Ecological engineering: from concepts to applications

Nodular diagnosis for ecological engineering of the symbiotic nitrogen fixation with legumes

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Abstract

As a major contributor to the reduced nitrogen pool in the biosphere, symbiotic nitrogen fixation by legumes plays a critical role in a sustainable production system. However this legume contribution varies with the physico-chemical and biological conditions of the nodulated-root rhizosphere. In order to assess the abiotic and biotic constrains that might limit this symbiosis at the agro-ecosystem level, a nodular diagnosis is proposed with common bean as a model grain-legume, and a major source of plant proteins for world human nutrition. The engineering of the legume symbiosis is addressed by participatory assessment of bean recombinant inbred lines contrasting for their efficiency in use of phosphorous for symbiotic nitrogen fixation. With this methodology, in field-sites chosen with farmers of an area of cereal-cropping in the Mediterranean basin, a large spatial and temporal variation in the legume nodulation was found. Soil P availability was a major limiting factor of the rhizobial symbiosis. In order to relate the field measurements with progress in functional genomics of the symbiosis, *in situ* RT-PCR on nodule sections has been implemented showing that the phytase gene is expressed in the cortex with significantly higher number of transcripts in P-efficient RILs. It is concluded that various tools and indicators are available for developing the ecological engineering of the rhizobial symbiosis, in particular for its beneficial contribution to the bio-geochemical cycle of N, and also P and C.

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1. Introduction

Farmers in most developed countries are recommended to minimize nitrogen fertilization for environmental sake whereas those in less developed countries cannot afford it in large agricultural areas. Thus, symbiotic nitrogen fixation (SNF) by legumes that already constitutes a major input into agricultural and natural ecosystems may provide an ecologically acceptable complement or substitute for mineral nitrogen fertilizers. However, P deficiency is a major limiting factor for legume production where legume N nutrition largely depends upon the rhizobial symbiosis, particularly in acidified or calcareous soils. 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.

A possible explanation is that legumes, especially common bean, have higher P requirements than non symbiotic plants [1]. Nevertheless to increase common bean production, it is possible to improve its SNF potential and expression under P deficiency [2,3]. Although the large genetic diversity of growth habits and duration, from bushy determinate to climbing indeterminate types, was not fully explored in the putative nine distinct gene pools of the species [4]. In this study we screened in glasshouse hydroaeroponic culture for genotypic diversity in P use efficiency (PUE) for SNF among common bean recombinant inbred lines (RILs) of the cross of the parental lines DOR364 and BAT477. The most contrasting RILs were tested for nodulation and growth in field with multi-location participatory testing in conventional cereal-legume rotation systems in South France. The relation with soil P availability and nodule phosphatase expression was considered.

2. Materials and Methods

2.1. Hydroaeroponic culture of nodulated common-bean in glasshouse

Common bean seeds of RILs (F8-9) from the cross of BAT477 and DOR364 were surface-sterilized in Ca hypochlorite 3%, washed with sterile distilled water. They were germinated in Petri dish with 0.8% Agar YEM media (Vincent, 1970). Selected uniform seedlings of each RIL were inoculated by soaking them 30 min into 100 ml rhizobial broth containing around 10^8 cells ml^{-1} of *Rhizobium tropici* CIAT 899. Both rhizobia and seeds were initially supplied by the Centro Internacional de Agricultura Tropical (Cali, Colombia).

Roots of inoculated seedlings were passed carefully through pierced rubber stopper, fixed with cotton fitted around the hypocotyl, and placed upon the topper of a 0.40 x 0.20 x 0.20 m vat receiving 15 plants before transplanting into a temperature-controlled glasshouse. They were individually transferred during 3rd week into 1 l serum bottle for individual-plant culture until harvest during 6th week when they had reached the flowering stage (R6-7).

They received 1 l pl^{-1} of the nutritive solution from Hernandez and Drevon [6], complemented with sterile distilled water and renewed every week, and aerated with 400 ml compressed air min^{-1} l^{-1} solution. The pH was maintained near 7 through the addition of 1 g CaCO$_3$ l^{-1} solution. The solution contained 2 and 1 mM urea starter N during the first 2 weeks and at transplanting into bottle, respectively. Thereafter, plants were grown with N-free nutrient solution. Every week, 75 or 250 µmol KH$_2$PO$_4$ pl^{-1} were applied for the deficient or sufficient P treatments, namely 75P and 250P. The vats and bottles were distributed in a block design.

