

Influence of inoculation and phosphorus regimes on symbiotic nitrogen fixation and phosphorus use efficiency of Algerian cowpea (*Vigna unguiculata* L. (Walp.)) landraces

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Abstract. To study the genotypic variation of cowpeas on plant growth and phosphorus (P) uptake which is a function of different P regimes in the soil rhizosphere, 6 landraces of cowpea commonly found in northern Algeria (NE4, NE10, NE11, NKT5, NKT7, NKB7) and 4 landraces from Sahara in southern Algeria (NAG4, NAG5, NAT2 and ND3) were studied during 2013–2014 in greenhouse. They were inoculated with *Mesorhizobium* sp. (S1), *Bradyrhizobium* sp. (S2) and co-inoculation (S1–S2) under three P treatments: no P supply (P0), soluble P (PP) and insoluble P (TCP). Only varieties commonly found in northern Algeria nodulated with soluble P (PP) and inoculation containing *Mesorhizobium* sp. (S1). As a result of the symbiosis, the use of S1 has significantly increased shoot dry weight by 22%, total P content by 20% and P use efficiency for symbiotic nitrogen fixation by 18% compared to no inoculation (T). The landraces from the northern of Algeria expressed a higher growth than those from Sahara in the south of Algeria, especially three of them (NE4, NE10 and NE11) who showed a high performance under all P regimes. We suggest that these three landraces may be useful for improving symbiotic nitrogen fixation in cowpeas when growth is limited by low-P soils and that they could contribute to sustainable farming systems through reduction of farmer's dependence on fertilizers.

Key words: Cowpea, Landraces, Nitrogen fixation, Phosphorus, Rhizobia, Symbiosis.

INTRODUCTION

Legumes as a source of symbiotic fixation of atmospheric nitrogen play a major environmental role in cropping systems, which may save nitrogen fertilizer (Graham, 2008). Cowpea (*Vigna unguiculata* L. (Walp.)) is an important leguminous crop that is used for live stocks as feed, as green vegetables, as well as dry beans for human consumption (Murillo–Amador et al., 2006; Goenaga et al., 2008). In Algeria cowpea is mainly consumed for home consumption and subsistence and considered to have therapeutic properties.

Symbiotic nitrogen fixation (SNF) by legume symbionts, or the ability to convert di-nitrogen to ammonia, occurs only within nodules, which are formed specifically in response to the bacterial symbionts (Beattie, 2007). In addition, SNF is highly sensitive to environmental stresses, such as phosphorus deficiencies (Drevon & Hartwig, 1997). The P requirement of legumes is increased when the legume is dependent on N₂ fixation (Leidi & Rodriguez–Navarro, 2000; Gentili & Huss–Danell, 2003). Nodule growth is

more sensitive to P deficiency than plant growth (Drevon & Hartwig, 1997). Thus, P deficiency leads to nodulation delay (Kouas et al., 2005). Indeed, SNF is an energetically expensive process which requires more inorganic P than mineral nitrogen assimilation (Vadez & Drevon, 2001).

Phosphorus (P) is one of the most important plant growth-limiting nutrient in soils besides nitrogen (Deubel & Merbach, 2005; Richardson et al., 2009). Low phosphorus fertility of the soils may be due to low total P content, particularly in soils with low organic matter content. The low availability of P is often due to its insoluble association with such cations as Ca, Al or Fe and its adsorption on surfaces of mineral phases (Alkama et al., 2012).

Ghalmi et al. (2010) identified the various landraces of cowpea cultivated in Algeria, mainly from two regions: Kabylia in northern Algeria (Tizi-Ouzou) and Saharian oases (El Gole'a, Adrar) in southern Algeria. They identified two different cultivar groups of cowpea: Melanophthalmus in Kabylia and Biflora in the Sahara.

Our investigation is the first study to determinate if the geographical origin of accession affects the phosphorus use efficiency (PUE) in Algerian cowpeas. The purpose of the present work was firstly to characterize different cowpea landraces for their ability to establish a symbiosis with rhizobia by checking the effectiveness and infectivity of the strains used, and secondly to investigate their efficiency in nitrogen fixation, and their tolerance to P deficiency in order to reduce production costs and dependence of farmers on soil fertilizers.

