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The nodule conductance to O_2 diffusion increases with high phosphorus content in the *Phaseolus vulgaris*-rhizobia symbiosis

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Abstract Although recent studies have addressed the effects of phosphorus (P) deficiency on nodule O_2 permeability, little attention has been given to the relationship between nodule P status and nodule permeability. To study these traits, four recombinant inbred lines, namely RILs 34, 83, 115, 147 and one local variety (Concesa) of common bean *(Phaseolus vulgaris*) were inoculated with RhM11 (a native rhizobial strain from Haouz area of Marrakesh), and grown in hydroaeroponic culture under P-sufficiency (250 μmol P plant^{-1} week^{-1}) versus P-deficiency (75 μmol P plant^{-1} week^{-1}) conditions. At the flowering stage, the biomass of plants and nodules and their P contents was determined after measuring O_2 uptake by nodulated roots (Conr) and nodule conductance to O_2 diffusion (g_n). The results showed that P-deficiency significantly decreased plant growth and nodulation, though there were differences between bean genotypes. P-deficiency also induced a decrease in nodule P content (31%) in both sensitive (83, 147) and tolerant lines (34, 115), a 42 and 27% reduction in shoots of sensitive and tolerant lines, respectively. These decreases were associated with significant variations in nodule surface and O_2 permeability among bean genotypes and P-nutrition. Under P-deficiency, g_n increased more for the sensitive (39%) than for the tolerant lines (27%). This increase was linked with a rise both in the P levels in nodules and shoots, as well as in the efficiency of symbiotic nitrogen fixation as determined by nodule-dependent biomass production for the sensitive lines. Furthermore, positive correlations were found between O_2 permeability, g_n and P content both in nodules and shoots (r^2=0.94** and r^2=0.96**). We conclude that nodule variations in Conr and g_n are related to nodule P content, and may contribute to the adaptation of energy metabolism in N_2-fixing bean nodules to P-deficiency.

Keywords Nodulation · Nodule O_2 conductance · *Phaseolus vulgaris* · Phosphorus · Rhizobia · Symbiosis

1 Introduction

Soil phosphorus (P) deficiency is among the most significant abiotic constraints for crop plants, including legumes. Sub-optimal supplies of P can lead to 15% yield losses for the common bean *(Phaseolus vulgaris* L.) which is the most important legume for human consumption (Shenoy and Kalagudi 2005). Low soil P availability is considered a major nutritional constraint to symbiotic nitrogen fixation (SNF) (Araújo et al. 1997). Under nitrogen-limiting conditions, legumes develop a symbiotic association with rhizobia and form nitrogen-fixing organs on their roots, or in some species on stems. SNF is a major process for N importation into most terrestrial ecosystems. The sensitivity of nitrogen-fixing plants to P-deficiency has been widely

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documented and is generally attributed to the large amount of P-dependent carbon and energy turnover required in nodule metabolism (Plaxton 2004; Schulze and Drevon 2005). Thus, N_2-fixing legumes usually require more P than plants dependent on mineral N fertilizers (Serraj & Adu-Gyamfi 2004).

In legumes, N_2-fixing root-nodules are, during development, strong P sinks and nodule P concentrations normally exceed those of roots and shoots thus indicating the important role of P in nodule functioning (Schulze and Drevon 2005). Although oxygenated leghemoglobin gradients may have a role in the fine control of O_2 levels in infected cells (Thumfort et al. 1999), a physical barrier to gas diffusion in the inner cortex seems to be the primary site for the regulation of nodule permeability to gas diffusion (Hunt and Layzell 1993; Galvez et al. 2000). It is reported that nodule O_2 permeability was increased under P-deficiency in soybean (Ribet and Drevon 1995) and common bean (Vadez et al. 1996). Under progressive P-deficiency, the number of smaller nodules increased in white lupin with more nodules found adjacent to cluster-root zones (Schulze et al. 2006). Alfalfa also adapts to P-deficiency by forming small nodules with high O_2 permeability which enables an increase in O_2 consumption per unit N_2 fixed and P content (Schulze and Drevon 2005). These authors claim that an increase in O_2 consumption creates an O_2 sink. This, in turn, induces the observed increase in nodule O_2 permeability and is associated with nodule cortical cell size mediated by osmoregulatory reactions (Serraj et al. 1995; Drevon et al. 1998).

The effects of environmental factors on nodule O_2 permeability have been examined using plants grown in hydroponic cultures; however, the relationships between the P status of the plants, nodule permeability and N_2-fixing activity are still poorly understood (Schulze 2004). The aims of the present study were to: (1) to improve our understanding of the effect of P-deficiency and rhizospheric O_2 concentration on nodule O_2 permeability in five bean genotypes differing in their tolerance to P-deficiency; (2) to study how inoculation with RhM_{11}, a rhizobial strain isolated from nodules of common bean plants collected in a farmer's fields in the Haouz area of Marrakesh-Morocco, affects nodule O_2 permeability and hence the conductance to the O_2 diffusion; and (3) to determine whether there is an interaction between O_2 permeability and both nodule and shoot P contents.

