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Insights into some key parameters involved in the variability of tolerance to phosphorus deficiency in the legume model *Medicago truncatula*

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Abstract

Phosphorus is a key limiting factor for plant growth. Several approaches are developed to mitigate the impact of P shortage on plants and to the selection of crops with high P mobilizing capacity from P-deficient soils. In this work, four *Medicago truncatula* genotypes (A17, TN8.20, TN1.11, and TN6.18) were compared for their efficiency to cope with P limiting conditions using several criteria. Significant differences between genotypes, P deficiency treatments, and the interaction of genotypes with P deficiency treatments were found. P limitation resulted in an important decrease in shoot biomass, P content, P use efficiency, and photosynthetic parameters. A significant variability was found between the four genotypes, with A17 and TN8.20 being the most tolerant genotypes to P deficiency. This was consistent with the better ability of these genotypes to acidify rhizosphere and stimulate the activity of acid phosphatase and its relative gene (*MtPAP1*). The expression of P transporter genes (*MtPT1*, *MtPT3*, and *MtPT5*) was induced by P deficiency, however, the overexpression of those genes was more pronounced in tolerant genotypes. Overall, our data indicate that A17 and TN8.20 are more efficient in mobilizing P under limiting conditions and could be cultivated in P-deficient soils as forage crops.

Keywords: legumes, *Medicago truncatula* genotypes, P deficiency, P uptake.

Introduction

Phosphorus (P) plays a crucial role in numerous plant functions including energy transfer, photosynthesis, carbohydrate production, nutrient movement within the plant and transfer of genetic characteristics, and its involvement in the antioxidant defense systems under stressful conditions (Kleinert *et al*. 2017, Roch *et al*. 2019, Saleem *et al*. 2020, Wang *et al*. 2021). However, P is one of the least available macronutrients in soils, particularly in calcareous ones. In these soils, most of P binds with various chemical forms of other elements, forming insoluble compounds such as iron-aluminum oxide complexes or calcium salts (Castro-Guerrero *et al*. 2016, George *et al*. 2016, Torri *et al.* 2017, Taalab *et al*. 2019).

Plants challenged with P deficiency exhibit several adjustments at different levels including a decrease in P consumption *versus* an increase in P recycling from various organs and an efficient P use for basic metabolic processes (Paz-Ares *et al.* 2022). Another plant adaptation strategy under low P availability is the acidification of the rhizosphere *via* H^+-ATPases and the induction and accumulation of extracellular purple acid phosphatases (PAP). These enzymes are responsible for the release of available Pi from organic complexes (Del Vecchio *et al.* 2014) due to their ability to dephosphorylate

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Abbreviations: cDNA - complementary deoxyribonucleic acid; C_i - intercellular CO_2 concentration; E - transpiration rate; F_v/F_m - maximal photochemical efficiency of PSII; g_s - stomatal conductance; PAE - phosphorus acquisition efficiency; P_N - net photosynthetic rate; PUE - phosphorus use efficiency; RNA - ribonucleic acid; RT-qPCR - reverse transcription quantitative PCR.
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organic P complexes present in the rhizosphere or in intracellular compartments (*i.e.*, scavenging of Pi from organophosphate compounds in the apoplasts) to release Pi (Tian and Liao 2015, Bhadouria *et al.* 2023) and thus, are one among the key actors for improving plant P nutrition. Both H^+-ATPases and PAP are recognized as major enzymes occurring at the interface root-rhizosphere (Touhami *et al.* 2020) and the stimulation of their activities generally results in an activation of several high affinity P transporters which are responsible for Pi uptake (Uhde-Stone 2017, Srivastava *et al.* 2018). Recently, numerous high-affinity PHT1 family transporters were identified in different plant species and were shown to be responsible for P uptake from the rhizosphere under P limitation (Huang *et al.* 2019, Xu *et al.* 2021).

Legumes have evolved complex strategies at both morphological, physiological, and biochemical levels to deal with P deficiency. Legumes can create symbiotic relationships with N_2-fixing rhizobia to facilitate acquisition of nutrients such as N and P. Thus, their utilization in agricultural management programs aiming at reducing the use of synthetic fertilizers represents an attractive agroecological approach for a sustainable agriculture (Valliyodan *et al.* 2017). Legumes are also seen as key species for P-deficient soils management to promote the ecosystem efficiency due to their ability to mobilize P mediated by specific P acquisition strategies (Kouas *et al.* 2009, Tang *et al.* 2021, Ajal and Weih 2022). For instance, the ability of plants to develop highly specialized morphological root traits in response to P deficiency is of major significance (Xia *et al.* 2020). However, the selection of crops with high tolerance to P low availability should consider that P deficiency tolerance is plant species/genotypes dependent (Kale *et al.* 2021).

Medicago truncatula has emerged as a model legume for the investigation of the main tolerance mechanisms to different environmental constraints such as drought, salt stress, nutrient shortage, and pathogenic agents (Mhadhbi *et al.* 2011, Kallala *et al.* 2019, Batnini *et al.* 2021). Accession A17 was documented to be tolerant to pathogens (*Fusarium* and *Rhizoctonia*), whereas TN1.11 was among the least tolerant accessions (Batnini *et al.* 2021). Under iron deficiency conditions, Kallala *et al.* (2019) reported that A17 and TN8.20 displayed high tolerance to low Fe availability contrasting with TN1.11 and TN6.18.