2.2. Nodular diagnosis in multi-location participatory field assessment

From the screening in the glasshouse, four contrasting RILs were chosen for the multi-location field comparison with the local cultivar in Lauragais (France, 43-13 ºN, 02-19 ºE, 130 mas) with 6 farmers of the SPHC (Syndicat des Producteurs de Haricot à Cassoulet) in 2003, 3 farmers in 2004 and 2005, and in most contrasting fields of 2 farmers in 2006 and 2007. In each field the RILs were sown in May in a 2-m single-row per RIL. This constituted a randomized block design, each block being one farmer's field. The RIL block was included within the local bean field in order to reduce border effects and to expose the RILs to similar environment and practices as the local bean cultivar, in particular with 0.5 m between rows.

Nodulation and growth of the RILs were measured in July, at the similar flowering stage as in the glasshouse experiment, i.e. when the first pod was 2 cm long. Sampling was performed by excavating 20 cm in depth and

around the root system of 10 plants per row, per block. Soil and plant samples were transferred in plastic bags and stored in a cold room until further analysis in laboratory.

Rhizospheric soil was sorted from field plants. The available P of the rhizospheric soil was measured by the Olsen method according to standard NF ISO 11263 after extraction by soil agitation with a solution of 0.5 NR sodium bicarbonate with pH 8.5. Plants were separated into shoot, root and nodules, and dried at 70 ºC for 2 days to constant weight.

Standard deviations were determined for all the traits. Analyzes of variance and regressions were performed using the general linear model procedure of the Statistical Analysis System package (SAS Institute, 2000). Fixed effects in the analysis of variance were genotype and treatment.

2.3. Nodule in situ *reverse transcription and PCR amplification*

Three mm diameter nodules were harvested from 5 week-old plants in glasshouse, and thoroughly washed with DEPC (diethyl pyrocarbonate) treated water. They were then fixed overnight at 4 °C in tubes containing freshly prepared PFA [2% (v/v) paraformaldehyde, 45% (v/v) ethanol and 5% (v/v) acetic acid] as modified from Koltai and Bird [7]. Fixed nodules were washed extensively to remove PFA with four changes of DEPC-treated water over 30 min under regular agitation (2x5 min + 2x10 min). Thereafter, the nodules were included in low-melting agar 9% (m/v), dissolved with filtered PBS 1x, and cut into samples of 50 microns-depth using a micro-cut unit. The nodules sections were transferred in tubes containing 0.5 ml of DEPC-treated water, and the rest of agar was removed by washing with DEPC-treated water heated to 60 °C three times.

The sections of fixed nodules were incubated with 40 µl RT mix [8x buffer (5x), 1.25 dNTP (10 mM), 0.4 dig-11-dUTP, 3 Primer rev (10µM), 6.7 DEPC-treated water], and heated to 65 °C for 5 min and then placed in ice for 2 min. Thereafter, the reverse transcriptase was added to each sample to a final concentration of 5 µl^{-1} and samples were incubated at 42 °C for 1h. After reverse transcription, the RT mix was removed, and the samples were washed three times with 100 µl DEPC-treated water. Negative controls (NRT) were prepared by omitting the reverse transcription. Thereafter, the PCR reaction was performed in 20 µl of DEPC-treated water and 20 µl of PCR mix [4x PCR buffer (10x), 1.2 $MgCl_2$ (50 mM), 0.8 dNTP (10 mM), 0.4 dig-11-dUTP, 1 Primer dir (10µM), 1 Primer rev (10cM), 0.2 Taq Poly (5U/µl), 10.4 DEPC-treated water] with 30 cycles thermocycling (95 °C for 3 min, 95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s; 72 °C for 2 min) and at 20 °C for 5 min. The PCR mix was removed after amplification and the samples were washed three times with PBS 1x.

