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The increase in O_2 nodule conductance under phosphorus deficiency varies among genotypes in *Medicago truncatula*

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ABSTRACT

The effect of phosphorus deficiency on plant growth, nodulation, and symbiotic nitrogen fixation as well as, the nodulated-roots oxygen consumption, nodule permeability and conductance to the oxygen diffusion of *Medicago truncatula–Sinorhizobium meliloti symbiosis* were studied. Three lines, namely TN6.18, originated from local populations, F83005.5 originated from Var (France) and Jemalong 6, a reference cultivar from Australia, were hydroponically grown in nutrient solution supplied with 5 µmol (P deficient) and 15 µmol (P sufficient: Control), under semi-controlled conditions in a glasshouse. A genotypic variation in tolerance to P deficiency was found: TN6.18 was the most tolerant line whereas F83005.5 was the most sensitive. The relative tolerance of TN6.18 was concomitant with the greater P requirement, the higher N_2 fixation, the stimulation of nodule respiration and the less increases of conductance to the oxygen diffusion in nodules tissues. The higher P use efficiency for nodule growth and for symbiotic nitrogen fixation was detected in the tolerant line. Results suggest that the tolerance to P deficiency seems to depend on thehost plant ability to reallocate P from both leaves and roots to their nodules. P is needed in high energy demand conditions to maintain adequate nodule activity and prevent negative effects of the O_2 excess on the nitrogenase.

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Introduction

Phosphorus (P) and nitrogen (N), are the most limited nutriment for vegetative growth (1, 2). In the case of legumes, N uptake is provided from atmospheric dinitrogen by the symbiotic association between the host plant and its microsymbiont partner. Thus, symbiotic nitrogen fixation (SNF) by legumes that already constitute a major input into agricultural and natural ecosystems may provide an ecologically acceptable complement or substitute for mineral nitrogen fertilizers (3). However, P deficiency is a major limiting factor for legume production where legume N nutrition largely depends on the rhizobial symbiosis, particularly in acidic and calcareous soils (4). Thus, low-soil P availability is a primary constraint to legume productivity in many low-input systems. It is also a limitation in high-input systems where soil chemistry converts the fertilizer P into less available forms so that high P fertilization is inefficiently applied.

P is an essential macronutrient for plant growth and development. It is a major component of essential structural molecules and is an important constituent for energy transformation and regulation of various enzymatic activities (5, 6). This element plays a role in many metabolic processes related to the aboveground organs, including but not limited to, energy generation, nucleic acid synthesis, photosynthesis, respiration, glycolysis, membrane synthesis and integrity, activation/inactivation of enzymes, redox reactions, signaling and carbohydrate metabolism (7, 8). As such, the low availability of P in soil imposes serious limitations on plant growth and development. Of particular importance, P deficiency directly reduces photosynthesis (9-11) which is closely connected with the symbiotic tissues. Such negative effects are predicted to have serious implications on the growth and functioning of the nodule because of the specific requirement of P for symbiotic N_2 fixation as an energy-requiring process.

*Medicago truncatula*was first proposed as a model by Barker *et al.* (12) to study rhizobia-legume symbiosis. Now, it is internationally recognized as a model legume for studying the general biology of legumes and for exploring the genetic and molecular aspects of N_2-fixing symbiosis in leguminous plants (13-15). This is due to its relatively small diploid ($2n = 16$) genome (approximately 375 Mbp), tractable genetic properties, high level of synteny with several other legumes of interest, and the availability of its genomic sequence (16), and a relatively short generation time (around 4 months seed to seed). Moreover, *M. truncatula* is closely related to the most agricultural important forage legume in the world, *M. sativa* L. *M. truncatula*

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is readily nodulated by the soil bacterium *Sinorhizobium meliloti* (17). Nowadays, the *S. meliloti*2011 strain is the most frequently used in studies relating to the biology and genomics of N_2 fixation (18-20). By using the *M. truncatula-Sm* 2011 symbiosis as a model system, significant advances have been achieved in understanding the nature of the symbiotic relationship (21-23).

The effectiveness of N_2 fixation in *M. truncatula-Sm*2011, under P deficiency, has not been well examined (9), despite the fact that P-deficiency is one of the major abiotic factors that adversely affect both nodule function and the growth of leguminous plants (24-26). Thus, a concerted effort is needed to better understand N_2fixation efficiency on a whole-plant basis in *M. truncatula* plant grown and nodule functioning under various environmental conditions, particularly in association with the model microorganism *S. meliloti.* Therefore, the main objective of the present study was to evaluate the symbiotic performance of the model *M. truncatula-S. meliloti* symbiosisin three lines, under low P supply conditions. The metabolic mechanism responsible for the regulation of nodule functioning under P deficiency was examined by addressing the possible inter-relationship between oxygen consumption and nitrogenase activity estimated by fixed nitrogen.

