

RESEARCH PAPER

# Growth and nodulation of symbiotic *Medicago truncatula* at different levels of phosphorus availability

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## Abstract

*Medicago truncatula* is an important model plant for characterization of P deficiency on leguminous plants at the physiological and molecular levels. Growth optimization of this plant with regard to P supply is the first essential step for elucidation of the role of P in regulation of nodulation. Hence, a study was carried out to address the growth pattern of *M. truncatula* hydroponically grown at different gradual increases in P levels. The findings revealed that *M. truncatula* had a narrow P regime, with an optimum P level (12  $\mu\text{M}$  P) which is relatively close to the concentration that induces P toxicity. The accumulated P concentration (2.7 mg g<sup>-1</sup> dry matter), which is normal for other crops and legumes, adversely affected the growth of *M. truncatula* plants. Under P deficiency, *M. truncatula* showed a higher symbiotic efficiency with *Sinorhizobium meliloti* 2011 in comparison with *S. meliloti* 102F51, partially as a result of higher electron allocation to N<sub>2</sub> versus H<sup>+</sup>. The total composition of free amino acids in the phloem was significantly affected by P deprivation. This pattern was found to be almost exclusively the result of the increase in the asparagine level, suggesting that asparagine might be the shoot-derived signal that translocates to the nodules and exerts the down-regulation of nitrogenase activity. Additionally, P deprivation was found to have a strong influence on the contents of the nodule carbon metabolites. While levels of sucrose and succinate tended to decrease, a higher accumulation of malate was observed. These findings have provided evidence that N<sub>2</sub> fixation of *M. truncatula* is mediated through an N feedback mechanism which is closely related to nodule carbon metabolism.

**Key words:** Asparagine, carbon and nitrogen metabolites, feedback, *Medicago truncatula*, nitrogen fixation, nitrogenase activity, nodulation, phloem, phosphorus availability, plant growth, symbiosis capacity, translocation.

## Introduction

Phosphorus (P) deficiency forms an important constraint for legume crop production, especially in tropical marginal countries (Bilyeu *et al.*, 2008; Calderón-Vázquez *et al.*, 2011). Most arable lands throughout the world are considered to be P deficient, and the global P reserves are expected to deplete rapidly during the few next decades (Lambers *et al.*, 2006; Ha and Tran, 2013). Accordingly, the crop growth and yield are expected to reduce greatly due to low P availability especially for legumes, since legume

nodules responsible for N<sub>2</sub> fixation have high P requirements (Sulieman and Tran, 2012). Yet, P is often the element that sets the limits for biological productivity (Sato and Miura, 2011; López-Arredondo and Herrera-Estrella, 2012). Legumes are well known to be a vital component of various agroecosystems, particularly in developing countries (Tran and Nguyen, 2009; Thao and Tran, 2012). Among the legumes, the model plant *Medicago truncatula* is widely believed to be one of the best systems for scientists

to develop efficient cultivars that are more tolerant to P deficiency and provide higher yields with less need for P fertilizers (Jain et al., 2007). Among the many reasons why *M. truncatula* was chosen as a model legume are its modest genome size of 500–550 Mbp, simple diploid genetics, short seed-to-seed generation time, workable levels of transformation, availability of excellent mutant populations, and large collections of diverse ecotypes (Young and Udvardi, 2009).

Unfortunately, up to now a comprehensive study focused on evaluating the growth of *M. truncatula* under a wide range of gradually increasing P supplies to optimize their growth in a hydroponic system, which is often used for growing *M. truncatula* for physiological and molecular studies, is still lacking. Most of the experiments performed were based on two extreme P levels (deficient versus sufficient) or by using several P levels with large intervals (Tang et al., 2001; Sulieman et al., 2010). Detailed knowledge of the effect of P nutrition on plant growth and development would enable the design of experiments to dissect the mechanism(s) regulating the relationship between symbiotic N<sub>2</sub> fixation and P supply (Sulieman and Tran, 2012). This, in turn, is the cornerstone for development of any future novel N<sub>2</sub>-fixing crops, which have high N<sub>2</sub> fixation capacity under P limitation, by genetic engineering. Indeed, genotypes with higher P efficiency, which are defined broadly as those having the ability to grow and yield with low P availability, would be extremely useful (Richardson et al., 2011).

Although the *M. truncatula* international community has focused intensively on the dissection of the genetic and molecular bases of the nodulation process in legumes, little is known about the symbiotic efficiency of *M. truncatula* × *Sinorhizobium meliloti* under normal or environmental limitations. The precise understanding of factors affecting *M. truncatula*–bacterial association will enable improvement of the symbiotic efficiency by genetic engineering. In this regard, it has recently been reported that the model *S. meliloti* 2011 strain formed a less effective symbiotic association with *M. truncatula* than with *M. sativa* under normal growing conditions (Sulieman and Schulze, 2010a), while the reverse was revealed under P deprivation (Sulieman et al., 2013). However, a comparison of *S. meliloti* 2011 and the most frequently used *S. meliloti* 102F51 strain in terms of symbiotic efficiency has not been performed with *M. truncatula* under various levels of P limitation.

Thus, in the present study, the growth of *M. truncatula* plants was initially optimized in a nutrient solution culture by considering a wide range of gradually increasing P levels. On the basis of the obtained results, the symbiotic performance of the model *S. meliloti* 2011 strain and the most frequently used *S. meliloti* 102F51 strain with *M. truncatula* was evaluated under low P supply conditions. Finally, the physiological mechanism responsible for the regulation of nitrogenase activity under P deficiency was examined by addressing the possible inter-relationship between various internal N and C metabolites under the same conditions.

## Materials and methods

### Plant materials and growth conditions

*Medicago truncatula* Gaertn. ‘Jemalong’ (line A17) was hydroponically grown in a controlled environment chamber at 23/18 °C with a 16 h photoperiod and a relative humidity of ~70%. Photosynthetic active radiation was ~360 μmol m<sup>-2</sup> s<sup>-1</sup> measured at the top of the canopy. Initially, seeds were chemically scarified in 95% sulphuric acid for 6 min, surface sterilized with 5% sodium hypochlorite for 3 min, cold-treated at 4 °C in water overnight, then sown in 1 mm diameter silica and irrigated manually with tap water until transplanting. For all studies, the transplanting date was designed as day 0.

Twelve days after planting, four plantlets were transferred to 3 litre plastic pots containing a vigorously aerated nutrient solution. The pots were arranged in a randomized design with four replicates for each treatment. The base nutrient solution had the following composition: 700 μM K<sub>2</sub>SO<sub>4</sub>; 500 μM MgSO<sub>4</sub>; 800 μM CaCl<sub>2</sub>; 1.3 μM H<sub>3</sub>BO<sub>3</sub>; 0.03 μM Na<sub>2</sub>MoO<sub>4</sub>; 0.3 μM ZnSO<sub>4</sub>; 0.7 μM MnCl<sub>2</sub>; 0.07 μM CoCl<sub>2</sub>; 0.3 μM CuCl<sub>2</sub>; and 3.3 μM FeNaEDTA (ferric monosodium salt of ethylenediamine tetra-acetic acid). The pH was adjusted with 0.25 mM MES [2-(N-morpholino) ethane-sulphonic acid]. Plants were supplied with (0.3 mM N) urea starter N at transplanting and at the first replacement of the solution. Thereafter, they were grown in N-free nutrient solution. The solution was renewed once a week during the first 14 DAT (days after transplanting) and was then changed twice a week to avoid depletion of the nutrients. Solution pH was adjusted daily with KOH to 6.5. Compared with other legumes, nodulated *M. truncatula* plants have a limited P range (up to 15 μM P) for growth and nodule development (Tang et al., 2001; Sulieman et al., 2010). Under concentrations of <1 μM P, previously no nodulation established in the cultivated plants (Sulieman et al., 2008). In the present study, P was applied at distinct P levels (1, 5, 9, 12, and 15 μM) when the seedlings were transplanted to the nutrient solution. KH<sub>2</sub>PO<sub>4</sub> was the source of P in the experiment. The nutrient solutions were supplemented with K<sub>2</sub>SO<sub>4</sub> to ensure equal potassium supply in all these P treatments.