2 Materials and methods

2.1 Plant material and growth conditions

The present study was conducted in a glasshouse under natural light with day/night temperatures of 28/20°C and a photoperiod of 16 h with an additional illumination of 400 μmol photon sm^{-2} s^{-1} and 70% relative humidity during the day. This study was carried out using four recombinant inbred bean lines, namely RILs 34, 83, 115, and 147 (originated from CIAT) and one common bean variety widely cultivated in Morocco (Concesa). In a previous study, lines 115 and 34 had been characterized as P-efficient whereas 147 and 83 had been categorized as P-inefficient based on plant growth and seed yield in relation to the availability of P (Drevon et al. 2010). The Concesa variety is frequently cultivated in Moroccan fields as it is known to be resistant to rust and provides high quality green beans.

Seeds were surface-sterilized with 3% calcium hypochlorite, then washed thoroughly in several changes of sterile distilled water. Thereafter, seeds were germinated for 4 days at 28°C on a roll of germination paper soaked with sterile distilled water. Plants were inoculated with the rhizobial strain RhM_{11}. The inoculum was prepared by growing the bacteria in Yeast Extract Mannitol medium at 28°C for 3 days to an approximate cell density of 10^9 mL^{-1}. RhM_{11} had been isolated from nodules of common bean plants collected from a farmer's fields in the Haouz area of Marrakesh, Morocco. The common bean plants inoculated with this local rhizobial strain showed a higher nodulation and an increase in phytase and phosphatase activities in nodules under P deficiency (Mandri et al. 2011).

The seedlings were transferred into hydroaeroponic culture consisting of vats filled with 40 L of nutrient solution (Vadez et al. 1996) which were arranged in a fully randomized block design. The roots of selected uniform seedlings were passed through the hole of a rubber stopper on the vat cover, and cotton wool was fitted at the hypocotyl level so that the root system was suspended in the nutrient solution. At the beginning of the third week of culture, plants were transferred to 1 L glass bottles containing nutrient solution and wrapped in aluminium foil to ensure a dark plant-rooting environment.

The composition of the nutrient solution used in the overall study consisted of P applied at 75 and 250 μmol P $plant^{-1}$ $week^{-1}$ as deficient and sufficient supply, respectively, in the form of KH_2PO_4. In addition the solution contained $MgSO_4$ (100 μM), K_2SO_4 (700 μM), $CaCl_2$ (1,650 μM), Fe-EDTA (16 μM), $MnCl_2$ (4 μM), H_3BO_3 (22 μM), $ZnSO_4$ (0.4 μM), $NaMoO_4$ (0.05 μM), and $CuSO_4$ (1.6 μM). Urea was added to a final concentration of 2 mmol $plant^{-1}$ to the nutrient solution only during the initial 15 days of growth to avoid N-deficiency during nodule development. Thereafter; the plants were grown in N-free nutrient solution. Weekly, the pH of nutrient solution was adjusted to around 7 by addition of 0.2 gL^{-1} $CaCO_3$. The medium was continuously aerated by an air flow of approximately 500 mL min^{-1} during the study.

2.2 Nodule O_2 uptake measurements

The system used for nodule O_2 uptake measurement is presented in Fig. 1. The consumption of O_2 by the nodulated roots (Conr) was measured with a Witt Logger Oximeter (Witt, France) at 35 days after sowing (DAS). One day before the measurement of Conr, the level of the nutrient solution in the bottle was reduced to one-third of the volume with the basal part of the root systems in the nutrient solution and the top, nodulated portion in the air above the nutrient solution. For measurements of gas exchange, the O_2 consumption of the biological material was followed by monitoring O_2 in a known volume of a serum bottle plus tubing (Jebara et al. 2005). O_2 consumption was detected by an O_2 sensor located in the Witt Logger oximeter which was connected to a data logger. Measurements were taken every 60 s. The gas surrounding nodulated roots was circulated to the O_2 sensor (which detected O_2 in the range of 0%–100% O_2) by a peristaltic pump that ensured homogeneous mixing and recirculation of air in the closed system at a flow rate of about 400 ml min^{-1}.