The effect of P deficiency on the behavior of different *M. truncatula-Sinorhizobium* associations has received increasing attention (Sulieman *et al.* 2013). However, to the best of our knowledge, the genotypic variability among *M. truncatula* genotypes for their response to P deficiency is poorly addressed, despite such a variability is of great interest since it might enable to identify tolerant genotypes for possible valorization in soils impacted by P deficiency. Besides, the identification of reliable morpho-physiological, biochemical, and molecular mechanisms governing plant response to P deficiency is essential to develop breeding programs aiming at improving P uptake in plants under challenging conditions. Considering the abovementioned elements, the present study compares the response of four *M. truncatula* genotypes under P starvation using physiological, biochemical, and molecular tools.

Materials and methods

Experimental design, plant growth conditions and treatments: *Medicago truncatula* Gaertn. is a leguminous plant species characterized by its small diploid genome ($2n = 16$) enabling it to be used as a plant model for legumes because of its relevant interests in N_2-fixing symbiosis investigated at both genetic and molecular levels (Ané *et al.* 2008). In this study, four genotypes of *M. truncatula* were used: TN1.11, TN6.18, A17, and TN8.20. Seeds were scarified and surface-disinfected for 5 min with H_2SO_4. After imbibition with distilled H_2O, seeds were kept at 4°C overnight in darkness. Ten seeds were germinated in Petri dishes for 2 d at 25°C as described by Mhadhbi *et al.* (2005). Six-day-old seedlings were then cultivated for further 7 d in a half-strength aerated nutrient solution (Vadez *et al.* 1996). Homogenous seedlings (8 plants) were selected and further cultivated under the following treatments: C = control (360 µM KH_2PO_4), DP = direct P deficiency treatment (10 µM KH_2PO_4), and IP = induced P deficiency by bicarbonate (360 µM KH_2PO_4 + 0.5 g L^{-1} $CaCO_3$ + 10 mM $NaHCO_3$). The bicarbonate-induced deficiency was intended to mimic calcareous soils in natural conditions. Full strength (Vadez *et al.* 1996) modified nutrient solution containing macronutrients: $MgSO_4$ (1 mM), KNO_3 (2 mM), K_2SO_4 (0.7 mM), $CaCl_2$ (1.65 mM), and micronutrients as a mixture of salts: $MnSO_4$ (6.6 µM), $CuSO_4$ (1.56 µM), $ZnSO_4$ (1.55 µM), $(Na)_2MoO_4$ (0.12 µM), $CoSO_4$ (0.12 µM) and H_3BO_3 (4 µM) and EDTA-K-Fe complex, was used. The pH of the nutrient solution was initially adjusted to 6.0 and reached 8.2 for bicarbonate treatment (IP). The experiments were carried out in a growth chamber under controlled conditions (16/24°C night/day temperature, 14-h photoperiod, 70 ± 5% relative humidity). The nutrient solution was continuously aerated and was changed every 5 d. After 27 d of treatment, plants were harvested. Leaves and roots were then separated, rinsed with distilled water, and oven dried for 48 h at 60°C. Afterward, the dry mass was determined. Moreover, leaves and roots were frozen in liquid nitrogen and kept at -80°C to be used for enzyme activity assays.

Extraction and determination of leaf and root P content, P acquisition efficiency (PAE), and P use efficiency (PUE): *M. truncatula* freshly harvested samples (leaves and roots) were washed with distilled water, dried for 72 h at 60°C, pulverized to powder and then digested in a concentrated nitric acid:perchloric acid (2.5:1, v/v) solution. The solution was then distilled with 20 mL of HNO_3 (N/7). Finally, the mixture was filtered with Whatman paper. Mineral P was determined using an atomic absorption spectrophotometer (Grusak 1995).

Phosphorus acquisition efficiency (PAE) and P use efficiency (PUE) were also calculated. PAE reflects

the capacity of roots to absorb P from the soil solution, and was estimated by rationing the amount of P taken up during the experiment to the mean root dry mass (RDM) as follows: PAE [µmol(P) mg^{-1} (RDM)] = (Q2 – Q1)/RDM, where Q2 – Q1 = the quantity of P [µmol] taken up in each plant between the initial and the final harvests (Houmani *et al.* 2015).

Phosphorus use efficiency (PUE) expressed dry mass increase rate per unit of P content [mg(RDM) $µmol^{-1}$(P)]. It was calculated as the ratio between the changes in biomass production and the changes in P accumulation over the treatment duration.

RNA extraction, cDNA synthesis, and real time PCR assay: Root samples were ground into a fine powder with liquid nitrogen. Total RNA was extracted according to Chang *et al.* (1993) with some modifications according to Abid *et al.* (2015). Briefly, 20 µl of β-mercaptoethanol was added into the extraction buffer and then the mixture was incubated at 65°C for 30 min (shake 30 s and pause 2 min). 600 µl of chloroform-isoamyl alcohol (24:1) was added to the homogenate and the mixture was centrifuged at 14 000 *g* for 10 min at room temperature. The supernatant was collected and total RNA was precipitated overnight at 4°C using lithium chloride (10 M) after which the tubes were centrifuged at 14 000 *g* for 30 min at 4°C. The RNA pellet was dissolved in 200 µl of RNase-free water. An equal volume of sodium acetate (3 M, pH 5.2) and 600 µl of absolute ethanol were added, set in -80°C for 30 min and then centrifuged for further 30 min at 14 000 *g* at 4°C. The upper aqueous phase was removed, and then the precipitate was washed in 600 µl 70% ethanol. After that, the RNA pellets were allowed to dry for 2 min and were resuspended in 20 µl RNase-free water and finally stored in at -80°C until use.