A dense water suspension of *S. meliloti* (102F51) was added to the solution at a rate of ~10<sup>8</sup> cells ml<sup>-1</sup> twice. This strain is known to lack uptake hydrogenase activity (Hup<sup>-</sup>), thus enabling the measurement of H<sub>2</sub> evolution for nitrogenase activity (Sulieman and Schulze, 2012b). Inoculum was prepared by growing the bacteria in yeast extract mannitol (YEM) medium at 28 °C for 4 d. Inoculation resulted in intensive nodulation, and the first nodules became visible ~8–11 d after the first inoculation. Plants were harvested at 63 DAT and were fractioned into shoots, roots, and nodules. The nodule samples were frozen immediately on dry ice and stored at –20 °C until subsequent analysis.

### Gas exchange measurements

A separate set of plants was grown as described above at two P levels: 1 μM (deficient P) and 12 μM (sufficient P). Nitrogenase (EC 1.7.99.2) activity was measured *in situ* as H<sub>2</sub> evolution using an open flow gas exchange system. *Medicago truncatula* plants were grown in glass cylinders as previously described by Sulieman and Schulze (2010b). The cylinder contained 250 ml of the above-mentioned nutrient solution and was inoculated with 1 ml of either the *S. meliloti* 102F51 or 2011 strain during the first 10 d at each solution change. Plants were supplied with (0.3 mM N) urea starter N for 3 weeks after transplanting. The solution was intensely aerated by an airflow of normal air of ~1 vol. min<sup>-1</sup> during the experiment. On the 63rd day of growth, the nodulated root system of the plants was sealed within their dark glass cylinders and connected to a computer-controlled, open-flow gas analysis system. This time frame corresponds to the maximum potential nitrogenase activity in *M. truncatula* and other plant species, such as soybean (Fabre and Planchon, 2000; Sulieman et al., 2013). Apparent nitrogenase activity (ANA) was measured as H<sub>2</sub> evolution in an N<sub>2</sub>:O<sub>2</sub> (80:20) mixture, and total nitrogenase activity (TNA) was determined as the

peak value of  $H_2$  evolution in an  $Ar:O_2$  (80:20) mixture. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as  $[1 - (ANA/TNA)]$  (Sulieman and Schulze, 2010a).

#### Phloem sap collection and analyses

At the end of the experiment, shoot branches were cut off at the bases and immediately re-cut under an Na-EDTA solution (5 mM, pH 6.0) (King and Zeevaert, 1974). The yield of the exudation was increased by a further cut of the branches at 2 mm from the previous cut 1 h after the beginning of the collection. Phloem sap collection was carried out for 4 h duration at the same time (09:00 h) in a humid climate chamber in the dark. At the end of the collection period, branch fresh weights (FWs) were measured and the phloem-EDTA solutions were frozen ( $-20^\circ C$ ) until subsequent analysis. For determination of the amino acid composition, phloem sap was analysed using a fluorescence detector (Waters, Milford, MA, USA).

#### Nodule sugars and organic acid analyses

Frozen nodule materials were ground to a fine powder and homogenized with a mortar and pestle in liquid N. Around 0.5 mg of each sample was extracted with 3 ml of 50% (v/v) ethanol for 20 min in a  $50^\circ C$  water bath. The solution samples were subsequently centrifuged at  $4^\circ C$  for 20 min at 8000 rpm (6810 g). The supernatants were immediately used for the high-performance liquid chromatography (HPLC) analyses after filtration (0.45  $\mu m$ ). The detection of various nodule sugars was carried out using a refractometer (Knauer, Berlin, Germany), while for organic acids the determination was carried using a photodiode array detector 996 (Waters).

#### Determination of N and P contents

Dry matter samples of various plant organs (shoot, root, and nodules) were ground to a fine powder and used for determination of P and N contents. The plant samples were weighed, ground, and the subsamples (0.3 g) were digested with a mixture of  $HNO_3$  and  $H_2O_2$  (30%) in a volumetric ratio (4:2) in a microwave oven. Phosphate in the extract was measured calorimetrically as previously described (Scheffer and Pajenkamp, 1952). Nitrogen was determined in a C/N analyser according the manufacturer's instructions (Vario EL, Elementar GmbH, Hanau).

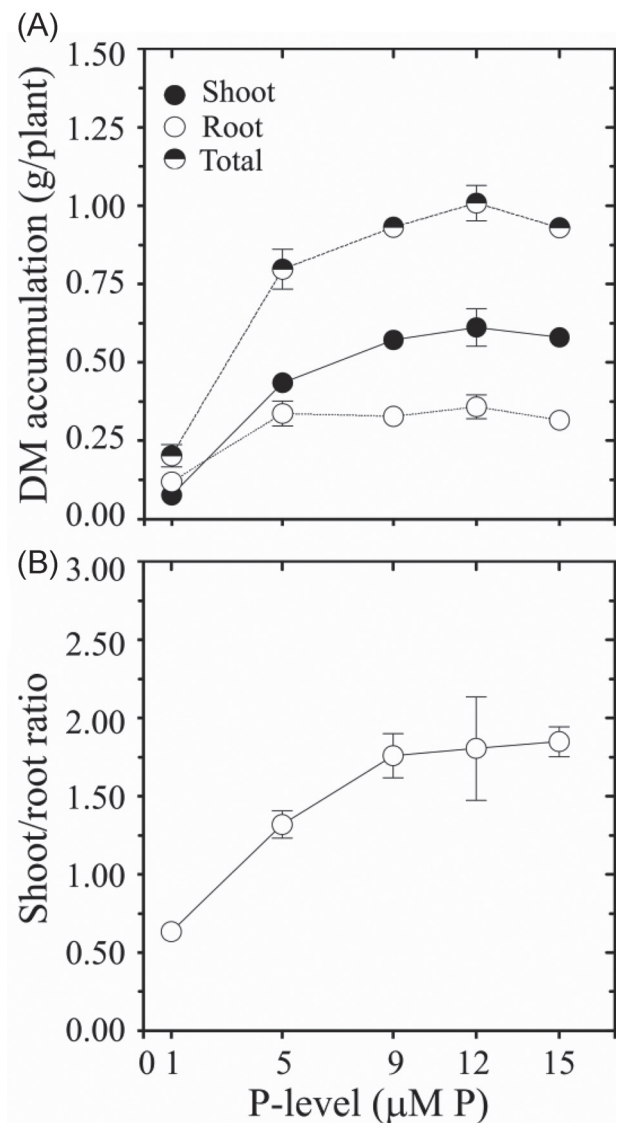
#### Statistical analysis

Statistical analyses were carried out using Sigastat analytical software (version 3.5, Systat software, Inc.: San Jose, CA, USA) to determine the significance of the observed differences at the 95% confidence level. Mean separation procedures were carried out using the Tukey's test.