MATERIALS AND METHODS

The present study was conducted during 2013–2014 in pots in a greenhouse at the Experimental Station of ENSA (Ecole Nationale Supérieure Agronomique), Algiers, (36° 30. 50'N). It included ten landraces of cowpeas (*Vigna unguiculata* (L.) (Walp.)) obtained from collection of ENSA (Ghalmi et al., 2010). Seeds characteristics are given in Table 1.

Table 1. Characters of Algerian cowpea accessions

Landraces	Geographical region	SC	EC	SSH	ST	SL	WHS
NKB7	Bejaia, North	Holstein	Absent	Kidney	Smooth	0.9	23.46
NKT7	Tizi Ouzou, North	Cream	Black	Kidney	Smooth	1.2	27.34
NKT5	Tizi Ouzou, North	Black	Absent	Ovoid	Smooth	0.5	7.83
NAG5	Adrar, Oasis	Black	Black	Globose	Rough	0.5	14.38
NAG4	Adrar, Oasis	Black	Absent	Ovoid	Smooth	0.4	9.49
NAT2	Adrar, Oasis	Brown	Absent	Ovoid	Smooth	0.4	10.82
NE4	El Kala, North	Cream	Black	Globose	Smooth	0.5	14.87
NE10	El Kala, North	Brown	Absent	Ovoid	Smooth	0.5	13.43
NE11	El Kala, North	Black	Absent	Ovoid	Smooth	0.6	15.33
ND3	Djanet, Oasis	Cream	Absent	Rhomboid	Smooth	0.4	10.1

SC Seed Color, EC Eye Color, SSH Seed Shape, ST Seed texture, SL Seed Length (cm) WHS Weight of Hundred Seeds (g).

Soil

The soil (obtained from the Institut Technique des Cultures Maraîchères Industrielles' experimental station, Algiers (36° 45. 24.3'N)) was selected for its low phosphorus content. It was sterilized and divided into 480 sterile pots with a capacity of 10 kg.

The soil has undergone physical–chemical analysis: (granulometry, pH, and electrical conductivity (CE), CaCO₃ content, Total–N, Total–P and Olsen–P), granulometrical and chemical soil properties of experimental soil are given in Table 2.

Table 2. Granulometrical and chemical soil properties

Clay %	Loam %	Sandy %	pH	CE Ms cm ⁻¹	CaCO ₃ %	Total–N g kg ⁻¹	Total–P mg kg ⁻¹	Olsen–P mg kg ⁻¹
20.3	21.5	58.2	7.1	3.725	13.54	0.7	235	20.3

Thus, Olsen–P value reveals that the initial soil (P0 regime) without P input has a low available P rate; 10 times lower than the total P content. This is related to the fact that the soil is calcareous and that much of the total P can be precipitated. The mechanisms of precipitation of P in calcareous soil have been studied previously by Tunesi et al. (1999). In this case P0 is considered as deficient P.

Bacteria

For this study, two isolates of bacteria (a fast–growing strain as *Mesorhizobium sp.* (S1), and slow–growing strain as *Bradyrhizobium sp.* (S2)) were isolated from northern Algeria' cowpea nodules and obtained from collection of ENSA, Algiers, Algeria. Isolates were cultivated on Yeast Extract Agar medium (YEAM), inoculums were prepared in YEAM without addition of Agar.

Culture conditions and experimental device

The ten landraces were grown under three different P regimes (deficient P (P0), soluble inorganic P as KH₂PO₄ (PP) 0.2 g per 10 kg of soil and insoluble Tricalcium phosphate as Ca₃ (PO₄)₂, 6H₂O (TCP) 0.7 g per 10 kg of soil). These P regimes were combined with four inoculation types (control without inoculant (T), inoculated with *Mesorhizobium sp.* (S1), *Bradyrhizobium sp.* (S2) or doubly inoculated (S1–S2)). The experiment was organized into a split–plot design with four replications for each combination of landraces, P regime and inoculation type, providing the 480 pots (10 x 3 x 4 x 4). The seeds of cowpeas were surface–sterilized using absolute ethanol for 10 seconds followed by the sodium hypochlorite at 12 ° for 5 minutes; followed by 10 washes in sterile distilled water, and then sown. The seedlings were inoculated by covering with 1ml of bacterial culture containing 10⁸bacteria mL⁻¹ and 10⁹bacteria mL⁻¹ respectively for *Mesorhizobium sp.* and *Bradyrhizobium sp.*

Data collection

At full flowering stage, which is spread out according to the precocity of the landraces, shoots were separated from roots at the cotyledon node, dried for 48 h at 75 °C and weights recorded. However, for landraces which has nodulated, nodules were detached from the roots, counted, dried and dry weight determined.

