioxidant status is usually accompanied by a decrease in plasma MDA levels. In this study, provision of nutmeg flesh extract through the ration increased the concentration of SOD and decreased MDA in the blood serum of broiler chickens. It is well known that the flesh of nutmeg contains bioactive substances such as phenolics and flavonoids, both of which are powerful antioxidants. These findings were consistent with previous study reporting that plant extracts such as *Echinacea purpurea* containing flavonoids and other bioactive substances, can increase SOD levels in broiler blood (Lee et al., 2012). The increase in SOD activity caused by nutmeg flesh extract administration indicates that broiler chickens have a strong free radical scavenging capacity, as evidenced by lower MDA concentrations. In this regard, Lee et al. (2012) and Sugiharto & Turrini (2022) confirmed that SOD can prevent the formation of free radicals that can cause cell damage.

CONCLUSIONS

Treatment with nutmeg flesh extract up to 1.5 mL kg^{-1} of feed to high density reared broiler chickens increased the hight of intestinal villi, total lactic acid bacteria and decreased total coliforms, while having no effects on the blood profile. Aside from the effect of high stocking density, administration of nutmeg flesh extract decreased MDA levels and increase daily weight gain of broilers.

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Selection value of initial material according to the main biochemical parameters of grain in new maize hybrids creation

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Abstract. Increasing demand for corn grain with improved quality indicators provides grounds for creating new breeding samples that can meet the requirements of society. To achieve this goal, the breeding program included inbred lines VK13, VK69, AE801, and AE392 - sources of mutations in the gene for endosperm structure *waxy* and *ae*, respectively. The article presents experimental data on the study of corn hybrids created by crossing with these sources of gene mutations and is characterized by a high content of protein, starch, and oil in the grain, as well as yield. According to the results of laboratory and field research, hybrid combinations with high protein content in grain were identified - 13.07%; starch - 72.6%; oils - 5.83%. In field conditions, the highest yield was 9.37 t ha^{-1}. Further research will determine the nature of the inheritance of these traits, and suggest ways of breeding work to improve grain quality.

Key words: breeding, hybrid, corn, protein, starch.

INTRODUCTION

In maize hybrid development, it is essential to know the parent's performance per se in different environments as well as the genetic base in populations to facilitate the selection of superior lines (Gayosso-Barragán et al., 2020).

The quality of maize grain is determined by biochemical parameters, such as the content of proteins, starch, and oil. The most effective and efficient method of improvement is breeding (Kuzmishina et al., 2014). Mutations of endosperm structure genes that significantly change the biochemical composition of grain are successfully used to create maize hybrids for specific purposes. A lot of research confirmed the effectiveness of using mutations of endosperm structure genes and their combinations to optimize the biochemical composition of grain (Boyer & Hannah, 2001).

The main component of maize grain is starch, which makes up about 70% of the total weight of the grain. It is represented by amylose and amylopectin. The high protein content of maize grain is usually positively correlated with the oil content. Maize grain, in comparison with other cereals, excluding oats, is characterized by the highest oil content (3.5–7%). The fats in the grain are unevenly distributed. The largest number (up to 60%) is concentrated in the embryo and only 0.61–0.73% is in the endosperm (Shorohov, 2007).

The creation of maize hybrids with improved grain biochemical composition depends on the availability of a reliable source material for breeding (Zemoida et al., 2019; Vasylenko et al., 2021). It must have a high genetically determined level of grain quality traits, stably reproduce this level in different climatic conditions of cultivation and be combined with productivity and other valuable economic traits (Silenko, 2011).

It is known that maize grain endosperm contains a complex mixture of starch granules and protein bodies. The physical structure of endosperm depends on the type of interaction between these compounds, and reserve proteins play an important role in the physical structure of mature grain (Pereira et al., 2008; Shcherbakov et al., 2017; Mazurenko et al., 2020). About 7.0–11.2% of protein accumulates in the endosperm, which is 81% of the grain mass, and 14.0–26.0% of the protein in the embryo, which is 11.7% of the grain mass. The concentration of protein in the aleurone layer is the highest, in its cells found up to 36% of protein to dry weight of endosperm (Wolf et al., 1972; Watson, 2003).

In maize grain with a floury texture, starch granules and protein bodies are located in the endosperm disorganized. In vitreous grains, the location of starch granules is more organized, and the intergranular spaces are perfectly filled. Reserve proteins are usually responsible for the relationship between starch grains and endosperm matrix proteins, thus affecting grain hardness (Gibbon & Larkins, 2005). Waxy maize, also known as sticky maize, has high economic, nutritional, and processing value. In recent decades, many adapted maize inbred lines have been developed for hybrid seed production by different selection methods. Because of the popularity of glutinous maize, waxy maize lines are frequently selected by maize breeders. An abundance of waxy maize germplasm has been obtained through decades of waxy maize breeding (Luo et al., 2020).

Maize oil is characterized by high energy content. The energy value of 100 g of maize oil is about 884 kcal. Good quality cooking oil is usually associated with an increased proportion of unsaturated and saturated fatty acids. Maize oil is low in saturated fatty acids and contains an average of 11% palmitic acid and 2% stearic acid, compared to relatively high levels of polyunsaturated fatty acids such as linoleic acid. Maize oil is relatively stable because it contains only a small amount of linolenic acid (0.7%) and has a high level of natural antioxidants (Val, 2009). The caloric content of the oil is 2.25 times higher than that of starch, and research on livestock feeding has shown a higher rate of weight gain per unit of feed for high-oil than for ordinary Maize (Lambert et al., 2004).

The current rapid expansion of the human population on earth, particularly in the less developed countries, raises the possibility of widespread, serious malnutrition and starvation for many unless agricultural technology can intervene with appropriate answers to these problems. Plant breeders have been charged with developing varieties with high yields and improved quality of grain (Prasanna et al., 2001). Therefore, substituting the normal maize for high-quality maize would substantially reduce the malnutrition problems of resource-poor people depending on maize as a staple food (Gemechu et al., 2016).

With a hybrid modeling, it is difficult to predict how one or another valuable economic trait will manifest itself in different combinations and growing conditions. As a rule, most morphological and adaptive traits have a complex nature of inheritance, which is polygenic. Therefore, the strategy of modern selection is an in-depth study of these traits, as a result of which it will be possible to control the processes of their production (Zhemoyda et al., 2019).

The research aimed to determine the suitability of inbred lines - sources of mutations in the gene of endosperm structure waxy and ae for the creation of new breeding samples of maize with high content of protein, starch, oil, and high yields.

MATERIALS AND METHODS

The purpose and objectives of the experiment are selection of source material (inbred lines) of maize for inclusion in crosses with sources of high protein, high starch, and oil content, followed by obtaining high-heterosis hybrids with high yields.

Field research was conducted during 2020–2021 in the research fields of the laboratory of the Department of Genetics, Breeding and Seed Production. prof. M. O. Zelenskyi NULES of Ukraine of a separate subdivision of NULES of Ukraine 'Agronomic Research Station', located in Bila Tserkva district of Kyiv region. The soil of the experimental site is typical, low-humus chernozem. Total atmospheric precipitation during the growing season between 29/04 and 10/10 was 301 mm in the 2020, and only 173 mm between 12/04 and 8/10 in 2021 growing season. There was much less atmospheric precipitation, but it fell on the main critical phases of the maize vegetation, which contributed to the normal maize cultivation (Fig. 1). Groundwater is at a depth of 1.5–2 m, this is the reason for additional irrigation was not carried out. Fertilizers and herbicides were not applied at the experimental sites. The predecessor of both years was winter wheat.

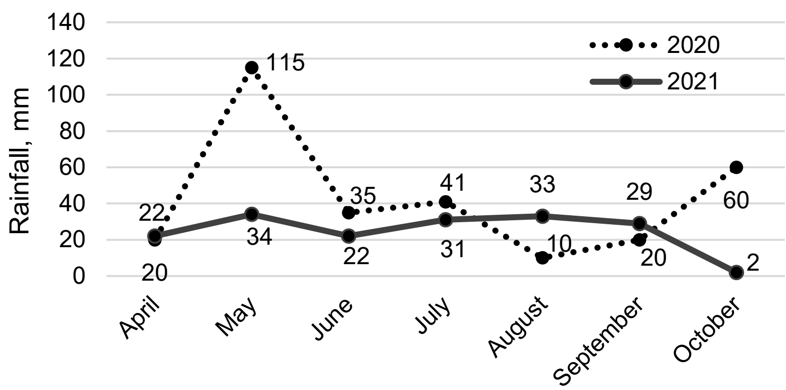

Figure 1. Average amount of atmospheric precipitation per month, mm (2020–2021).

The accounting area for each analyzing sample was a double row of a 7 m long plot, spacing between from row to row was 70 cm and from plant to plant was 17 cm. (Dospehov, 2012). The research plots were located according to the RCBD design in triple duplication.

Research material: 65 experimental maize hybrids created as a result of test crosses; testers VK13, VK69 (sources of mutation of the endosperm structure gene - *waxy*), AE801, AE392 (sources of mutation of the endosperm structure gene - *ae*); inbred lines - source material of researching hybrids.

Evaluation of experimental maize hybrids in terms of protein, starch, oil, and yield was performed according to the method of the qualifying examination of plant varieties on suitability for distribution in Ukraine (Tkachyk, 2017). FOSS 'Infratec 1241 Grain Analyzer' was used to determine the main biochemical indicators of maize grain quality. The principle of device operation is based on infrared spectrophotometry. Uncrushed and unprocessed grain was used for analysis (Tkachyk, 2017). The content of protein, starch, and oil is shown as a percentage of grain dry matter.

To determine the biochemical parameters, five sources were randomly selected from each plot. After drying in the laboratory, the cobs were threshed by hand. The obtained grain was cleaned of impurities on sieves, after which the analysis was performed.

The division of all hybrids into groups according to the content of protein, starch, and oil in the grain was performed according to the description reference book for the *Zea mays* L species. (Kyrychenko et al., 2009).

To carry out mathematical and statistical data processing, the studied hybrids were compared with each other in four groups formed according to their maternal form - the tester. The group with the VK13 tester included 24 hybrids; with tester VK69 - 19 hybrids; with tester AE801 - 14; with AE392 tester - 8 hybrids. The conditional standard was the average value of the indicator (protein, starch, oil, and yield) within the group.

Heterosis was estimated based on mid-parent heterosis (MPH) using the following formulae:

$$MPH (\%) = 100 \times (F1 - MP)/MP$$

To determine the significance of the difference between the means, the criterion of least significant difference (LSD) was used, which shows the marginal deviation for the difference of the sample means. If the actual difference is greater than the *LSD* it is significant.

Statistical data processing was performed using Microsoft Excel 2016 in combination with XLSTAT. The error of the results of statistical analysis is $P < 0.05$.

RESULTS AND DISCUSSION

The maternal components for experimental maize hybrids creation were four inbred lines: VK13 and VK69 - sources of mutations in the gene structure of the *waxy* endosperm structure; lines AE392 and AE801 are sources of mutations in the gene of the endosperm structure *ae* (Shiyanova et al., 2015). These inbred lines were characterized by high protein, starch, and oil content (Zhemoida et al., 2020) (Table 1).

According to the results of laboratory analyses of the main biochemical components conteined in the grain, all the studied experimental hybrids were grouped by the percentage of protein, starch, and oil.