## Results

### Effect of P supply on plant biomass production

Decreasing P supply in the growing medium was followed by a significant reduction ( $P \leq 0.001$ ) in the dry matter (DM) accumulation of *M. truncatula* plants. Shoot and root growth was increased proportionally with increasing levels of P supply, leading to improved total DM production, with the highest total DM value recorded at a P level of 12  $\mu M$  (Fig. 1A), suggesting that 12  $\mu M$  is the optimal P concentration for the growth of *M. truncatula* plants in a hydroponic system. Under low P levels ( $<12 \mu M$ ), shoot DM production was more affected when compared with that of the roots, which, unlike the shoots, had similar growth rates at 5, 9, and 12  $\mu M$



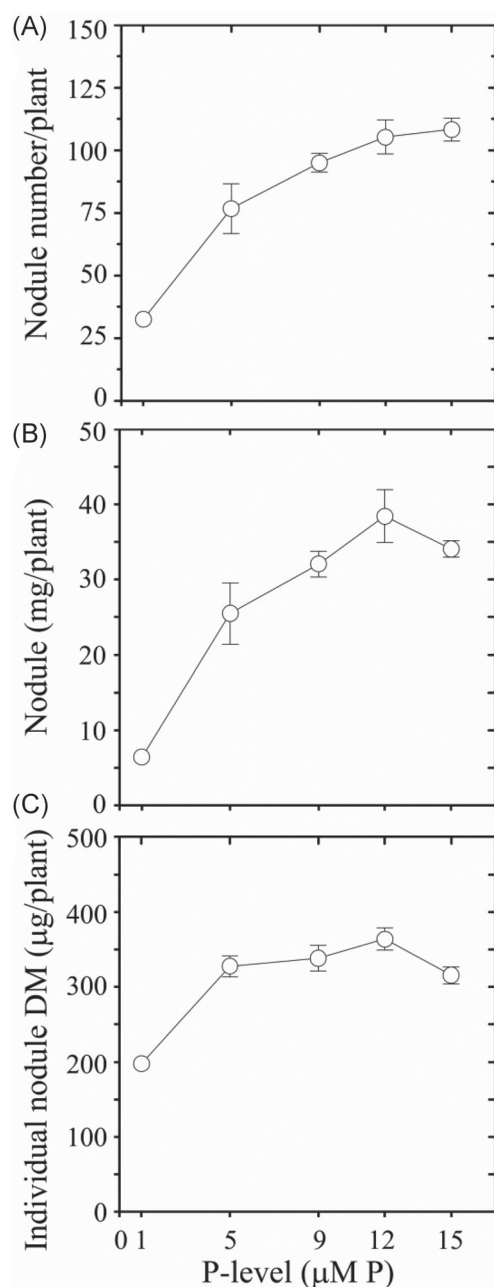
**Fig. 1.** Effect of P supply on (A) dry matter (DM) formation and (B) the shoot/root ratio of *M. truncatula* plants hydroponically grown for 9 weeks with supplementation with the indicated concentrations of P. Data are the means of four replicates, with error bars representing SE values.

P (Fig. 1A). Consequently, the shoot/root ratio ( $g g^{-1}$ ) was increased with an increase in the P supply (Fig. 1B).

### Effect of P supply on nodulation

Nodules were first recognized at 5 DAT and were clearly visible on the plant root system between 8 and 10 DAT. All nodulation parameters investigated were significantly ( $P \leq 0.001$ ) affected by P supply. Nodule number showed a strong tendency to increase as the P level in the growing medium was increased up to 15  $\mu M$  (Fig. 2A). Total nodule dry weight was markedly increased with increasing P supply, and reached the maximum at a P level of 12  $\mu M$  (Fig. 2B). Dry matter weight of the individual nodules exhibited a similar pattern to the total nodule DM production (Fig. 2C).





**Fig. 2.** Effect of P supply on (A) the number of nodules, (B) total nodule dry matter (DM) accumulation, and (C) individual nodule DM formation in *M. truncatula* plants hydroponically grown for 9 weeks with supplementation with the indicated concentrations of P. Data are the means of four replicates, with error bars representing SE values.

#### Effect of P supply on N and P concentrations

P concentrations in both shoot and root were significantly increased ( $P \leq 0.001$ ) with the increase in P level supplied to the nutrient solution, and the P concentration in the roots exceeded that of the shoots at all ranges of P supplementations (Table 1). It is important to note that a P concentration of 15  $\mu\text{M}$  was found to produce P toxicity symptoms in *M. truncatula* plants. The P toxicity symptoms started with a yellowing of the leaf margins between the second and third

weeks after full nodule development, which quickly turned into necrotic tissues (Fig. 3). As for total P content, it was observed that the total P content of the shoots showed a sharp increment with the increase in the level of supplemented P, while that of the roots reached the peak level at 12  $\mu\text{M}$  P and decreased thereafter.

With respect to N assimilation, the N content ( $\text{mg plant}^{-1}$ ) of the whole plant and various plant organs was increased with increased P level in the culture medium (Table 1). In contrast, the N concentration ( $\text{mg g}^{-1}$ ) was found to be significantly decreased ( $P \leq 0.001$ ) with increasing P supply and a strong effect was revealed for the shoots. The C/N ratios were remarkably decreased under low P supply, and the strongest response was observed for the shoot followed by the root and nodules (Table 1). In addition, decreasing P supply resulted in a significant increase ( $P \leq 0.001$ ) in the N/P ratio. At the lowest supplied P concentration (1  $\mu\text{M}$ ), the ratio of the shoot was twice that of the root (Table 1).

#### Effect of P supply on phloem free amino acids

The response of various free amino acids in the phloem sap to different levels of P is shown in Fig. 4A. The levels of all free amino acids analysed were found to be increased with the decrease in supplementation of P to the nutrient solution. The highest increase was noticed for the amide asparagine at the lowest P supply of 1  $\mu\text{M}$ , which was 17-fold higher in concentration when compared with that obtained at 12  $\mu\text{M}$  supplied P (Fig. 4A). The total free amino acid concentration of the phloem sap was also highly increased with decreasing P supply in the plant culture solution (Fig. 4B). These data suggested that the levels of amino acids, both individually and in total, were negatively correlated with the levels of P supply. In addition, consistent with its highest accumulation at a low P level (1  $\mu\text{M}$ ), asparagine was found to constitute a significant amount, ~28%, of the total amino acids under this severe P-deficient condition (Fig. 4C).