Total P concentration

Plant P uptake corresponded to the total P taken up by plants during plant growth. Total P concentration in plant (shoot, root and nodules) was determined by green malachite method after digestion by nitric and perchloric acids according to Valizadeh et al. (2003). It was calculated as follows: P uptake (total P content) = [P concentration in shoot (mg g^{-1}) \times shoot dry weight (g)] + [P concentration in root (mg g^{-1}) \times root dry weight (g)] + [P concentration in nodule (mg g^{-1}) \times nodule dry weight (g)] / 1,000.

Phosphorus use efficiency (PUE)

PUE represents the efficiency of use of phosphorus by the plant. There are several ways to design and calculate PUE, from physiological point of view to agronomic point of view (Baligar et al., 2011; MacDonald et al., 2011). In this study, we chose to calculate a physiological PUE which is the ratio between the dry weight of the whole plant and the concentration of phosphorus in the plant (Vadez & Drevon, 2001).

Statistical analyzes

Effects of inoculation and P nutrition on plant growth, nodulation, P concentration in plant and plant P uptake were tested using multi-way analyses of variance (ANOVA) with R software, version 3.0.2 (R Core Team, 2013), using Rcmdr package (Fox, 2005) and RcmdrPlugin.EZR (Kanda, 2013), considering landraces, inoculation treatments and soil P regimes as factors. The means were compared by Tukey's multiple comparison test at 0.05 probability. Graphics were performed using RcmdrPlugin.FactoMineR (Husson et al., 2014) and RcmdrPlugin.KMggplot2 (Triad & Kengo, 2013).

RESULTS AND DISCUSSION

Plant Growth expression under different P regimes and inoculation types

In order to assess the effects of P deficiency on growth and SNF, the dry weight of the different landraces was evaluated considering shoot measurement under different P regimes and different inoculation types (Fig. 1). The ANOVA results of shoot dry weight are reported in Table 3.

Table 3. ANOVA results of sDW

	Sum Sq	Df	F value	Pr(> F)
Factor1.P regime	319.6	2	293.8886	< 2.2e-16 ***
Factor2.Landraces	10.6	9	2.1617	0.024105 *
Factor3.Inoculation	68.8	3	42.2055	< 2.2e-16 ***
Factor1 X Factor2	28.1	18	2.8712	9.461e-05 ***
Factor1 X Factor3	12.0	6	3.6657	0.001512 **
Factor2 X Factor3	16.4	27	1.1156	0.317789
Factor1XFactor2XFactor3	19.9	54	0.6771	0.959624
Residuals	195.7	360		

, * Significant difference at $p < 0.01$ and $p < 0.001$, respectively.

The results showed that the P regime, landraces and the inoculation type significantly affected the shoot dry weight (sDW). There were also interaction effects between P regime and landraces and P regime and inoculation type.

sDW varied between 1.4 and 3.2 g sDW pl⁻¹ for NKT5 and NE11 without inoculation (Fig. 1, T), between 1.2 and 3.1 g sDW pl⁻¹ for NAG5 and NE11 for inoculation S2 (Fig. 1, S2), between 2 and 4.5 g sDW pl⁻¹ for NAG4 and NE10 for inoculation S1 (Fig. 1, S1) and between 1.8 and 3.3 g sDW pl⁻¹ for NAG5 and NE4 for co-inoculation S1–S2 (Fig. 1, S1–S2). Landraces NE10 and NE4 expressed the highest production potential with more than 4 g sDW pl⁻¹ under soluble P regime (PP). Growth under TCP varied between 1.3 pl⁻¹ for landraces from Saharian region and 3 g sDW pl⁻¹ for landraces from the north of Algeria. For all P regimes, the highest sDW were found for the landraces NE4, NE11, and NE10, from the north of Algeria.