Table 1. Content of the main components of quality in a grain of maize inbred lines - source material of analyzing hybrids (2020–2021)

Inbred line name	Nutrient content, %					
	Protein		Starch		Oil	
	2020	2021	2020	2021	2020	2021
	Maternal form					
AE801	13.8 ± 0.5	13.7 ± 0.3	66.8 ± 0.7	66.2 ± 1.1	5.9 ± 0.2	5.9 ± 0.1
AE392	12.0 ± 0.3	12.5 ± 0.2	70.3 ± 0.8	71.9 ± 0.6	4.4 ± 0.9	4.6 ± 0.1
VK69	10.8 ± 0.2	11.5 ± 0.3	70.7 ± 0.7	72.1 ± 0.9	5.3 ± 0.1	5.5 ± 0.1
VK13	12.2 ± 0.2	12.2 ± 0.5	70.9 ± 0.6	71.9 ± 0.8	4.2 ± 0.1	4.1 ± 0.1
	Paternal form					
CO255	12.9 ± 0.4	12.6 ± 0.3	67.9 ± 0.7	67.4 ± 0.7	4.0 ± 0.0	3.9 ± 0.1
FV243	11.8 ± 0.4	11.3 ± 0.2	69.8 ± 0.7	71.8 ± 0.9	4.2 ± 0.1	4.2 ± 0.1
G255	13.2 ± 0.8	12.8 ± 0.5	68.2 ± 0.7	70.3 ± 0.6	4.2 ± 0.1	4.1 ± 0.1
Q170	12.0 ± 0.2	11.5 ± 0.2	71.4 ± 0.8	71.7 ± 1.1	3.9 ± 0.1	4.1 ± 0.1
HLG1203	11.4 ± 0.2	11.5 ± 0.3	70.4 ± 0.6	70.5 ± 0.9	3.9 ± 0.1	3.9 ± 0.1
UHK678	10.5 ± 0.2	10.5 ± 0.3	68.0 ± 0.5	68.8 ± 0.8	4.2 ± 0.1	4.1 ± 0.1
UHK686	12.2 ± 0.5	11.8 ± 0.4	71.7 ± 0.6	70.2 ± 0.7	3.7 ± 0.1	3.5 ± 0.1
AK157	11.1 ± 0.3	10.6 ± 0.1	70.8 ± 1.1	69.9 ± 0.6	4.3 ± 0.1	4.3 ± 0.1
AK159	10.0 ± 0.3	9.8 ± 0.3	70.0 ± 1.0	69.6 ± 0.6	4.4 ± 0.1	4.2 ± 0.2
VK19	12.0 ± 0.2	11.5 ± 0.2	64.8 ± 0.5	69.8 ± 0.7	5.3 ± 0.8	4.9 ± 0.1
VK32	12.3 ± 0.4	12.0 ± 0.3	69.5 ± 0.8	72.3 ± 0.8	3.7 ± 0.1	3.6 ± 0.1
VK37	11.5 ± 0.3	11.7 ± 0.3	70.8 ± 0.9	69.7 ± 0.9	3.9 ± 0.1	3.8 ± 0.1
AE746	13.6 ± 0.3	12.9 ± 0.3	68.7 ± 0.9	65.5 ± 0.4	5.6 ± 0.2	5.4 ± 0.2
AE800	10.4 ± 0.4	10.8 ± 0.3	72.1 ± 0.6	70.1 ± 0.8	4.9 ± 0.2	4.9 ± 0.1
Conditional standard	11.9	11.7	69.6	69.6	4.5	4.4
$LSD_{0.05}$	0.94	0.73	1.61	1.30	0.22	0.24

There were four groups in terms of protein content: 18 samples were included in the group with less than 9.0% protein content; the group with a protein content of 9.1–10.0% - 24 samples; the group with a protein content of 10.1–12.0% - 21 samples and the group with a high protein content of 12.1–15.0% - 2 samples (Fig. 2).

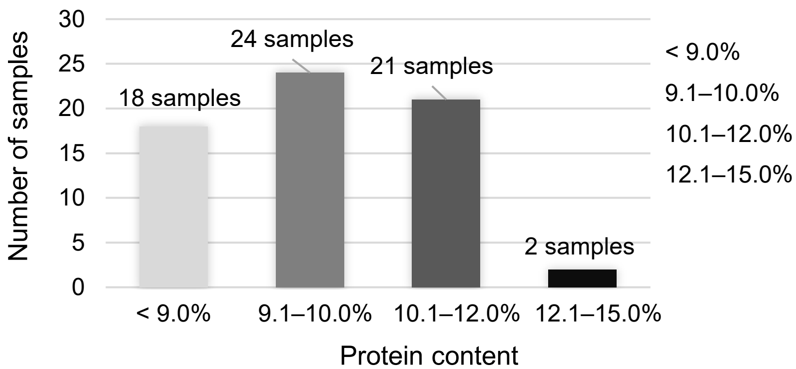

Figure 2. Distribution of hybrids by protein percentage.

According to the starch content, 65 experimental hybrids were divided into III groups: Group I with a starch content of 61–65%, which included 3 samples; Group II with a starch content of 66–70% - 45 samples and Group III with a starch content > 70% - 17 samples (Fig. 3).

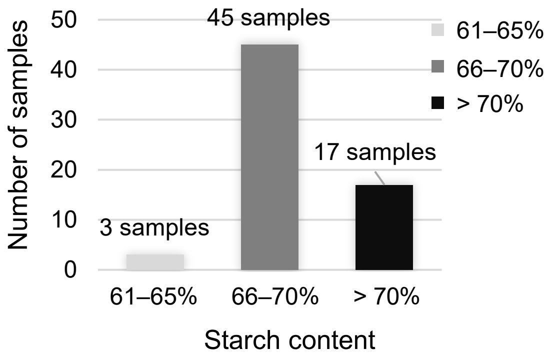

Figure 3. Distribution of hybrids by starch percentage.

According to the oil content, III groups were also formed: with a content of 2.6–3.8% - 7 samples; with a content of 3.9–5.0% - 52 samples, and a group with an oil content of 5.1–7.0% - 6 samples (Fig. 4).

According to analyzing results of the group of experimental hybrids using the maternal form VK13, it was found that the average protein content for 2 years varied between 7.97–13.07%, with standard deviation (*s*) 0.547, starch - 65.9–72.6% ($s = 0.8519$), oil - 3.9–5.27% ($s = 0.2393$).

Significantly average high protein content was found in hybrids VK13xCO255 (13.07%) and VK13xVK37 (12.60%). A very high content of starch in grain was observed in hybrids: VK13xUHK678 (72.60%) and VK13xUHK686 (72.57%). High oil content was formed by hybrids VK13xVK37 (5.27%) and VK13xUHK686 (5.07%).

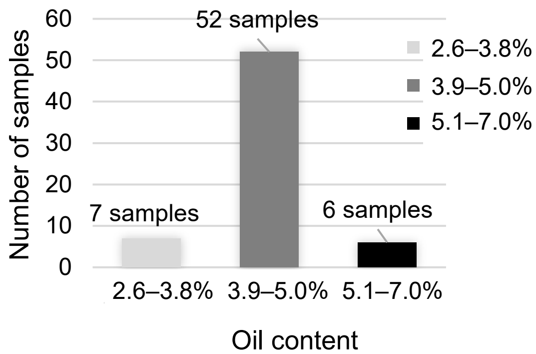

Figure 4. Distribution of hybrids by oil percentage.

In 2020 hybrid VK13xVK37 were characterized by a significant positive degree of MPH in terms of protein and oil content; VK13xUHK686 - by oil content; VK13xUHK678 - starch content. In 2021, a positive manifestation of MPH was found in hybrids VK13xVK37 and VK13xUHK678 in oil content (Table 2).

Average protein content in the grain over the years of research of the analyzed hybrids group, the maternal form of which was the inbred line VK69, varied between 8.03–9.83% ($s = 0.5026$), starch - 67.50–72.80% ($s = 0.9322$), oil - 3.67–5.27% ($s = 0.3293$).

According to biochemical indicators, high, significantly higher than the conditional standard, the oil content in the grain formed by a hybrid VK69xVK13 (5.27%), and very high starch content was characterized by a hybrid VK69xFV243 (72.80%).

Table 2. Description of hybrids with maternal form VK13 (2020–2021)

Hybrid formula	Content in the grain, % / MPH, %			Yield, t ha^{-1}
	Protein	Starch	Oil	
	2020			
VK13xCO255	13.1 ± 0.2 / 7.57	68.2 ± 0.4 / –0.39	4.5 ± 0.1 / 9.40	6.85
VK13xVK37	12.5 ± 0.2 / 10.29	67.3 ± 0.8 / –6.42	5.3 ± 0.1 / 30.44	5.79
VK13xUHK678	9.2 ± 0.2 / –18.56	73.1 ± 0.8 / 5.17	4.7 ± 0.1 / 10.38	4.64
VK13xUHK686	8.3 ± 0.2 / –32.18	73.1 ± 0.8 / 2.43	5.0 ± 0.1 / 25.87	8.74
VK13xAK159	10.4 ± 0.2 / –6.76	68.5 ± 0.8 / –1.35	4.2 ± 0.1 / –3.00	5.41
VK13xFV243	9.6 ± 0.2 / –20.40	67.4 ± 0.8 / –4.23	4.7 ± 0.1 / 11.82	6.48
VK13xAE746	10.8 ± 0.3 / –16.68	64.9 ± 0.6 / –7.09	4.7 ± 0.2 / –5.47	4.39
VK13xVK19	11.2 ± 0.2 / –7.94	66.9 ± 0.6 / –4.43	4.9 ± 0.2 / 3.35	4.01
Conditional standard	10.4	69.1	4.5	5.77
$LSD_{0.05}$	0.68	4.62	0.34	0.42
	2021			
VK13xCO255	13.0 ± 0.4 / 1.25	66.9 ± 0.6 / –5.43	4.4 ± 0.1 / 10.00	6.45
VK13xVK37	12.7 ± 0.3 / 0.77	69.5 ± 0.8 / –0.39	5.2 ± 0.2 / 33.22	4.47
VK13xUHK678	10.2 ± 0.3 / –10.76	72.1 ± 0.7 / 2.52	4.5 ± 0.2 / 11.06	5.70
VK13xUHK686	7.6 ± 0.2 / –36.39	72.1 ± 0.9 / 1.46	5.1 ± 0.1 / 35.36	6.52
VK13xAK159	9.7 ± 0.3 / –11.91	67.2 ± 0.5 / –6.38	4.3 ± 0.1 / 4.84	6.79
VK13xFV243	9.5 ± 0.3 / –18.92	70.3 ± 0.4 / –2.08	4.6 ± 0.2 / 11.90	8.52
VK13xAE746	10.6 ± 0.2 / –15.65	66.9 ± 0.4 / –2.56	4.6 ± 0.1 / –2.55	4.97
VK13xVK19	11.1 ± 0.3 / –6.10	68.3 ± 0.7 / 3.68	5.0 ± 0.2 / 10.84	4.87
Conditional standard	10.3	69.4	4.5	6.03
$LSD_{0.05}$	0.70	4.37	0.29	0.40

In 2020, in terms of starch content in grain, the highest manifestation of MPH was found in hybrid VK69xAK159, in terms of oil content - hybrid VK69xVK13. In 2021, a positive manifestation of MPH in terms of starch content in grain was found in hybrid VK69xVK19, and oil content - in hybrid VK69xVK13 (Table 3).