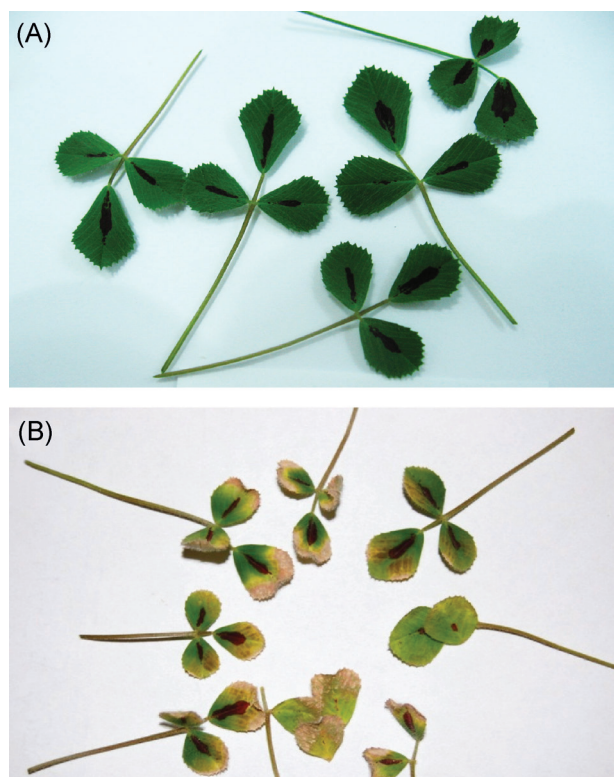
#### Effect of P supply on accumulation of organic acids and sugars in nodules

A remarkable different feature in the contents of nodule organic acids was observed as shown in Fig. 5A. *Medicago truncatula* had a strong tendency to accumulate a large share of its organic acids as succinate under sufficient P-conditions (Fig. 5A). Citrate showed a similar pattern to succinate, although the concentration of this organic acid was comparatively lower at every concentration of P supplementation. Under severe P deficiency (1  $\mu\text{M}$  P), there was a significant reduction in the concentration of succinate with a pronounced shift to the synthesis of malate and at a lower level to that of fumarate. These data indicated that both malate and fumarate tended to accumulate under lower P treatments. For sugar metabolites, sucrose, glucose, and fructose were the major compounds detected in nodules, and sucrose was found to be the dominant compound under sufficient P supply conditions (Fig. 5B). Severe P deficiency (1  $\mu\text{M}$  P) resulted in a sharp reduction in the concentration of sucrose, while the rest

**Table 1.** Effect of P supply on P and N assimilation and N/P and C/N ratios of *M. truncatula* plants grown for 9 weeks in a nutrient solution supplemented with various P concentrations.

	P concentration in nutrient solution ( $\mu\text{M P}$ )				
	1	5	9	12	15
P concentration in DM ( $\text{mg g}^{-1}$ )					
Shoot	$0.67 \pm 0.01$ a	$0.87 \pm 0.03$ a	$1.52 \pm 0.08$ b	$2.02 \pm 0.13$ c	$2.71 \pm 0.18$ d
Root	$1.22 \pm 0.04$ a	$1.60 \pm 0.10$ a	$2.26 \pm 0.14$ b	$2.59 \pm 0.10$ cb	$2.88 \pm 0.20$ c
P content ( $\text{mg plant}^{-1}$ )					
Shoot	$0.05 \pm 0.01$ a	$0.38 \pm 0.03$ a	$0.87 \pm 0.06$ b	$1.22 \pm 0.12$ cb	$1.57 \pm 0.12$ c
Root	$0.15 \pm 0.03$ a	$0.53 \pm 0.04$ b	$0.74 \pm 0.02$ bc	$0.92 \pm 0.08$ c	$0.91 \pm 0.08$ c
N/P ratio (%/%)					
Shoot	$51.31 \pm 1.49$ a	$17.00 \pm 0.61$ b	$7.36 \pm 0.12$ c	$5.81 \pm 0.46$ c	$4.43 \pm 0.33$ c
Root	$25.51 \pm 0.74$ a	$15.68 \pm 1.27$ b	$11.09 \pm 0.69$ c	$10.25 \pm 0.12$ c	$9.73 \pm 0.53$ c
N concentration in DM (%)					
Shoot	$3.46 \pm 0.13$ a	$1.46 \pm 0.04$ b	$1.12 \pm 0.05$ c	$1.15 \pm 0.02$ c	$1.18 \pm 0.04$ bc
Root	$3.09 \pm 0.02$ a	$2.47 \pm 0.07$ b	$2.50 \pm 0.16$ b	$2.66 \pm 0.12$ b	$2.77 \pm 0.05$ ab
Nodules	$4.68 \pm 0.07$ a	$4.15 \pm 0.14$ b	$4.18 \pm 0.06$ b	$3.95 \pm 0.05$ b	$4.23 \pm 0.01$ b
N content ( $\text{mg plant}^{-1}$ )					
Shoot	$2.66 \pm 0.58$ a	$6.36 \pm 0.49$ b	$6.40 \pm 0.46$ b	$7.03 \pm 0.62$ b	$6.86 \pm 0.26$ b
Root	$3.69 \pm 0.63$ a	$8.33 \pm 1.04$ b	$8.14 \pm 0.38$ b	$9.43 \pm 0.81$ b	$8.73 \pm 0.45$ b
Nodules	$0.30 \pm 0.03$ a	$1.07 \pm 0.19$ b	$1.34 \pm 0.07$ c	$1.52 \pm 0.14$ c	$1.44 \pm 0.04$ c
Total	$6.64 \pm 1.19$ a	$15.76 \pm 1.33$ b	$15.88 \pm 0.83$ b	$17.98 \pm 1.11$ b	$17.03 \pm 0.45$ b
C/N ratio (%/%)					
Shoot	$12.30 \pm 0.51$ a	$28.96 \pm 0.84$ b	$37.88 \pm 1.64$ c	$36.54 \pm 0.84$ c	$35.44 \pm 1.07$ c
Root	$13.69 \pm 0.19$ a	$17.16 \pm 0.46$ b	$17.40 \pm 1.11$ b	$16.02 \pm 0.77$ ab	$15.46 \pm 0.06$ ab
Nodules	$9.80 \pm 0.09$ a	$10.59 \pm 0.25$ b	$10.42 \pm 0.18$ ab	$10.83 \pm 0.10$ b	$10.49 \pm 0.13$ ab

Data presented are the means  $\pm$ SE of four replicates. Different letters (a, b, c, and d) within a row indicate a significant difference from each other in all combinations (Tukey's test,  $P \leq 0.001$ ).

