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Discrimination against ^{15}N among recombinant inbred lines of *Phaseolus vulgaris* L. contrasting in phosphorus use efficiency for nitrogen fixation

Mohamed Lazali[a,b,c,*], Adnane Bargaz[d], Georg Carlsson[d], Sidi Mohamed Ounane[b], Jean Jacques Drevon[a,*]

[a] Institut National de la Recherche Agronomique, UMR Ecologie Fonctionnelle & Biogéochimie des Sols et Agroécosystèmes, INRA-IRD-CIRAD-SupAgro, Place Pierre Viala 34060 Montpellier, France
[b] Ecole Nationale Supérieure Agronomique (ENSA), Département de phytotechnie, Avenue Hassan Badi, El Harrach 16200 Alger, Algeria
[c] Université de Khemis Miliana, Faculté des Sciences de la Nature et de la Vie & des Sciences de la Terre, Route Theniet El Had, Soufay 44225 Ain Defla, Algeria
[d] Swedish University of Agricultural Sciences (SLU), Department of Biosystems and Technology, Box 103, SE-23053 Alnarp, Sweden

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ABSTRACT

Although isotopic discrimination processes during nitrogen (N) transformations influence the outcome of ^{15}N based quantification of N_2 fixation in legumes, little attention has been given to the effects of genotypic variability and environmental constraints such as phosphorus (P) deficiency, on discrimination against ^{15}N during N_2 fixation. In this study, six *Phaseolus vulgaris* recombinant inbred lines (RILs), i.e. RILs 115, 104, 34 (P deficiency tolerant) and 147, 83, 70 (P deficiency sensitive), were inoculated with *Rhizobium tropici* CIAT899, and hydroaeroponically grown with P-sufficient (250 μmol P plant^{-1} week^{-1}) versus P-deficient (75 μmol P plant^{-1} week^{-1}) supply. Two harvests were done at 15 (before nodule functioning) and 42 (flowering stage) days after transplanting. Nodulation, plant biomass, P and N contents, and the ratios of ^{15}N over total N content (^{15}N/Nt) for shoots, roots and nodules were determined. The results showed lower ^{15}N/Nt in shoots than in roots, both being much lower than in nodules. P deficiency caused a larger decrease in ^{15}N/Nt in shoots (−0.18%) than in nodules (−0.11%) for all of the genotypes, and the decrease in shoots was greatest for RILs 34 (−0.33%) and 104 (−0.25%). Nodule ^{15}N/Nt was significantly related to both the quantity of N_2 fixed ($R^2 = 0.96^{***}$) and the P content of nodules ($R^2 = 0.66^{*}$). We conclude that the discrimination against ^{15}N in the legume N_2-fixing symbiosis of common bean with *R. tropici* CIAT899 is affected by P nutrition and plant genotype, and that the ^{15}N/Nt in nodules may be used to screen for genotypic variation in P use efficiency for N_2 fixation.

Introduction

The relative abundance of the stable nitrogen isotope ^{15}N in the biosphere varies as a result of isotopic discrimination during physical, chemical and biological processes (Hauck, 1973). In legume-rhizobia symbiosis, N_2-fixing root-nodules are often enriched in ^{15}N as compared to above-ground plants (Shearer and Kohl, 1986). However, the abundance of ^{15}N may be affected by abiotic and biotic factors. The latter may include the genotypic diversity both of the host and the rhizobial symbiont as well as the corresponding nodule activities such as nodule respiration (Bargaz et al., 2011).

Discrimination against ^{15}N during N_2 fixation and export of fixed N from the nodules to the shoots has been observed in perennial *Trifolium* species (Steele et al., 1983; Ledgard, 1989; Carlsson et al., 2006), annual *Lupinus* species (Bergersen et al., 1988) and *Glycine max* (Kohl et al., 1983; Shearer et al., 1984; Bergersen et al., 1986). Differences in the ^{15}N signature between parts of a single plant have been attributed to preferential acquisition of ammonium or nitrate (Evans, 2001), nitrate reduction in roots or shoots (Pate et al., 1993, Unkovich et al., 2000), N_2 fixation in nodules (Shearer et al., 1984) and to such abiotic factors as drought (Robinson et al., 2000). These changes in discrimination against ^{15}N among nodulated-legume

Abbreviations: ^{15}N/Nt, ratio of ^{15}N over total N content; DAT, days after transplantion; EURS, efficiency in use of the rhizobial symbiosis; N, nitrogen; nDW, nodule dry weight; P, phosphorus; PUE, phosphorus use efficiency; RIL, recombinant inbred line; sDW, shoot dry weight; SNF, symbiotic nitrogen fixation.