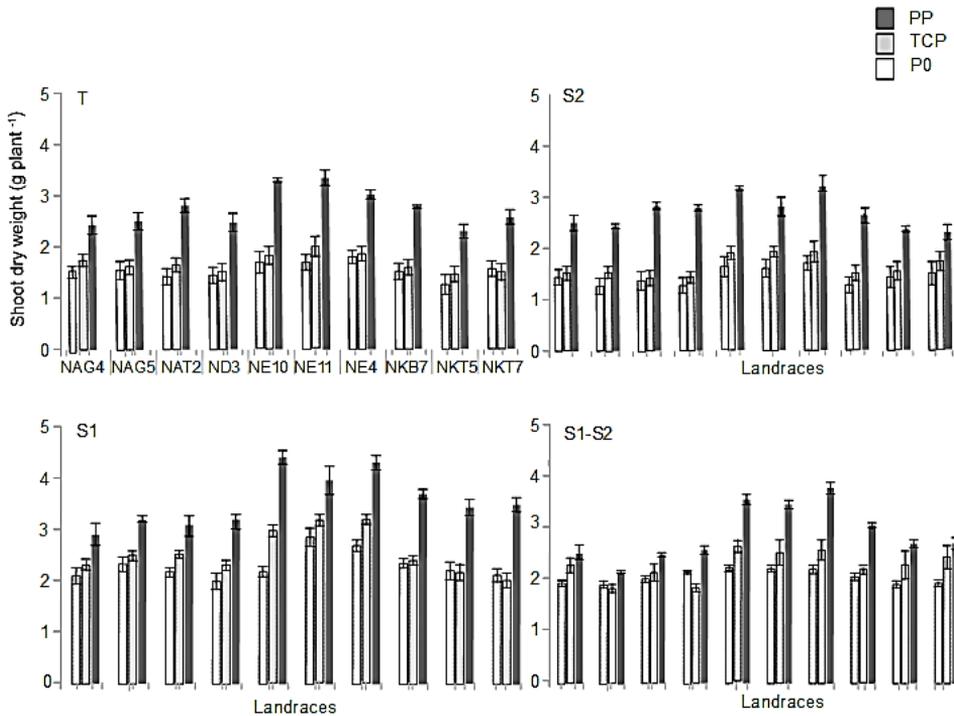


Figure 1. Effect of P regimes on shoot dry weight for landraces under control without inoculation (T), inoculation with (S2), inoculation with (S1) and co-inoculation (S1–S2). Data are means and SD of 4 harvested plants.

As indicated by the ANOVA in Table 3 and by Fig. 2, a, P regimes and inoculation significantly affected the shoot biomass. For a given inoculation type, the soluble P regime (PP) resulted in a significant increase in the shoot biomass compared to insoluble P (TCP) and deficient P (P0) regimes. Also, for a given P regime, the shoot dry weight significantly increased with inoculation with the fast growing strain *Mesorhizobium sp.* (S1) or S1–S2, compared to no inoculation (T) or inoculation with the slow growing strain *Bradyrhizobium sp.* (S2). Then, inoculation S1 increased shoot dry weight not only for PP regime but also for TCP and P0 regimes by 22% and 30% respectively (Fig. 2, a)