Successive measurements were performed with an initial pO_2 of 21, 25, 30, 35, 40 and 45 kPa O_2 corresponding to 250, 298, 358, 417, 476 and 536 µmol $O_2 L^{-1}$, respectively. Nodulated root O_2 uptake was determined by measuring the rate of decline in O_2 concentration in the gas surrounding the nodulated roots during a 20 min period after closing the system. O_2 consumption of the nodulated-roots was calculated as Conr = $\Delta pO_2(V/24.2)(60/t)$, with ΔpO_2 = pO_2 initial—pO_2 final in % of atmospheric pressure; V in L, volume of the gas phase under the experimental conditions; t in min, time between the initial and final measurement of O_2; 24.2 in L, volume of 1 pure gas mol under the experimental conditions (Schulze and Drevon 2005) Conr is expressed in µmol O_2 consumed $plant^{-1}$ h^{-1}. Moreover, nodule permeability (mm^3 h^{-1}) was defined as the slope of the linear part of the regression of Conr (µmol O_2 h^{-1}) as a function of external O_2 concentration (µmol O_2 L^{-1}) corresponding to the tested pO_2 in Fig. 3 (Schulze and Drevon 2005).

2.3 Harvest and measurement of shoot, root and nodule parameters

Plants were harvested 42 days after transplanting (DAT), after gas exchange measurements and separated in shoots, roots and nodules. Nodule distribution was determined including surface area (cm^2) and diameter (mm) using automated image analysis software WinRhizo 2005b (Regent Instruments. Inc., Canada). The plant and nodule dry weight was determined after drying for 3 days at 70°C. Dry samples were ground for shoot and nodule P content determination. 50 mg subsamples of shoots and nodules were digested in concentrated HNO_3 in a microwave oven (Ethos, Milestone) at 40 bars for 15 min. The phosphorus content was determined using the vanado-molybdate method (AFNOR 1969); the optical density was determined at 460 nm with a variant Cary 1E spectrophotometer.

The efficiency of the rhizobial symbiosis (EURS) was estimated by the slope of the linear regression (y = ax + b), where "a" corresponds to the EURS as (g pDW—g pDW_0) g^{-1} nDW and "b" corresponds to the plant biomass production without nodules (g pDW_0) and (Zaman-Allah et al. 2007).

2.4 Statistical analysis

The experimental design was a randomized complete block. The growth values and the parameters related to nodulation were means of nine replicates per treatment per genotype. Four replicates were used for P determination and five for both O_2 uptake by nodulated roots O_2 uptake and O_2 conductance. The data were analysed using

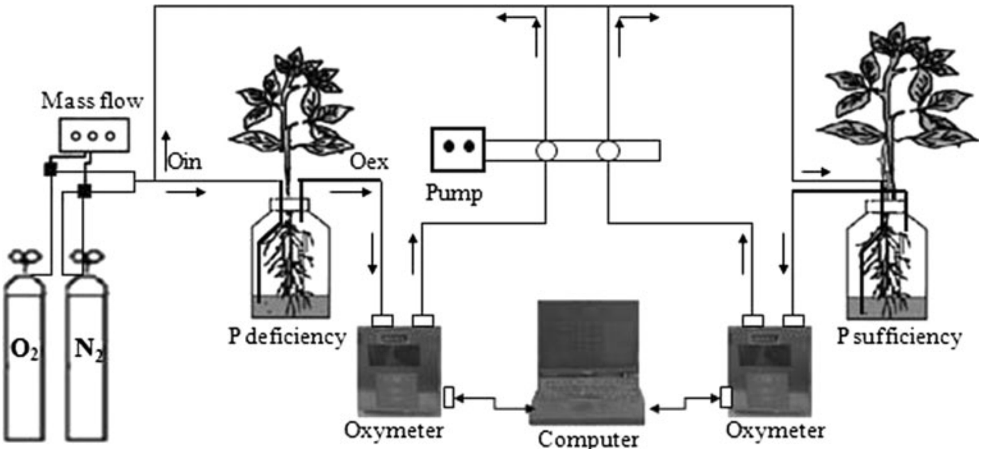

Fig. 1 Experimental set-up for measuring O_2 uptake by nodulated roots of common beans

ANOVA and subsequent comparison of means was performed using Fisher's LSD test at 5% probability. The relationship between different parameters was tested by regression analysis.

3 Results

3.1 Nodule and shoot P contents

The level of P-deficiency applied in this work induced a significant reduction of nodule and shoot P contents (Fig. 2). We noticed large genotypic differences in nodule P content in response to P-deficiency. Concesa showed the lowest nodule P levels whatever the P supply (Fig. 2a). The decrease in nodule P contents under P-deficiency as compared to P-sufficiency was significantly higher in the lines 147 (46%) and 34 (35%) than in the lines 115 (28%) and 83 (17%).

