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Effect of phosphorus deficiency induced in calcareous soil on plant growth, phosphorus use efficiency and acid phosphatase activity of *Medicago truncatula.*

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Abstract

Calcareous soils, characterized by a higher pH, are frequent in the North West of Tunisia. Large concentrations of calcium carbonate in calcareous soils result in accumulation of high levels of bicarbonate ions, which complex with phosphate, resulting in phosphorus deficiency (induced P deficiency) for plants. The impact of calcareous soil on plant growth, photosynthetic activity and acid phosphatase activity was explored in two lines of *Medicago truncatula*: TN6.18 and Jemalong. Calcareous soil significantly restricted shoot growth only in Jemalong (-45 % of the control). When grown on calcareous soil, root length was stimulated, this effect being more pronounced in TN6.18. Under calcareous soil, net CO_2 assimilation declined more in Jemalong (-40 % of the control) than in TN6.18 (-20 % of the control). CO_2 accumulation was increased in Jemalong (+35% of the control) plants grown in calcareous soil. The acid phosphatase activity was higher in plants cultivated under calcareous soil. This increased phosphatase activity was more pronounced in TN6.18, which showed higher accumulation of Pi in shoots and roots than Jemalolng. In the light of these results, the present study proposes acid phosphatase as a useful candidate for improving Pi acquisition and utilization under calcareous soil.

1. INTRODUCTION

Calcareous soils contain high levels of calcium carbonate (CaCO3) that affects soil properties related to plant growth, such as soil water relations and the availability of plant nutrients. They are common in the arid areas of the earth (FAO, 2016), occupying more than 30% of the earth's surface, and their CaCO3 content varies from just detectable up to 95% (Marschner, 1995). Calcareous soils, characterized by a high pH, are frequent in the Mediterranean area, including Tunisia. Large concentrations of calcium carbonate in calcareous soils result in accumulation of high levels of bicarbonate ions, which complex with phosphate, resulting in phosphorus deficiency (induced P deficiency) for plants. P deficiency is more critical in highly withered soils of tropics and subtropics, as well as in calcareous/alkaline soils of the Mediterranean basin (Hinsinger 2001). According to Kouas et al (2005), P limiting restricted plant growth and symbiotic nitrogen fixation in common bean. Phosphorus plays an important role in an array of cellular processes, including maintenance of membrane structures, synthesis of biomolecules and formation of high-energy molecules. It also helps in cell division,

enzyme activation/inactivation and carbohydrate metabolism (Razaq et al. 2017). Plants are known to involve several mechanisms to increase their P absorption efficiency, such as the modification of soil exploration by roots to increase the P absorption area (Kouas et al., 2009, De Souza Campos et al., 2019). For instance, phosphorus deficiency in the soil induces various morphological changes in plant roots, including the formation of root hairs (Zhou et al., 2016; Mo et al., 2019). In addition to alterations in root architecture, other changes include acidification of the rhizosphere, exudation of low molecular weight organic acids, and secretion of acid phosphatases and photosynthesis-related enzymes (Kouas et al., 2009, Vengavasi and Pandey 2016). Acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) can hydrolyse a range of organic P compounds (Zhu et al., 2020), and these enzymes are more abundant in the rhizosphere when plants are P-starved (Pang et al., 2018, Touhami et al, 2020). The production of phosphatase is a potential way for plants to enhance P availability, as a large proportion of soil P (up to 80%) occurs in organic forms (Richardson et al. 2001).

Medicago truncatula is a model plant widely used to study genomics of legume plants because of its small diploid genome size and relatively easy transformation (Wang et al. 2011). There are different ecotypes (lines) of M. truncatula with wide genetic variations (Ellwood et al. 2006). In the present study, we compared the effect of calcareous soil on the growth, photosynthetic parameters and acid phosphatase activity in two lines of M. truncatula: Jemalong and TN6.18.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

This study focused on two lines of *M. truncatula* one hand, the line TN6.18 is native to Thela (North West of Tunisia), a region where calcareous soil is frequent. On the other hand, the line Jemalong (from Australia) is frequently used as a reference line by the scientific community working on *M. truncatula*. Seeds were scarified and sown individually in 4 L plastic pots filled with 4.5 kg of a fine, mixed calcareous (sampled in the region of Hammamet, Tunisia) or non-calcareous soil (sampled in the region of Soliman, Tunisia). The culture was carried out in a glasshouse under natural light. Soil moisture was maintained close to field capacity. The main soils characteristics are given in Table 1. At the beginning of flowering, 90 days after germination, plants were harvested, and separated into shoots and roots.