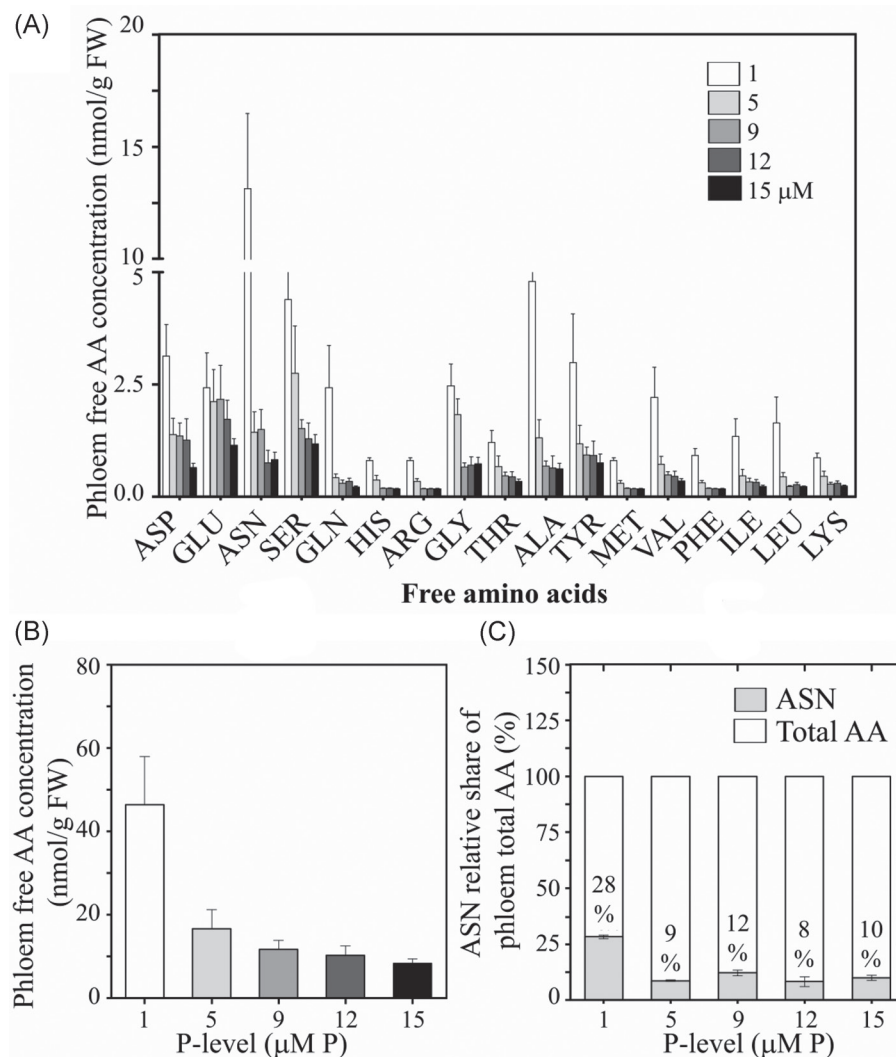
**Fig. 3.** *Medicago truncatula* leaves with (A) normal and (B) P toxicity symptoms. The plants were grown in nutrient solution at a P

of the sugars remains relatively unaffected at any supplied P level (Fig. 5B).

#### Effect of P supply on the performance of *S. meliloti* 102F51 and 2011 strains with differential N-fixing activity

A gas exchange trial experiment was carried out to examine the effect of P deficiency ( $1 \mu\text{M P}$ ) on the performance of two symbiotic partners, *S. meliloti* 102F51 and 2011 strains, which are known to have a different  $\text{N}_2$ -fixing capacity in combination with Jemalong A17 (Suliman and Schulze, 2010a). It was found that P deficiency severely inhibited plant growth and development of nodules (Table 2) as well as N and P assimilation (Table 3) when *M. truncatula* plants were inoculated with either the *S. meliloti* 102F51 or 2011 strain. When both combinations were compared under the P limitation level, it was revealed that the negative effect of P deficiency was more pronounced in terms of the plant growth and nodule development of the *S. meliloti* 102F51–*M. truncatula* combination relative to *S. meliloti* 2011–*M. truncatula*. Application of  $1 \mu\text{M P}$  resulted in an  $\sim 8.6$ - and  $7.4$ -fold reduction in shoot DM accumulation in plants inoculated

concentration of  $12 \mu\text{M}$  or  $15 \mu\text{M}$ , respectively. Toxicity started with a yellowing of the leaf margins, which quickly turned into necrotic tissues.

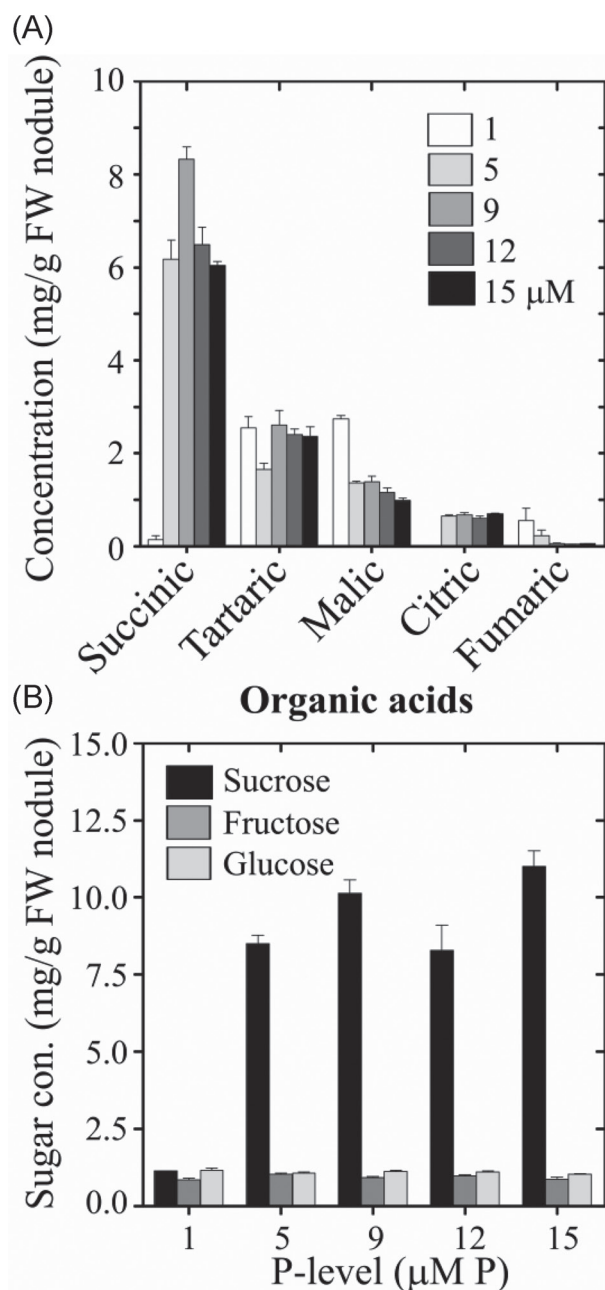


**Fig. 4.** Effect of P supply on (A) phloem free amino acid composition, (B) phloem total free amino acid composition, and (C) relative share of phloem asparagine of the total amino acids in *M. truncatula* plants hydroponically grown for 9 weeks with supplementation with the indicated concentrations of P. Data are the means of four replicates, with error bars representing SE values. FW, fresh weight; AA, amino acids; ALA, alanine; ARG, arginine; ASN, asparagine; ASP, aspartic acid; GLU, glutamic acid; GLN, glutamine; GLY, glycine; HIS, histidine; ILE, isoleucine; LEU, leucine; LYS, lysine; MET, methionine; PHE, phenylalanine; SER, serine; THR, threonine; TYR, tyrosine; VAL, valine.