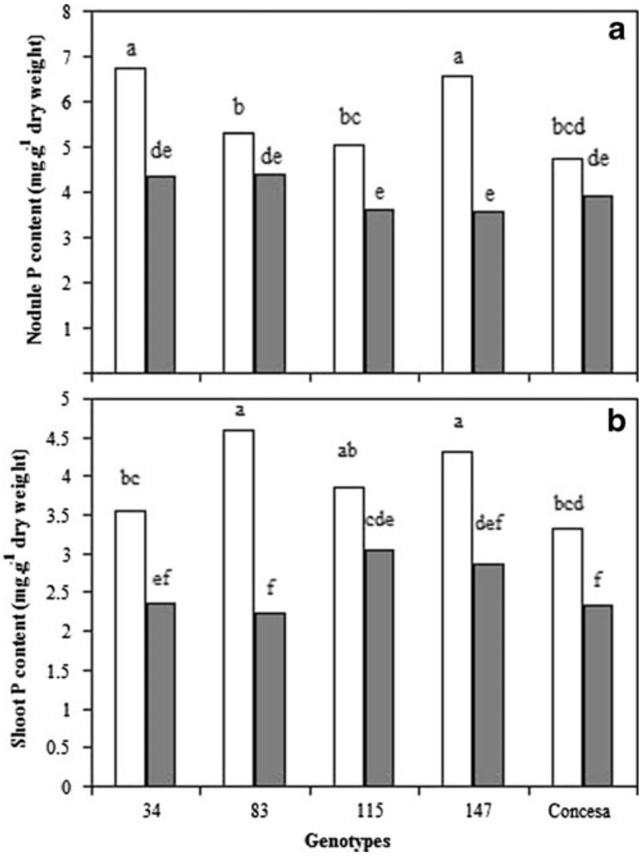

Fig. 2 Effect of P-deficiency (*filled square*) versus P-sufficiency (*empty square*) on nodule and shoot P contents of five common-bean genotypes inoculated with RhM_{11}. Data are means of five replicates harvested at 42 days after transplanting. Mean values followed by the same letter are not significantly different at $P<0.05$

As in nodule P contents, shoot P contents markedly decreased under P-deficiency for all bean genotypes in this study (Fig. 2b). Under P-sufficiency, the sensitive line 83 had the highest level of accumulated P in shoots (4.5 mg g^{-1}), whereas it showed the maximum decrease of 51% under P-deficiency. In addition, constraint-deficiency caused a slight decrease of 21% in the shoot P contents of the tolerant line 115 compared to the remaining beans that showed a comparable decrease (ca. 33%) in P contents. Overall, under P-sufficiency, P contents of nodules were almost twice as high as those of shoots.

3.2 O_2 uptake in response to pO_2 and nodule permeability

The response of O_2 uptake by nodulated roots to variation of rhizospheric pO_2 (μmol O_2 L^{-1}) was measured in order to assess the effect of P-deficiency on nodule permeability, following the principles described by Jebara et al. (2005). The data in Fig. 3 show that a raise of pO_2 induced a significant increase in nodulated-root respiration (Conr) up to the critical oxygen pressure (COP = external pO_2 beyond which the O_2 diffusion within nodules is no longer stimulatory for respiration). Thus, the COP could be calculated as the first derivative of the curvilinear regression of Conr as a function of pO_2. Independently of P supplies, similar COP values of 417±11 μmol O_2 L^{-1} were found lines 34, 83, 147, and in Concesa. However for line 115, the COP could be observed only under P-deficiency; under P-sufficiency the nodulated-root respiration steadily increased with increasing pO_2.

P-deficiency induced a large decrease in Conr for the sensitive line 147 independent of the pO_2. This was also true for line 34 but only between 358 and 417 μmol O_2 L^{-1} whereas for line 83 and Concesa there were no significant differences in Conr between P supplies. In parallel, independently of P supplies, the Conr of the tolerant line 115 was slightly stimulated by increases of pO_2 and varied between 295 and 1,106 μmol O_2 $plant^{-1}$ h^{-1} under ambient and high applied pO_2, respectively. Thus at ambient pO_2 (250 μmol O_2 L^{-1}), the Conr varied between both lines and P supplies, although it was significantly reduced under P-deficiency only for the sensitive lines 147 and 83 and in Concesa in comparison with the tolerant lines 115 and 34.

3.3 Nodule mass and conductance to O_2 diffusion

Differences in the response of nodule dry weight, number and surface to P-deficiency are shown in Fig. 4. Less genotypic variability was observed for both biomass and surface of nodule than for nodule number (Fig. 4a).