Materials and Methods

This study was conducted with three *Medicago truncatula* lines. The local TN6.18 originated from the population of Tala (Kasserine region). This line was purified and characterized in the "Laboratory of Legumes" (CBBC BorjCedria, Tunisia). The F83005.5 originated from Var (France) provided by T. Huguet (CNRS-INRA Toulouse, France) and the Jemalong A6, a cultivar from Australia and the Jemalong for the international genomic program on *M. truncatula*. Seeds were scarified, surface sterilised and pre-germinated in agar (0.9%). They were transferred in 250 mL glass bottles wrapped with aluminum foil to maintain darkness in the rooting environment. The roots of selected uniform seedlings were gently passed through the hole of a rubber stopper on the bottleneck, and cotton wool was fitted at the hypocotyl level to maintain the root system suspended in the liquid nutrient solution. The latter contained 0.7 mM K_2SO_4; 1 mM $MgSO_4$,$7H_2O$; 1.65 mM $CaCl_2$ for macronutrients, and 6.6 µM Mn; 4 µM Bo; 1.5 µM Cu; 1.5 µM Zn; 0.1 µM for micronutrients and 22.5 µM for iron. It was supplied with KH_2PO_4 four times per week with 5 µmol (P deficient) and 15 µmol (Control). During the first 2 weeks, i.e. before nodule function, the nutrient solution was complemented with 1 mM of urea. This starter nitrogen source prevents nitrogen deficiency which would occur between the exhaustion of cotyledon storage and the establishment of symbiosis. In every single plant, it was initially added 1mL of *Sinorhizobium meliloti* 2011 inoculum containing approximately 10^8 cells mL^{-1}. The nutrient solution was thereafter, renewed every 2 weeks without urea and rhizobia inoculum. The nutrient solution pH was maintained at 7.0 by adding 0.2 g L^{-1} $CaCO_3$. It was aerated with a flow of 400 mL min^{-1} of filtered air *via* a compressor and "spaghetti tube" distribution system. Plants were grown in a temperature-controlled glasshouse with night/day temperatures of *circa* 20/28 °C and a 16 h photoperiod with additional lights of 400 µmol PAR m^{-2} s^{-1}.

Nodulated-root O_2 consumption

One day before the measurement, the level of the nutrient solution was lowered to one-third of the volume of the culture bottle for the majority of nodules to be in direct contact with the gaseous phase (27). The circulation of the gaseous phase in the circuit from the nodulated-root environment through the oximeter was driven by a peristaltic pump with a flow of 400 mL min^{-1} that favoured the homogenisation of the O_2 concentration in the gas and liquid phases. The measurement consisted in registering with an oximeter (Abiss, La Verpillère, France), the pO_2 decreased during successive confinement periods with varying initial pO_2, as previously described in detail by Jebara and Drevon (28). A steady decrease in pO_2 corresponded to stationary nodulated-root respiration and constant nodule permeability. After 5 min registration during steady state, the device was flushed with a new N_2-O_2 gaseous mixture established in less than 10 min with mass-flow meters (Tylan, F38296, LaVerpillère). For each 5 min confinement, the O_2 consumption by the nodulated root (Conr), could be calculated as follows:

$Conr = \Delta pO_2 (V/24.2) (60/t)$ with ΔpO_2 = initial – final pO_2 in % of the atmospheric pressure; V = volume of gas phase in L; 24.2 = volume of 1 mol pure gas in experimental conditions in L; t = time between initial and final O_2 measurement in min, i.e. 5. Conr was expressed in µmol O_2 consumed per h per plant.

Plant harvest and measured parameters

The plants were harvested after the gas-exchange measurements and separated into shoots, roots and nodules. Shoot, root and nodule dry weight (DW) was determined after drying for 3 days at 70°C.

Since *M. truncatula* nodules are indeterminate, with unlimited growth, they are often multilobular, each lobe representing an N_2-fixing zone. Therefore in this work, the nodules number included lobe number. Each unit nodule N_2-fixing zone was considered as a cylinder of 1 mm diameter and 1 mm length (27), for the calculation of the whole nodule surface as follows:

$S = n \prod D H$ with n, the nodule number per plant; D, fresh nodule diameter; H, length of the fresh nodule. The nodule surface was expressed in μm^2. The efficiency in utilisation of the rhizobial symbiosis (EURS) was estimated by the slope of the regression model of shoot biomass as a function of nodule biomass, as shown in Figure 4. For a linear adjustment curve, i.e. y=ax+b, b corresponds to the shoot biomass production without nodules (g sDW_0), and a corresponds to the EURS as (g sDW - g sDW_0) g^{-1}nDW.

Nitrogen content was determined using the Kjeldahl Method (29): 20-25 mg gunpowder, from the different organs,was digested in hot concentrated H_2SO_4, and there was distilled with the excess of NaOH, and finely measured with the variation of oxydo-reduction colorated solution. Nitrogen uptake was calculated as the difference between N quantities (mmol per plant) measured at final harvest and the amount determined in two weeks old plants.