with *S. meliloti* 102F51 and *S. meliloti* 2011, respectively. When the amount of fixed N and the nitrogenase activity were quantified in plants inoculated with either *S. meliloti* 102F51 or 2011, it was observed that the supplementation with 1  $\mu$ M P significantly reduced the fixed N amount and nitrogenase activity relative to 12  $\mu$ M P treatment (Table 4). This finding suggested that a low level of P supply had a negative impact on  $N_2$  fixation. The comparatively higher reduction in plant growth and nodulation for *S. meliloti* 102F51-inoculated plants compared with *S. meliloti* 2011-inoculated plants under low P was also reflected in the gas measurements (Table 4). Although the reductions in ANA, TNA, and EAC were much higher in the *S. meliloti* 102F51 combination than in the *S. meliloti* 2011 combination, a significant level was reached only for TNA. The reduction in the daily fixed N under low P was in line with the gas measurements for inoculation with *S. meliloti* 102F51, while the

specific activity for nitrogenase showed a higher tendency to increase in the case of inoculation with *S. meliloti* 2011 when compared with *S. meliloti* 102F51 under a limited P level (Table 4). Overall, the *S. meliloti* 102F51–*M. truncatula* combination revealed more sensitivity to P deficiency when compared with *S. meliloti* 2011-inoculated plants, while the latter combination was more adapted to low P by increasing the specific fixed N level. As a result, *S. meliloti* 2011-inoculated plants were relatively better in terms of plant growth and N% (Table 2). On the other hand, under P-sufficient conditions, the *S. meliloti* 102F51-inoculated plants had a higher nitrogenase activity, and subsequently more fixed N, in comparison with *S. meliloti* 2011-inoculated plants (Table 4). In agreement with this finding, these plants produced more biomass and a higher N concentration, suggesting that *S. meliloti* 102F51 could form a better combination with *M. truncatula* than *S. meliloti* 2011 under sufficient P supply.





**Fig. 5.** Effect of P supply on (A) concentrations of organic acids and (B) concentration of sugars in nodules of *M. truncatula* plants hydroponically grown for 9 weeks with supplementation with the indicated concentrations of P. Data are the means of four replicates, with error bars representing SE values. FW, fresh weight.

## Discussion

P deficiency is one of the critical limiting factors, adversely affecting nodulation and  $N_2$  fixation, and thereby legume growth and productivity, worldwide (Tesfaye *et al.*, 2007). Plants engaged in symbiotic  $N_2$  fixation are known to have high P demand (Vance *et al.*, 2003; Sulieman *et al.*, 2013). This is related to the high energy that is required for nitrogenase to function at a high level. Other processes that contribute to a plant's high requirement for P include signal transduction,

enzyme activation–inactivation, C partitioning, plastid function, and membrane biosynthesis (Graham and Vance, 2000).

The present study has considered a comprehensive experimental approach aimed at addressing three main objectives that have a close inter-relationship. The first aim was to test the crop's performance at several gradually increasing P levels and to develop a proper hydroponic system for growing *M. truncatula* plants. Such a system will help in producing plants with high growth rates and enable accurate physiological and molecular studies to be performed. Only one study has been available to date using this platform, and this examined only four levels of P supply with a wide interval between the levels, where the highest concentration was almost double the lowest concentration (Tang *et al.*, 2001). Thus, this study might not provide exactly optimal P supply conditions for *M. truncatula* plants. In the present approach, the gradual increases in P supply to the symbiotic plants resulted in a proportional increase in the DM accumulation for the growing plants up to 12  $\mu M$  P and started to decrease thereafter (Fig. 1A). Thus, the results suggested that 12  $\mu M$  P is the optimal level for growing *M. truncatula* plants in solution culture. These results apparently indicated that *M. truncatula* possesses an optimal P level which is relatively close to the level that induces P toxicity (Fig. 3). P toxicity in *M. truncatula* is an unusual phenomenon as it occurs at a P concentration (2.7  $mg\ g^{-1}$  DM for shoots) that is considered comparatively low to most other leguminous and non-leguminous plant species (Table 1). For example, the P concentrations at which toxicity symptoms developed were reported to be 15  $mg\ g^{-1}$  DM in white lupin (Keerthizinghe *et al.*, 1998), 17.2–22  $mg\ g^{-1}$  DM in yellow lupin (Warren and Benizian, 1959), 14.5  $mg\ g^{-1}$  DM in *Arabidopsis* (Delhaize and Randall, 1995), 20–28  $mg\ g^{-1}$  DM in barley (Green *et al.*, 1973), 8.4–13.7  $mg\ g^{-1}$  DM in wheat (Loneragan *et al.*, 1966), and 6–8  $mg\ g^{-1}$  DM in black gram (Bell *et al.*, 1990). Due to this fact, the phenomenon of P toxicity, although not common and not widely recognized in nature, deserves more investigation for this model plant that might suffer a disruption in the ability to cope with a high P concentration in the solution.

On the basis of the results obtained from the initial growth optimization trial, it was possible to compare the performance of two symbiont strains *S. meliloti* 102F51 and 2011, which are reported to have different symbiotic capabilities with *M. truncatula* (Sulieman and Schulze, 2010a), under P deprivation conditions. This premise was necessary for identifying the better symbiotic partner for *M. truncatula* between both strains, as well as characterizing the reasons for the suboptimal efficiency for *M. truncatula*  $\times$  *S. meliloti* under low P supply. Indeed, little is known about how *M. truncatula*  $\times$  *S. meliloti* is affected by P deficiency at the whole-plant level (Sulieman *et al.*, 2013). The present data revealed that under conditions of suboptimal P supply (1  $\mu M$  P), the symbiotic performance of *M. truncatula*–*S. meliloti* 102F51 plants was more sensitively affected when compared with that derived from combination with *S. meliloti* 2011 (Tables 2, 3). As a result, *S. meliloti* 2011-inoculated *M. truncatula* plants were comparatively more adapted to low P supply, showing higher plant growth and shoot N%

**Table 2.** Dry matter and nodulation of *M. truncatula* plants inoculated with *S. meliloti* 2011 or *S. meliloti* 102F51 and grown for 9 weeks in a nutrient solution supplemented with either a deficient (1  $\mu$ M) or sufficient (12  $\mu$ M) P level.

	<i>S. meliloti</i> 2011		<i>S. meliloti</i> 102F51	
	1 $\mu$ M P	12 $\mu$ M P	1 $\mu$ M P	12 $\mu$ M P
DM (mg plant <sup>-1</sup> )				
Shoot	104.6 $\pm$ 11.7 b	780.0 $\pm$ 23.8 c	98.7 $\pm$ 13.8 b	848.7 $\pm$ 47.6 a
Root	150.2 $\pm$ 24.9 b	443.1 $\pm$ 39.8 a	149.3 $\pm$ 18.6 b	458.6 $\pm$ 40.4 a
Nodules	2.2 $\pm$ 0.4 b	61.5 $\pm$ 25.2 a	1.9 $\pm$ 0.4 b	56.9 $\pm$ 23.5 a
Total	257.0 $\pm$ 34.2 b	1284.7 $\pm$ 77.6 a	249.9 $\pm$ 32.2 b	1364.2 $\pm$ 65.7 a
Shoot/root (mg mg <sup>-1</sup> )	0.7 $\pm$ 0.1 b	1.8 $\pm$ 0.1 a	0.7 $\pm$ 0.0 b	1.9 $\pm$ 0.1 a
Nodule number plant <sup>-1</sup>	12.0 $\pm$ 4.5 b	60.3 $\pm$ 8.8 a	9.8 $\pm$ 1.3 b	48.8 $\pm$ 13.5 a

Data presented are the means  $\pm$ SE of four replicates. Different letters (a, b, and c) within a row indicate a significant difference from each other in all combinations (Tukey's test,  $P \leq 0.001$ ).

**Table 3.** N and P assimilation and C/N ratio of *M. truncatula* plants inoculated with *S. meliloti* 2011 or *S. meliloti* 102F51 and grown for 9 weeks in a nutrient solution supplemented with either a deficient (1  $\mu$ M) or sufficient (12  $\mu$ M) P level.