Under P-deficiency, the percentage of decrease of nodule biomass ranged between 30 and 40% in all the tested genotypes. However, nodule numbers in these genotypes

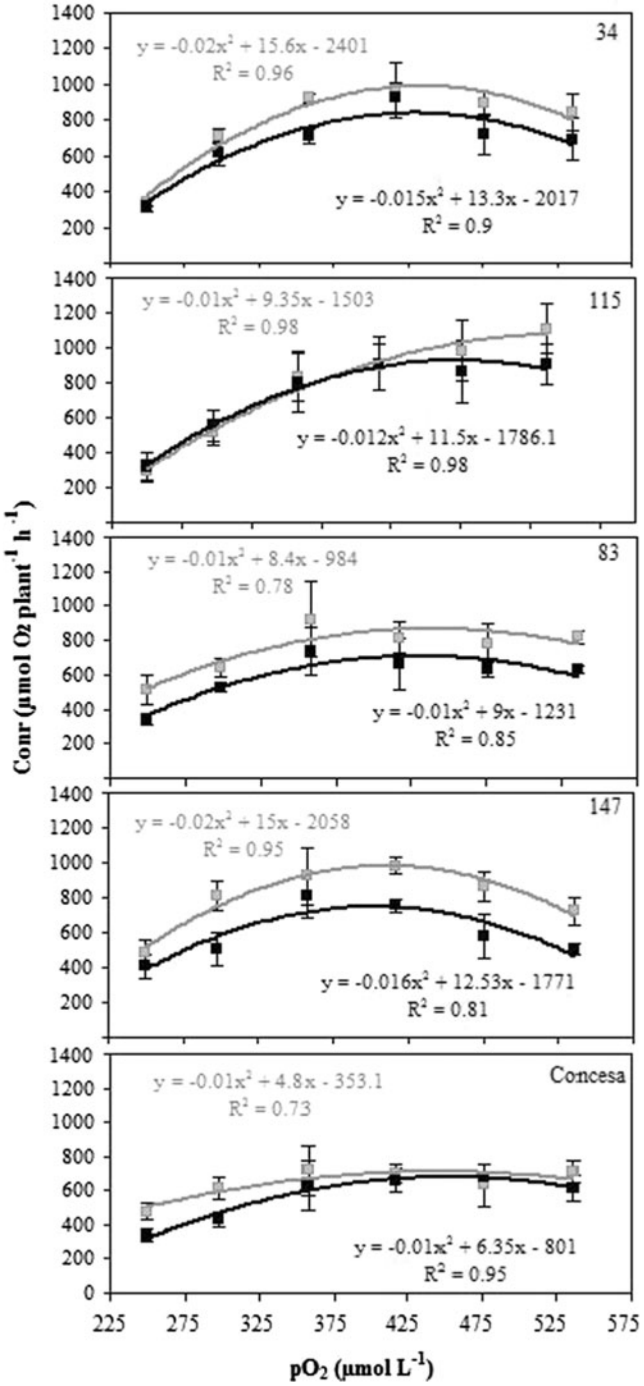

Fig. 3 Response of nodulated-root O_2 consumption to increases in rhizospheric pO_2 in five common-bean genotypes inoculated with RhM_{11} and grown at P-sufficiency (*empty square*) versus P-deficiency (*filled square*). Data are means and standard deviations of five replicates harvested at 42 days after transplanting