Homogenate samples of 20-25 mg gunpowder, from the different organs,were digested with a mixture of HNO_3 and H_2O_2 (30%) in a volumetric ratio (4:2). P concentration in the extract was measured calorimetrically by the molybdenum-vanadate method, as previously described (30).

Statistical procedures.

The analysis of variance and the standard deviation of the means were performed with the statistical software (Version 5; StatSoft, France) to determine the significance (at $P < 0.05$) of differences in biomass data for symbiotic effectiveness. The statistical analysis of the regression model of nodulated-root respiration as a function of external pO_2 was performed with covariance analysis. If the correlation between nodulated roots O_2 consumption and O_2 concentration was significant, the mean values and standard deviation of the model parameters were calculated. For each parameter, values (means of 6 replicates ± S. D.) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

Results

Growth and nodulation

Figure 1A shows genetic differences among studied lines under P sufficiency for shoot growth estimated by dry material production. TN6.18 was the most fodder-productive line, whereas the reference Jemalong was the least, and F83005.5 was intermediate. Under P deficiency, shoot production was decreased for the three lines, with TN6.18 (-22%) and F83005.5 (-56%) as the least and most affected, respectively.

Results in Figure 1B shows that P deficiency reduced root production by 20 % in TN6.18, 32% in Jemalong and 54% in F83005.5. The ratio of shoot-upon-root parts (Fig. 1C) was the same for TN6.18 and Jemalong but lower than for F83005.5. Under P deficiency, it was not significantly changed whatever the line.

Under P sufficiency, nodule growth was equal in TN6.18 and Jemalong and the largest in F83005.5 (Fig. 2A). Under P deficiency TN6.18 and F83005.5 lines showed a contrasting inhibition of their nodule growth by 58 % and 87 % respectively; whereas inhibition was close to 77% in Jemalong.

This decrease in nodule biomass was coupled with a decrease in nodule number under P deficiency (Fig. 2B). F83005.5 and Jemalong were significantly the most and least affected under P sufficiency, whereas Jemalong produced the most nodules.

Phosphorus content and use efficiency

Data in figure 3 shows the P content in various plant organs of the three studied lines. P contents in both shoots and roots were significantly decreased with P deficiency supplied to the nutrient solution. The P content in the roots exceeded that of the shoots independently of the P supply (Fig. 3A&B). In nodule tissues, it was more than four times more important than in shoots in all lines. Under P deficiency), all lines displayed significant decreases in P content in nodules (Fig. 3C).

P shortage restricted significantly P status in plant tissues (Fig. 3C), TN6.18 being however the less affected. In this local tolerant line, the reduction in P content ranged from -34% for shoots and nodules to -44% in roots, while it varied from -40% for shoots and nodules to -55% for roots in the Jemalong. F83005.5 showed an intermediate behavior

Data in figure 4 shows the P use efficiency for nodule dry matter production (PUE/NDW as g Nod DW g^{-1} Nod P) and for the SNF (PUE/SNF as g N fixed g^{-1} Nod P) in the three *M. truncatula* lines under P sufficiency *vs* deficiency in the nutrient solution. The PUE/NDW was higher under P deficiency than P sufficiency for all lines (Table

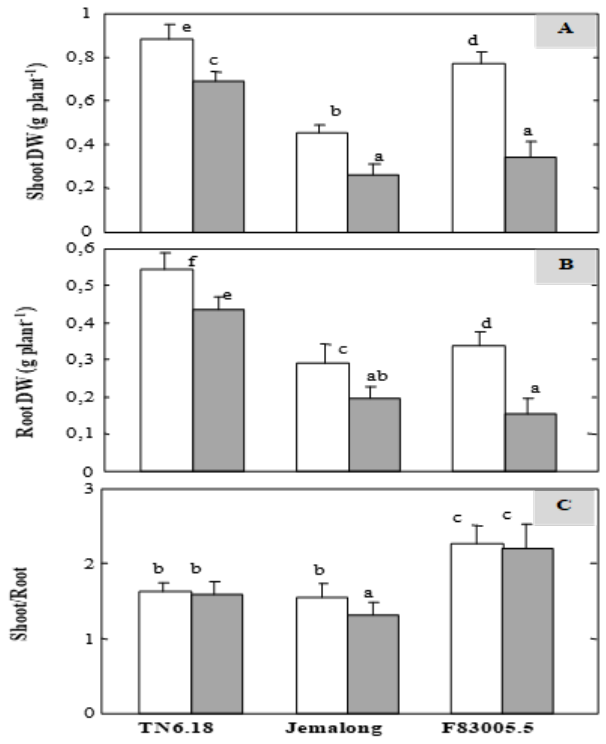

Figure 1. Variation of shoots **(A)** and roots biomass **(B)** and shoot-to-root ratio (**C**) in three *Medicago truncatula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