According to the 2 years average results, analyzed grain quality of hybrids, the maternal form of which was the inbred line AE801, it was found that variation in the percentage of protein was 8.7–11.27% (s = 0.5592), starch - 65.0–70.83% (s = 0.9711), oil - 3.67–5.13% (s = 0.2786).

Table 3. Description of hybrids with maternal form VK69 (2020–2021)

Hybrid formula	Content in the grain, % / MPH, %			Yield, t ha^{-1}
	Protein	Starch	Oil	
	2020			
VK69xVK13	9.4 ± 0.2 / –20.90	69.4 ± 0.3 / –2.00	5.3 ± 0.2 / 10.19	6.06
VK69xCO255	9.4 ± 0.2 / –20.75	70.4 ± 0.5 / 1.54	4.3 ± 0.1 / –8.16	6.24
VK69xG255	9.7 ± 0.3 / –19.76	70.0 ± 0.4 / 1.49	4.3 ± 0.1 / –9.73	10.57
VK69xUHK686	7.8 ± 0.3 / –32.57	70.9 ± 0.5 / –0.34	3.6 ± 0.1 / –21.06	8.20
VK69xVK19	9.8 ± 0.3 / –14.23	69.9 ± 0.6 / 0.31	4.3 ± 0.1 / –18.09	5.56
VK69xAE746	9.1 ± 0.4 / –25.42	66.3 ± 0.9 / –4.96	4.6 ± 0.1 / –16.15	6.19
VK69xFV243	8.4 ± 0.2 / –26.19	72.4 ± 0.6 / 2.17	4.6 ± 0.1 / –3.81	4.39
VK69xAK159	8.6 ± 0.2 / –17.92	72.3 ± 0.3 / 2.70	3.6 ± 0.2 / –25.78	4.97
Conditional standard	8.8	69.7	4.3	6.13
$LSD_{0.05}$	0.75	5.41	0.30	0.51

	2021			
VK69xVK13	9.9 ± 0.2 / –15.28	70.6 ± 0.4 / –1.85	5.3 ± 0.1 / 10.40	4.91
VK69xCO255	8.9 ± 0.5 / –25.88	71.4 ± 0.8 / 2.33	4.3 ± 0.1 / –8.86	5.97
VK69xG255	10.0 ± 0.2 / –17.73	68.5 ± 0.4 / –4.49	4.1 ± 0.1 / –15.25	8.18
VK69xUHK686	8.3 ± 0.1 / –29.06	68.5 ± 0.4 / –3.63	3.8 ± 0.1 / –16.34	6.08
VK69xVK19	9.3 ± 0.5 / –19.39	69.4 ± 0.9 / 5.04	4.5 ± 0.3 / –14.91	5.82
VK69xAE746	9.4 ± 0.4 / –28.19	68.7 ± 0.4 / –0.06	4.6 ± 0.2 / –23.62	5.41
VK69xFV243	8.3 ± 0.2 / –26.95	73.1 ± 0.2 / 2.61	4.5 ± 0.1 / –7.00	5.77
VK69xAK159	7.8 ± 0.2 / –27.12	70.4 ± 0.8 / 0.52	3.4 ± 0.1 / –30.19	5.47
Conditional standard	8.9	69.4	4.3	6.01
$LSD_{0.05}$	0.68	4.85	0.34	0.41

Within this group of hybrids, there are three combinations: AE801xFV243 (11.18%), - with high protein content; very high content of starch grain was observed in hybrid AE801xVK32 (70.83%), hybrid AE801xAE746 was characterized by significantly higher than the standard protein and oil content (11.27% and 5.13%, respectively).

In 2020, MPH was detected in the hybrid AE801xVK32 by starch content. In 2021, positive MPH was detected in hybrids AE801xVK19, AE801xVK32, and AE801xCO255 (Table 4).

Table 4. Description of hybrids with maternal form AE801 (2020–2021)

Hybrid formula	Content in the grain, % / MPH, %			Yield, t ha^{-1}
	Protein	Starch	Oil	
	2020			
AE801xAE746	11.0 ± 0.4 / –19.79	66.7 ± 0.4 / –1.60	5.1 ± 0.1 / –11.03	2.82
AE801xVK32	8.8 ± 0.2 / –32.75	70.6 ± 0.9 / 2.19	3.8 ± 0.2 / –20.69	5.14
AE801xCO255	10.1 ± 0.3 / –24.84	68.5 ± 0.2 / –1.35	4.5 ± 0.2 / –9.82	3.82
AE801xAK159	9.9 ± 0.3 / –14.52	68.2 ± 0.3 / 1.12	4.2 ± 0.1 / –19.64	5.53
AE801xVK19	9.0 ± 0.3 / –30.55	65.4 ± 0.3 / –0.56	3.8 ± 0.1 / –31.54	5.02
AE801xFV243	11.5 ± 0.3 / –9.55	67.1 ± 0.4 / –1.73	4.7 ± 0.1 / –8.02	6.27
AE801xVK13	9.6 ± 0.2 / –26.14	68.2 ± 0.5 / –1.01	4.2 ± 0.1 / –16.84	5.14
AE801xAE800	9.4 ± 0.2 / –22.64	65.1 ± 0.5 / –3.33	4.8 ± 0.1 / –12.47	3.27
Conditional standard	9.8	67.5	4.2	5.45
$LSD_{0.05}$	0.53	5.27	0.24	0.48
	2021			
AE801xAE746	11.5 ± 0.4 / –13.83	64.2 ± 0.4 / –2.41	5.1 ± 0.1 / –8.83	3.18
AE801xVK32	9.3 ± 0.4 / –27.74	71.0 ± 0.6 / 4.03	3.8 ± 0.1 / –19.58	4.42
AE801xCO255	10.2 ± 0.4 / –22.76	68.3 ± 0.5 / 5.25	4.2 ± 0.1 / –15.14	4.65
AE801xAK159	9.6 ± 0.3 / –21.79	67.8 ± 0.5 / –1.56	3.9 ± 0.1 / –21.22	5.75
AE801xVK19	8.7 ± 0.2 / –30.91	69.8 ± 0.5 / 7.44	3.9 ± 0.1 / –27.08	4.20
AE801xFV243	10.9 ± 0.2 / –14.42	69.6 ± 0.4 / 0.90	4.2 ± 0.2 / –15.84	6.09
AE801xVK13	10.4 ± 0.3 / –19.66	66.7 ± 0.5 / –3.40	4.6 ± 0.2 / –6.58	8.72
AE801xAE800	8.5 ± 0.2 / –30.72	65.3 ± 0.3 / –7.09	4.4 ± 0.2 / –19.65	4.18
Conditional standard	9.6	67.6	4.2	6.02
$LSD_{0.05}$	0.69	4.71	0.33	0.46

The inbred maize line AE392, which has a high oil content (Zhemoida et al., 2020) acted as the maternal form of a group of experimental hybrids, including the hybrid AE392xHLG1203 with a significantly higher oil content compared to competitors in its group - 5.83%). According to the obtained data from laboratory analyses, it should be noted that the protein content in this group of hybrids was 9.9–10.8% (s = 0.5757), starch 67.10–68.27% (s = 0.575), oil 4.37–5.83% (s = 0.2204) - average values for years of research.

In this group of hybrids, MPH was manifested only in terms of oil content in grain: 2020 - in hybrids AE392xCO255, AE392xHLG1203, AE392xAK157; in 2021 - hybrids AE392xVK19, AE392xCO255, and AE392xHLG1203 (Table 5).

Table 5. Description of hybrids with maternal form AE392 (2020–2021)

Hybrid formula	Content in the grain, % / MPH, %			Yield, t ha^{-1}
	Protein	Starch	Oil	
	2020			
AE392xHLG1203	10.4 ± 0.2 / –11.08	67.4 ± 0.5 / –0.36	5.6 ± 0.2 / 35.00	3.45
AE392xCO255	10.4 ± 0.3 / –16.67	68.3 ± 0.5 / –1.24	4.8 ± 0.2 / 14.08	4.65
AE392xAK157	9.7 ± 0.4 / –16.23	67.7 ± 0.5 / –4.13	5.1 ± 0.1 / 17.48	6.55
AE392xAE800	10.4 ± 0.4 / –7.33	68.8 ± 0.3 / –1.97	4.3 ± 0.2 / –7.83	3.75
AE392xQ170	10.5 ± 0.3 / –13.11	68.4 ± 0.3 / –0.69	4.6 ± 0.1 / 9.36	2.75
AE392xVK19	10.5 ± 0.3 / –12.81	66.1 ± 0.7 / –3.67	5.1 ± 0.2 / 5.80	3.31
AE392xAE746	10.8 ± 0.4 / –15.74	67.2 ± 0.2 / –4.09	4.0 ± 0.1 / –19.90	2.96
AE392xUHK686	7.9 ± 0.4 / –34.07	66.0 ± 0.7 / –7.03	4.2 ± 0.1 / 2.72	3.91
Conditional standard	10.1	68.0	4.7	3.92
$LSD_{0.05}$	0.69	6.53	0.50	0.31
	2021			
AE392xHLG1203	10.7 ± 0.3 / –10.96	66.6 ± 0.5 / –9.95	6.1 ± 0.2 / 42.71	2.73
AE392xCO255	11.2 ± 0.3 / –11.10	67.7 ± 0.8 / –2.86	5.1 ± 0.1 / 18.60	3.70
AE392xAK157	10.1 ± 0.2 / –12.93	68.8 ± 0.5 / –2.96	4.9 ± 0.1 / 11.91	5.90
AE392xAE800	10.7 ± 0.4 / –8.48	66.3 ± 0.9 / –8.06	4.4 ± 0.1 / –7.54	3.72
AE392xQ170	9.7 ± 0.3 / –19.74	68.0 ± 0.6 / –8.13	4.2 ± 0.1 / –2.75	3.55
AE392xVK19	10.5 ± 0.4 / –12.21	68.1 ± 0.3 / 1.81	6.4 ± 0.3 / 34.24	2.99
AE392xAE746	9.9 ± 0.2 / –21.83	68.3 ± 0.3 / 0.05	4.4 ± 0.1 / –12.56	3.88
AE392xUHK686	8.1 ± 0.2 / –33.91	68.8 ± 0.4 / –3.28	4.2 ± 0.1 / 4.68	3.17
Conditional standard	10.1	67.3	5.0	3.71
$LSD_{0.05}$	0.75	6.17	0.43	0.33

Endosperm mutants favorably change the consistency of maize grain, but they also cause certain undesirable consequences, as is expected of most mutants. Since the kernel weight is reduced due to less density per unit volume as starch is loosely packed with a lot of air spaces, there is a corresponding decline in the yield (Toro et al., 2003; Singh & Venkatesh, 2006).

Simultaneously with the study of the main biochemical parameters, the yield of experimental hybrids was also determined. A comparison of hybrids by yield level in 2020–2021 was conducted separately for each group formed by maternal forms.

The average yield of the group of hybrids with the maternal form VK13 was 5.99 t h^{-1}. The highest yield in this group was formed in the hybrid VK13xUHK686 - 7.63 t h^{-1}, in general, the yield varied between 2.93–7.63 t h^{-1}.