The quantity and quality of RNA were assessed spectrophotometrically using a *NanoDrop® ND1000* spectrophotometer. Total RNA was treated with 5 U RNase-free DNase I (*Thermo Fisher Scientific*, USA) for 20 min at 37°C. *Turbo-I First Strand cDNA Synthesis Kit* (*Biomatik*, Wilmington, DE, USA) was used to generate cDNA with 5 µg of total RNA from root tissues as indicated in the manufacture's protocol. The RT-qPCR was carried out in a *7300 Real-Time PCR Detection System* (*Applied Biosystems*, Foster City, USA) with the following program: 95°C denaturation for 10 min, then 40 cycles of denaturation at 95°C for 30 s, annealing/elongation at 60°C for 1 min. The reactions were performed in 30 µl volume containing 2 µl of first strand cDNA, 200 µM each of gene specific primers (Table 1 Suppl.), 15 µl *Maxima SYBR Green/ROX qPCR Master Mix* (2X) (*Biomatik*) and 12 µl nuclease-free water. Each gene was normalized to the internal ubiquitin levels. All reactions were performed in triplicate and a melting curve analysis of amplification products was performed at the end of each PCR by slow heating from 65°C to 95°C at 0.5°C/s and continuous monitoring of the fluorescence signal.

The heat map was generated using *R package* (http://www.r-project.org/) to compare the changes in amount of transcripts of the four selected genes.

Plant acidification potential: Acid phosphatase (EC 3.1.3.2) activity was assayed using 200 mg of root fresh material mixed in 100 mM Na-acetate buffer (pH 5.0) containing 6 mM β-mercaptoethanol, 0.1 mM phenylmethylsulphonylfluoride (PMSF), and 10% (w/w) polyvinylpyrrolidone (PVP). The mixture was centrifuged at 12 000 *g* for 30 min at 4°C. Supernatants were collected and used for acid phosphatase activity determination in an assay mixture containing 100 mM Na-acetate buffer (pH 4.8), 5 mM *p*-nitrophenylphosphate, and the enzyme. The mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 0.1 M NaOH. Acid phosphatase activity was measured spectrophotometrically at 410 nm by monitoring the *p*-nitrophenol released (Zribi *et al.* 2015). Activity of acid phosphatase excreted into the external solution was assayed after 24 h of root exudation using *p*-nitrophenyl phosphate (*p*NPP) as a substrate.

The plant acidification capacity was also estimated by weekly monitoring the nutrient solution pH during the experiment using a *Radiometer PHM 84* pH meter.

Chlorophyll determination, gas exchange, and chlorophyll fluorescence: *SPAD 502* meter, as a rapid and non-destructive method was used to assess the chlorophyll content of fully expanded leaves. The photosynthetic gas-exchange parameters, such as stomatal conductance (g_s), net CO_2 assimilation rate (P_N), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured using an open type and portable photosynthesis system (*LCA-4*, *Bio-Scientific*, Great Amwell, Herts, UK). The measurements were determined on the youngest and fully expanded top leaves. Chl fluorescence was monitored using a modulated chlorophyll fluorimeter (*OS1-FL*, *Opti-Sciences*, Tyngsboro, MA, USA). After a dark-adaptation period of 30 min, the minimal Chl fluorescence (F_0) was determined by a weak red light pulse (6 s). Maximum fluorescence of dark-adapted state (F_m) was measured during a subsequent saturating pulse of white light [8 000 µmol(photon) m^{-2} s^{-1} for 0.8 s]. The maximal photochemical efficiency of PSII (F_v/F_m) was expressed as: $F_v/F_m = (F_m – F_0)/F_m$.

Statistical analysis: Two-way *ANOVA* was performed using the *SPSS 18* program, and means were separated according to Tukey test at $P<0.05$. Data shown are means of six replicates per treatment for plant growth, leaf and root P content, and pH acidification and three replicates per treatment for acid phosphatase activity and gene expression.

Results

Effect of P deficiency on plant growth: Analyzing biomass production revealed a significant decrease for both shoots and roots upon P deficiency conditions, and that this decline was genotype-dependent. TN6.18 and TN1.11 were more impacted by P shortage as compared to A17 and TN8.20 (Fig. 1). Reductions of shoot dry mass (DM) reaching *ca.* 43% and 34% were registered in

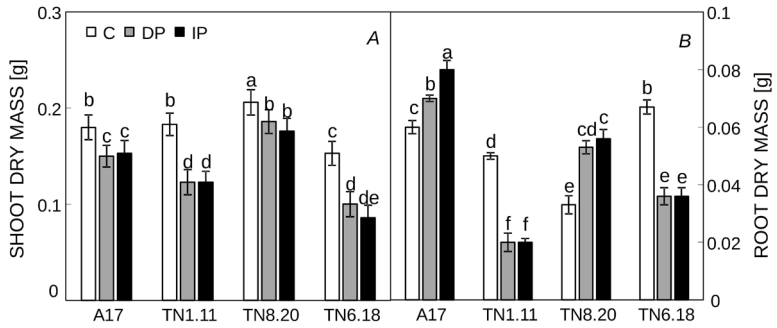

Fig. 1. Shoot (*A*) and root (*B*) dry mass of *M. truncatula* genotypes grown for 27 d with either in a control nutrient solution (C), under direct P deficiency (DP), or under induced P deficiency (IP). Data are the means of six replicates (for statistical output *see* Table 2 Suppl.).