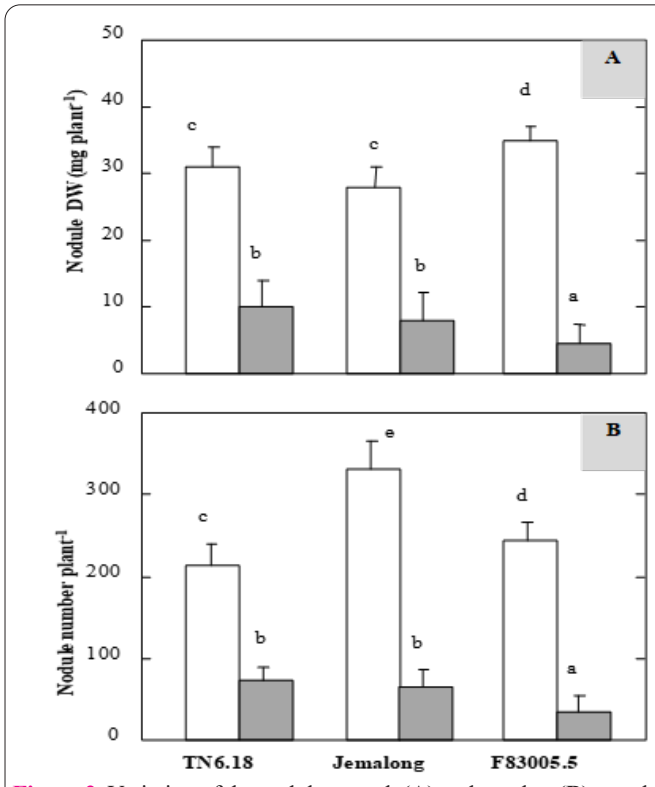

Figure 2. Variation of the nodule growth **(A)** and number **(B)** per plant in three *Medicago truncatula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

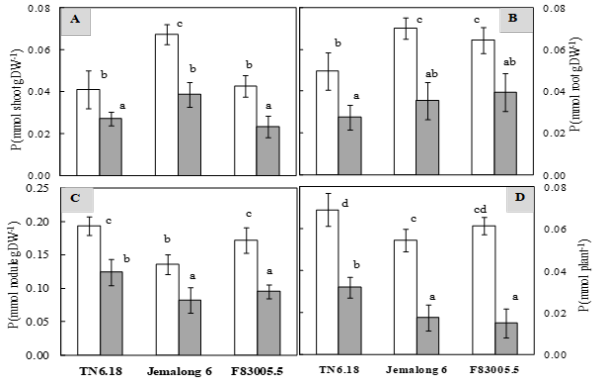

Figure 3. Variation of inorganic phosphorus concentration (mmol g DW^{-1}) in plant organs: shoots (A), roots (B) and nodules (C), and total P (mmol $plant^{-1}$) (D) in three *Medicago truncatula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

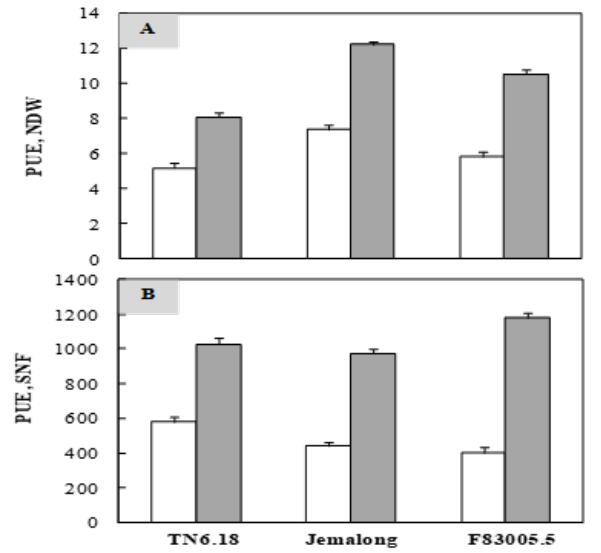

Figure 4. Effects of phosphorus availability in nutrient solution on phosphorus use efficiency for nodule dry matter production (A) (PUE, NDW: g nodule DW per g Nod P) and for the symbiotic nitrogen fixation (B) (PUE, SNF: g N fixed per g Nod P) in three *M. truncalula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

1). Under P deficiency, the Jemalong was characterized by the significantly highest PUE/NDW with 12.2 ± 0.1 g Nod DW g^{-1} Nod P whereas, TN6.18 show the lowest value of 8.1 ± 0.1 g NDW $mmol^{-1}$Nod.P. Also, under P sufficiency, the highest PUE/NDW was observed in Jemalong with a mean of 7.4 ± 0.1 g NDW $mmol^{-1}$Nod.P. compared to 5.2 to 5.8 g NDW $mmol^{-1}$Nod.P for TN6.18 and F83500.5, respectively (Fig. 4).

The PUE/SNF was increased by P deficiency in all studied lines, with the most stimulation in the sensitive line F83005.5 (200 %), and the least in the most tolerant line, TN6.18 (77 %). Under sufficient P, this parameter was the highest in TN6.18 with 581 ± 15 mmol N per mol Nod. P. compared to 442 ± 6 to 402 ± 13 mmol N mol^{-1} Nod. P in Jemalong and F83005.5 respectively.