The highest average yield of 6.46 t h^{-1} was shown by the group whose maternal form of hybrids was the inbred line VK69. Within this group, the yield of hybrids varied between 4.20 and 9.37 t h^{-1}. The highest yield was recorded in the hybrid VK69xG255 - 9.37 t h^{-1}.

In the group of hybrids with the parent component AE801, the average yield was 5.74 t h^{-1} and was in the range of 3.0–8.63 t h^{-1}. The best yield in this group was the hybrid AE801xVK13 - 8.63 t h^{-1}.

The yield of the hybrids group with the parent component AE392 varied between 3.09–6.23 t h^{-1}, and the average value was 3.92 t h^{-1}. The highest yield in this group was formed by the hybrid AE392xAK157 - 6.23 t h^{-1}.

CONCLUSIONS

The use of inbred lines VK13, and VK69 - as sources of mutations in the gene structure of the endosperm structure *waxy* and AE801 and AE392 - mutations *ae* affects the creation of hybrids with improved grain quality and high yields.

As a result of the analysis of experimental data on the content of new hybrids of the main biochemical components in maize grain, the following are distinguished within their groups:

– high in protein: VK13xCO255 - 13.07%, VK13xVK37 - 12.60%, AE801xAE746 - 11.27%, AE801xFV243 - 11.18%;

– with a high starch content: VK13xUHK678 - 72.60%, VK13xUHK686 - 72.57%, VK69xFV243 - 72.80%, AE801xVK32 - 70.83%.

– with high oil content: VK13xUHKK686 - 5.07%, VK13xVK37 - 5.27%, VK69xVK13 - 5.27%, AE801xAE746 - 5.13%, AE392xHLG1203 - 5.83%.

According to the level of yield, hybrids were identified: VK69xG255 with a yield of 9.37 t h^{-1} and AE801xVK13 with a yield of 8.63 t h^{-1}.

According to the set of valuable economic indicators hybrids VK13xCO255 - high protein content and yield; VK13xVK37 and AE801xAE746 - simultaneously high content of protein and oil; VK13xUHK686 - high content of starch, oil, and yield.

Inbred maize lines CO255, UHK686, VK37, FV243, AE746, and others are a valuable breeding material (parent component) for the creation of new hybrids with a set of improved grain quality indicators.

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Duration of low temperature changes physiological and biochemical attributes of rice seedling

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Abstract. The low temperature (LT) is detrimental to growth of rice seedling during boro season in Bangladesh. An experiment was conducted in growth chamber during June to October, 2021 using BRRI dhan29 as planting material. The aim of this experiment was to determine the effect of variable duration of LT on growth, physiological and biochemical traits of rice seedling and to determine the age of seedling that can tolerate cold effect. In this experiment 5, 10, 15 and 20 days old seedling (DOS) were exposed to 6 °C for 1, 2, 3 and 4 days. Seedlings were also grown under room temperature (25 °C, RT) which consider as control treatment. Seedlings were grown in plastic trays filled with mixture of soil and cowdung. The experiment was conducted following completely randomized design with 3 replications. Data on shoot length and weight, root length and weight, chlorophyll (Chl), carotenoids, malondialdehyde (MDA) and proline contain were determined after 5 days of temperature sock. The results revealed that the LT was injurious to younger rice seedling when they were exposed to LT for 1 to 2 days. The shoot and root length as well as their dry weight were reduced under low temperature. Further, the Chl and carotenoid content of younger rice seedlings degraded within 2 days of LT exposure. On the contrary, the proline and MDA content of rice seedlings increased to reduce the harmful effect of under LT. It could be concluded that the rice seedlings could tolerate the detrimental effect of LT when they attain at least 15 days.

Key words: cold injury, lipid peroxidation, photosynthetic pigment, stress.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple foods and extensively cultivated worldwide (Fairhurst & Dobermann, 2002; Shelton et al., 2002). More than half of the world's populations derives a significant proportion of their caloric intake from rice consumption. Asia is the major rice growing area where 90% of the worlds′ rice is grown (Rashid & Yasmeen, 2017). Bangladesh, a part of Asia, has a large agrarian base with 76% of total population living n the rural areas and 90% of the population directly related with agriculture. Agriculture contributes 14.1% of national GDP, where rice sector pays one-half (BRRI, 2021). Therefore, rice has a significant role in socio-economic condition of Bangladesh and it is imperative to increase rice production in order to meet the food demand in future.

Any stress condition either biotic or abiotic inhibits plant growth, reduced bio-production and changes physiological condition (Nilsen & Orcutt, 1996). The alteration in the plant system due to stress condition depends on the intensity and duration of the stress environment (Godbold, 1998). Different abiotic stress like drought, high and low temperature, salinity, submergence etc. cause an enormous loss each year worldwide due to reduction in crop productivity and crop failure. They cause water reduction and osmotic changes in the cellular level, suppress the activities of cellular molecules, and can result in reduced growth and extensive losses in agricultural production (Xiong et al., 2002). Abiotic stress in fact is the principal cause of crop failure worldwide, dipping average yield for most major crops by more than 50% (Eshghi et al., 2010). Among the stresses, temperature stress act as an abiotic stress factor that has a strong impact on the survival, growth, reproduction and distribution of plants in large area of the world (Boyer, 1982). Each plant has its unique set of temperature requirements, which are optimum for its proper growth and development. Every single plant is characterized by a certain genetically fixed level of resistance to LT, which reduces its metabolic activity. This level of resistance can vary among individual plants and species. The LT or cold is one of the major stresses that limit growth of rice seedling because rice is sensitive to chilling stress.

Rice is grown though out the year, but boro season (winter season) is the major rice growing season in Bangladesh. This season starts in the month of November and ends in April. Seedlings are grown in the seed bed during the month of November to December, when the air temperature remains below 20 °C. Therefore, rice seedling faces a low temperature (LT) stress in the seedling stage. The LT injury is a common problem during boro season in Bangladesh (BRRI, 2021) because air temperature goes below the critical temperature in many areas of the country (Rashid & Yasmeen, 2017). Therefore, seedlings of boro rice experienced very low temperature during the month of December and January. Germination rates becomes below and rice seedlings get yellowish color with stunted growth (Mukhopadhyay et al., 2004; Tuteja et al., 2012). It also causes a series of morpho-physiological and biochemical changes in young rice plant (Hasegawa et al., 2000). These changes also known as acclimation response (Hughes & Dunn, 1996) because the changes in molecular and cellular level occurs in the plant body to overcome and survive under stress condition.

The reduction in seedling growth of rice due to LT is one of the major problems in tropical and subtropical areas. However, the occurrence of low temperature stress during the early growth stages of rice inhibits seedling establishment, eventually leads to non-uniform crop maturation and dramatically reduce its production (Aghaee et al., 2011). As the cold environment has numerous adverse effects on raising rice seedling as well as on rice production. In boro season, sever cold damages young rice seedlings every year in northern part of Bangladesh. This damage is largely due to acute dehydration associate with freezing. Farmers were not satisfied with the rice seedling due to poor growth and low quality because seedling mortality occurs after transplanting during boro season. Moreover, rice transplanting delayed due to cold weather during boro season. Overall, due to exposure to LT, the physiology of crop changes like total chlorophyll (Chl) content reduction, limitation of photosynthetic activity, and oxidative stress. LT causes irreversible injury in leaves, such as necrosis (Ye et al., 2009), chlorosis (Andaya & Mackill, 2003), reduction of crop survival rate, retard growth, and block the synthesis of proteins, lipids, and carbohydrates (Liu et al., 2013). Senberga et al. (2018) also reported that low temperature plays a vital role in seed germination of faba bean and root zone temperature needed at least 8 °C for germination and symbiotic development.

The objectives of this study were to determine the effect of variable duration of LT on growth, physiological and biochemical traits of rice seedling and to determine the age of seedling that can tolerate cold effect. Seedling height, dry matter production, pigment content, proline and malondialdehyde (MDA) contents were tested in this study.

MATERIAL AND METHODS

Experimental Site and Treatments

An experiment was conducted in growth chamber under laboratory condition of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur during June to October, 2021. BRRI dhan29 was used as planting material. In this experiment 5, 10, 15 and 20 days old seedling (DOS) were exposed to LT (6 °C) for 1, 2, 3 and 4 days. Seedlings were also grown under room temperature (25 °C, RT) which was considered as control treatment. The experiment was conducted following completely randomized design with 3 replications.

Seed Sowing

Seedlings were grown in plastic trays filled with mixture of soil and cow dung. The size of each tray was 48×48 cm and contains 36 chambers. About 25 seeds were sown in each chamber. The ratio of soil and cowdung was 3:1. Before filling the trays, the mixture of soil was sieved to remove clods. At first, the trays were placed in a plain land. The one fourth of each chamber of trays was filled with clod free soil materials. The rice seeds were sown on the trays at five days intervals to get seedlings of 5, 10, 15 and 20 days old. The seeds were sown in a planned way so that each tray contains seedlings of 5, 10, 15, and 20 days old. After sowing, the seeds were covered with clod free soil mixture and irrigated to keep the seed moistened. The trays were irrigated at 2 times every day using plastic bottle.

Treatment Imposition

When the rice seedlings gained desirable age, the trays were transfer to growth chambers for 2 hours to impose temperature sock. After exposure to desirable temperature, the trays were removed from growth chamber to room temperature.

Data Collection

The data regarding various relevant parameters were collected accordingly during the experimental period. Data on shoot (SL) and root length (RL); shoot (SW) and root weight (RW), Chl, carotenoids, proline and MDA contents were determined after 5 days of temperature sock. Seedlings were collected from trays and washed with tap water.

After washing the seedlings, the were gently wiped with a tissue paper. The SL and RL of five seedlings were measured and averaged to get SL and RL of a rice seedling. The root and shoot of rice seedling were taken after oven dry at 80 °C for 72 hours. Strength of seedling was calculated from the following formula:

$$\text{Seedling strength (mg cm}^{-1}) = \frac{\text{Shoot weight (mg)}}{\text{Shoot length (cm)}} \quad (1)$$

The formulae for computing Chl a, b and total carotenoids were determined by the method of Yang et al. (1998). Chl content was determined on fresh weight (FW) basis extracting with 80% acetone by using double beam spectrophotometer. The acetone-water mixture (4:1) was used as a solvent. The absorbance maxima were read at 663.0 nm for Chl a, 645.0 nm for Chl b and 470.0 nm for carotenoids. Contents of Chl a, Chl b and total carotenoids were calculated from the following equations:

$$\text{Chl a (mg g}^{-1}\text{ FW)} = [12.7\ (D663) - 2.69\ (D645)] \times [V/1{,}000 \times W] \quad (2)$$

$$\text{Chl b (mg g}^{-1}\text{ FW)} = [22.9\ (D645) - 4.68\ (D663)] \times [V/1{,}000 \times W] \quad (3)$$

$$\text{Total Chl (mg g}^{-1}\text{ FW)} = [20.2\ (D645) + 8.02\ (D663)] \times [V/1{,}000 \times W] \quad (4)$$

$$\text{Total carotenoids (mg g}^{-1}\text{ FW)} = \frac{[1{,}000\ (D470) - 2.27\ (\text{Chl a}) - 81.4\ (\text{Chl b})]}{227} \quad (5)$$

where, D (663,645,470) = Optical density of the Chl a, b and carotenoids extract at wavelength of 663, 645 and 470 nm, respectively. V = Final volume (mL) of the 80% acetone with Chl extract and W = Weight of fresh leaf sample in g. Proline content in leaf of rice seedling estimated. The 0.5 g of fresh weight of leaf was taken for proline estimation and subsequently proline was estimated according to (Bates et al., 1973).