TN6.18 upon exposure to induced (IP) and direct P (DP) deficiency, respectively. Root biomass was differently affected by P starvation. In TN1.11 and TN6.18, root DM decreased upon both DP and IP deficiencies. By contrast, A17 and TN8.20 root DM was significantly increased by DP and IP deficiencies as compared to control plants, with a marked increase registered in TN8.20 (+69%) (Fig. 1*B*). The increase in root biomass production in A17 and TN8.20 upon low P availability suggested their ability to enhance Pi mobilization under low Pi supply (for statistical output *see* Table 2 Suppl.).

Leaf and root P content: P content in the different organs of *M. truncatula* generally decreased upon P deficiency stress irrespective of the studied genotype (Fig. 2). Low P availability restricted P uptake by roots and its translocation to shoots (Fig. 2). Interestingly, some differences were detected between the four genotypes. TN1.11 and TN6.18 genotypes showed the strongest reductions as compared to A17 and TN8.2 (-41 and -42% in roots and -45 and -61% in leaves, for TN1.11 and TN6.18, respectively) (for statistical output *see* Table 2 Suppl.).

Effect of P deficiency on acid phosphatase activity in roots or released into the nutrient solution: The activity of acid phosphatase in roots or released into the nutrient solution was significantly enhanced in all genotypes either by direct (DP) or induced (IP) P deficiency (Fig. 3*A*).

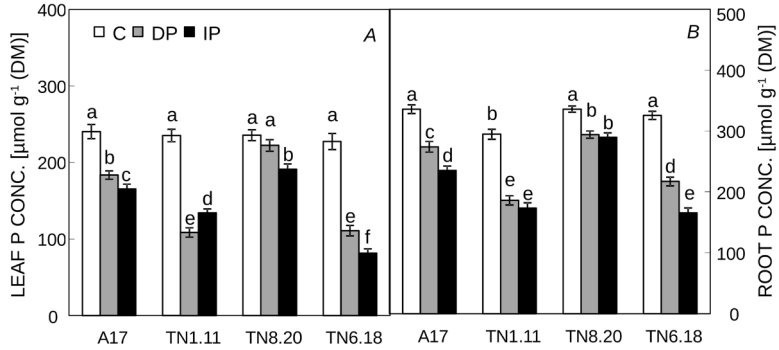

Fig. 2. Leaf (*A*) and root (*B*) P content of *M. truncatula* genotypes grown for 27 d with either a control nutrient solution (C), under direct P deficiency (DP), or under induced P deficiency (IP). Data are the means of six replicates (for statistical output *see* Table 2 Suppl.).

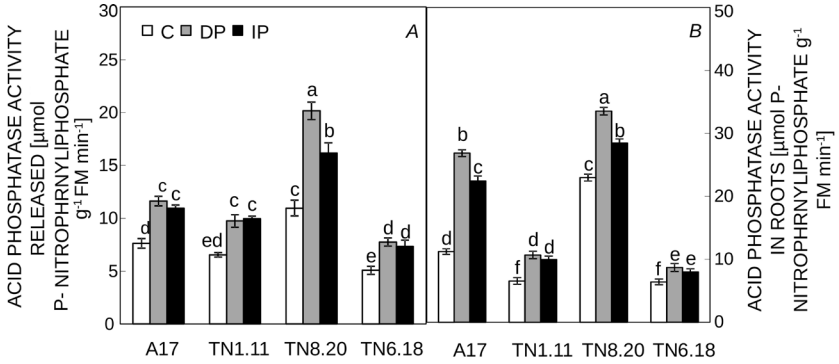

Fig. 3. Acid phosphatase activity released into the culture solution (*A*) and in roots (*B*) of *M. truncatula* grown for 27 d with either a control nutrient solution (C), under direct P deficiency (DP), or under induced P deficiency (IP). Data are the means of three replicates (for statistical output *see* Table 2 Suppl.).

In both cases, the increase was more pronounced in A17 and TN8.20 as compared to TN1.11 and TN6.18. It is noteworthy that the highest increase in acid phosphatase in roots or released into the nutrient solution was detected in plants subjected to direct P (DP) deficiency. In fact, the observed increase in root phosphatase activity was 141 and 45% in A17 and TN8.20, respectively. A similar trend was observed for the released phosphatase into the nutrient solution (+71 and +84% in A17 and TN8.20, respectively) (for statistical output *see* Table 2 Suppl.).

Effect of P deficiency on chlorophyll content, gas-exchange parameters and chlorophyll fluorescence: Overall, chlorophyll index (as SPAD values) was significantly reduced by P deficiency, despite the reduction was genotype-dependent. TN1.11 and TN6.18 showed the lowest chlorophyll content under direct and induced P deficiency (55% and 58% for TN1.11 and TN6.18, respectively; Fig. 4*A*). Regarding leaf gas-exchange parameters, the stomatal conductance (g_s), the net photosynthetic rate (P_N), the transpiration rate (E), and the intercellular CO_2 concentration (C_i) were significantly reduced by P starvation (Fig. 4*B-E*). Interestingly, TN1.11 and TN6.18 showed the highest reductions as compared to A17 and TN20.6. P deficiency also affected significantly F_v/F_m ratio with a significant decline especially in TN1.11 (Fig. 4*F*, Table 2 Suppl.).