Before detection of amplified cDNA, the samples were incubated in 100 µl of blocking solution [BSA 2% with triton 0.3% (3 µl.ml^{-1}) for 30 min under gentle agitation in darkness at 37 °C. Then the blocking buffer was removed and replaced by 100 µl of acid phosphatase-conjugated anti-dioxygenin diluted 1:1000 in BSA (2%). The samples were incubated 90 min at room temperature and then washed three times (5 min, 10 min and 10 min) in filtered PBS (1x) to remove excess antibody. Detection was carried out using the enzyme-labeled fluorescent ELF-97 endogenous phosphatase. ELF substrate was diluted 1:40 in the detection buffer, vigorously shaken and filtered through a 0.22-µm filter [8]. Samples were incubated in 20 µl ELF substrate-buffer solution for 20 min in the dark and transferred to washing buffer for 3 washings of 1 min were performed, then mounted and observed immediately with gray-scale view camera using image J software for analysis.

3. Results and discussion

3.1. Screening for N$_2$-dependent growth in controlled environment under low P

In a preliminary screening, a large diversity of adaptation to deficient P supply was found in hydroaeroponics under glasshouse with RILs 5, 6, 7, 12, 26, 28, 34, 60, 64, 75, 104 and 115 that were tolerant to deficient P, in contrast with RILs 1, 4, 11, 24, 27, 29, 32, 37, 38, 62, 73, 83, 124 and 147 that were sensitive, as illustrated in Fig. 1A. This experiment was repeated with most contrasting RILs of the above screening with RILs 7, 28, 75 and 115 being significantly more efficient than RILs 83, 124 and 147 (Fig. 2A). The RIL 115 confirmed to be one of the most efficient RILs with a mean N$_2$-dependent growth of 8 g DW plant^{-1} corresponding to a yield potential higher than 4 t grain ha$^{-1.}$ The shoot / root ratio decreased under P deficiency since there was no decrease in root growth under deficient P supply except for RILs 104 and 147.
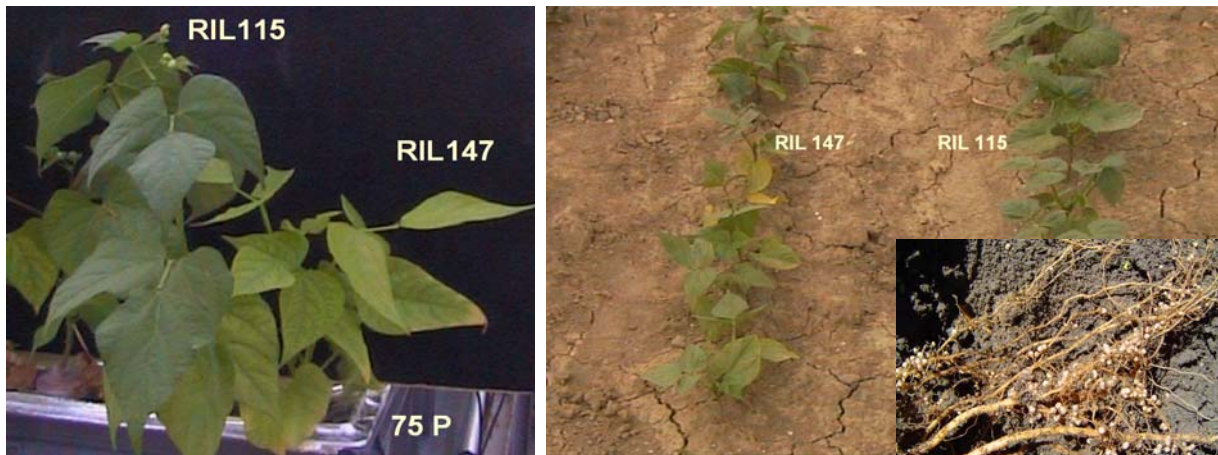
Fig. 1. Contrasting recombinant inbred lines 115 and 147 in (A) glasshouse hydroaeroponics under deficient P, farmer's field in Lauragais (B) with limitation in N_2 fixation for 147, (C) with extensive nodulation for 115
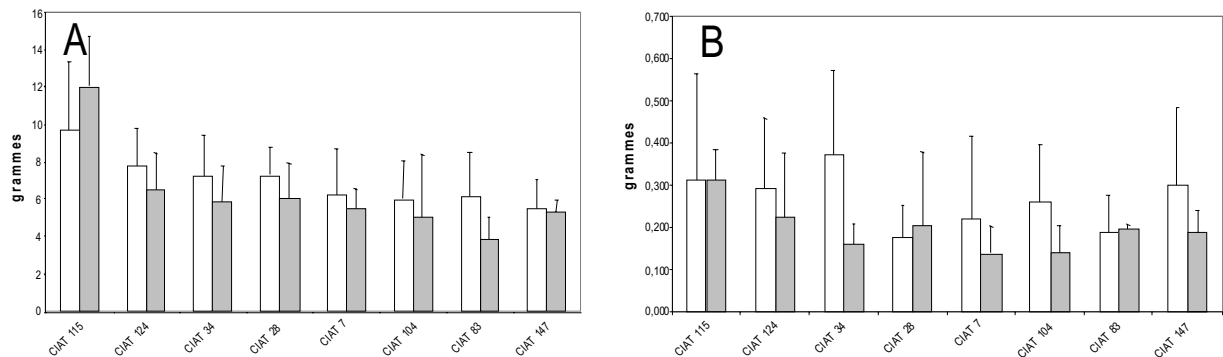