Table 1. Main characteristics of soil used to test sensitivity to lime

Main characteristics	Non calcareous soil	Calcareous soil
Sand (%)	77.50	62.00
Silt (%)	18.20	22.50
Clay (%)	4.00	14.00
pH	7.00	8.50
Total carbonates (%)	7.30	38.00
Active lime (%)	2.50	13.50
Organic matter (%)	1.04	0.76
C (%)	0.60	0.61
N (mg g^{-1})	1.87	1.64
P (g g^{-1})	0.24	0.19
K (mg g^{-1})	95.00	82.00

2.2 Leaf Gas Exchange and Chlorophyll Content

Net CO_2 assimilation and stomatal conductance were recorded with a portable photosynthesis system (LCA4). Measurement conditions were as follows: 1200 µmol mm^{-2} s^{-1} photosynthetically active radiation (PAR), 378 µmol mol^{-1} ambient CO_2 concentration and 28±2 °C leaf temperatures. Measurements were carried out in the morning between 10:00 and 12:00 (10 replicates per treatment). Data were automatically collected every minute after the photosynthesis rate had stabilized. Leaf chlorophyll content was spectrophotometrically determined according to Torrecillas et al (1984) from 100 mg fresh leaf tissues extracted in dark for 72 h in 80 % acetone. Extract absorbance was measured at 649 and 665 nm.

2.3 Acid Phosphatase Assay

Leaves and roots (0.1 g) were ground separately in a mortar with an extraction mixture consisting of 0.1 M acetate buffer (1 $g.ml^{-1}$ buffer), 6 mM ß-mercaptoethanol, 0.1 mM phenyl methyl sulfonyl fluoride, and 6 g insoluble polyvinylpolypyrrolidone. The homogenate was centrifuged at 30,000 × *g* at 4°C for 30 min. The reaction mixture contained 100 mM sodium acetate buffer (pH5.8), 5 mM p-nitrophenyl phosphate, and the enzyme extract in a total volume of 0.5 ml. After 30-min incubation at 30°C, the reaction was stopped by

the addition of 1 ml 0.5 M NaOH. Acid phosphatase activity was measured at 405 nm by monitoring the p-nitrophenol released.

2.4 Pi Determination

Samples (25 mg DW of old leaves) from each plant were digested in HNO_3 (0.5%, w/v). The inorganic phosphorous released was quantified by the molybdovanadate method at 460 nm.

2.5 Statistical Analysis

A one-way analysis of variance, using the AV1W MSUSTAT program with orthogonal contrasts and mean comparison procedures, was performed to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD; $P<0.05$).

3. RESULTS

3.1 Effect of calcareous soil on Plant Growth

Under non-calcareous soil (control), shoot dry weight (DW) was higher in two lines of *M. truncatula* (Table 2), but this parameter was significantly restricted only in Jemalong when grown on calcareous soil (-45 % of the control). Under calcareous soil, root DW was not affected in both ecotypes, while the root length appeared to be stimulated, this effect being more pronounced in TN6.18 (+35% of the control).

Table 2. Effect of calcareous soil on shoot DW, root DW and root length of two lines of *M. truncatula,* Jemalong and TN6.18. For each parameter, values (means of 9 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

Main characteristics	TN6.18	Jemalong
Shoot DW g.plant^{-1}		
Non calcareous soil	0.94a	1.21b
Calcareous soil	0.85a	0.65c
Root DW g.plant^{-1}		
Non calcareous soil	0.074a	0.08a
Calcareous soil	0.089a	0.077a
Root length cm.plant^{-1}		
Non calcareous soil	21a	19a
Calcareous soil	32b	22a

Table 3. Effect of calcareous soil on total chlorophyll content, net CO_2 assimilation, stomatal conductance and the internal CO_2 concentration (Ci) of two lines of *M. truncatula,* Jemalong and TN6.18. For each parameter, values (means of 9 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

Main characteristics	TN6.18	Jemalong
Total chlorophyll (mg.g^{-1} FW)		
Non calcareous soil	1.65a	1.75a
Calcareous soil	1.38a	0.9c
Net CO2 assimilation, µmol m^{-2}s^{-1}		
Non calcareous soil	9.5a	10.5a
Calcareous soil	7.8a	6.5a
Stomatal conductance, mol m^{-2} s^{-1}		
Non calcareous soil	0.12a	0.14a
Calcareous soil	0.095b	0.06a
The internal CO2 concentration (Ci) µmol,mol^{-1}		
Non calcareous soil	229.2	215.3
Calcareous soil	243.5	2.95.8