Rhizosphere acidification: P deficiency-induced changes in external medium pH were compared in the four genotypes differing in their ability to mobilize P under low availability of this element in the external medium. A strong decrease in the nutrient solution pH was observed with significant differences depending on P tolerance of the investigated genotypes. The lowest pH values were found in A17 and TN8.20 plants under direct P deficiency (DP) (from 7.2 to 5.5 and 4.7, respectively) (Table 1). This also suggested that A17 and TN8.20 were more efficient in mobilizing Pi under low Pi availability *via* the acidification of the external medium. Yet, the presence of sodium bicarbonate in the free-P nutrient solution (IP) suppressed the release of proton into the nutrient solution, leading to pH values relatively closed to the control plants (Table 1).

Effect of P deficiency on expression of *MtPAP1* and some Pi transporter genes (*MtPT1*, *MtPT3*, and *MtPT5*): Purple acid phosphatase family are encoded by several genes depending on the plant species. In the present investigation, heat map depicting changes in the expression patterns of *MtPAP1* gene (a gene encoding a putative membrane acid phosphatase) in response to P deficiency showed that *MtPAP1* expression was induced by P deficiency, especially in A17 (Fig. 5).

Plant phosphorus acquisition involves many low and high Pi transporters that operate at the root plasmalemma. To assess whether these transporters are induced by P deficiency under the conditions used in the present study, the relative expressions of three P transporters were investigated and data were visualized using heat map. Our data showed that the studied genotypes showed different gene expressions under P deficiency (Fig. 5). *MtPT1* was significantly induced by both DP and IP conditions especially in A17 genotype. By contrast, *MtPT3* and

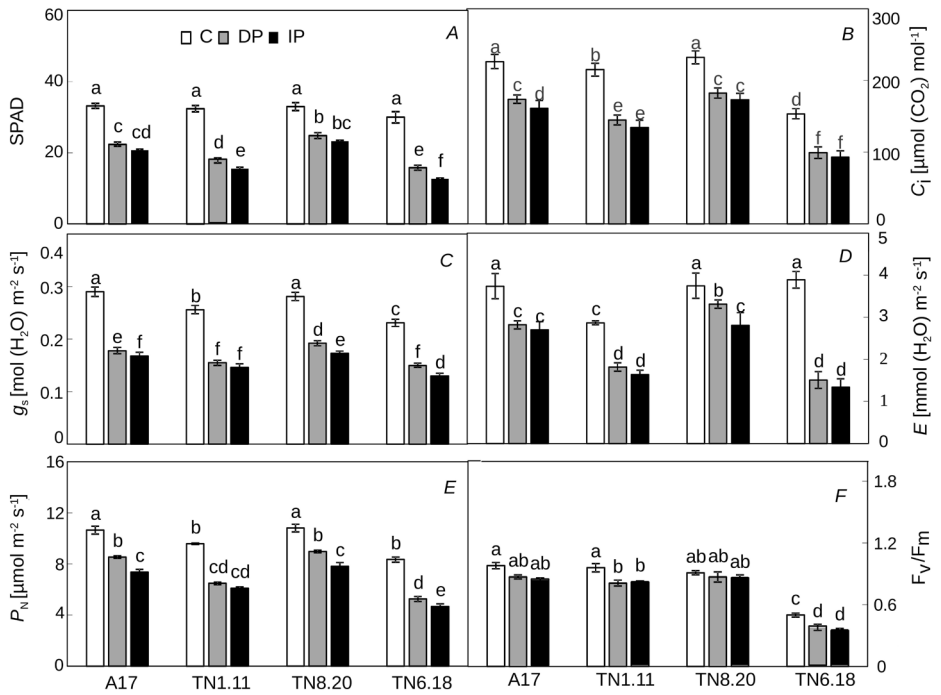

Fig. 4. SPAD (*A*), intercellular CO_2 concentration (C_i) (*B*), stomatal conductance (g_s) (*C*), transpiration rate (E) (*D*), net photosynthetic rate (P_N) (*E*), and F_v/F_m (*F*) of *M. truncatula* grown for 27 d with either a control nutrient solution (C), under direct P deficiency (DP), or under induced P deficiency (IP). Data are the means of six replicates (for statistical output *see* Table 2 Suppl.).

Table 1. pH values of the culture media (pH_i = pH initial; pH_f = pH final) during the 4 weeks treatment of *M. truncatula* genotypes grown during 27 d on a control nutrient solution (C), under direct P deficiency (DP) or induced P deficiency (IP). Means of six replicates ± SEs. Values followed by *different letters* are significantly different at $P<0.05$ according to Tukey test.