* Corresponding authors at: Institut National de la Recherche Agronomique, UMR Ecologie Fonctionnelle & Biogéochimie des Sols et Agroécosystèmes, INRA-IRD-CIRAD-SupAgro, Place Pierre Viala 34060 Montpellier, France.
Tel.: +33 0 4 99 61 23 32; fax: +33 0 4 99 61 21 19.
E-mail addresses: m.lazali@yahoo.fr (M. Lazali), drevonjj@supagro.inra.fr (J.J. Drevon).

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parts with decreasing N_2 fixation are probably due to shifts in internal ^{15}N partitioning resulting in the specific ^{15}N distribution patterns among nodules, roots and shoots.

Unlike non-nodular tissues, nodules of a wide range of legume species are strikingly enriched in ^{15}N (Shearer et al., 1980). The extent of nodule ^{15}N enrichment has been reported to be affected by the nodules' metabolic activity and the N_2 fixation efficiency of rhizobial strains (Bergersen et al., 1986; Ledgard, 1989). Metabolically active (N_2-fixing) nodules of soybean were significantly enriched in ^{15}N, whereas nodules from plants grown under N-rich conditions exhibited little ^{15}N enrichment (Shearer et al., 1984). Additionally, the degree of ^{15}N discrimination during transport of fixed N varied among combinations of *Trifolium* genotype and *Rhizobium leguminosarum* bv. *trifolii* species (Carlsson et al., 2006), which indicates that there was a discrimination against ^{15}N during N_2 reduction by nitrogenase or the diffusion of N_2 into the infected nodular tissues.

In soybean plants totally dependent on N_2 fixation in symbiosis with rhizobial strains differing in N_2 fixation efficiency, Kohl et al. (1983) found that the symbiotic efficiency was positively correlated to nodule ^{15}N enrichment. It was attributed to an effect of stronger ^{15}N discrimination in the export of N from nodules than in incorporation of N in new nodule tissues, and that the synthesis of nodule biomass utilized recently fixed N within the same nodule (Kohl et al., 1983). Differences in abiotic factors, such as light, mineral nutrition, and moisture may also cause differences in ^{15}N discrimination (Ledgard, 1989).

In common bean, the respiration within the nodule cortex is closely related to P status and N_2 fixation activity (Salsac et al., 1984; Ribet and Drevon, 1995; Vadez et al., 1996; Schulze and Drevon, 2005). Furthermore, genotypic variability among recombinant inbred lines (RILs) of common bean has been shown to be involved in the adjustment of the nodule respiration and P deficiency tolerance (Vadez et al., 1996; Jebara et al., 2005; Bargaz et al., 2011). The increase in nodule O_2 permeability under P deficiency (Ribet and Drevon, 1995; Vadez et al., 1996, Alkama et al., 2012) would predict a lower discrimination against ^{15}N entry within nodules as a result of lower resistance against gas diffusion. Thus, the aims of the present study were: (1) to improve our understanding of the effect of P deficiency on discrimination against ^{15}N in six common bean RILs differing in their tolerance to P deficiency and (2) to determine whether there are interactions between the $^{15}N/Nt$ ratio in plants and both N_2 fixation and efficiency in use of the rhizobial symbiosis. The following hypothesis was tested: discrimination against ^{15}N increases under P sufficiency, because of higher resistance for N_2 molecules to enter nodules with lower gas permeability with either one or two than without ^{15}N atoms.