Data Analysis

The data were analyzed using computer software CropStat 7.2 and the graphs were prepared using excel program. Treatment means were separated using *LSD* at 5% level of probability.

RESULTS AND DISCUSSION

Shoot Length

The growth of shoot depends on various environmental factors. Air temperature is one of them, which significantly controls the range of plant growth. Each plant has its optimum growth temperature but 18–21 °C temperature is considered as optimum for most of the temperate plants for subsequent growth and development (Junttila, 1986). The results of this experiment showed that the interaction of LT and seedling age (SA) had a significant effect on shoot length (SL) of rice seedlings (Table 1). The SL of 5 to

Table 1. Effect low temperature on shoot length (cm) of rice seedling

Seedling age (days)	Exposure to low temperature for				
	0 day (control)	1 day	2 days	3 days	4 days
5	13.7	12.3	12.0	12.6	11.3
10	14.2	14.8	14.7	12.4	13.7
15	14.4	14.5	14.6	14.9	13.6
20	15.8	13.8	14.6	14.8	13.8
$LSD_{0.05}$					
SA	0.932				
TD	1.041				
SA × TD	2.073				
CV (%)	9.01				

SA = Seedling age and TD Temperature duration.

20 DOS of rice reduced significantly when they were treated with LT. Under LT, however, the reductions in SL of younger seedling (5 to 10 DAS) were more prominent than older one. The SL of 5 DOS was 13.7 cm when it was treated with 25 °C temperature (control), which reduced to 11.3 cm when LT exerted 4 days. Similarly, SL of 10 DOS reduced by 12% when they were experienced with LT upto 3 days. On the contrary, SL varied from 15.8 to 13.8 cm when 20 DOS were treated with LT upto 0 to 4 days. It revealed that aged seedlings were less affected by prolong LW. Zhao et al. (2020) stated that LT exhibited a significant inhibitory effect on growth of rice seedling. Chl content is an important index to evaluate photosynthesis capacity. Previous study showed that LT inhibited the activities of Chl biosynthesis enzymes (Nagata et al., 2007). Fyson & Sprent (1982) stated that seedling is exposed to temperature delayed nodulation in bean which is associated with the slower plant growth.

Shoot Weight

The production of percent shoots dry weight decreased in response to low environmental temperature. Low temperature severely reduces the dry weight content of plant (Hnilickova et al., 2002). In the present study, LT exerted a significant effect on shoot dry matter (SDM) of rice seedlings (Table 2). The SDM of 5 DOS varied from 6.4 to 4.3 mg plant^{-1} when treated with LT at 0 to d days. Similarly, the range of SDM was 5.33 to 7.61, 5.68 to 7.3, and 4.6 to 7.4 mg plant^{-1} in case of 10, 15 and 20, respectively when they are treated with LT at 0 to 4 days. This indicated that the prolong LT was detrimental to SDM of rice seedlings. Plant growth reduced under LT condition due to limited Chl content. The effect of LT for higher duration inhibited the greening process and seedling growth. Therefore, accumulation of SDM hampered significantly under LT. Ben-Haj-Salah & Tardieu (1995) also reported that LT inhibited the leaf cell division and elongation results lower SDM.

Table 2. Effect low temperature on shoot dry matter (mg plant^{-1}) of rice seedling

Seedling age (days)	Exposure to low temperature for 0 day (control)	1 day	2 days	3 days	4 days
5	6.4	5.5	5.1	5.0	4.3
10	7.6	6.1	5.7	6.7	5.3
15	7.2	5.5	5.8	5.9	5.7
20	7.4	6.1	6.1	7.1	4.6
LSD$_{0.05}$					
SA	1.756				
TD	2.195				
SA × TD	3.388				
CV (%)	7.0				

SA = Seedling age and TD Temperature duration.

Seedling Strength

Seedling strength was also decreased significantly due to LT treated trays (Table 3) in all aged seedling. In case of 5 DOS, the strength of seedlings was 0.47, 0.45,

Table 3. Effect low temperature on strength (mg cm^{-1}) of rice seedling

Seedling age (days)	Exposure to low temperature for 0 day (control)	1 day	2 days	3 days	4 days
5	0.47	0.45	0.42	0.40	0.38
10	0.54	0.41	0.38	0.54	0.39
15	0.50	0.38	0.40	0.39	0.42
20	0.47	0.44	0.42	0.48	0.33
LSD$_{0.05}$					
SA	0.295				
TD	0.334				
SA × TD	0.656				
CV (%)	8.0				

SA = Seedling age and TD Temperature duration.

0.42, 0.50 and 0.38 mg cm^{-1} when they were treated at 0, 1, 2, 3 and 4 days, respectively. However, the strength of seedlings reduced drastically in case younger seedling (5 DOS) than older one (20 DOS).

Root Length

For subsequent growth and development of plants, there should have optimum environmental temperature as the activation of various functional enzymes needs proper temperature along with other requirements. Proper root growth of maximum plant species requires 27–30 °C soil as well as environmental temperature (Drennan & Nobel, 1998). Any deviation from this optimum level of temperature leads to the decrease root growth. In this study, root length (RL) of rice seedling was significantly smaller when they were exposed to longer duration of LT (Table 4). In case of 5 DOS, the RL was 3.9 cm under control which reduced to 2.5 cm under 4 days treated trays. However, RL of 10 to 20 DOS was also reduced due increased duration of LT from 0 to 4 days. Roots are responsible to acquire water and nutrients to improve plant productivity (Klein et al., 2020). However, roots are the most sensitive part of the plant to temperature (Munyon et al., 2020). The results of these experiment also showed that the root system of rice seedling reduced significantly under LT. Senberga et al. (2018) also found that primary root length of faba bean also reduced significantly due to low temperature.

Table 4. Effect low temperature on root length (cm) of rice seedling

Seedling age (days)	Exposure to low temperature for 0 day (control)	1 day	2 days	3 days	4 days
5	4.0	3.5	2.8	2.7	2.5
10	3.8	3.3	3.1	2.7	2.7
15	2.7	2.7	2.5	2.8	2.8
20	2.5	2.3	2.4	2.8	2.7
*LSD*0.05					
SA	0.624				
TD	0.682				
SA × TD	1.371				
CV (%)	30.4				

SA = Seedling age and TD Temperature duration.

Root Weight

The effect of LT on dry matter partitioning between shoot and roots is difficult to predict because temperature may affect directly water and nutrient uptake as well as other physiological and biochemical processes inside the plant body. However, root dry matter (RDM) of rice seedlings significantly increased when they were exposed to LT from 0 to 4 days (Table 5). In case of 5 DOS, the RDM was 1.2 mg plant^{-1} under control which was 1.4 mg plant^{-1} under 4 days treatment. Similar trend was also true for 10 DOS. However, RDM of older rice seedling (15 to 20 DOS) reduced dramatically when they experienced with LT. Root weight ratio of different bean varieties were reduced under 4 °C (Senberga

Table 5. Effect low temperature on root dry matter (mg plant^{-1}) of rice seedling

Seedling age (days)	Exposure to low temperature for 0 day (control)	1 day	2 days	3 days	4 days
5	1.2	0.73	1.2	1.3	1.4
10	1.1	1.2	1.3	1.9	1.5
15	0.43	0.87	1.6	1.6	1.3
20	0.43	0.28	0.50	0.19	0.76
*LSD*0.05					
SA	2.104				
TD	2.353				
SA × TD	4.711				
CV (%)	25.1				

SA = Seedling age and TD Temperature duration.

et al., 2018). Root development not only the indicator of plant growth, but also demonstrates the effect of microorganisms in the soil that the seedling is exposed to, as delayed nodulation in bean due to low temperature has been associated with the slower plant growth (Fyson & Sprent, 1982).

Chl a Content:

Chl content of rice seedling varied significantly when they are exposed to various duration of LT (Figs 1 to 3). The LT showed a sever effect on Chl a content with the increasing of duration. Chl a content of 5 to 20 DOS varied 1.8 to 2.2 mg g^{-1} fresh weight (FW) under control, while it was 0.7 to 1.04 mg g^{-1} FW under 4 days duration. Similarly, the range of Chl a was 1.2 to 2.0, 1.1 to 1.8 and 1.0 to 1.7 mg g^{-1} FW under 1, 2 and 3 days of LT exposure (Fig. 1). LT stress is one of the most important factors that limit photosynthetic activities of plants by reducing the pigment content in the plants. It has been reported that Chl a and b content decreased in plants when they were subjected to cold stress (Yadegari et al., 2007).

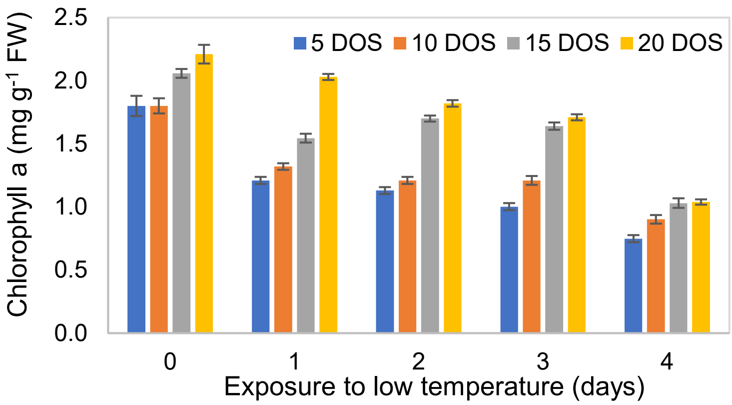

Figure 1. Effect low temperature on chlorophyll a content of rice seedling.
Var indicates mean value ± standard error.

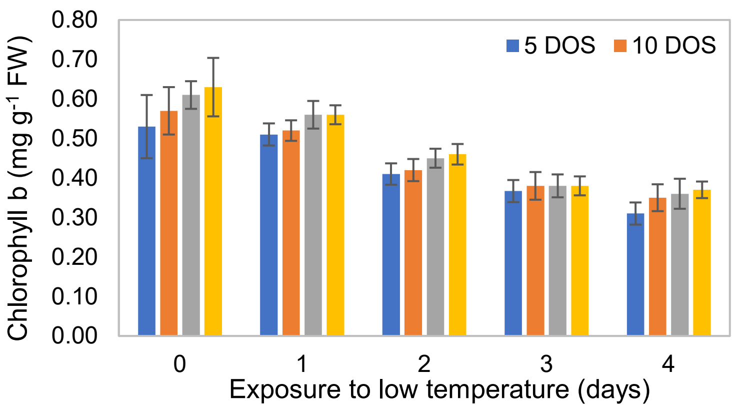

Figure 2. Effect low temperature on chlorophyll b content of rice seedling.
Var indicates mean value ± standard error.

Chl b Content

The concentration of Chl b in rice seedlings were also affected due to various duration of LT. The Chl b content ranged from 0.53 to 0.63 mg g^{-1} FW under control and 0.51 to 0.56 mg g^{-1} FW under 1 day exposure to LT (Fig. 2). Similarly, the content of Chl b in rice plant reduces gradually when they were exposed to longer duration of LT. The decrease in the Chl b content under LT stress might be due to suppression of Chl biosynthesis, which probably by inhibiting the activities the activities of Chl biosynthesis enzymes (Nagata et al., 2007).