Symbiotic nitrogen fixation and nodule efficiency

The SNF was estimated by the nitrogen content in plant tissues (mmol per plant) calculated as the difference between N quantities measured at final harvest and in two-weeks old plants (Fig. 5). Data shows that under P sufficiency, the TN exhibited the most important activity with more than 3.4 mmol N fixed per plant whereas, the reference line had the less active nodules with only 1.6 mmol N fixed per plant.

P shortage led to a significant reduction of the SNF in all lines, this negative impact is however more pronounced in F83005.5 exceeding 80%, whereas it did not reach 50% in the TN6.18 (Fig. 5). This behaviour was concomitant with significant stimulation of the nodule efficiency estimated by the quantity of N fixed per g DW nodule. The increase was most pronounced in the most sensitive line F83005.5 with 60% compared to only 13 % in the tolerant TN6.18. In the Jemalong, nodule efficiency was increased by 33 % under deficient P as compared to sufficient doses.

Efficiency in the utilisation of the rhizobial symbiosis

In order to assess whether the efficiency in utilization of the *S. meliloti* 2011 rhizobia varied among the three lines, the shoot biomass of each individual plant was plotted as a function of its nodule weight at harvest. Data in figure 6 shows that a significant correlation between both parameters was found for all lines whatever the P supply. The slope of the linear regression significantly decreased under P deficiency for the local TN6.18 line from 17.71 to 8.19 g shoot DW g^{-1} nod DW. It did not change in the Jemalong.

Under optimum P, a significant difference in EUSR was found among genotypes. Nodule efficiency for TN6.18 and F835005.5 was in the range of 18 g sDW g^{-1}nDW. This was more efficient than in the Jemalong.

O_2 uptake and nodule conductance

The response of nodulated-root O_2 uptake to variation

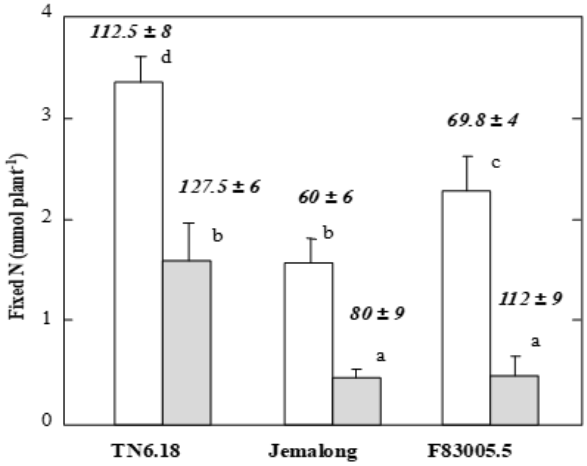

Figure 5. Variation of the symbiotic nitrogen fixation (mmol N per plant) measured in three *M. truncatula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Numbers in histograms correspond to nodule efficiency for nitrogen fixation calculated by the ratio of the total nitrogen fixed in the whole plant by nodule dry weight (mmol N per g nodule DW) for the period of the treatment. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

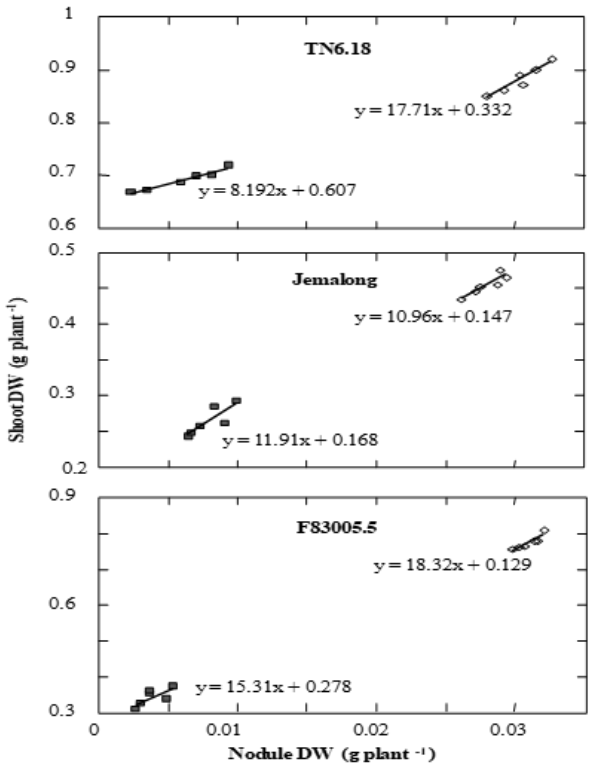

Figure 6. Variation of the efficiency in the use of the rhizobial symbiosis (EURS) in three *M. truncatula* lines grown under sufficient (white diamond) *versus* deficient (grey square) P supply. The plants were 45 days old. Results are individual values of six replicates.

of rhizosphericpO_2 was measured in order to assess the effect of P deficiency on nodules permeability, and the subsequent inhibition of nitrogenase-linked respiration. The measurement for nodule permeability, i.e. the slope of the linear response of oxygen consumption by nodulated-root (Conr) as a function of pO_2, was limited to the 15-25 kPa O_2interval since it was previously shown that the nodule respiration of *M. truncatula* was stimulated steadily by raising rhizosphericpO_2 between 15 and 25 kPa O_2, whereas it had less or no stimulatory effect above 25 kPa O_2(26).