Materials and methods

Biological material and growth conditions

Experiments were conducted in a glasshouse under natural light with 30/25 °C day/night temperature and a 16 h photoperiod with an additional illumination of 400 μmol photons $m^{-2}\,s^{-1}$ and 70% relative humidity during the day. In this study, six recombinant inbred lines were used: RILs 147, 115, 104, 83, 70 and 34. These RILs were selected from a previous screen among 100 progenies of the crossing, in 1996, of two parental RILs, namely BAT477 and DOR364, from the International Center of Tropical Agriculture (CIAT, Cali, Colombia). Under conditions of P deficiency, RILs 115, 104 and 34 were characterized as tolerant genotypes, while RILs 147, 83 and 70 as sensitive genotypes respectively, based on plant growth and seed yield in relation of SNF-derived to P availability (Drevon et al., 2011). Seeds were surface-sterilized with 3% calcium hypochlorite for 10 min and rinsed by 5 washings with sterile distilled water. They were subsequently pre-germinated in sterile distilled water for 4 days at 28 °C. The seedlings were inoculated with the reference strain *Rhizobium tropici* CIAT899 grown in liquid yeast extract mannitol medium at 28 °C for 3 days to a density of approximately 10^9 cells mL^{-1}. Thereafter, the roots of selected uniform seedlings were passed through the hole of a rubber stopper on the cover of 40 L vat for hydroaeroponic culture according to Hernandez and Drevon (1991). A cotton wool was fitted at the hypocotyls level to maintain the root system suspended in the nutrient solution.

Based on the work of Vadez et al. (1999), P was supplied weekly in the form of KH_2PO_4 as 75 or 250 μmol $plant^{-1}$ $week^{-1}$ for deficient and sufficient P supply, respectively, to the following nutrient solution: $CaCl_2$ (1650 mM); $MgSO_4$ (1000 mM); K_2SO_4 (700 mM); Fe-EDTA (8.5 mM Fe as sequestrene); H_3BO_3 (4 mM); $MnSO_4$ (6 mM); $ZnSO_4$ (1 mM); $CuSO_4$ (1 mM); Na_2MoO_4 (0.1 mM). Urea was supplied as 2 mmol $plant^{-1}$ into the nutrient solution during the initial 2 weeks of growth to avoid N deficiency during nodule development. Thereafter, the plants were grown in N-free nutrient solution in order to depend exclusively on N_2 fixation. The pH in the nutrient solution was maintained around 7 by addition of 0.2 g L^{-1} $CaCO_3$ and the solution was aerated by an air flow of ambient air compressed of 400 mL min^{-1}.

Measurements of shoot, root and nodule biomass, symbiotic N_2 fixation and P contents

In each treatment, a subset of the plants was harvested at the onset of nodulation, 15 days after transplanting (DAT), and the remaining plants were harvested at 42 DAT. The shoot was separated from the root at the cotyledonary node and nodules were separated from roots and counted. Each plant fraction was weighed after drying for 48 h at 70 °C.

To assess the efficiency in use of the rhizobial symbiosis (EURS), i.e. the ratio of symbiotic nitrogen fixation (SNF)-dependent growth of shoot per unit of nodule biomass, the values of the shoot biomass accumulation between 15 and 42 DAT were plotted as a function of those of nodule biomass, with the slope of the linear regression being considered an estimate of the EURS (Lazali et al., 2013). The SNF was calculated as the difference between plant N content (g $plant^{-1}$) at 42 and 15 DAT, i.e. the quantity of N accumulated during the SNF-dependent growth (Kouas et al., 2005).

Total P content in nodules was measured in dry matter aliquots of 50 mg digested with concentrated HNO_3 in a microwave oven (ETHOS, Milestone) at 40 bars for 15 min. Total P content was determined with the vanado-molybdate method (AFNOR, 1969) by measuring the absorbance at 460 nm with a variant Cary 1E spectrophotometer.

$^{15}N/Nt$ determination

Dry samples were ground to a fine powder in a ball mill for determination of ^{15}N content. ^{15}N analysis was performed on aliquots of 2 mg dry weight (DW) for nodules and 2.5 mg DW for shoots and roots. The isotopic composition of N_2 from the samples was measured with mass spectrometer (Isoprime, Cheadle, UK) and expressed as the ratio of ^{15}N over total N content in the analyzed sample ($^{15}N/Nt$).

In order to assess the discrimination against ^{15}N during N_2 fixation, the $^{15}N/Nt$ ratio of SNF-derived N was calculated from the difference between values at 42 and 15 DAT, as following: $(^{15}N_{42\ DAT} - {}^{15}N_{15\ DAT})/[(^{14}N_{42\ DAT} - {}^{14}N_{15\ DAT}) + (^{15}N_{42\ DAT} - {}^{15}N_{15\ DAT})]$. Since the sampling at 15 DAT was performed just before the appearance of functioning nodules, the $^{15}N/Nt$ ratio in nodules was analyzed only at 42 DAT.