Genotypes	Treatments	pH_i	1 week pH_f	2 weeks pH_f	3 weeks pH_f	4 weeks pH_f
A17	C	6	6.20 ± 0.1^{b}	6.93 ± 0.1^{b}	7.11 ± 0.2^{b}	7.21 ± 0.5^{b}
	DP	6	6.43 ± 0.1^{b}	6.21 ± 0.3^{c}	6.80 ± 0.5^{bc}	5.50 ± 0.3^{d}
	IP	8	8.09 ± 0.2^{b}	7.96 ± 0.3^{a}	8.25 ± 0.1^{a}	7.96 ± 0.2^{a}
TN1.11	C	6	6.13 ± 0.3^{b}	7.01 ± 0.2^{b}	6.97 ± 0.4^{bc}	6.25 ± 0.4^{bc}
	DP	6	5.86 ± 0.4^{bc}	6.90 ± 0.5^{b}	7.25 ± 0.1^{b}	5.93 ± 0.1^{cd}
	IP	8	8.25 ± 0.5^{b}	7.88 ± 0.2^{a}	8.07 ± 0.3^{a}	8.06 ± 0.3^{a}
TN8.20	C	6	6.20 ± 0.5^{b}	7.13 ± 0.1^{b}	7.30 ± 0.4^{b}	7.21 ± 0.6^{b}
	DP	6	5.80 ± 0.4^{bc}	6.30 ± 0.1^{c}	7.14 ± 0.2^{b}	4.70 ± 0.2^{d}
	IP	8	8.10 ± 0.1^{b}	7.80 ± 0.5^{a}	8.20 ± 0.5^{a}	8.00 ± 0.3^{a}
TN6.18	C	6	6.20 ± 0.2^{b}	6.50 ± 0.3^{bc}	6.80 ± 0.1^{bc}	6.78 ± 0.3^{bc}
	DP	6	6.34 ± 0.3^{b}	6.10 ± 0.2^{c}	7.20 ± 0.3^{b}	6.32 ± 0.2^{c}
	IP	8	8.70 ± 0.6^{a}	7.50 ± 0.4^{a}	8.40 ± 0.6^{a}	8.40 ± 0.5^{a}

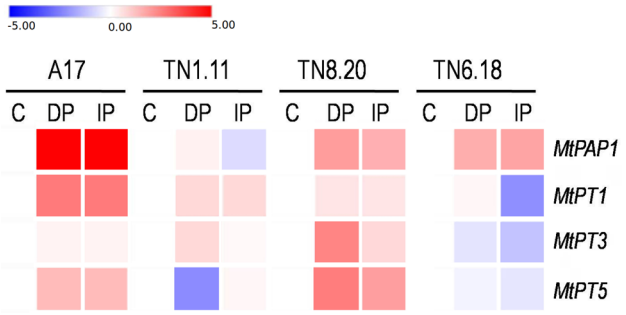

Fig. 5. Heat map representation of the effects of P deficiency on the gene expression level in leaves of *M. truncatula* genotypes grown for 27 d with either a control nutrient solution (C), under direct P deficiency (DP) or under induced P deficiency (IP). *Red* and *blue* indicate higher and lower expression values, respectively. Intensity of the colors is proportional to the absolute value of log2 of the fold difference in expression.

MtPT5 were overexpressed under direct P shortage (DP) conditions in TN8.20 which exhibited the highest expression.

Discussion

P deficiency seriously limits plant growth and crop production around the world (Han *et al.* 2022). However, plants grown in P-limiting environments have evolved physiological and biochemical adaptations to enhance Pi uptake from the P deficient medium (Lambers 2022). Annual *Medicago* species can be considered as forage crops and suffer, especially under the current climate change conditions, by several abiotic stresses. In the current study, the differential behavior of four contrasting *M. truncatula* genotypes to P deficiency was investigated using several morpho-physiological, biochemical, and molecular tools allowing us to discriminate between efficient and inefficient genotypes for P-efficiency mobilization under low availability of this element in the external medium.

Medicago species, generally grown on neutral or alkaline soils, are sensitive to P deficiency and exhibit several physiological and biochemical adaptations to such a constraint (He *et al.* 2020). Stress perception by the different plant organs is the primary step of stress response by plants (Vives-Peris *et al.* 2020). Shoots and particularly leaves are the main seat of photosynthesis while roots are the key organs involved in the uptake of water and nutrients (Javeed *et al.* 2022). Under our experimental conditions, a significant decrease in the shoot plant biomass was detected in P deficient plants in all genotypes, and to more extent in TN1.11 and TN6.18, which are hence, sensitive to P deficiency, whereas A17 and TN8.20 appeared to be more tolerant. Our data confirmed previous findings on the same species indicating a decrease in plant dry matter under decreasing Pi supply (Sulieman *et al.* 2013). Similarly, Chea *et al.* (2021) pointed a significant reduction in plant height and biomass by 60 - 80% under P deficiency in potatoes. Root dry mass was reduced by P deficiency in TN1.11 and TN6.18 while being increased in A17 and TN8.20 grown under the same conditions. It has been reported that abiotic stresses induce changes in root morphology to different levels. The increase in root dry matter detected in P deficient A17 and TN8.20 genotypes resulted in an increase of the surface area for P absorption under these conditions. According to Yeh et *al.* (2020), the increase in the total root surface area allowed the roots to explore the maximum ground area and thus promoting Pi uptake without significant energy loss (Yeh *et al.* 2020). Thus, genotypic differences in P uptake from P-deficient soils can be a consequence of the ability of the genotype to improve root growth. Overall, plants respond to P deprivation in the root rhizosphere in several ways including the allocation of more carbon to roots rather than to shoots, thereby increasing root to shoot ratio, hair

Table 2. Phosphorus acquisition (PAE) and use (PUE) efficiencies of *M. truncatula* genotypes grown during 27 days on a control nutrient solution (C), under direct P deficiency (DP) or induced P deficiency (IP). Means of six replicates ± SEs. Values followed by *different letters* are significantly different at $P<0.05$ according to Tukey test.

