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Variability in symbiotic nitrogen fixation among white landraces of common bean from the Iberian peninsula

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Abstract
Common bean is considered to have a low capacity of symbiotic nitrogen fixation (SNF) with rhizobia in Europe. However, genetic variability for SNF among bean germplasm has been found in the regions of origin of this species. The objective of this research was to search for European bean germplasm with high SNF capacity. For this purpose, the N_2-dependent growth of various bean accessions, belonging to the great northern, caparron, white kidney and canellini market classes, was compared in glasshouse, under optimized symbiotic conditions with a *Rhizobium tropici* inoculant. In the studied germplasm, genotypic variation was found for (i) nodulation with PHA-0184, PHA-0267, PHA-0290, PHA-0034 and PHA-0276 having the highest nodule biomass, (ii) N_2-dependent plant-growth with PHA-0013, PHA-0014, PHA-0290 and PHA-0838 having the highest shoot biomass and (iii) the ratio of the shoot growth as a function of nodule growth with PHA-0034, PHA-0053, PHA-0184, PHA-0267 and PHA-0276 having the highest value. It is concluded that the accessions PHA-0013, PHA-0014, PHA-0034 and PHA-0053 from the Iberian peninsula could be recommended for further improvement of the SNF potential in the common bean varieties currently grown in Europe.

Keywords: Breeding, genetic variability, nitrogen fixation, *Phaseolus vulgaris* L., Rhizobiaceae, symbiosis

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is a traditional and important worldwide edible crop originated from the Andean and Mesoamerican areas. This crop has been a relevant source of protein in human diet for centuries, especially where animal protein is very expensive and sometimes unsafe or unhealthy. Thus, common bean is extensively grown by farmers in all continents and its cultivation as a staple food extends into marginal areas (Vadez et al., 1999). In addition, the increase of the vegetarian diets among Europeans could lead to an overall increase in grain legumes consumption in Europe. Indeed, with the earth's populations increasing 1.4% annually, unprecedented increases in crop production will be needed if the current levels of dietary protein and caloric intake are to be maintained.

Such an importance is emphasized by the fact that the area of fertile agricultural land is shrinking, thus forcing agriculture onto more marginal land where nutrient availability may be jeopardized by soil chemical and physical conditions (Rengel, 2002).

Nitrogen availability is often a limiting factor for crop productivity, particularly in developing countries where nitrogen fertilisers are either unavailable or unaffordable (Graham, 1981). The recommendations in both tropical and temperate areas are that farmers would apply to crops at least minimal doses of nitrogen. Moreover, the use of chemical N must be limited for the preservation of the environment, particularly in Europe where rates of N fertilization have increased steadily (Vance, 1998; Fink et al., 1999), in addition to manure recycling (Graham and Vance, 2000) and significant N deposition from the atmosphere (Goulding et al., 1998).

Biological nitrogen fixation through symbiotic, associative and free-living microbial systems, already contributes towards a major and sustainable input into agriculture. The symbiotic nitrogen fixation (SNF) still

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provides more nitrogen to the agricultural ecosystems worldwide than the total amount of N fertiliser applied. This system could constitute an ecologically acceptable alternative to the high application of N fertilisers, particularly in Europe, and an economic alternative to the limited access of developing countries to N fertilisers.

Despite the ability of common bean to enter intimate association with nitrogen-fixing symbionts, the productivity of this crop is often limited by nitrogen deficiency under agronomic conditions (Rosas et al., 1998). Research directed toward increasing SNF to overcome this constraint in a cheaper and sustainable way has emphasized the need to improve both the plant genotype and the rhizobial strain components of the symbiosis (Kipe-Nolt et al., 1993). Many common bean cultivars are poor N_2 fixers in comparison with other legumes (Bliss, 1993; Isoi and Yoshida, 1991). However, there is evidence of genotypic variability for traits associated with SNF potential (Bliss, 1993; Park and Buttery, 1989; Pereira and Bliss, 1989; Pereira et al., 1989a,b) including with relatively low P supply (Vadez et al., 1999).

Although, previous research did not explore the large genetic diversity within the various gene-pools of the common bean domestication in America (Singh, 1991). Furthermore, selection has produced breeding lines able to fix high levels of N_2 and increase bean production, particularly in low fertility soils (Miranda and Bliss, 1991). However, many different bean cultivars are grown worldwide or even in one region of a single country, on the basis of distinct regional preferences which relate to cropping system, day length, plant type and seed characteristics, including consumers preferences based on seed shape and color and cooking quality (Bliss, 1993).