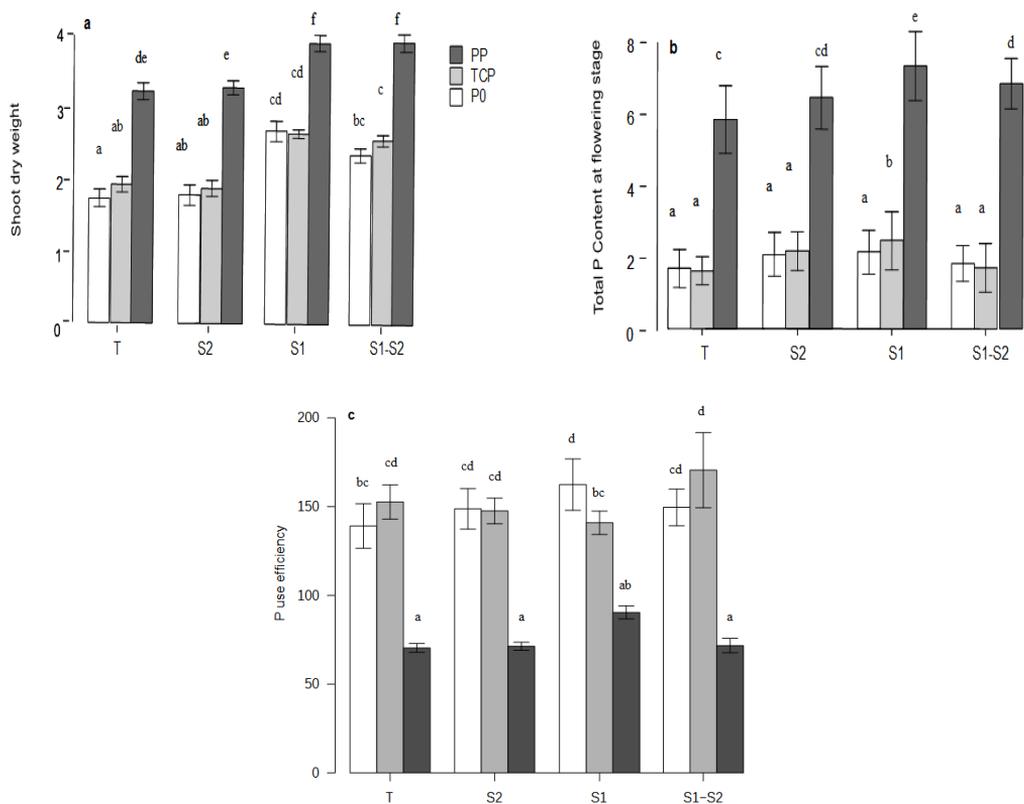


Figure 2. Effect of P regimes (P0, TCP and PP) and inoculation types (T, S2, S1 and S1–S2) on shoot dry weight (g plant⁻¹) (a), on total P content (mg P g plant⁻¹ DW) (b) and on P use efficiency (P⁻² dry weight mg⁻¹ P) (c). Values are the means of 40 replicates. Bars indicate standard errors. Mean values labelled with the same letter were not significantly different at $p < 0.05$.

Variability in phosphorus use efficiency (PUE) response for SNF

In order to evaluate the PUE of landraces, a first evaluation of the impact of P deficiency was performed by measuring the total P content of the plants at flowering stage.

Table 4 shows the ANOVA results for total P content in the plants. This P content was significantly influenced by the P regime, the inoculation type, and the landraces, with also an interaction between P regime and inoculation. Under soluble P regime (PP), total P content increased, by more than 20% and 16% respectively for inoculation with S1 and co-inoculation with S1–S2 compared to no inoculation (T) (Fig. 2, b). Under insoluble P regime (TCP), inoculation with S1 significantly increased P content by 20% compared to the other inoculation types (Fig. 2, b).

Furthermore, in response to P deficiency, plants can adjust their internal P requirement by optimizing the metabolic phosphorus use efficiency (PUE) by producing overall more biomass per unit of P consumed, which in fixing more N₂ per unit of P for a SNF-dependent legume (Vadez & Drevon, 2001). In Table 5 are reported the ANOVA results of physiological PUE.

From this ANOVA, P regimes and inoculation type significantly affected the P use efficiency. There was also an interaction between P regimes and inoculation type.

The inoculation with *Mesorhizobium sp.* (S1) significantly increased the PUE by about 8% and 11% respectively for soluble P (PP) and (P0) compared to other inoculation type (Fig. 2, c). Whereas, the co-inoculation S1–S2 significantly increased the PUE by more than 10% under insoluble P. (Fig. 2, c) shows a strong decrease of PUE for PP regime compared to P0 regime and TCP regime.