Total Chl Content:

The LT showed a sever effect on total Chl content of rice seedling. Under LT, the rice seedlings became yellow due to degradation of Chl. The younger seedlings were more affected when the duration of LT increases. The total Chl content of control treatment varied 2.3 to 2.8 mg g^{-1} FW, while it was 1.1 to 1.4 mg g^{-1} FW under 4 days of LT (Fig. 3). It was observed that LT induced a significant decrease in the content of photosynthetic pigment fraction (Chl a and b) as a result of the content of total Chl content in the leaves. The result was also agreed with the findings of Habibi et al. (2011).

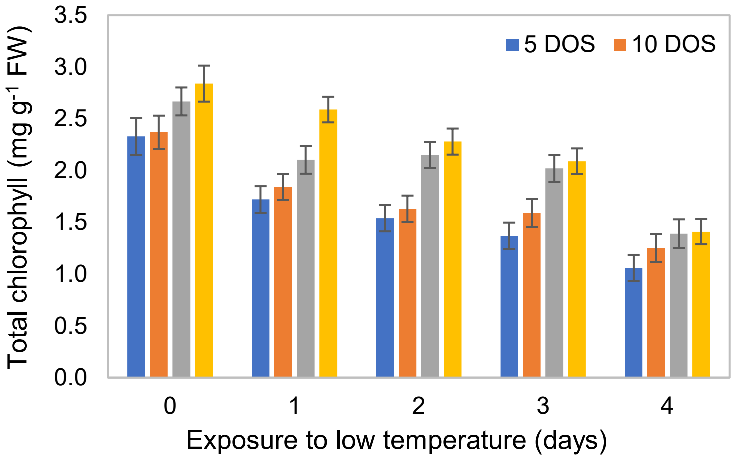

Figure 3. Effect low temperature on total chlorophyll content of rice seedling.
Var indicates mean value ± standard error.

Carotenoid Content

Carotenoid content of rice seedlings varied significantly due to various duration of LT (Fig. 4). The concentration of carotenoids in rice seedling drastically decreased when they were exposed to LT from 1 to 4 days. However, the 5 to 10 DOS affected severely regarding carotenoids content from 1 to 4 days LT treatment. Moreover, the carotenoids content of 5 DOS was 0.5 mg g^{-1} FW, while it was 0.20, 0.21, 0.20 and 0.091 mg g^{-1} FW at 1, 2, 3 and 4 days LT treatment, respectively. The content of this pigment of 10 to 20 DOS also reduced significantly under 1, 2, 3 and 4 days LT treatment as compared to control. The LT reduced carotenoids level in plants was also reported by Zhao et al. (2020).

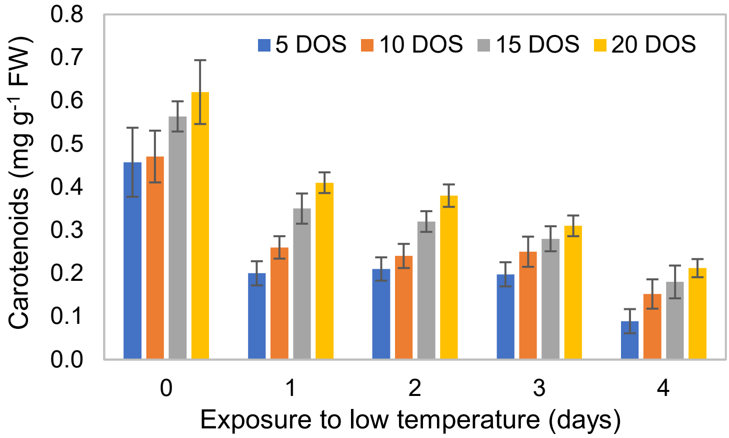

Figure 4. Effect low temperature on carotenoids content of rice seedling.
Var indicates mean value ± standard error.

Proline Content

The proline content dramatically increased in younger rice seedlings (15 to 20 DOS) when they exposed to LT for longer duration (Fig. 5). In case of 5 DOS, the proline content varied from 0.011 µg g^{-1} FW in control, which increased to 0.025, 0.026, 0.028 and 0.033 µg g^{-1} FW at 1, 2,3 and 4 days exposure to LT. Proline accumulation in plants is one of the most popular approach used by the plants to deal with LT stress. Proline is an osmolyte which helps plants to survive under LT through cold acclimation (Ritonga & Chen, 2020). In this experiment, it was observed that more proline accumulated in the younger seedlings. The result also supported by the findings of Habibi et al. (2011).

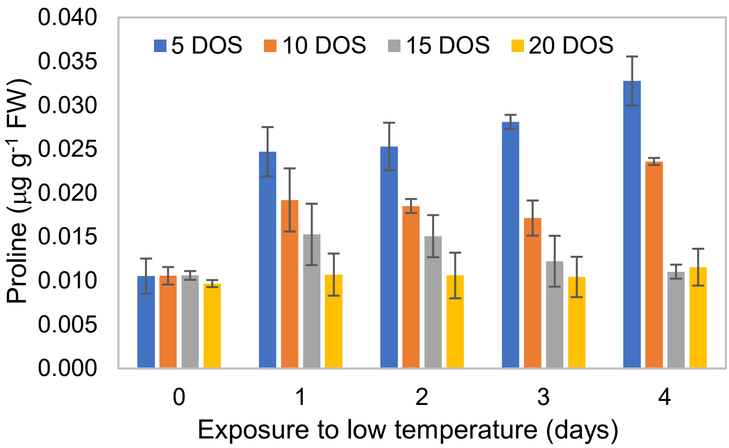

Figure 5. Effect low temperature on proline content of rice seedling.
Var indicates mean value ± standard error.

MDA Content

The MDA content dramatically increased in rice seedlings when they exposed to LT for longer duration (Fig. 6). However, in case 5 DOS, the proline content varied from 9.2 to 13.5 µ mol g^{-1} FW, while it ranged from 7.8 to 13.6 µ mol g^{-1} FW in case of 10 DOS under 1 to 4 days of LT. The amount of MDA increases in the rice seedlings when they were treated with longer duration of LT. This result indicated that the LT stress induced reactive oxygen species accumulation and caused higher amount of MDA in rice plant. The results also supported by the findings of Zhao et al. (2020).

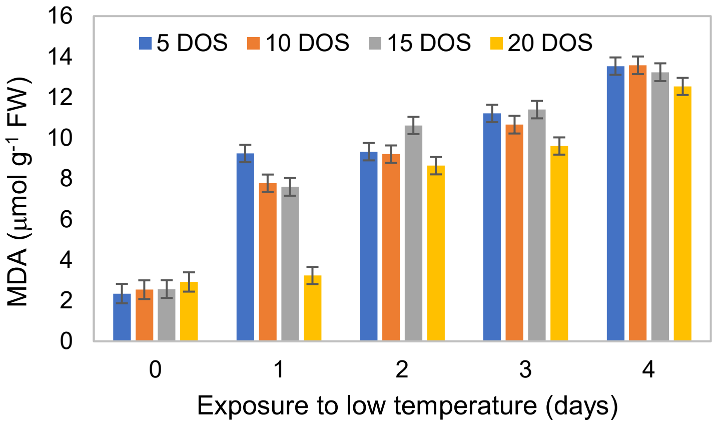

Figure 6. Effect low temperature on MDA content of rice seedling,
Var indicates mean value ± standard error.

CONCLUSIONS

The present study indicated that low temperature (LT) was injurious to younger rice seedling when they were exposed to LT for 1 to 2 days. The shoot and root length as well as their dry weight reduced, chlorophyll and carotenoid content of younger rice seedling within 2 days of LT exposure. On the contrary, the proline and MDA content of rice seedlings increased to reduce the harmful effect of under LT. Based on this study, it might be concluded that the rice seedlings could tolerate the detrimental effect of LT when they attain at least 15 days.

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Enrichment of growing media using biochar, compost, and nanosilica for the cultivation of *Oryza sativa* L.

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Abstract. This study investigated the effect of growing media composition on the growth and productivity of *Oryza sativa* var. Inpago 7 cultivated in polybags at Sumedang, West Java, Indonesia, for 126 days. A randomized block design was applied with nine combinations of growing media. Each treatment consisted of five polybags and was replicated three times. Plant height, number of leaves, and tillers were measured at 15, 30, and 45 days after transplanting to observe the growth parameters. While, for estimation of productivity were recorded the weight of dry grain harvest (DGH), the weight of dry milled grain (DMG), and the weight of 100 grains (g) were recorded for each treatment for estimation of productivity. The results showed that the highest number of panicles (24 panicles per plant), the weight of dry harvested grain (21.74 g per plant), and weight of dry milled grain (17.83 g per plant) were obtained when cultivated with a growing medium containing soil, biochar, synthetic fertilizer, urea and sprayed with nano-silica. The growing media composition did not influence the weight of 100 grains because it is more influenced by genetic characteristics. An appropriate combination of soil, biochar, synthetic fertilizer, urea, and sprayed with nano-silica is recommended as a growing media for cultivating *O. sativa* L. var. Inpago 7.

Key words: biochar, growing media, nanosilica, *Oryza sativa*.

INTRODUCTION

Rice (*O. sativa* L.) is a major and important crop in Asia as well as in Southern Europe and The United States of America (Nair, 2019) with a global annual productivity of 518 tons (Vijayakumar et al., 2005). The Food and Agricultural Organization suggested that by the year 2025, the world population (about 8.5×10^9 people), will require substantial enhancement in agricultural production to satisfy the demand (Singh et al., 2018). As the population grows, the need for rice continues to increase, while the amount of arable land for cultivation crops is limited (Pratiwi et al., 2020). Rice is produced from approximately 8.1 million ha of land in Indonesia in 2013. Sustainable management of that land for rice production is very crucial to meet national rice demand

(Purwanto et al., 2017). Recently, the main problems of tropical agricultural soils are the depletion of soil fertility and the decline in the productivity of agriculture crop due to the reducing of organic matter and imbalances nutrients inside the soil (Agegnehu et al., 2017). One of the factors that determine the productivity and physiological growth of *O. sativa* L. is the growing medium. Various studies have been carried out to investigate the effect of different growing media on the growth and yield of different plants (Manh & Wang, 2104; Mehmood et al., 2017). The substrate or a combination of substrates used to grow seedlings is important to provide plants with mechanical supports, water, and mineral for their growth and development (Radha et al., 2018). The application of growing media either in excess or less than optimum dose affects both yield and quality of the harvested rice. Hence proper management of crop nutrition, particularly the composition of the growing medium, is of immense importance to optimize plant growth and meets nutritional needs (Tubana et al., 2016).