	<i>S. meliloti</i> 2011		<i>S. meliloti</i> 102F51	
	1 $\mu$ M P	12 $\mu$ M P	1 $\mu$ M P	12 $\mu$ M P
P concentration in DM (mg g <sup>-1</sup> )				
Shoot	0.53 $\pm$ 0.1 b	0.85 $\pm$ 0.1 a	0.53 $\pm$ 0.1 b	0.95 $\pm$ 0.2 a
Root	0.88 $\pm$ 0.1 b	1.30 $\pm$ 0.1 a	0.85 $\pm$ 0.1 b	1.38 $\pm$ 0.1 a
Nodules	2.18 $\pm$ 0.1 b	3.45 $\pm$ 0.1 a	2.12 $\pm$ 0.1 b	3.39 $\pm$ 0.1 a
Total P (mg)	0.19 $\pm$ 0.0 b	1.45 $\pm$ 0.2 c	0.18 $\pm$ 0.0 b	1.63 $\pm$ 0.1 a
%N in DM				
Shoot	2.64 $\pm$ 0.5 b	2.09 $\pm$ 0.1 a	2.47 $\pm$ 0.1 b	2.17 $\pm$ 0.2 a
Root	2.35 $\pm$ 0.1 c	1.98 $\pm$ 0.2 a	2.59 $\pm$ 0.2 b	1.95 $\pm$ 0.1 a
C/N ratio (%/%)				
Shoot	17.0 $\pm$ 3.3 b	20.4 $\pm$ 1.0 a	18.1 $\pm$ 1.0 b	19.5 $\pm$ 1.5 a
Root	17.8 $\pm$ 1.2 b	21.0 $\pm$ 2.7 a	16.4 $\pm$ 1.0 b	21.2 $\pm$ 1.6 a

Data presented are the means  $\pm$ SE of four replicates. Different letters (a, b, and c) within a row indicate a significant difference from each other in all combinations (Tukey's test,  $P \leq 0.001$ ).

**Table 4.** Nitrogenase activity, electron allocation, and N<sub>2</sub> fixation of *M. truncatula* plants inoculated with *S. meliloti* 2011 or *S. meliloti* 102F51 and grown for 9 weeks in a nutrient solution supplemented with either a deficient (1  $\mu$ M) or sufficient (12  $\mu$ M) P level.

	<i>S. meliloti</i> 2011		<i>S. meliloti</i> 102F51	
	1 $\mu$ M P	12 $\mu$ M P	1 $\mu$ M P	12 $\mu$ M P
Nitrogenase activity:				
ANA ( $\mu$ mol H <sub>2</sub> plant <sup>-1</sup> h <sup>-1</sup> )	0.27 $\pm$ 0.2 b	2.26 $\pm$ 0.7 a	0.32 $\pm$ 0.2 b	2.93 $\pm$ 0.4 a
TNA ( $\mu$ mol H <sub>2</sub> plant <sup>-1</sup> h <sup>-1</sup> )	0.74 $\pm$ 0.4 a	4.64 $\pm$ 1.1 c	0.92 $\pm$ 0.2 b	7.24 $\pm$ 1.0 a
EAC	0.64 $\pm$ 0.1 b	0.49 $\pm$ 0.2 a	0.68 $\pm$ 0.1 b	0.59 $\pm$ 0.0 a
Fixed N (mg N 24 h <sup>-1</sup> )	0.11 $\pm$ 0.1 b	0.53 $\pm$ 0.3 c	0.13 $\pm$ 0.1 b	0.97 $\pm$ 0.2 a
Specific fixed N ( $\mu$ g N mg nodule <sup>-1</sup> h <sup>-1</sup> )	1.96 $\pm$ 0.4 b	0.39 $\pm$ 0.1 a	3.08 $\pm$ 0.6 b	0.83 $\pm$ 0.2 a

Data presented are the means  $\pm$ SE of four replicates. Different letters (a, b, and c) within a row indicate a significant difference from each other in all combinations (Tukey's test,  $P \leq 0.001$ ).

under P deficiency (Tables 2, 3). In accordance with these findings, P deficiency resulted in a 15% and 31% increment in EAC as measured for plants inoculated with *S. meliloti* 102F51 and 2011, respectively (Table 4). These findings suggested that the high efficiency in N<sub>2</sub> fixation observed in

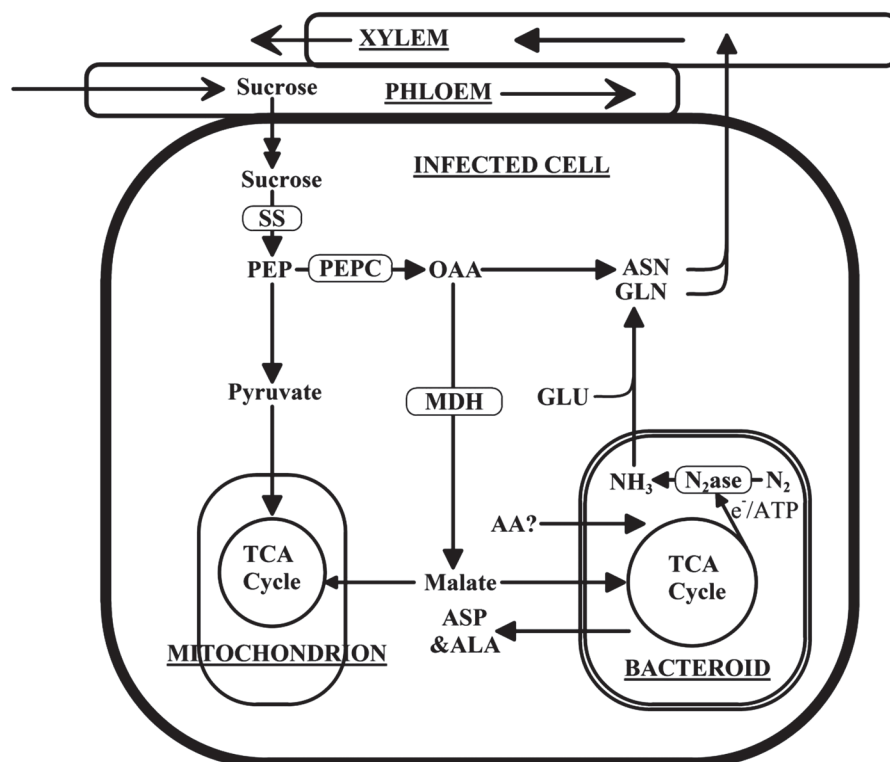
*S. meliloti* 2011-inoculated *M. truncatula* under P deficiency were due to the higher increase in specific activity of nodules (N<sub>fixed</sub> per unit nodule biomass), partially as a result of a high relative efficiency (electrons allocated to N<sub>2</sub> versus H<sup>+</sup>) (Table 4).