3.2 Chlorophyll content and photosynthetic parameters

The results in Table 3 showed that total chlorophyll (Chl) content was higher in plants cultivated in non-calcareous soil than those in calcareous soil. Calcareous soil decreased this parameter in both lines, but this effect was more marked for Jemalong (-50 % of the control) than for TN6.18 (-18% of the control). Under calcareous soil conditions, the net CO_2 assimilation also declined (Fig 3b), this tendency being, however, more pronounced in Jemalong (-40 % of the control) than in TN6.18 (-20 % of the control). Regarding the stomatal conductance (gs), a similar behavior was observed compared to that of net CO_2 assimilation. The internal CO_2 concentration (Ci) was measured and the results showed that the plants cultivated in calcareous soil were characterized by higher accumulation of CO_2 compared to the control plants (Table 3), this Tendency was more pronounced in Jemalong (+30% of the control).

3.3 Phosphorus content

Plant P status was restricted by calcareous soil only in Jemalong (Fig. 1.). In this line, the reduction in P content ranged from (- 40%) for shoots and to (-30% of the control) in roots. Under the same conditions, TN6.18 accumulated more inorganic phosphorus in shoots than Jemalong.

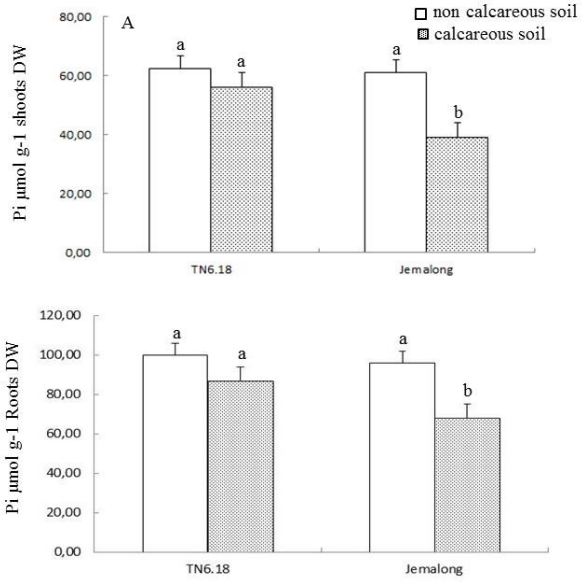

Fig. 1. Effect of calcareous soil on Pi concentration in shoots and roots of two lines of *M. truncatula,* Jemalong and TN6.18. For each parameter, values (means of 9 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

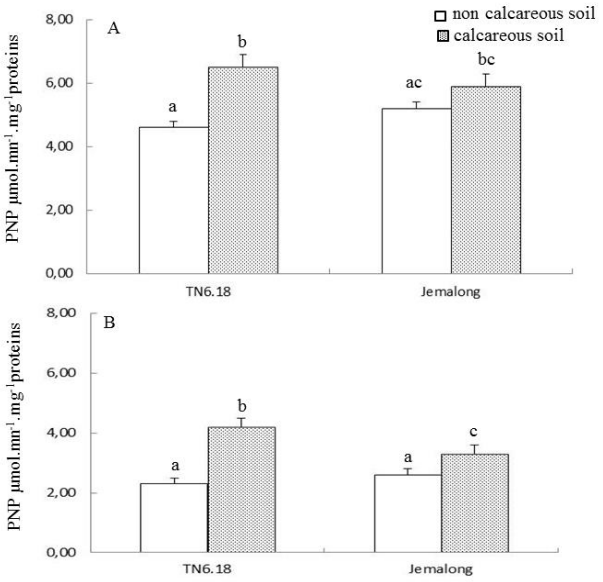

Fig. 2. Effect of calcareous soil on acid phosphatase activity in shoots (A) and roots (B) of two lines of *M. truncatula,* Jemalong and TN6.18. For each parameter, values (means of 4 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

3.4. Acid phosphatase activity

Regarding the mechanisms involved in the acquisition and use of P, the activity of acid phosphatase was measured (Fig. 2.). The calcareous soil increased acid phosphatase activity in leaves of TN6.18 (+ 30 % of the control). Under these same conditions, this parameter was increased in roots, this effect being more pronounced in TN6.18 (+45 % of the control) as compared to Jemalong (+22 % of the control).