Over the past decades, many studies have been carried out to replace the soil in a growing media with superior quality, inexpensive, readily available, and environmentally friendly substrates (Kather, 2015; Tubana et al., 2016; Radha et al., 2018). Biochar is a carbon-rich material that has been considered as a promising alternative as a replacement of soil as a growing medium (Novák et al., 2020). Biochar is a soil conditioner produced from the thermal decomposition of organic materials through pyrolysis, and it has the potential to increase crop yields. It can improve plant growth and soil quality. The advantage of biochar addition on cultivation process were attributed to improved soil properties, such as decrease in soil bulk density, and subsequent increase in porosity and water holding capacity, increase in the cation exchange capacity which enhances the retention of basic nutrients, increased uptake of N and its availability in soil, adsorption of soil phytotoxins, liming effects, and increased plant nutrient concentration (Wacal et al., 2019). Nemati et al. reported that biochar increases pH and decreasing nutrient leaching up to 11% (Nemati et al., 2015). The study also highlighted that biochar is a good alternative for replacing perlite and peat moss in the growing medium. In another study conducted by Parr, biochar also serves as nutrient retention whose can improve the accessibility of nutrients into plants (Parr & Sullvian, 2015).

In addition to biochar, compost has been widely used as a soil conditioner because it contains a high organic matter and possesses physical and chemical properties that can improve the fertility and aggregate structure of the soil (Wasis & Fathia, 2010; Khater, 2015). Application of biochar to nutrient poor soils could be one of the potential options to enhance agriculture productivity (Xu et al., 2017). The plant residue biochar, an alternative organic supplement to chemical fertilizers, could be viable and sustainable way to enhance crop yield (Agegnehu et al., 2017). The use of biochar in agriculture can enhance agriculture soil fertility, adverse impact of chemicalization on soil fertility (Kim et al., 2017), soil microbial biodiversity (Luo et al., 2017), and agriculture productivity (Agegnehu et al., 2017).

Khater (2015) has investigated compost's physical and chemical properties produced from cattle manure, herbal plant residues, and sugar cane residue (Kather et al., 2015). The total organic matter in the compost varies from 28.6 to 41.2%, with a carbon to nitrogen ratio of 14.2:1 to 18.5:1 that lies in the ideal range of ready to use compost for growing plants (Rosen et al., 1993). According to Gab (2014) and Ramos (2017), compost can improve the structure of soil than decreases crust formation, surface runoff, and erosion. The application of compost increases porosity, water retention, and hydraulic conductivity of the soil.

Silica is a non-essential nutrient that is very beneficial for plants to hold water in a dry environment, bind other nutrients so that the nutrient content in the soil is not lost, and water reserves will be maintained its quantity. The supply of Si can provide a good crop yield because it can increase cell strength and endurance. Si makes leaves more erect in the effect of high nitrogen fertilization to increase photosynthesis. It plays a role in increasing growth, increasing photosynthesis, transpiration efficiency and evaporation, increasing leaf strength, chlorophyll concentrations per leaf area and product quality. The use of nano-silica will make fertilizers more easily absorbed by plants and more efficient than conventional chemical fertilizers (Prihastanti et al., 2018).

This study was carried out to investigate the potential application of biochar and compost as constituents of the growing medium used to cultivate rice. Biochar and compost were mixed with soil at pre-determined compositions and added with nano-silica to promote plant growth and increase plant turgidity. Optimum composition and appropriate dose of the growing medium is of economic importance to increase plant productivity and farmers' income. Hence, this study aimed to determine the effect of variations in the growing medium composition using biochar, compost, and nano-silica on the growth and productivity of *O. sativa* var. Inpago 7.

MATERIALS AND METHODS

Materials

The seeds of *O. sativa* var. Inpago 7 was purchased from Indonesian Center for Rice Research, Sukamandi, West Java. The growing media used in this study consisted of ultisol soil (0.20% nitrogen; 0.061% phosphorus; 1.29% potassium; 0.003% silicon monoxide), leaf compost (0.79% nitrogen; 2.56% phosphorus; 0.023% potassium; 0.0004% silicon monoxide), biochar (0.69% nitrogen; 0.035% phosphorus; 0.10% potassium; 0.11% silicon monoxide), liquid nano-silica (1.3% silicon dioxide) (brand name: Tenaz), synthetic fertilizer from (15% nitrogen:15% phosphorus:15 % potassium) (brand name: Mutiara), and urea (45% nitrogen).

Cultivation of *O. sativa* var. Inpago 7

O. sativa L. var. Inpago 7 was cultivated using polybags (diameter: 30 cm, height: 40 cm) in a screen house at Institut Teknologi Bandung - Jatinangor Campus, Sumedang, West Java, Indonesia with an 5 of 27–29 °C, relative humidity of 72–75% and wind speed of 0.4–0.6 m s^{-1}. A randomized block design was applied in this study with nine different treatments on the composition of the growing media, as shown in Table 1 where the composition for T8 is typically

Table 1. Composition of the growing media used for the cultivation of *O. sativa* var. Inpago 7

Code	Composition
T1	Soil: biochar: compost (2: 1: 1) + nano-silica
T2	Soil: biochar (2: 2) + synthetic fertilizer + urea + nano-silica
T3	Soil: biochar (2: 2) + nano-silica
T4	Soil: compost (2: 2) + sprayed nano-silica
T5	Soil + synthetic fertilizer + urea + sprayed nano-silica
T6	Soil: biochar (2: 2) + sprayed nano-silica
T7	Soil: compost (2: 2)
T8	Soil + synthetic fertilizer + urea
T9	Soil: biochar (2: 2)

applied by farmers in the studied location, hence considered as the control in this study. Each treatment consisted of five pieces of polybags containing two seeds. Every treatment was replicated three times, and the average values were reported. Initially, the seeds were germinated on wet damp-sack, and the growing medium was mixed according to the composition shown in Table 1 before being placed inside the polybags.

After the seeds had germinated, the seeds were sown using trays until the leaf reached a size of ± 10 cm, then transplanted into polybags that have been filled with the growing media. Every plant was provided with 2.7 g of synthetic fertilizer (equivalent to 300 kg ha^{-1}), 1.35 g of urea (equivalent to 150 kg ha^{-1}), and 2.7 mL of nano-silica (equivalent to 2 L ha^{-1}). The plants were watered once a day as suggested by the System Rice Intensification method (Thakur et al., 2010; Widiasri et al., 2021). The growth performance of *O. sativa* L. var. Inpago 7 were measured at 15, 30, and 45 days after transplanting (DAT). The parameters of growth of performance were plant height, number of leaves, and number of tillers. A meter ruler was utilized to measure the height of plant starting from the bottom of the shoot which is located at the surface of the soil until the end of the tallest leaf in the plant's foliage (Mensah & Frimpong, 2018). The number of leaves and tillers of the *O. sativa* L. var. Inpago 7 were counted manually (Japakumar et al., 2021). The biomass was harvested after 126 days by cutting the panicles containing grain. The weight of dry grain harvest (DGH), the weight of dry milled grain (DMG), and the weight of 100 grains (g) were recorded for each treatment for estimation of productivity.

Statistical analysis

The software that was utilized to carry out the statistical analysis for this study is IBM SPSS. Statistical analysis was conducted using Analysis of Variance (a = 0.05) and Duncan's Multiple Range Test (a = 0.05) to compare the differences between the average values of each treatment.

RESULTS AND DISCUSSION

Effects of growing media composition on plant growth

Height of the plant, number of leaves, and number of tillers were recorded periodically, and the results are shown in Table 2–4. Plant height is an indicator of plant growth and an important agronomic trait directly related to the harvest index and yield potential. The total number of elongated internodes and the length of each elongated internodes determine plant height with paddy usually has 4–6 elongated internodes (Zhang et al., 2009; Zhong et al., 2020). Table 2 shows that variations in the growing media composition have significant effects on plant height.

After 15 and 30 days of transplanting, T1 and T5 gave better effects on the average plant height than the control (T8) and other compositions, whereas, after 45 days of transplanting, rice grown in T2, T5, and T7 reached its highest average height at the end of the vegetative period: 59.72 cm, 57.69 cm, and 57.94 cm, respectively. The increase in plant height was in line with the abundant amount of nitrogen element in the media because the plants were supplied with synthetic fertilizer and urea, which results in an increase in vegetative growth with more nitrogen supply to the plants. The plant height recorded in this study lies in the range of plant height of 55 to 79 cm for *O. sativa* L var.

Ciherang cultivated at the same location as reported by Widiasri et al. (2021).

The average number of leaves for each treatment after 15, 30, and 45 days of transplanting are presented in Table 3. Leaves are the most noticeable parts of a plant; they are the predominant photosynthetic organs and pivotal for carbon fixation (Even, 2018). Some leaf parameters, such as shape, number, size, thickness, direction, and chloroplast level, are very important factors influencing a plant's biomass formation and success. Variation in the growing media composition at the beginning phase had no significant effects on the number of leaves. The effect of began to appear at 30 and 45 days of transplanting. Even though the effect was not significantly different for T4, T5, and T7 after 30 days of transplanting as well as T1, T4, T5, and T8 after 45 days of transplanting, the number of leaves increased for T2 after 45 days of transplanting as compared to the control (T8).

Table 2. Effect of growing media composition on the plant height of *O. sativa* var. Inpago 7

Treatment	Plant height (cm)		
	15 days	30 days	45 days
T1	30.01[a]	38.61[ab]	49.46[ab]
T2	39.43[c]	51.70[e]	59.72[c]
T3	30.56[ab]	36.09[a]	46.42[a]
T4	32.89[ab]	41.81[abc]	55.12[abc]
T5	40.21[c]	48.68[de]	57.69[bc]
T6	30.96[ab]	38.28[ab]	48.56[a]
T7	33.60[ab]	44.31[cd]	57.94[bc]
T8	34.72[b]	43.51[bcd]	54.33[abc]
T9	30.38[ab]	36.31[a]	47.32[a]

*The average number followed by superscript of the same letter in each column is not significantly different based on *Duncan's Multiple Range Test* (a = 0.05).

Table 3. Effect of growing media composition on the number of leaves of *O. sativa* var. Inpago7

Treatment	Number of leaves		
	15 days	30 days	45 days
T1	4.40[a]	10.37[ab]	37.13[abc]
T2	11.77[a]	31.47[c]	73.27[e]
T3	3.70[a]	8.47[a]	23.13[a]
T4	6.67[a]	20.77[abc]	42.11[bcd]
T5	6.77[a]	22.63[bc]	48.50[cd]
T6	6.17[a]	12.10[ab]	28.54[ab]
T7	6.80[a]	22.40[bc]	55.47[d]
T8	5.80[a]	15.83[ab]	35.70[abc]
T9	5.43[a]	13.60[ab]	23.54[a]

*The average number followed by superscript of the same letter in each column is not significantly different based on *Duncan's Multiple Range Test* (a = 0.05).

Table 4. Effect of growing media composition on the number of tillers of O. sativa var. Inpago 7

Treatment	Number of leaves		
	15 days	30 days	45 days
T1	1.77[a]	4.97[ab]	14.71[bcd]
T2	4.93[a]	14.63[c]	23.03[e]
T3	1.20[a]	3.50[a]	7.97[a]
T4	2.43[a]	8.77[ab]	17.60[cd]
T5	3.00[a]	9.83[b]	16.84[cd]
T6	2.13[a]	5.03[ab]	9.79[ab]
T7	2.63[a]	9.30[b]	18.78[de]
T8	2.37[a]	7.09[ab]	12.59[abc]
T9	2.27[a]	5.83[ab]	8.95[a]

*The average number followed by superscript of the same letter in each column is not significantly different based on *Duncan's Multiple Range Test* (a = 0.05).