Since nitrogenase activity is widely known to consume the largest amounts of energy to drive  $N_2$  reduction, it remains necessary for *M. truncatula* to regulate this process tightly, particularly under P limitation conditions (González *et al.*, 2001; Sulieman *et al.*, 2010). To discover the reasons behind the decline in nitrogenase activity at different P levels, phloem sap extraction was used for the detection of the shift in amino-N composition over the whole set of P concentrations (Fig. 4A). Phloem sap extraction is one of the most appropriate methods used for studying the long-distance translocation in legumes and for characterization of the potential signal(s) involved in nitrogenase control, namely whole-plant N feedback regulation (Atkins and Smith, 2007; Sulieman and Tran, 2012). This method is reliable and has been preferentially used in the study of the regulation of  $N_2$  fixation in several species, including soybean (Neo and Layzell, 1997), white clover (Høgh-Jensen *et al.*, 2002), white lupin (Hartwig and Trommler, 2001), and *M. truncatula* (Sulieman *et al.*, 2010). Most of the previous interpretations of nitrogenase down-regulation were based on the remarkable accumulation of free amino acids in the nodules (Schulze and Dreven, 2005) or the roots (Almeida *et al.*, 2000) of P-stressed plants. The results obtained in this report support the whole N feedback mechanism which is inversely related to the P supply in the growing medium. The possible scenario under limiting P conditions could be explained as follows: the remarkable reduction in the host plant growth (Fig. 1A, Table 1) would result in a reduction in N demand for the newly fixed N, a component which is known to be highly expensive in term of C energy (Fischinger *et al.*, 2006). As a result, a shoot-derived signal would probably be sent to the nodule through the phloem to induce a down-regulation of nitrogenase activity (Sulieman, 2011). The apparent higher concentrations of the total amino acids in the phloem sap of P-stressed plants strongly support this hypothesis (Fig. 4B). Moreover, the higher N concentrations in the shoot and nodule fractions and the apparent modifications in the C/N and N/P ratios for those fractions provide further support for this interpretation (Tables 1, 3). With the aid of phloem amino acid feeding and  $^{15}N$  application to *M. truncatula* growing plants, it was possible to show that this mechanism is valid and that N demand is the main regulator of nitrogenase activity (Sulieman and Schulze, 2010b; Sulieman, 2011). The higher total amino acid concentrations in the phloem sap of P-stressed plants remarkably resulted from higher shares of asparagine (Fig. 4A, C). This amide was found to be significantly increased in nodulated plants under different environmental conditions such as nitrate application (Bacanawo and Harper, 1997), defoliation (Hartwig and Trommler, 2001), drought (Larrainzar *et al.*, 2009), and salt stress (Fougère *et al.*, 1991). As a result, it is suggested that asparagine might be the shoot signal or a precursor that interacts with nodule machinery and down-regulates the nitrogenase activity (Sulieman and Tran, 2012; Sulieman *et al.*, 2013). Previous phloem feeding of asparagine to intact nodulated plants strongly supports this explanation (Sulieman *et al.*, 2010). The artificial phloem feeding with 3.0 mM asparagine resulted in a greater increase in the nodule concentration of this amide while concomitantly reducing nitrogenase activity.

It was previously reported that there is a close association between the whole-plant N feedback mechanism and the nodule C metabolism (Larrainzar *et al.*, 2009). Several investigations reported that the N signal strongly influences the nodule C supply by affecting certain key enzymes, including sucrose synthase (SS) and phosphoenolpyruvate carboxylase (PEPC) (Fig. 6), which consequently affect the concentration of the main organic acids supplied to functioning bacteroids (González *et al.*, 2001; Minchin and Witty, 2005). In this study, investigation of the nodule organic acid profile revealed that different P concentrations had strong effects on the patterns and concentrations of various organic acids (Fig. 5A). Unlike several other leguminous species, succinic acid was found to be the main constituent of the organic acids in nodules of *M. truncatula* that can supply bacteroids with the required energy (Sulieman and Schulze, 2010a; Sulieman *et al.*, 2013). The concentration of this organic acid was significantly reduced under severe P deficiency (1  $\mu M$  P supply), which might subsequently lead to a lower nitrogenase activity (Table 4). The relatively higher levels of malate and to a lesser extent of fumarate suggest a compensatory mechanism under P deprivation conditions (Fig. 5A). Malate is believed to be one of the major substrates entering the  $N_2$ -fixing bacteroids (Schulze *et al.*, 2002). This dicarboxylic acid is the major energy source for the bacteroids and plant mitochondria in different species (Fig. 6), and is used for  $NH_4^+$  assimilation as the C skeleton in the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway (Stütt *et al.*, 2002). Le Roux *et al.* (2008) reported a pronounced increase in total malate levels in nodules subjected to P deficiency. The P deficiency-induced level of this organic acid was associated with reduced  $N_2$  fixation and inversely related to the amino acid concentration in nodules; that is, available organic C was reduced for N assimilation. The higher level of malate accumulation under P deficiency was suggested to be mainly directed for solubilizing soil-bound phosphate (Hammond and White, 2008). On the other hand, severe P deficiency was found mainly to reduce the concentration of sucrose without altering the contents of the rest of the sugars examined (Fig. 5B). Sucrose is known to be the main imported carbohydrate source from the shoot to the nodules (Schulze *et al.*, 2002) (Fig. 6). In contrast, other reports have previously shown that P limitations were associated with a substantial accumulation of sugars in all tissues and particularly in nodules (Olivera *et al.*, 2004; Hammond and White, 2008). One possible explanation could be that the photosynthetic capacity, which is known to be quite stable over a wide range of decreasing P supply, might be harshly affected under such severe P starvation conditions (1  $\mu M$  P supply). The stability of the sucrose concentration above this critical P level provides support for this explanation (Fig. 5B). Increasing evidence also suggested that sucrose might be involved in sensing and signalling the P status of different plants (Hammond and White, 2008).

Indeed, exposure of *M. truncatula* to P deficiency had a dramatic effect on amino acid and organic acid metabolism, and, as a result, nitrogenase activity. The present results suggest that the amino acid flow from the shoots to nodules is



**Fig. 6.** Simplified representation showing major metabolic routes of C and N metabolism and the key enzymes involved in nodules of an amide-producing legume. AAT, aspartate aminotransferase; ALA, alanine; ASN, asparagine; ASP, aspartic acid; GLN, glutamine; MDH, malate dehydrogenase;  $N_2$ ase, nitrogenase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; SS, sucrose synthase.

closely associated with nodule C metabolism in *M. truncatula* under limited P conditions. This apparently was related to the nodule organic acid metabolism, including succinic acid, the main C source for providing energy for the functioning bacteroids (Sulieman *et al.*, 2013). The existence of the close association between N and C metabolites in regulating the level of current nitrogenase activity has been revealed by several reports (González *et al.*, 2001; Minchin and Witty, 2005). However, the precise nature of the shoot-borne signal that links the perception of the P alteration and the signal transduction pathway, which leads to the changes in C metabolism, remains to be elucidated. Additionally, other factors, such as amino acid cycling or  $O_2$  supply, may also be involved in the regulation, as discussed in other reports (Schulze and Drevon, 2005; Sulieman and Tran, 2012, and references therein). The present study with the model *M. truncatula* can also be applied for studying P demand of legume crops, such as soybean, during nodulation in controlled conditions. The results gained from studies conducted in a controlled environment could then be used for estimation of the P demand of crops in the field, as evidenced by the report on potassium uptake by sugar beet and wheat in the field (El Dessougi and Claassen, 2001). Overall, the results of this study have provided a basic foundation for in-depth molecular dissection of the relationship between P availability and  $N_2$  fixation capacity in legumes, ultimately leading to development of efficient  $N_2$ -fixing legume crops, such as soybean.

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