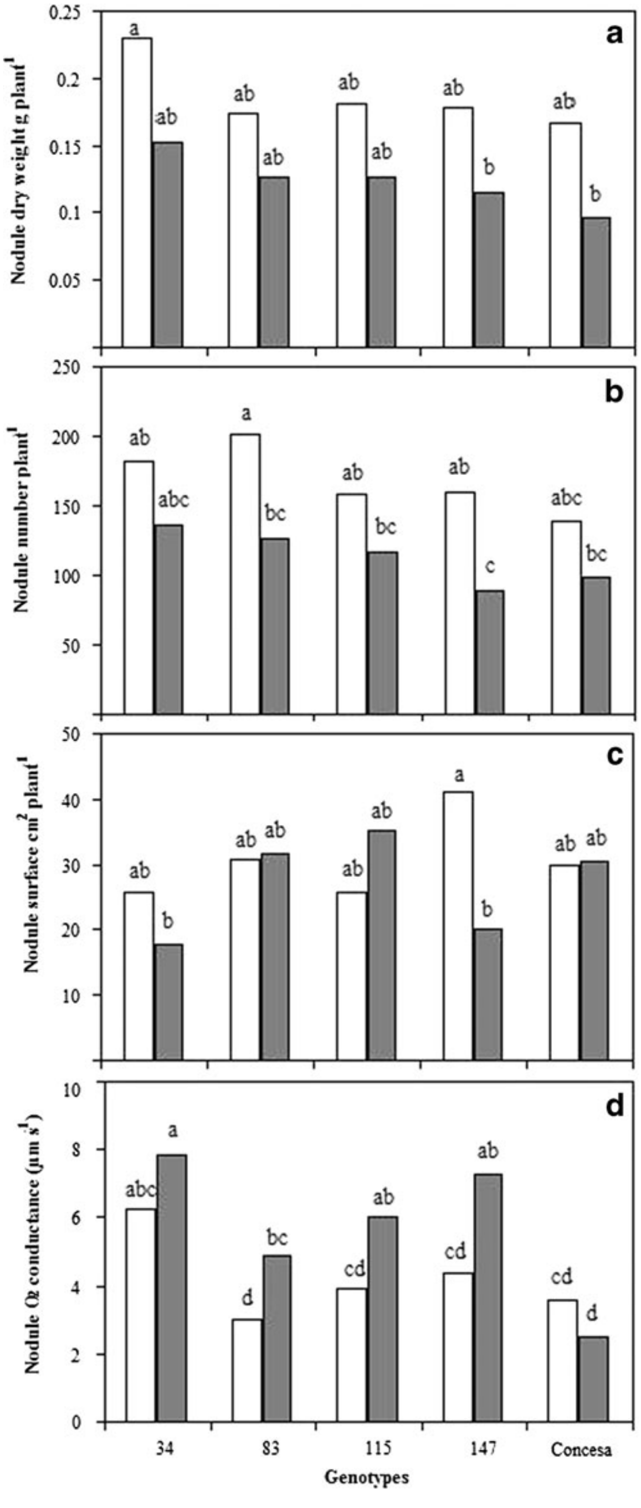

Fig. 4 Effect of P-deficiency (*filled square*) versus P-sufficiency (*empty square*) on dry weight (**a**), number (**b**), surface (**c**) and nodule conductance to O_2 diffusion (**d**) of five common-bean genotypes inoculated with RhM_{11}. Data are means of five replicates harvested at 42 days after transplanting. Mean values followed by the same letter are not significantly different at $P<0.05$

showed more variation depending both on the genotype and the P-level (Fig. 4b). Although nodule numbers were not significantly different under P-deficiency, they were lower for the tolerant lines 115, 34 and Concesa. However, nodule number was significantly less for the two P-deficient sensitive lines 147 (by 44%) and 83 (by 37%). The data in Fig. 4c showed that P-deficiency induced a significant decrease of 51% in nodule surface of the sensitive line 147. In contrast, under the same conditions, this parameter was not significantly affected in the remaining genotypes compared to their respective controls.

The nodule conductance, defined as the nodule permeability per unit of nodule-surface, could be calculated by dividing the permeability values in Fig. 3 by the nodule area. The data in Fig. 4d show that, under P-deficiency, nodule O_2 conductance increased significantly more in the lines 83, 147 and 115 than in line 34 and Concesa. Nodules of the latter genotype registered the lowest conductance to O_2 diffusion independent of the P supply.

3.4 Relationship between P contents in nodules and shoots and permeability to O_2 diffusion

Although both nodule and shoot P contents were severely reduced under P-deficiency, the correlation between these last parameters was weak (r^2=0.44; result not shown). Interestingly under P-sufficiency, positive correlations were found between both nodule or shoot P contents and nodule O_2 permeability and to a lesser extent, nodule conductance to O_2 diffusion (Fig. 5). However, there was no significant correlation between those parameters under P-deficiency.

3.5 Shoot biomass and the efficiency of the rhizobial symbiosis

The EURS was evaluated by the testing regression slope of the correlation between shoot and nodule biomass (Zaman-Allah et al. 2007) as illustrated in Fig. 6. The data revealed a significant difference between both treatments and genotypes. Independent of the P supply, shoot and nodule biomass of lines 34, 83 and 147 were positively correlated (up to r^2=0.6) under P-deficiency. The EURS was 66 and 40% higher under P-deficiency than under P-sufficiency in the sensitive lines 147 and 83, respectively. However, the EURS was reduced under P-deficiency in the other genotypes.

Altogether, the data obtained show large genotypic variability between the tested genotypes regarding the effect of P-deficiency on plant growth, with a significant reduction for all bean genotypes except for the tolerant line 115.

4 Discussion

In this study, the observation of an increase in nodule O_2 conductance of 1 μm s^{-1} for each increase of 1 mg Pg^{-1} nodule P (Fig. 5a), is to our knowledge the first description of a correlation between O_2 conductance and P content of a legume nodule. However, this correlation was observed here only under P sufficiency, and for relatively high nodule P contents between 5 and 7 mg g^{-1} (Fig. 2). This increase in nodule O_2 conductance and the subsequent increase in nodule respiration are not directly associated with symbiotic nitrogen fixation since the P supply is not a limiting factor of these processes under P sufficiency. This statement is confirmed by the absence, under P sufficiency, of any relation between nodule P content and the efficiency of the rhizobial symbiosis for plant growth, estimated from the slope of the regression between biomasses of shoot and nodules (Fig. 6). Thus, P toxicity within nodules might be