		PAE [µmol(P) mg^{-1}(RDM)]	PUE [mg(RDM) μmol^{-1}(P)]
A17	C	4.83 ± 0.2^{a}	6.56 ± 0.4^{d}
	DP	3.21 ± 0.3^{d}	8.63 ± 0.5^{a}
	IP	2.93 ± 0.2^{e}	8.16 ± 0.4^{b}
TN1.11	C	4.92 ± 0.4^{a}	5.69 ± 0.3^{e}
	DP	2.11 ± 0.3^{f}	4.92 ± 0.4^{ef}
	IP	0.94 ± 0.1^{g}	4.24 ± 0.2^{f}
TN8.20	C	5.03 ± 0.4^{a}	5.92 ± 0.3^{de}
	DP	4.38 ± 0.3^{d}	7.89 ± 0.5^{b}
	IP	4.34 ± 0.3^{d}	7.24 ± 0.4^{c}
TN6.18	C	4.26 ± 0.2^{b}	5.67 ± 0.3^{e}
	DP	1.98 ± 0.2^{f}	5.34 ± 0.2^{e}
	IP	0.67 ± 0.1^{g}	5.68 ± 0.3^{e}

density (Lynch and Brown 2001), and the development of lateral root (Mollier and Pellerin 1999).

There is a close relationship between the plant biomass production and the photosynthetic activity. Our results showed that the observed restriction in plant growth under P deficiency conditions was associated with a decrease in the photosynthetic activity (chlorophyll content and almost all gas-exchange parameters). Phosphorus deficiency reduces plant growth *via* an inhibition of the photosynthetic machinery since P is a crucial element of the structure of DNA, RNA, phospholipids, NADP, ADP, and ATP, and thus plays an essential role in plant metabolic processes, notably photosynthesis. P is also a key component of chloroplasts and thereby affects the structure and function of these organs (Li *et al.* 2022, Noor *et al*. 2022). Thus, among the harmful effects of P deficiency, a decrease in leaf net photosynthesis rate, chlorophyll synthesis (Meng *et al*. 2021), and ribulose 1,5-bisphosphate (RuBP) activity (Verlinden *et al*. 2022) were previously documented. In addition, P deficiency disrupts the electron transport chain causing an impairment of electron transport from the donor side of photosystem II to the acceptor side of PS (Carstensen *et al*. 2018), which is further suggested with our finding. Using F_v/F_m as an indicator of maximum photochemical efficiency of PSII under stressful conditions (Xing *et al*. 2010) revealed a significant decrease of this ratio upon P deficiency, suggesting an alteration in the PSII functional integrity. It is worth mentioning that the effects of P deficiency on photosynthesis were more pronounced in TN1.11 and TN6.18, again reflecting the sensitivity of both genotypes to P deficiency. In contrast, A17 and TN8.20 are able to maintain their photosynthetic activity under low P supply *via* the mobilization of P from different cellular organelles. In this regard, our data confirm previous findings on faba bean, peanut, and citrus (M'sehli *et al*. 2018, Patel *et al*. 2020, Meng *et al*. 2021).

To maintain an adequate rate of photosynthesis, plants need to use efficiently P under limiting P conditions. In this regard, some species are able to maintain phosphorus use efficiency under low P supply while other species attempt to increase the uptake of this element under such conditions (Beroueg *et al*. 2021). In both cases, a decrease in plant growth and an increase in internal Pi remobilization were previously described. It has been reported that plants cope with nutrient deficiency by increasing nutrient use efficiency, defined as the quantity of biomass produced per unit of nutrient absorbed (Dixon 2020). In our case, PUE was increased only in A17 and TN8.20 genotypes indicating that those genotypes were more efficient in mobilizing P under limiting conditions (Table 2). Such a finding is of great significance since improving crop phosphorus use efficiency is currently considered as a promising solution to many agriculture challenges. Javeed *et al*. (2022) revealed that in podzolic soils, enhancing PUE can play an important role in different cropping systems/crop production practices to meet ever-increasing demands in food, fiber, and fuel. The same authors attributed the increase in PUE to the stimulation of seed P reserve remobilization, PAE, plant internal P utilization efficiency (IPUE), or both for sustainable P management strategies.

P uptake and P use efficiency are generally regulated by several mechanisms allowing the plant species to improve P bioavailability in the rhizosphere. Those mechanisms include the acidification of the rhizosphere and the stimulation of enzymes involved in P acquisition (Baccari and Krouma 2023). Our data showed that A17 and TN8.20 presented the lowest pH values under direct Pi shortage conditions (Table 1). Similar data were obtained in *Arabidopsis thaliana* subjected to Pi deficiency and revealed an increase in the acidification capacity and the H^+-ATPases activity (Lei *et al*. 2016). Under our experimental conditions, the rhizosphere acidification was concomitant with the induction of acid phosphatase activity in both root extracts and root exudates. The enhancement of acid phosphatase activity promotes the transformation of organic phosphorus into inorganic form enabling

the plant to mobilize Pi from the external medium (Touhami *et al*. 2020). In other legume species, the activity of this enzyme was enhanced in nodules under Pi starvation (Lazali and Drevon 2014). Our results corroborate previous findings on other plant species such as cotton (Asif *et al.* 2023) for which, low P stimulated many enzymes involved in P metabolism especially in tolerant cotton genotypes, a result which was not observed in sensitive ones. Likewise, enhanced acid phosphatase activities and P uptake were observed in manure-applied corn silage growing in podzolic soils in a boreal agroecosystem (Ali *et al.* 2019).