Fig. 2. Biomass of shoot (A) and nodules (B) of contrasting recombinant inbred lines of the cross of BAT477 x DOR364 in glasshouse hydroaeroponic culture under sufficient (white) versus deficient (grey) P supply. Data are means and standard deviation of 10 replicates at flowering stage

The ratio of shoot / nodule was calculated on the basis of the shoot biomass minus 1.8 gDW that corresponded to the growth of the plant without nodulation (i.e. the growth exclusively due to the supply of N by the seed and the starter urea in the hydroaeroponic culture). Since the level of additional growth depended upon the amount of N_2 fixed and the efficiency of its utilisation for growth, this ratio was considered as an estimate of the efficiency in use of the rhizobial symbiosis (EURS). Under P sufficiency, RILs 34 and 115 were the most efficient by contrast with RILs 28 and 83 that expressed a significantly lower EURS (Fig. 2B). Under P deficiency, RILs 7 and 115 had the highest EURS, by contrast with RIL124 that shows the lowest EURS.

3.2. Field observation of contrasting RILs in conventional cereal cropping systems

The contrasting RILs 115 and 104 *versus* 124 and 147 were tested with a participatory approach in fields in Lauragais. In 2004, the highest and lowest values for mean shoot growth of the RILs in one field, were 19 and 9 g SDW pl^{-1}, respectively for field 3 and 2, with mean growth around 15 g SDW pl^{-1} in other fields. However the RIL104 shows generally a lower growth than RIL115 whatever the field. The RIL 124 shows a significantly lower growth than RIL115 in field 3. A leaf yellowing was observed with RIL147 compared to RIL115 in field 2 (Fig.

1B), that resulted in significantly lower growth of RIL147 (7 g SDW pl^{-1}) than RIL115 (12 g SDW pl^{-1}) at flowering stage. Nodulation (Fig. 1C) was the lowest in field 2, with less than 2 mg NDW pl^{-1}, with a large proportion of plants harbouring no detectable nodules. It was the highest in field 3 with a maximum nodule mass of 54 mg NDW pl^{-1}, corresponding to more than 50 nodules per plant. Thus, nodulation varied considerably among fields. Nevertheless, there was no significant correlation between nodulation and growth in any field by contrast with observations during some previous years in this reference zone [9] or in hydroaeroponics [10].

This field tests were repeated in 2005 in 4 fields of Lauragais where nodulation was again quite variable spatially. In field 2 the nodulation was as low as in 2004 except for RIL104 that harboured a mean of 13 mg NDW pl^{-1}. The later was associated with a lower growth for RIL104 (4 g SDW pl^{-1}) than for other RILs (6 g SDW pl^{-1}), and it was significantly lower than in 2004. In field 4 the growth was similar among RILs, with a low nodulation of 10 mg NDW pl^{-1}. In field 5, nodulation was contrastingly much higher than in other fields, with nodule mass varying between 300 mg NDW pl^{-1} in RIL115 and 50 mg NDW pl^{-1} in RIL147. Although the growth was higher for RIL147 (10 g SDW pl^{-1}) than RIL115 (8 g SDW pl^{-1}), and there was no relation between growth and nodulation. In field 6, results were intermediate.