4. DISCUSSION

Our study showed that for Jemalong compared to TN6.18, the plant growth was significantly decreased in plants grown in the calcareous soil compared with those cultivated under non-calcareous soil. Some studies have shown that phosphorus deficiency has a depressive effect on leaf emergence and expansion, most likely due to the key role played by P on vacuolar Pi reserves in creating the cellular osmotic potential that constitutes a favorable condition for conservation, cell turgor and auxes (Talbi et al., 2015). Given that P is a component of phospholipids, the low availability of such element would limit membrane development and cell growth. Likewise, with the decrease in plant growth for Jemalong grown in calcareous soil, we also noticed a significant reduction in net CO_2 assimilation and stomatal conductance. The result showed that reduction in growth could result from a depressive effect of P deficiency on photosynthesis (Rao and Terry, 1989). However, P deficient plants showed a significant reduction in net photosynthetic rate independently of the internal concentration of CO_2 (Hernandez etal 2007). In the case of P deficiency, the lowering in photosynthesis is associated with a reduction in total leaf area resulting from a reduction in leaf expansion and number (Fredeen et al., 1989). Kavanova et al. (2006) showed that P deficient plants of *Lolium perenne* reduced leaf elongation rate by 39% due to a decrease in cell production rate [cell division (-19%) and final cell length (-20%)]. Furthermore, Pi in the chloroplast stroma functions as a substrate for ATP synthesis. Carstensen et al. (2018) indicated that P deficiency significantly reduces Pi levels and significantly reduces ATP production in isolated thylakoids. When the Pi substrate is lacking due to low P availability, ATP synthase activity decreases, which reduces ATP production in the stroma and reduces CO_2 fixation. Reduced ATP synthase activity reduces the flow of protons from the thylakoid lumen to the chloroplast stroma, which causes lumen acidification (Carstensen et al., 2018)

In plants grown in calcareous soil, TN6.18 had increased root length, while the biomass remained constant. This property reflects a capacity of this line to increase the specific length of its roots, which increases the exchange surface between roots and the growing medium. Increased root production without a proportional increase in their biomass is an effective P acquisition strategy through root proliferation (Peng et al., 2018; Mo et al., 2019). This characteristic has been considered in several studies as an important parameter for the selection of genotypes tolerant to phosphorus deficiency (Zhang et al., 2019; Yang et al., 2020). The results shown in TN6.18 cultivated in calcareous soil reveal a higher accumulation of Pi in shoots than in Jemalong. This suggests that the tolerant line TN6.18 is characterized by enhanced allocation of Pi from the roots to the shoots. This result was observed in previous work (Kouas et al., 2009) which showed an accumulation of Pi in shoots of the tolerant line BAT 477. Regarding the activity of acid phosphatase, calcareous soil increased this activity in shoots, more so in TN6.18. This behavior, related to the ability of plants to transfer P from inactive sites to active sites, creates an adaptive strategy to P deficiency. According to Gao et al. (2017) and during phosphate (Pi) starvation or leaf senescence, the accumulation of intracellular and extracellular acid phosphatases increases in plants in order to scavenge organic phosphorus (P). Under P deficiency, plants have the ability to remobilize P from inactive metabolic sites in senescent leaves and vacuoles to young leaves (Scachtman et al., 1998). Remobilization of P from leaves and stems and its allocation to reproductive tissues has been observed in beans (Kouas et al., 2009). In plants cultivated in calcareous soil, the acid phosphatase activity was more important in roots, especially in TN6.18. This behavior suggests that the acid phosphatases are involved in the remobilization and acquisition of P from soil (Yin et al., 2019). In terms of adaptive strategies, plant acid phosphatases are believed to play a vital role in Pi mobilization (Wang and Liu, 2018). It has been documented that P deficiency enhances the expression levels of most plant acid phosphatases in the roots under P-deficient conditions (Zhang et al., 2010). Similarly, Pi starvation enhanced transcript levels of this enzyme in maize, *Arabidopsis*, chickpea and soybean (Gonzalez-Munoz et al., 2015; Bhadouria et al., 2017; Venkidasamy et al., 2019; Zhu et al., 2020).

The phosphorus deficiency induced in calcareous soil causes significant changes in P content, plant growth and net photosynthesis as well as stomatal conductance in *M. truncatula*. The tolerant line TN6.18 cultivated in calcareous soil maintains its growth, which results from the preservation of photosynthetic activity, significant accumulation of Pi in shoots and roots with increased acid phosphatase activity. This suggests that this enzyme is involved in phosphorus acquisition and use efficiency. Thus, breeding cultivars with high P efficiency and optimizing field P management practices are necessary to maintain sustainable agricultural development.

5. CONCLUSION

In conclusion, this study indicates that the better tolerance of TN6.18 to P limitation in calcareous soil as compared to Jemalong could be explained by the capacity of TN6.18 to maintain (i) a higher chlorophyll content and photosynthetic activity, in concomitance with (ii) a higher acid phosphatase activity in roots and shoots to preserve an adequate phosphorus nutrition regardless of lacking P in calcareous soil.

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