Table 4. ANOVA results of total P content

	Sum Sq	Df	F value	Pr(>F)
Factor1.P regime	2294.8	2	2175.1601	< 2.2e-16 ***
Factor2.Landraces	10.2	9	2.1409	0.02561 *
Factor3.Inoculation	35.1	3	22.1865	3.327e-13 ***
Factor1 X Factor2	3.8	18	0.4038	0.98678
Factor1 X Factor3	37.1	6	11.7201	5.245e-12 ***
Factor2 X Factor3	8.7	27	0.6078	0.94052
Factor1XFactor2 XFactor3	16.6	54	0.5835	0.99151
Residuals	189.9	360		

*, *** Significant difference at $p < 0.05$ and $p < 0.001$, respectively.

Table 5. ANOVA results of PUE

	Sum Sq	Df	F value	Pr(>F)
Factor1.P regime	554021	2	60.6961	< 2e-16 ***
Factor2.Landraces	78297	9	1.9062	0.04996 *
Factor3.Inoculation	9845	3	0.7191	0.54111
Factor1 X Factor2	39945	18	0.4862	0.96337
Factor1 X Factor3	64143	6	2.3424	0.03120 *
Factor2 X Factor3	77921	27	0.6324	0.92467
Factor1XFactor2 XFactor3	96135	54	0.3901	0.99997
Residuals	1642999	360		

*, **, *** Significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Plant nodulation

Dry matter and number of nodules were evaluated in the different landraces (Fig. 3).

No nodules were found without supply of P (P0) and with supply of insoluble P (TCP P regime), and nodulation was only observed under soluble P regime (PP). This shows that nodulation does not occur in P deficiency conditions.

The landraces and the inoculation type affected significantly the nodule dry weight (nDW) as indicated by the ANOVA results of nodule dry weight for the 6 landraces having nodules under PP regime (Table 6).

Table 6. ANOVA results of nodule dry weight

	Sum Sq	Df	F value	Pr(>F)
Factor1.Landraces	0.5913	9	2.5197	0.008013 **
Factor2.inoculation	0.7487	3	9.5710	3.912e-06 ***
Factor1 X Factor2	0.8432	27	1.1976	0.229169
Residuals	11.4732	440		

, * Significant difference at $p < 0.01$ and $p < 0.001$, respectively.

The nodulation only occurred for landraces from the north of Algeria, namely; NE10, NE11, NE4, NKT7, NKB7, and NKT5, and for the inoculations including *Mesorhizobium sp.* (S1 or S1–S2). For these northern landraces nodule dry weight nDW was found to vary between 50 and 180 mg nDW pl^{-1} for double inoculation with S1–S2 and between 80 and 230 mg nDW pl^{-1} for inoculation with S1 (Fig. 3, a).

Within the landraces that provided nodules, the nodule number was also influenced by the inoculation type and the cowpea landraces as indicated by Fig. 3, b, for inoculation with S1, the 6 landraces showed high nDW for S1. On the other hand, for double inoculation S1–S2, NE10, NE4 NKB7, and NKT7 significantly showed a higher nodule number than NE11 and NKT5.

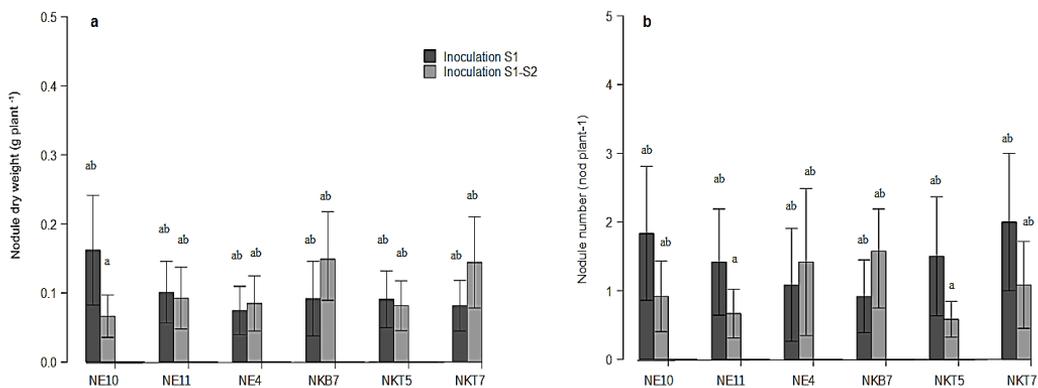


Figure 3. Effect of landraces and inoculation on nodule dry weight ($g\ plant^{-1}$) (a) and nodule number (b) for PP regime. Values are the means of 4 replicates. Bars indicate standard errors. Mean values labeled with the same letter were not significantly different at $p < 0.05$.