During 2006, the farmers of the SPHC proposed to change the protocol with a few meters unsown on the 6 rows where RILs were sowed. Unfortunately, this generated a heterogeneity that resulted in much lower growth of common bean in this sub-plot, except in field 3 from which data of growth and nodulation at flowering stage were processed. RIL147 and 115 show similar growth of 15 g SDW pl^{-1} as that in 2004. This was associated with a significantly higher nodulation for RIL115 (120 mg NDW pl^{-1}) than RIL147 (45 mg NDW pl^{-1}), confirming the good potential of the field 3 for nodulation.

During 2007, the comparison was restricted to RILs 115 and 147 in contrasting fields 1 and 2 with 3 replicated blocks in each field. The mean growth was the highest in field 1 with similar growth of 25 g SDW pl^{-1} for RILs 115 and 147. In field 2 the growth of RIL115 was 18 g SDW pl^{-1} which was significantly higher than the 15 g SDW pl^{-1} of RIL147. Nevertheless in both fields nodulation was very low, confirming that it may vary largely between years as previously observed in field 1 with the locally grown cultivar [9].

3.3. Physiological characterization of contrasting RILs

In order to study the mechanisms of the RIL effects on soil P bioavailability, a rhizotron system was developed with 1 mm soil-layer on which the nodulated legume was transferred after 2 weeks in hydroaeroponic pre-culture, as described by Kouas et al. [11]. A significant decrease of 1 and 1.5 unit of soil pH was found, with RIL115 under 250 and 75 P, respectively (Fig. 3). This acidification may result in a rhizospheric stimulation of P solubilization by the most efficient genotype, namely RIL115.
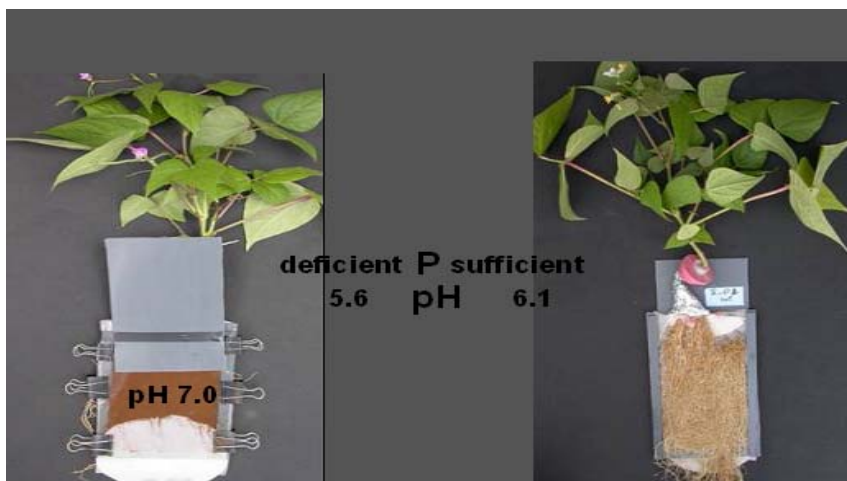

Fig. 3. Rhizotron methodology showing common-bean lines at flowering stage after transfer from hydroaeroponics at 4 week growth onto neutral Cazevieille soil, with subsequent change in soil pH depending upon P supply