In this study we screened for SNF potential among popular common bean landraces of the germplasm collection maintained at the MBG-CSIC (Misión Biológica de Galicia – CSIC, Pontevedra, Spain) (Table 1). These landraces are from the Andean and Mesoamerican centers of origin, belonging to the great northern, caparron, white kidney and canellini market classes (Santalla et al., 2001). The objective of this paper is to identify contrasting varieties which may be useful to further improve SNF potential in common bean.

Table 1. Common bean accessions with growth habit (I: determinate bush, II: indeterminate bush, III: indeterminate semi-climber or prostrate, IV: indeterminate climber) and international market class.

Accessions	Growth habit	Market class
PHA-0001	I	Canellini
PHA-0013	II	White kidney
PHA-0014	II	White kidney
PHA-0018	IV	White kidney
PHA-0034	I	Canellini
PHA-0053	I	Canellini
PHA-0080	II	Canellini
PHA-0098	II	Great northern
PHA-0115	IV	Canellini
PHA-0152	IV	White kidney
PHA-0173	III	Canellini
PHA-0184	III	White kidney
PHA-0185	IV	White kidney
PHA-0187	III	White kidney
PHA-0191	I	Canellini
PHA-0256	III	Canellini
PHA-0257	I	Canellini
PHA-0267	II	White kidney
PHA-0273	IV	Great northern
PHA-0276	IV	Canellini
PHA-0290	IV	White kidney
PHA-0324	III	White kidney
PHA-0331	IV	Caparron
PHA-0341	III	White kidney
PHA-0459	I	Canellini
PHA-0511	IV	Canellini
PHA-0525	II	Canellini
PHA-0584	I	White kidney
PHA-0600	IV	Canellini
PHA-0838	IV	White kidney
Linex	I	Canellini
Moggette	I	Canellini

2. Material and Methods

Plant and bacterial material

Thirty common bean accessions from the Iberian peninsula were chosen in the MBG-CSIC (Pontevedra, Spain) collection (Table 1). These landraces constitute a part of a core collection incorporating germplasm from the Mesoamerican and Andean origins according to previous works by De Ron et al. (1997), Escribano et al. (1998), Rodiño (2001), Rodiño et al. (2001) and Santalla et al. (2002). They belong to the great northern, caparron, white kidney and canellini international bean market classes (Santalla et al., 2001). Two common bean commercial lines, Linex and Moggette from Vilmorin (Angers, France), were used as controls.

The reference rhizobial was *Rhizobium tropici* CIAT 899 preserved in tubes at 4°C on Yeast Mannitol Agar media (YEM) (Vincent, 1970). The *R. tropici* inoculants were prepared in liquid YEM solution into an Erlenmeyer flask with agitation during 2 days at 28°C in darkness. Inoculation was performed by soaking common-bean seedlings during 30 min with 100 ml of inoculant containing approximately 10^8 cells ml^{-1}.

Hydroaeroponic cultivation of nodulated beans

Seeds of bean were sterilized in 3% calcium hypochlorite, washed with sterile distilled water and germinated in trays containing perlite moistened with deionized water, in a dark growth chamber at 28°C. The 4

day old seedlings were inoculated and passed carefully through a plastic tube in a pierced rubber stopper. They were fixed with cotton fitted around the hypocotyle, and mounted on the topper of a 0.40 × 0.20 × 0.20 m vat receiving 15 plants. They were grown in a temperature-controlled glasshouse in 15 l of the nutritive solution revised by Vadez et al. (1996), that was complemented with distilled water to the 40 l volume of the vat. They received an intense aeration by compressed air at a flow of 400 ml min^{-1} l^{-1} solution, and 3 mmol urea per plant, as a starter N described previously by Hernandez and Drevon (1991).

Biomass measurements and statistical analysis

Plants were harvested at the flowering stage, between 42 and 47 days after sowing, depending upon growth rate in glasshouse. Shoot, nodule and root components were separated and dried at 70°C for 2 days to constant weight and each fraction were weighted. The analysis of variance was performed according to Fischer (1990) with each container of 15 plants constituting one block. The fixed effect was the landrace. A preliminary screening, namely a pre-screen experiment, was realized with one repetition of the 30 accessions, from which 11 accessions were chosen for subsequent screening. The latter was performed with three blocks, i.e. 3 replicates per accession, and repeated at 2 sowing dates. The least significant difference (LSD) was used to rank differences between landrace means. Correlation and regression analysis were performed using the general linear model procedure of the SAS package (SAS Institute, 2000).