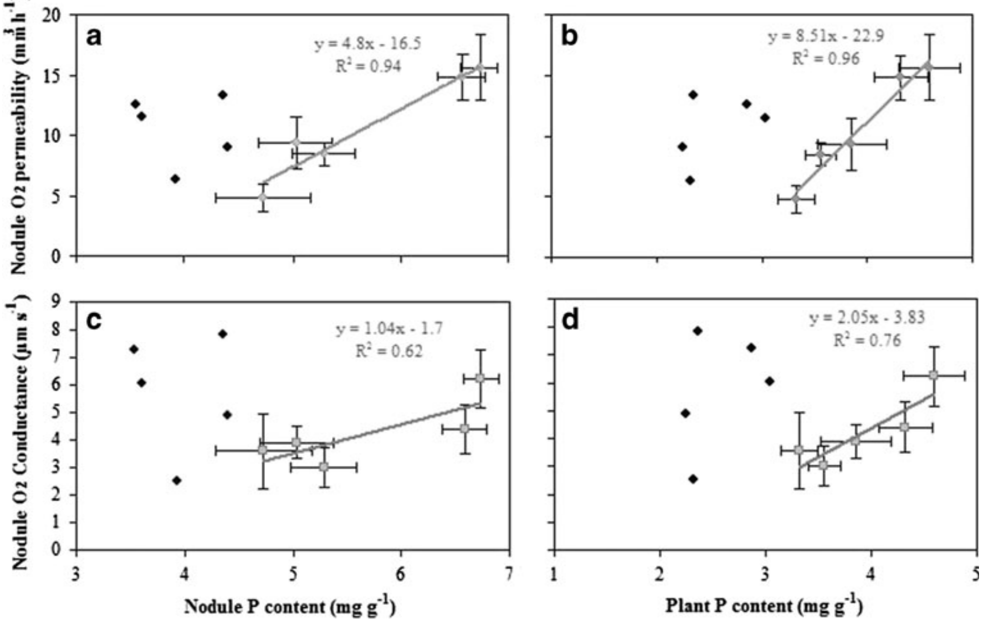

Fig. 5 Relationship between nodule O_2 permeability (**a** and **b**) and conductance to O_2 diffusion (**c** and **d**) and both nodule and shoot P contents of five-common bean genotypes inoculated with RhM_{11} and grown under P-deficient (*filled square*) versus P-sufficient (*empty square*) supply. Data are means standard deviations of five replicates harvested at 42 days after transplanting