Efficiency in use of the rhizobial symbiosis for plant growth

In order to assess the efficiency in use of the rhizobial symbiosis (EURS), the values of the shoot biomass were plotted against their corresponding nodule biomass. The slopes of the regressions were considered as an estimate of the EURS. This was achieved under PP regime (the only one having provided nodules) with S1 inoculation type (Fig. 4, a) and co-inoculation S1–S2 (Fig. 4, b). We first proceeded by grouping together all the landraces having provided nodules (i.e the northern landraces). Then we proceeded for each landrace. Fig. 4, a shows, for all the northern landraces together, that shoot and nodule biomass of northern landraces were positively correlated (up to $R^2 = 0.77$) under inoculation type S1. Whereas Fig. 4, b shows a positive correlation between shoot and nodule biomass up to $R^2 = 0.46$. However, by considering landraces individually, only landrace NE10 showed a positive correlation between sDW and nDW under inoculation S1 with $R^2 = 0.81$ (Fig. 5).

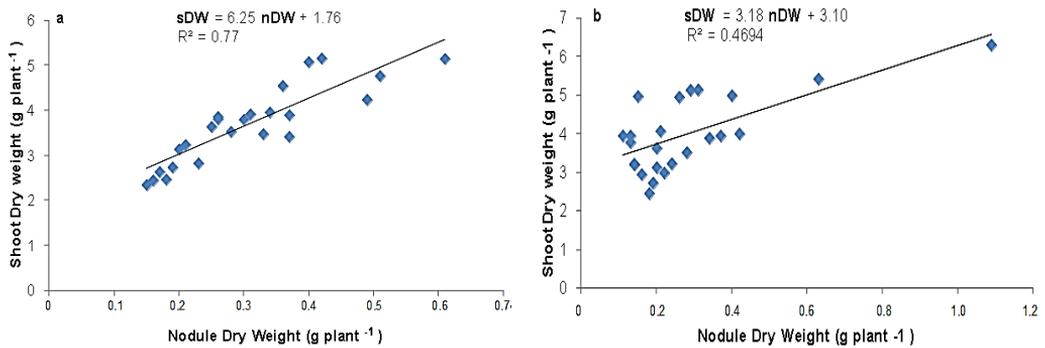


Figure 4. Global relationship between nodule dry weight and shoot dry weight for northern landraces under PP regime for inoculation S1(a) and co-inoculation S1–S2 (b). Data are means standard errors of 24 replicates.

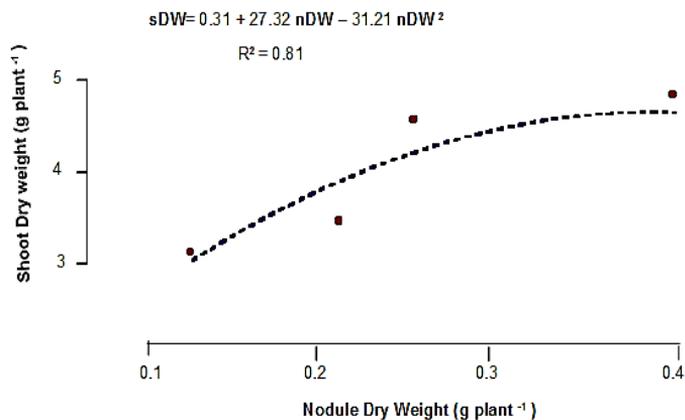


Figure 5. Correlation between nodule dry weight and shoot dry weight for NE10 under inoculation S1 and PP regime. Data are means standard errors of 4 replicates.

GENERAL DISCUSSION

The aim of this research was to investigate the ability of ten different cowpea landraces to establish a symbiosis with rhizobia and their efficiency in nitrogen fixation. Nodulation did not occur for all landraces, the process was observed only for northern landraces under PP regime and inoculations including S1 (Fig. 3). Thus, *Mesorhizobium sp.* (S1) was better at infecting than *Bradyrhizobium sp.* (S2). The lack of infectivity of S2 can be explained by the fact that legume root nodules may contain a range of bacteria that are unable to nodulate their hosts (Sprent, 2009).