Nodulated root oxygen consumption (Conr) was O_2-dependent (Fig. 7). The external pO_2 induced a significant increase in Conr regardless of P deficiency only in the TN, whereas, the Conr was not significantly different whatever the P supply in the other lines.

Data in Figure 8A shows that P shortage increased significantly the nodulated-root O_2 uptake at the ambiantalpO_2 of 21 kPa O_2 only in TN6.18, but not in F83005.5, whereas it was even a slight decrease (-11%) in Jemalong. With P sufficiency, Jemalong showed a significantly higher O_2 uptake of 240 µmol O_2 h^{-1} $plant^{-1}$ than the mean of only 130 µmol O_2 h^{-1} $plant^{-1}$ for the two other lines (Fig. 8A).

In Figure 7B, data show that P deficiency significantly decreased the nodule permeability, i.e. the slope of the regression models of nodulated-root O_2 uptake as a function of pO_2, for the three lines. The reduction of nodule permeability was between 12% in Jemalong to 56% in F83005.5. The local TN6.18 line showed an intermediate inhibition (-32%) of its nodule permeability to O_2. Under P sufficiency, a significant difference was also found among lines: nodule permeability for TN6.18 and Jemalong was in the range of 12 mm^3 h^{-1} compared to 17 mm^3 h^{-1} for F83005.5 (Fig. 8B).

The nodule conductance (Fig. 8C), was calculated as the nodule permeability per unit nodule area. With optimum P (15 µM), there was no genotypic variation of nodule conductance in the range of 4.4–4.6 µm s^{-1}. Underliming P nutrition (5 µM), this parameter was increased significantly by 6.7 folds in TN6.18; 11.5 folds in Jemalong and 30.2 folds in F83500.5.

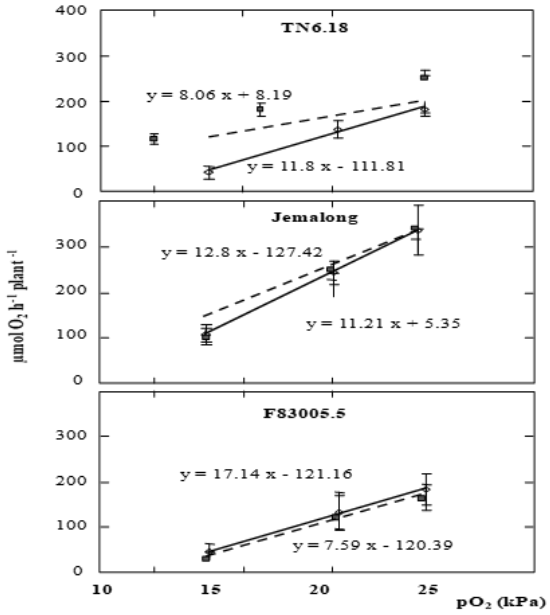

Figure 7. Effect of rhizosphere pO_2 on the oxygen uptake by nodulated-root of three *M. truncatula* lines grown under sufficient (white diamond) *versus* deficient (grey square) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D.

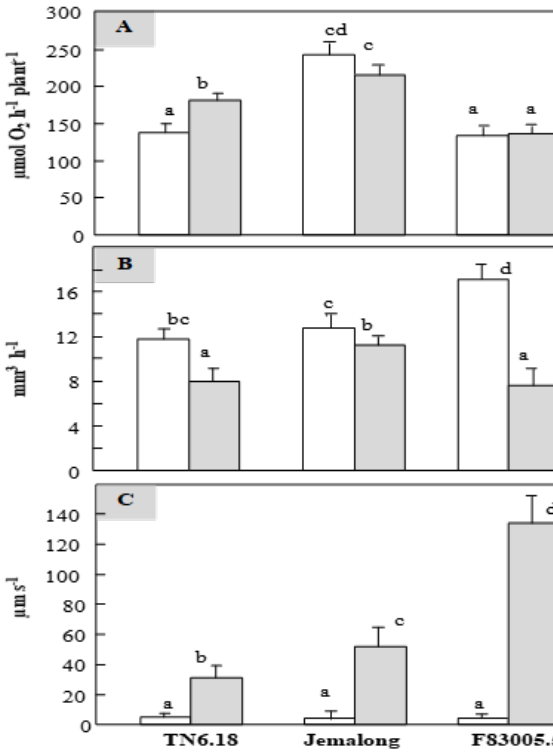

Figure 8. Variation of the O_2 consumption by the nodulatedroot (Conr) at ambiantal pO_2 (A), nodule permeability (B) and conductance to the oxygen diffusion (C) in three *Medicago truncatula* lines grown under sufficient (white) *versus* deficient (grey) P supply. The plants were 45 days old. Results are the mean of six replicates ± S.D. The same letters are not significantly different at 5% according to Fisher's LSD test.