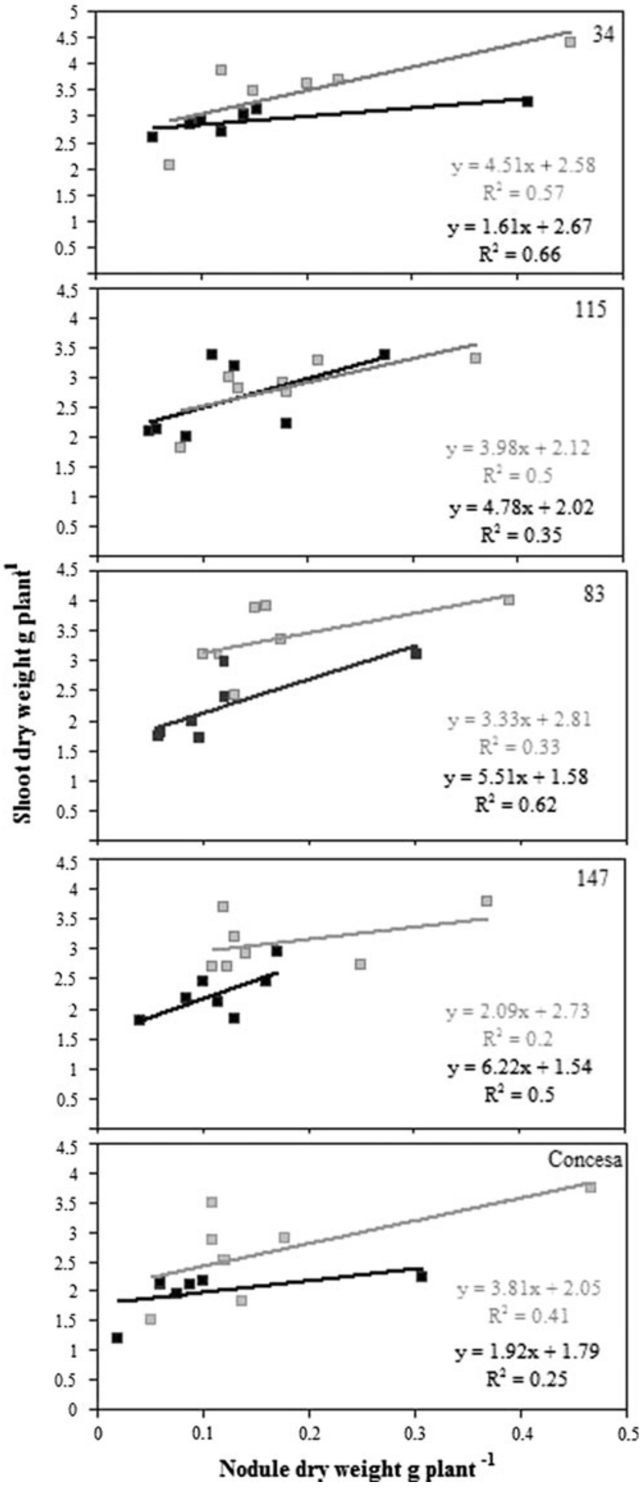

Fig. 6 Efficiency of the rhizobial symbiosis of five common bean genotypes grown under P-sufficient (*empty square*) versus P-deficient (*filled square*) supply. Data represent individual values of plant harvested at 42 days after transplanting

involved in the correlation of high nodule P contents with an increase in nodule respiration that is not linked with N_2 fixation. This hypothesis agrees with the correlation of nodule conductance also with high P contents in nodules and P levels in shoot reaching values as high as 5 mg g^{-1} (Fig. 5). The higher P contents in nodules than in shoots (Fig. 2), suggest that nodules may have acted as P sink to prevent toxic accumulation of P in the shoot under the P-sufficiency conditions used in this study.

Inorganic P accumulation in the nodule cortex was shown with ^{31}P-NMR studies (Rolin et al. 1989). Nodule P accumulation in cortical cell may interact with the osmoregulation of nodule O_2 conductance (Schulze and Drevon 2005). Indeed, the accumulation of inorganic P in vacuoles of the inner cortex would induce an increase in the size of these cells which has been previously associated with an increase in nodule conductance (Drevon et al. 1998). The subsequent increase in O_2 flux normally couples to O_2 respiration with ATP generation, since for each N_2 reduced at least 16 ATP are consumed (Salsac et al. 1984). However, under P sufficiency, N_2 fixation would not respond since the N demand of the plant is satisfied. Therefore the increase in respiration would not be coupled to an increase in N_2 fixation. Alternatively, it could stimulate a protective respiration to prevent a rise in O_2 concentration within nodules that is known to be toxic for nitrogenase. This hypothesis is supported by the high levels of antioxidants in the nodule cortex (Dalton et al. 1998). It is also consistent with the observed genotypic differences in nodule O_2 conductance (Figs. 3 and 4d) that depend also on nodule surface and the partitioning of P between nodules and shoots.

This investigation follows up previous studies of Vadez et al. (1999) who described variability in both nodule P content and tolerance to P deficiency among common bean genotypes (Vadez et al. 1996). Thus under P-deficiency, the decreases in nodule P and nodule surface of the sensitive genotypes are in agreement with the significant increase in their conductance to the O_2 diffusion. This has been shown in previous studies on these common bean genotypes (Jebara et al. 2005; Bargaz et al. 2010) and on alfalfa (Schulze and Drevon 2005). However, the tolerant lines that were able to moderate their decrease in P contents under P-deficiency, and allocate a larger percentage of plant P to the nodules, also maintained their nodule conductance to O_2 diffusion (Fig. 4d). This is consistent with the high correlation between plant N accumulation and both nodule mass and their P content that have been observed previously in common bean (Vadez et al. 1999; Christiansen and Graham 2002) and soybean (Rotaru and Sinclair 2009). In addition, the higher efficiency of the rhizobial symbiosis under P-deficiency for the lines 83, 147 and 34 than for the tolerant line 115 or Concesa (Fig. 6) may correspond to higher nodule demands for respiration, in relation to nodulation and biomass yield with the increase in nodule conductance to O_2 diffusion for N_2 fixation discussed above. Thus, under P-deficiency, nodules of the sensitive lines require more respiration than those of the tolerant lines. Although the critical role of P in the growth and activity of nodules has been well discussed (Tang et al. 2001), the genotypic variations linking O_2 permeability and nodule P content in response to P-deficiency are not yet fully understood.

In conclusion, the increase nodule O_2 conductance under P sufficiency could be attributed to an excess of nodule P content. Differences in P accumulation among genotypes points to the need for further investigations of mechanisms responsible for P distribution in nodule and its relation with nodule O_2 conductance that is assumed to regulate the N_2 fixation process (Minchin 1997; Schulze 2004). Understanding the role of nodule O_2 conductance and P metabolisms in response to environmental constraints may be important in developing strategies to improve the efficiency of root-nodule respiration for symbiotic nitrogen fixation under adverse conditions.

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