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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.

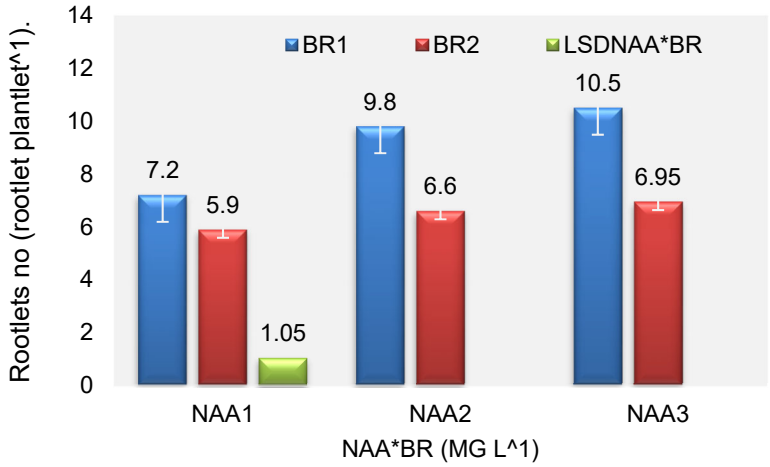

Figure 1. Effect of different concentrations of NAA and BR on the average number of rootlets of Chia plantlets in vitro.

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 $rootlet^{-1}$.

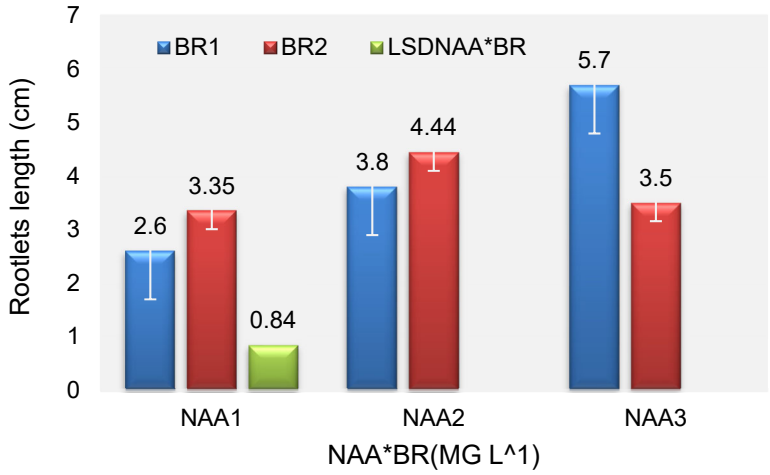

Figure 2. Effect of different concentrations of NAA and BR on the average rootlet length of Chia plants in vitro.

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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