Discussion

In this study, with N_2 fixing *M. truncatula*, the nodulated-roots oxygen consumption in the 3 lines increased with the external pO_2, suggesting that the nodule metabolism is limited by oxygen availability within the nodule-infected zone. This result can be explained by the existence of a physical limitation to the oxygen diffusion and the role which would play in the internal and external cortex of nodules to conserve conditions necessary for maintaining nitrogenase activity (31, 32). The increase in nodule respiration under P deficiency (Fig. 7) agrees with the finding by Ribet and Drevon (33), Kouas et al. (34) and Alkama et al. (24) for*Phaseolus vulgaris* showing that P deficiency increased the nodule conductance to O_2diffusion. The higher increase in nodule conductance under P deficiency for F83500.5 than for TN6.18 (Fig. 8C) agrees with previous results obtained by Vadez et al (31) and Kouas et al. (34) for *P. vulgaris*. This tendency of nodule conductance under P deficiency might result from:(i) The enhanced O_2 limitation following the activation of the wasteful O_2 alternative respiration in nodules; (ii) The direct effect of P deficiency on nodule O_2 conductance which induces alternative respiratory pathways so that harmful excess oxygen can be scavenged. Nodule cortex permeability to O_2, and consequently nodule O_2 content, has also been described to be affected by P availability (35, 36). Previous studies conducted with soybean, common bean and alfalfa exposed to low P reported an increase in cortex permeability (33, 37). However, in common bean (35), nodule O_2 conductance changes of genotypes were compared under P shortage, and two genotypes showed decreased nodule conductance while the other had no changes. Furthermore, nodule O_2 conductance is affected by the nodule shape variance (few large nodules or many small nodules) when the nodule permeability is the same, which could partially explain the genotypic variance in nodule conductance in common bean under P limitation (35). Given the negative impacts of an increase in O_2 permeability to nodule functioning, it stands to reason that legumes have several adaptations in place to reduce the effects of O_2permeability;these adaptive mechanisms can be structural or functional.

Schulze & Drevon (37) and Drevon et al (38) suggested that osmoregulatory changes in nodules cortical cells may be responsible for the increased O_2 diffusion under P deficiency and may be related to ensuring sufficient adenylate levels for high N_2 fixation. Other studies concluded that nodule O_2 conductance could be regulated by the variations of the intercellular spaces in the inner cortex of nodules under drought and salinity (27, 39, 40). According to this data, under P deficiency the nodules of TN6.18 were characterized by a low intercellular space then the nodule conductance to O_2 was less stimulated as compared to the sensitive line F83005.5. In addition, in *M. truncatula* the majority of the nodule aquaporins are indeed up-regulated under P deficiency(41), suggesting the involvement of aquaporins and their role in water conductance, as a mechanism for the cortical O_2 barrier. It would be important to report that water conductance was associated with K^+ movements from the cortex to infected cells due to the high demand for intracellular rhizobia that need K^+ for the maintenance of the osmotic status and turgor, cation/anion balancing, and control of membrane polarization (42) then the increasing of O_2 diffusion.

According to Wei etLayzell (43), the movement of K^+ from the cortex to the infected zone of nodules in *Glycine max* increased the nodule O_2 diffusion. Under P deficiency and especially in sensitive lines, the removal of K^+ from the nodule cortex can expedite the loss of water from cortical cells to the xylem and consequently large intercellular spaces then a higher O_2 diffusion. In addition, recent studies showed that P deficiency reduces the phospholipids components of cell membranes in exchange for sulfolipids and galactolipids (6, 44, 45). In this condition probably TN6.18 characterized by a low removal of membrane phospholipids can contribute to a low increase in nodule O_2 conductance.