The effect of growing medium composition on the number of tillers is shown in Table 4. After 15 days of transplanting, the growing media compositions had no significant effects on the number of tillers. The formation of new tillers started after 25 days of transplanting. Plants from T2 had a higher number of tillers with an average of 23 tillers after 45 days after being planted compared to the control (T8) and other treatments. As such may be attributed to more nitrogen supply to the plants during the active tillering stage. Previous studies have demonstrated that the application of nitrogen significantly increased the number of effective tillers and uptake of nitrogen by

O. sativa L up to the level of 200 kg of nitrogen per hectare (Meena et al., 2002; Meena et al., 2003).

Based on the results obtained in this study, T2 (soil: biochar (2:2) + synthetic fertilizer + urea + nano-silica) is the combination of growing media that produces the best growth of *O. sativa* L. with a plant height of 59.72 cm, 73 leaves, and 23 tillers. The presence of synthetic fertilizer and urea in the growing media at the beginning of the cultivation period is very important to promote plant growth because synthetic fertilizer and urea can be easily absorbed by the plants as essential nutrients (Leghari et al., 2016, Radchenko et al., 2021). Synthetic fertilizer is part of the essential nutrients needed for meristematic and physiological activities such as leaf formation, roots, as, dry matter production, and others, leading to efficient water translocation and nutrition, interception of solar radiation, and carbon dioxide. This can improve the process of larger photosynthesis from adequate assimilation to subsequent translocation to various channels (Jaliya et al., 2008).

Nitrogen is a constituent of amino acids and chlorophyll that is vital to accelerate plant height, phosphorus acts as a storage and transfer of energy for the entire metabolism that leads to the formation of roots in plants, and potassium acts as an enzyme activator to assists transport of assimilated products from the leaves to the entire plant tissue (Leghari et al., 2016). The addition of compost in the growing media had no significant effects at the beginning of the plant growth because the compost generally has a low concentration of nitrogen, phosphorus, and potassium as well as macro and micronutrients in comparison to commercial fertilizer (Kather, 2015). Compost provides a better effect as s soil conditioner because of its ability to retain water and improve the aggregate of soil as well increase the biological activity of soil (Manh & Wang, 2014, Kather, 2015).

The provision of biochar in a growing media positively affected growing plant due to the increased nutrition available to the plants. The silica content in biochar could increase plant growth especially the growth of stems and tillers. The number of tillers was influenced by the availability of nutrients in the soil, and the growth of tillers was strongly influenced by nitrogen, especially in the initial phase. Hence, the addition of biochar in the growing media improve soil quality to promote a better plant growth particularly the root and shoot (Liu et al., 2017; Pratiwi et al., 2020). The addition of biochar could increase the resistance of nitrogen element by preventing nitrate discharges which can increase soil nitrification thereby increasing the availability of nitrogen for the plants (Darusman et al., 2017).

The presence of nano-silica in the growing media had significant effects on plant growth. Provision of nano silica fertilizer by sprinkling to the leaves aims can lead to better absorption by the plants for optimum growth (Prihastanti et al., 2018). The plant cell wall acts as a barrier to insert foreign agents into plant cells. The nanoparticles having a smaller diameter compared with the pore diameter of cell wall can easily pass through the pores of the walls. The nanoparticles on the leaf surface enter the plant through the pores or villus base and then transported to different tissues (Nair et al., 2010). Silica plays a role in increasing growth, increasing photosynthesis, transpiration efficiency, and evaporation, increasing leaf strength, chlorophyll concentration per leaf area and product quality (Hwang et al., 2005). When silica is reduced, the amount of chlorophyll will decrease and consequently the photosynthesis of plants will decrease.

In addition, silica also has a role in the chain of photosynthesis and prevents chlorophyll degradation (Lawlor et al., 2002).

Previous studies have shown that nano-silica positively influences the structure and biosynthesis of cell walls for grassy plants and increases plant height significantly (Marxen & Klotzbucher, 2016). In addition, nano-silica also plays a role in the process of leaf formation, as demonstrated by Marxen & Klotzbucher, 2016). Silica also plays an important role in the upright leaf state and can provide more areas of leaves to light, thereby increasing the efficiency of plant photosynthesis. Silica makes leaves more erect in the effect of high nitrogen fertilization to increase photosynthesis. Sufficient use of silica may reduce the likelihood of wilting plants under drought conditions due to decreased permeability of water vapor from leaf epidermal cell walls. It also affects phosphorus fixation so that its availability increases (Prihastanti et al., 2018).

Effects of growing media composition on biomass productivity

After 100 days of cultivation, the plants were harvested, and the biomass productivity was determined in terms of the number of panicles, the weight of dry harvested grain (DHG) per plant, the weight of dry milled grain (DMG) per plant, and the weight of 100 grains (Table 5). The highest number of panicles (24 panicles per plant), the weight of DHG (21.74 g), and weight of DMG (17.83 g) were obtained when the plants were cultivated with a growing medium containing soil, biochar, synthetic fertilizer, urea, and nano-silica (T2) that also produced the highest number of tillers during the growth period as compared to the control (T8) and other treatments. The panicles were produced by productive tillers that possess flag leaves, indicating that the generative growth had begun. The significant increase in the number of panicles per plant with biochar treatments could be increase the weight of DHG and weight of DMG per plant. The results in this study indicate that application of biochar and nano silica have a capacity to increase plant development, water, and nutrients absorption, and subsequently maximize yield-related traits and productivity (Hafez et al., 2021).

Table 5. Effects of growing media composition on the average number of panicles, weight of dry grain harvested, weight of dry milled grain, and weight of 100 grains of *O. sativa* L. var Inpago 7 after 100 days of transplanting

Treatment	Number of panicles per plant	Weight of DHG per plant (g)	Weight of DMG per plant (g)	Weight of 100 grains (g)
T1	21.32^{bc}	15.04^{abc}	11.48^{abc}	2.23^{a}
T2	24.07^{c}	21.74^{c}	17.83^{c}	2.17^{a}
T3	9.20^{a}	5.86^{a}	4.35^{a}	2.29^{a}
T4	20.47b^{c}	11.22^{ab}	8.59^{ab}	1.54^{a}
T5	19.40b^{c}	13.64^{abc}	10.85^{abc}	2.31^{a}
T6	11.47^{a}	7.59^{a}	5.68^{a}	2.13^{a}
T7	22.67^{c}	20.04^{bc}	15.75^{bc}	2.22^{a}
T8	15.33^{ab}	14.53^{abc}	11.19^{abc}	2.28^{a}
T9	10.55^{a}	7.72^{a}	5.86^{a}	2.29^{a}

* The average number followed by superscript of the same letter in each column is not significantly different based on *Duncan's Multiple Range Test* (α = 0.05); DHG (dry harvested grain), DMG (dry milled grain).

The weight of DHG was significantly higher compared to plants cultivated with different growing media compositions with an average DHG < 20 g per plant. The percentage of grain weight loss from DHG to DMG for T2 was an average of 17.95%, lower than an average of 22.78% for the overall treatments. The lowest number of panicles (9 panicles per plant), the weight of DHG (5.86 g), the weight of DMG (4.53 g) were obtained when the plants were cultivated with a growing medium consisting of soil, biochar, and nano-silica (T3) that also produced the lowest number of tillers during the growth period. Such low values of tillers and panicles because the plants were not provided with synthetic fertilizer and urea as sources of nutrients during the growth period.

The weight of 100 grains for all treatments showed insignificant results, indicating that the different growing media compositions did not affect the overall grain size. The size of rice grains represented by the weight of 100 grains was more influenced by the genetic characteristics of the plant compared to the growing media composition used in this study. It has been reported that the variety of Inpago 7 should be able to produce ± 2.45 g per 100 grains (www.litbang.pertanian.go.id, 2002). The results obtained in this study were slightly lower because the plants were harvested after 100 days, 11 days sooner than the standard cultivation time for *O. sativa* var. Inpago 7 and consequently, the formation of the grains was not optimized.

Growing media that contain enough nutrients shows a better effect on the growth and productivity of *O. sativa* var. Inpago 7 in comparison to the plants that were cultivated with growing media that had not sufficient nitrogen, phosphorus, and potassium elements. In addition, the application of sprayed nano-silica also promotes better plant growth and increases biomass productivity. This is in line with the previous study by Marxen that the weight of rice grains for plants provided with 17.3 milligrams of silica per hectare increased by 35% compared to the plants that were not provided with silica (Marxen & Klotzbucher, 2016). The plants could absorb 37% of the provided silica (0.4 mg Si ha^{-1}) through their straws, positively affecting DHG and DMG. Increasing of biomass and yield on growing media with added biochar in line with the previous study by Jeffery et al. that addition biochar in soil or growing media can increased crop growth, thus significantly enhancing crop production in terms of either yield or biomass (Jeffery et al., 2011), and according to research conducted by Chen et al., who found that biochar increased grain per panicle through promoting rice grain (Chen et al., 2013). The potential improvement in crop yield from the addition of biochar has been explained by several mechanism, such as improved soil properties, favourable surroundings of root growth and modified soil nutrient status (Si et al., 2018).

According to Menna et al. Si fertilizer are applied in several crops for increased productivity and sustainable production. Addition of silica have suggested growth effect which increased biomass, yield, pollination, and yield parameters. It boosted up crop growth and accumulation of more photo-assimilates from source to sink and consequently, it led to higher grain yield (Meena et al., 2014). Ahmed et al. found that the increase in Si leads to increase in leaf area index, specific leaf weight, chlorophyl content, leaf dry weight, root dry weight, total dry weight and remarkable decrease in leaf water potential and shoot to root ratio (Ahmed et al., 2011). Due to a synergistic effect, applied Si has the potential to raise the optimum rate of N, thus helping to enhance yields. Si plays an important role in hull formation in rice and grain quality. Si deficiency always reduces the number of panicles per square meter and percentages of filled grains. It has significant effect on the percentage of filled spikelet and the number of spikelet per panicle, most carbon in the rice grain comes from photoassimilate produced in leaves (especially the flag leaf) during the grain-filling period (Detmann et al., 2012). Barley grains that were harvested from a silicon-fertilized plot had better germination than grains form no silicon fertilizer applied plots. Application of Si fertilizer accelerated citrus growth by 30–80%, speeded up fruit maturation by 2–4 weeks, and increased quantity. Sorghum also accumulates silica in the phytoliths. It enhances the crop quality, yield, growth and protects the plant from various biotic and abiotic hurdles (Meena et al., 2014).

CONCLUSIONS

In brief, growing media composition influenced the growth and productivity of *O. sativa* var. Inpago 7. The highest number of panicles, the weight of dry harvested grain, and dry milled grain were obtained when the plants were cultivated with a growing medium containing soil, biochar, synthetic fertilizer, urea, and nano-silica that also produced the highest number of leaves and tillers during the growth period (15 days, 30 days, and 45 days). The results showed that the highest number of panicles (24 panicles per plant), the weight of dry harvested grain (21.74 g per plant), and weight of dry milled grain (17.83 g per plant) were obtained when cultivated with a growing medium containing soil, biochar, synthetic fertilizer, urea and sprayed with nano-silica. Nevertheless, the weight of 100 grains was not influenced by the growing media composition, which highlights that the overall grain size was more influenced by the genetic characteristics of the plant compared to the growing media composition used in this study. An appropriate combination of soil, biochar, synthetic fertilizer, urea, and sprayed with nano-silica is recommended as a growing media for cultivating *O. sativa* L. var. Inpago 7.

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