3. Results

Pre-screening experiment

In a preliminary screening, the landraces were evaluated without replications. From data of shoot biomass in the Fig. 1A, the landraces PHA-0341, PHA-0013, PHA-0018, PHA-0276, PHA-0184, PHA-0098 and PHA-0256 constituted a group with a significantly higher growth than the contrasting group of PHA-0034, PHA-0525, PHA-0152, PHA-0115, PHA-0014, PHA-0185, PHA-0257, PHA-0173 and PHA-0324, with mean values of 13.06±2.33 and 2.22±0.97 g shoot dry weight $plant^{-1}$, respectively. There was no obvious relation with the growth habits as shown in Table 1. The root data in the Fig. 1B, where landraces have been ranked like in Fig. 1A, showed less variation than shoot data. Plants with high shoot growth had generally high root growth.

By contrast, there was much more variation in nodule mass per plants, including within each of both groups defined above (Fig. 1C). Thus, the highest nodule biomasses were found in PHA-0256 (>1,200 mg nodule dry weight $plant^{-1}$), PHA-0014, PHA-0276 and PHA-0191 (>600 mg nodule dry weight $plant^{-1}$), by contrast with the lowest nodule biomasses in PHA-0018, PHA-0267, PHA-0600, PHA-0080, PHA-0331, PHA-0115, PHA-0257 and PHA-0324 (<200 mg nodule dry weight $plant^{-1}$). This variation in nodule mass per plants resulted from large variation in nodule number per plant (Fig. 2A). In general, the plants with high nodule number had also high nodule mass as illustrated by the significant regression of Fig. 2B, with a mean individual nodule mass of 0.9 mg $nodule^{-1}$.

In order to test whether nodulation was the determinant factor of plant growth in this pre-screening, we searched for relation between shoot growth and nodulation, either mass or number (Figs. 3A and 3B). Three groups of individuals could be selected: (i) a large central group characterized by a significant regression of shoot growth as a function of nodule mass (shoot dry weight = 0.01 nodule dry weight + 4.7 R=0.76); (ii) a second group characterized by a significantly lower shoot/nodule mass ratio (shoot dry weight = 0.002 nodule dry weight + 1.6 R=0.47); and (iii) a third group characterized by significantly high shoot constant-value (shoot dry weight = 0.012 nodule dry weight + 10.4 R=0.76).

Shoot and root growth

Two groups of contrasting landraces were selected from the above pre-screening for further comparison in hydroaeroponic cultivation with replicates. The data in the Fig. 4A show that the landraces PHA-0290, PHA-0838, PHA-0013, PHA-0014, PHA-0184, PHA-0341 and Linex had a significantly higher shoot biomass (9.4±0.84 g shoot dry weight $plant^{-1}$) than PHA-0053 (4.3±2.2 g shoot dry weight $plant^{-1}$). In general, the plants with high shoot biomass had high root growth, as illustrated in the Fig. 4B with landraces being ranked like in the Fig. 4A.

Nodulation in glasshouse

The data in the Fig. 5A show that there was wide variation in nodule mass per plants. PHA-0184 and PHA-0267 PHA-0290, PHA-0034 and PHA-0276 (310±58 mg nodule dry weight $plant^{-1}$) had a significantly higher nodule biomass than PHA-0018 and Moggette (38±10 mg nodule dry weight $plant^{-1}$). This large variation in nodule biomass was due partly to large variation in nodule number per plant (Fig. 5B). Thus, PHA-0276 and PHA-0184 had the highest nodule number with more than 150 nodules per plant. Contrastingly, the landraces with less than 50 nodules per plant were PHA-0018 and Moggette.