Under P deficiency the higher decrease in nodule P content (Fig. 3C) and the lower N_2 fixation than for the tolerant line (Fig. 6), and the greater P requirement for nodules than for shoot or roots especially in TN6.18 (Fig. 3C) agrees with the previous studies reporting that the P content was higher in nodules than in shoots for *Phaseolus vulgaris* (46, 47)*, Acaciamangium*(48) and *Glycine max* (33). The high requirement of P for nodules might be related to the high energy requirement of the symbiotic nitrogen fixation (49). In addition, the higher nodule growth for TN6.18 than for F835005.5under P deficiency (Fig.2 A) suggests that the accumulation of P in nodules stimulates the growth and functioning of this organ. Alternatively, the higher tolerance of TN6.18 than F83005.5 under P deficiency, as established by symbiotic nitrogen fixation (Fig. 5) might be explained, since nitrogenase activity is widely known to consume the largest amounts of energy to catalyse N_2 reduction. Finally, to maintain a higher nitrogenase activity it is acceptable to reduce nodule O_2 conductance in tolerant line TN6.18. According to Rotaru and Sinclair (50) symbiotic N_2 fixation has a higher P requirement for maximum activity because of the high energy requirements for the reduction of atmospheric N_2 by the nitrogenase system indicating the important role of P in nodule functioning (51) and its regulation by O_2 permeability (37). In this work, the growth of plants, estimated by the quantity of biomass production, was higher in TN6.18 than in F83005.5 under P deficiency. In the same way, 5 µmol P severely reduced essential processes such as nodule growth, nodulation and symbiotic nitrogen fixation. Yet, the plant response was genotype-dependent, since this negative effect was more pronounced in F83005.5. The good adaptation of TN6.18 to P deficiency was attributed also to the higher P use efficiency for nodule growth and for symbiotic nitrogen fixation (Fig. 4), this result was confirmed previously in bean plants by Kouas et al. (34) and Alkama et al. (24).

Our results in Figure 9 showed a significant correlation between symbiotic nitrogen fixation (SNF) and Pi content in nodules (R^2= 0.96), suggesting that the high tolerance of TN6.18 to P deficiency, as compared to F83005.5, is associated with better capacity to maintain SNF and P content in nodules under low P supply. Vardien et al. (52) indicated that in low P conditions, nodules develop very flexible P recycling and internal P conservation mechanisms, rather than enhanced mechanisms aimed at acquiring external P. Within this context, according to Esfahani et al. (53), when exposed to low Pi conditions, chickpea plants tend to reallocate P from both leaves and roots to their nodules. Additionally, the better tolerance of TN6.18 under P deficiency due to the higher fixed nitrogen use efficiency for shoot

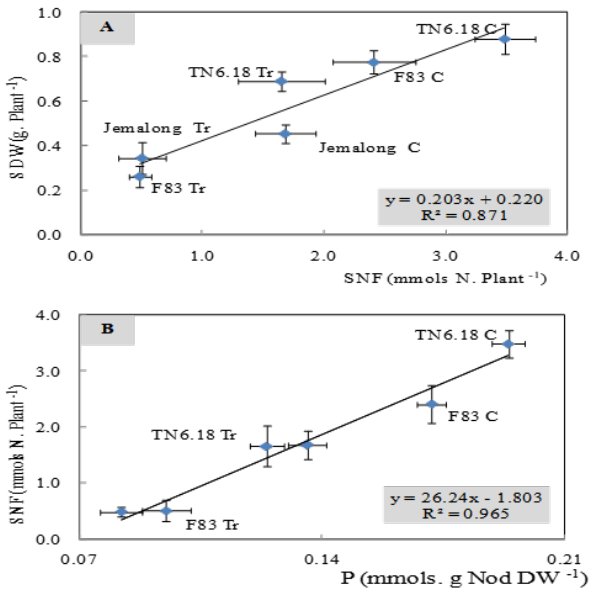

Figure 9. Relationship between symbiotic nitrogen fixation (SNF; mmols N. Plant^{-1}) and shoot dry weight (SDW; g. Plant^{-1}) (A) and between nodule phosphorus content and SNF (B) of three *M. truncatula* lines cultivated during 45 days under sufficient (C) and deficient P (Tr) supp.

growth (Fig. 9), in order to maintain the supply of energy by CO_2 assimilation.

The effects of P deficiency on the photosynthetic parameters as well as chlorophyll fluorescence and assimilating pigments will be envisaged to confirm this hypothesis. Previous studies conducted in N_2-fixing plants exposed to P deficiency (53-57) suggest that the main processes limiting nodule functioning were the accumulation of nitrogenous compounds and the nodule oxygen (O_2) permeability.

Conclusions

In conclusion, this work demonstrates that 5 µmol P severely reduced essential processes such as plant and nodule growth, nodulation and symbiotic nitrogen fixation. The tolerance of TN6.18 to P deficiency is associated with the higher P use efficiency for nodule growth and symbiotic nitrogen fixation. plants tend to reallocate P from both leaves and roots to their nodules. The greater P requirement for nodules and the higher N_2 fixation especially in TN6.18 might be related to the high energy requirement of the symbiotic nitrogen fixation. The P deficiency increased the nodule conductance to O_2 diffusion. The higher increase in registered for F83500.5 (sensitive) than the tolerant line TN6.18. To maintain a higher nitrogenase activity, the tolerant line TN6.18 tends to reduce its nodule O_2 conductance in order to prevent the negative effects of the O_2 excess on the nitrogenase.

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Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the study described in this manuscript.

Consent for publications

The author read and proved the final manuscript for publication.

Author's contribution

S. Aydi: Conceptualization, data curation, methodology, writing - original draft, validation. S. Sassi Aydi: Validation, writing - review & editing. S. Kouas: Formal analysis, discussion reviewing. R. Rahmani: Methodology, data curation. C. Abdelly: Supervision, validation, writing - review & editing.

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