Depret & Laguerre (2008), reported that different rhizobial genotypes may be better at infecting legume roots and producing effective nodules at different stages of host development. However, the nodulation for SNF with co-inoculation S1–S2 for PP showed a slight decrease compared to S1, which may have resulted in competition established between both of rhizobia strains. Wielbo et al. (2007) demonstrated that

competitiveness is affected by edaphic factors and soil nutrients, in this case by P availability.

No nodulation of *Mesorhizobium sp.* (S1) under TCP and P0 may be explained by a low tolerance to low levels of P for the fast-growing strains (Raman et al., 2006).

Landraces from oasis in Saharian region showed no nodulation with the two rhizobial strains that we tested so low SNF capacity. This might be attributed to their original domestication promoting a high level of specificity for the symbiosis rhizobia-cowpea genotype. In fact S1 and S2 are strains isolated from northern landraces, where they are adapted.

The nodulation and as a result, the SNF in the cowpea landraces, was found to be influenced by P deficiency. Thus, P deficiency particularly affects the rhizobial symbiosis (Attar, 2014). In fact, P appears to be essential for both nodulation and nitrogen fixation (Rai, 2006).

The increased plant growth under PP shown in Fig. 1 is likely attributed to enhanced P nutrition (Fig. 1). In addition to nitrogen-fixation, enhancement of legume symbioses is often manifested by an increase in the phosphorus content of the plant (Vessey, 2003).

The increase of shoot dry weight with S1 under P0 and TCP could result from a phosphatase release by S1, which can contribute to increase P availability to the plant. This could explain the increase of P content observed with S1 under P0 and TCP (cf. Fig. 2 b).

The greater sDW for NE4, NE11, and NE10 landraces under all P regimes compared to others landraces may be related to enhanced phosphorus use efficiency. In fact, one of the strategies employed by plants in response to P deficiency consists in optimizing the metabolic phosphorus use efficiency (PUE). This is achieved by producing higher overall biomass per unit of P consumed, through fixing more N₂ per unit of P for an SNF-dependent legume (Vadez & Drevon, 2001).

Our findings presented in figure 4 also revealed a positive correlation for inoculation S1 and co-inoculation (S1-S2) under PP regime between the biomass of symbiotic nodules nDW and the shoot dry weight sDW. This correlation may indicate that the symbiotic nitrogen fixation is effective under P sufficient conditions. The increase in EURS suggests high regulation between EURS and the plant P requirement, probably in relation to the high energy requirement of the SNF process (Lazali et al., 2013). This finding is in agreement with previously published data (Attar, 2014).

CONCLUSIONS

The presented research was focused on the influence of inoculation and phosphorus regimes on symbiotic nitrogen fixation and phosphorus use efficiency of Algerian cowpea landraces. It was determined that:

- Soluble P enhances the growth of plants, their total P content and nodulation.
- The fast-growing *Mesorhizobium sp.* (S1) is better at infecting and effectiveness than the slow-growing *Bradyrhizobium sp.* (S2).
- S1 increased shoot dry weight under P0 and insoluble P (TCP). This could be due to releasing of phosphatase by S1, which can contribute to enhance P availability.

- Only northern landraces showed nodulation with soluble P regime and inoculations with fast growing strains *Mesorhizobium* sp. (S1). As the soil was sterilised before inoculation and as the tested strains came also from the northern region, this suggests that there are probably regional adaptations between the rhizobia strains and the cowpea landraces.
- The strain S1 significantly increased the phosphorus use efficiency by 8% under P soluble conditions.
- The landraces NE4, NE11 and NE10 exhibited a higher performance under all P regimes with S1 inoculation. Therefore, these landraces may be used as a source of genetic diversity for cowpea production to improve PUE for SNF potential, and their adaptation to the climatic conditions of the South could be tested.

However, further studies on field experiments are needed to identify whether similar results may be observed with a more extensive array of cowpea landraces and rhizobia potentially adapted to southern landraces whether their potential differences in PUE are associated with genotype–rhizobia interaction in adaptation to low–P soils.

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