The Olsen P in the rhizospheric soil in Lauragais 2005-07 varied from a mean of 15 mg P Kg^{-1} soil in fields 2, 4 and 5 to more than 30 mg P Kg^{-1} soil in fields 1, 3 and 6. Also a large spatial variation within each field was found: e.g. from 6 to 21 mg P Kg^{-1} soil in field 4, and from 24 to 65 mg P Kg^{-1} soil in field 3. In 2007, the Olsen P was significantly higher in the rhizosphere of RIL 115 than 147 in two fields.
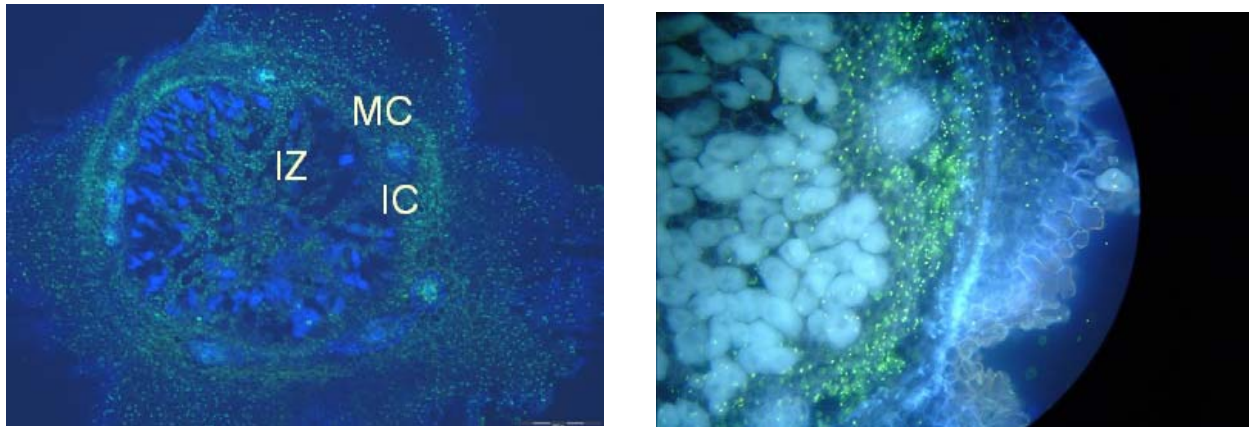

Fig. 4. Nodule sections of RIL115 showing *in situ* RT-PCR signal in green for (A) acid phosphatase and (B) phytase. IC, inner cortex; MC middle cortex; IZ, infected zone

Acid phosphatase (AP) primers were designed from leaf *Phaseolus vulgaris* AP sequence, for localization on nodule section. The green signal in Fig. 4A shows that *PvAP* was widely expressed in nodule cortex, both inner (IC) and middle (MC), and to less extent in non-infected cells of the infected zone (IZ). Since nodule phytase activity was found to increase under P deficiency [12], primers of phytase were designed from *Glycine max* and *Medicago truncatula* sequences, for phytase localization on nodule section. The green signal in Fig. 4B shows that phytase was widely expressed in nodules, and essentially localized in nodule cortex, both inner and middle.

4. Conclusions and prospects

The selection procedure in glasshouse hydroaeroponic culture maximizing the rhizobial symbiosis under highly controlled P supply is an innovative approach to select for the use of P by nodulated legumes. It complements other approaches that are focused on the acquisition of P. These RILs can be used to (i) assess the adaptation of grain legumes in low N&P soils of agro-ecosystems, with common bean as a model, (ii) identify soils where P offer is deficient for the legume N$_2$-dependent growth, (iii) search for mechanisms and genes from the improvement of efficiency in use of P for N$_2$ fixation, (iv) improve local or commercial varieties of common bean for adaptation to low P availability. The seeds of these 6 RILs are ready to identify soils where P deficiency is a limiting factor of SNF and the subsequent yield of grain legumes.

Six Recombinant Inbred Lines (RILs) of common bean (*Phaseolus vulgaris*) have been selected among the progenies of the cross between BAT477 and DOR364 for their efficiency in the use of P for symbiotic N$_2$ fixation. Adaptation to low P availability was partially due to a lower reduction of nodule number and mass for tolerant than for sensitive RILs. However in some exceptional RILs, the adaptation to deficient P was associated with a large growth of individual nodules, which partly compensated for the decrease in nodule number. Another compensation process was the increase in nodule activity. The later could be estimated by the ratio of plant mass per unit mass of nodule (namely the efficiency in use of the rhizobial symbiosis), and by the respiration linked to nitrogenase activity [13].

Further development of the improvement of EUP for SNF of legumes can contribute to stabilize the legume yield. It consists in using the RILs in a differential approach to identify mechanisms and genes responsible for the difference in the use of P for N$_2$ fixation, with such a methodology as Supersage (in cooperation with Frankfurt University), and with *in situ* RT-PCR and hybridisation. The RILs are also used in ecosystem research to assess the role of the EUP for SNF in adaptation of legumes to low fertility soils. The contribution of our work to sustainable

agriculture reveals the role of P biogeochemical cycles in the legume rhizosphere and their benefit for the associated or rotated crops. The use of the RILs in participatory assessment with producers in multilocation tests within agro-ecosystems is pursued for transfer of knowledge and generation of technology.

Acknowledgments

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