The enhancement of acid phosphatase activities by P deficiency was confirmed by determining the expression of genes encoding this enzyme, *MtPAP*. *MtPAP1* was increased by P deficiency especially in A17 and TN8.20 genotypes. Similarly, Li *et al.* (2011) reported a marked increase in the activity of root acid phosphatase and the expression of its relative gene (*MfPAP1*) in *M. falcata* under P-deficiency conditions. Besides, Wang *et al.* (2020) demonstrated that Pi deficiency induced the expression of purple acid phosphatase (PAPs) in root nodules, which leads to an enhanced acquisition and utilization of P in these organs. Thus, plant genotypes with higher abilities to stimulate acid phosphatase activities could be recognized as a sustainable solution for forage in P-deficient soils by increasing the phosphorus acquisition efficiency (PAE). The increase in acid phosphatase activities was generally concomitant with higher activity of many transporters involved in P uptake from the rhizosphere. According to the literature, different genes encoding several P transporters were previously described. Most *PHT1* genes are induced by Pi starvation in plant roots and are implicated in Pi uptake from soil or Pi translocation within plant tissues or cells (Chen *et al.* 2023). In this study, three genes involved in P transport (*MtPT1*, *MtPT3*, and *MtPT5*) were investigated. Our data depicted also an increase in the relative expression level of *MtPT1*, *MtPT3* (low Pi affinity), and *MtPT5* (high Pi affinity) under limiting P supply in the tolerant genotype (TN8.20) while genotype with lower tolerance to P shortage remained unaffected. An *et al.* (2023) revealed that Pi-starvation-enhanced expression of *PHT1* genes in *Stylosanthes guianensis* roots and that overexpression of *SgPT1* from this plant can increase Pi uptake and enhance root growth in transgenic plants.

In legumes, the enhanced expression of P transporters is crucial to maintain P acquisition under low Pi availability (Uhde-Stone 2017). A close relationship between internal cell P content and *PHT1* expression was documented in several plant species (Grün *et al.* 2018). According to Liu *et al.* (2011), *PHT1*, *PHT2*, *PHT3*, and *PHT4* are the main genes involved in plant adaptation to low P conditions. In *M. truncatula*, 7 of the 11 genes belonging to the phosphate transporter 1 (*PHT1*) family were expressed in roots and were induced by low-phosphate stress in nodules (Cao *et al.* 2021). Using a functional analysis of the phosphate transporter gene *MtPT6*, the same authors revealed that this latter was induced in the different parts of *M. truncatula* (shoots, roots, and nodules) under low-phosphate stress, suggesting that it may play a role in Pi uptake from soil and its transport from nodules to other tissues. Other transporters mediating P acquisition and maintenance of P homeostasis were identified in *M. truncatula* and most of them were induced by P starvation (Sun *et al.* 2012). The induction of P transporters by P deficiency was shown in several plant species such as *A. thaliana* for which the expressions of *AtPHT1;1* and *AtPHT1;4* were higher in roots under low-phosphate stress (Karthikeyan *et al.* 2002). In fact, in the same plant, more than 600 genes were induced under P deprivation and resulted in an enhancement of P uptake (Misson *et al.* 2005). More recently, Nguyen *et al.* (2019) investigation on the symbiotic association *M. truncatula*-mycorrhizal fungi revealed that *MtPT4* transporter expressed in roots was vital for P uptake. Ma and Chen (2021) reported an antagonistic interaction between phosphorus and nitrogen and plant growing under P deficiency conditions tend to upregulate Pi transport but downregulate NO_3^- transport.

Overall, the parameters investigated in this study provide strong evidence and shed light on a significant role of roots in understanding the mechanisms used by *M. truncatula* to deal with P deficiency (Fig. 1 Suppl.) and allow us to select genotype with high performance regarding their response to the lack of P in the medium.

Conclusions

Data inferred from this work provide insights into several physiological and metabolic changes involved in P deficiency tolerance of *M. truncatula* genotypes, based on morpho-physiological, biochemical, and molecular traits. A genotypic variability to P deficiency occurred among the studied *M. truncatula* genotypes: overall, two genotype groups contrasting in their P deficiency tolerance were identified: relatively tolerant genotypes (A17 and TN8.20) and sensitive ones (TN1.11 and TN6.18). The better behavior of A17 and TN8.20 under P shortage was linked with several physiological, biochemical, and molecular adaptations, notably the ability of plants to improve root biomass production, the increase of P acquisition efficiency, the ability to maintain an adequate rate of photosynthesis, the enhancement of rhizosphere acidification, acid phosphatase activity, and the expression of some P high affinity transporters. The selection of tolerant genotypes to P deficiency offers the opportunity for their use in soils with low P availability and it is a suitable approach for sustainable agriculture by minimizing the use of chemical fertilizers, especially in the context of the current climate change.

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