There was a significant regression of nodule mass as a function of nodule number for all the lines with a mean nodule mass of 1.4 mg $nodule^{-1}$ (Fig. 5C). This significantly higher mean individual nodule mass than in the pre-screening (0.9 mg $nodule^{-1}$ in Fig. 2B) was due to a lower mean nodule number per plant. An exception was PHA-0053 with large nodules associated with a low nodule

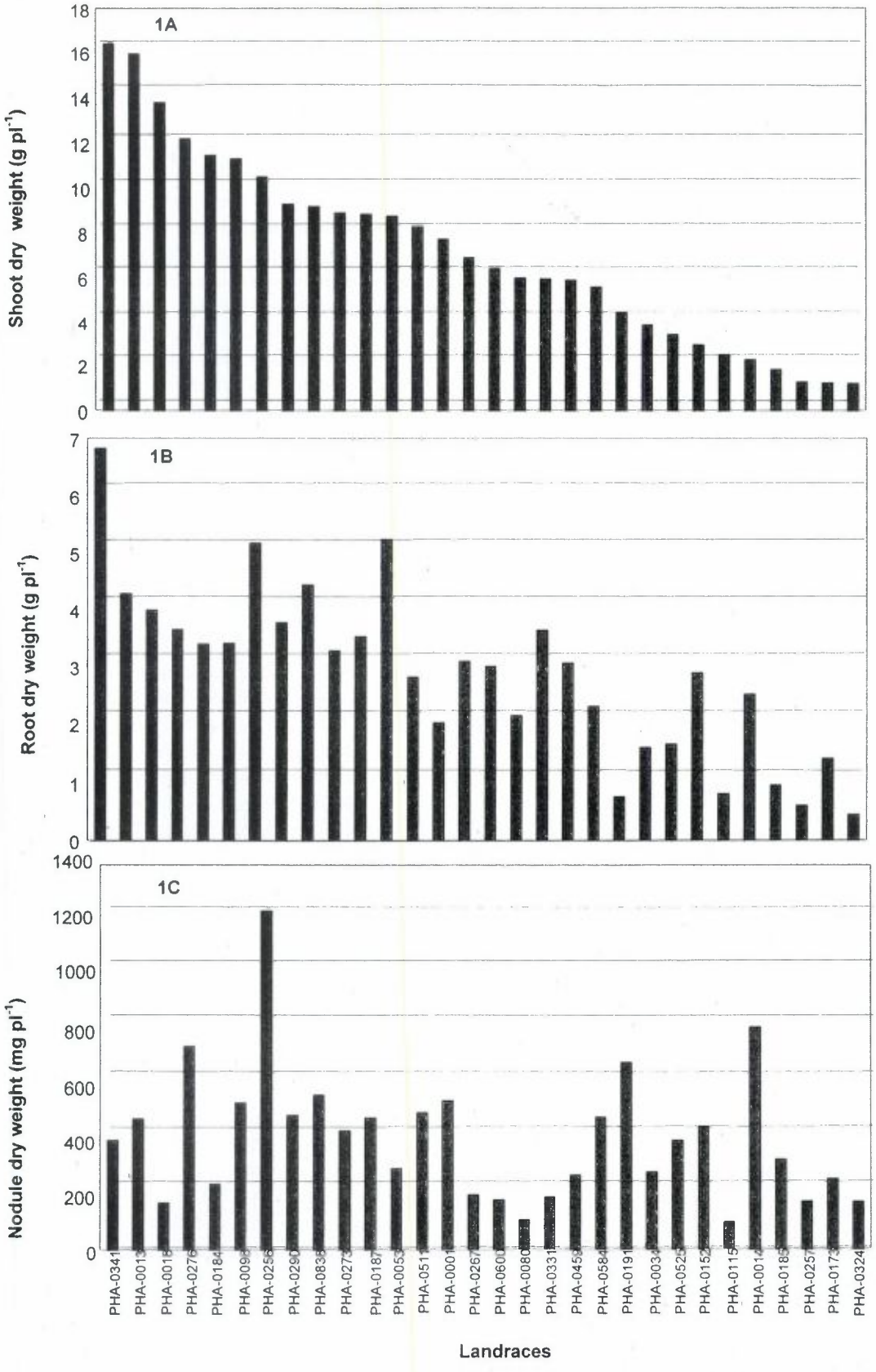

Figure 1. Shoot (g dry weight plant^{-1}) (1A), root (g dry weight plant^{-1}) (1B) and nodule (mg dry weight plant^{-1}) (1C) biomass of 30 landraces in the preliminary screening experiment in hydroaeroponic growth under glasshouse. Data are individual values for accession. Plant harvested at 45 days after sowing.

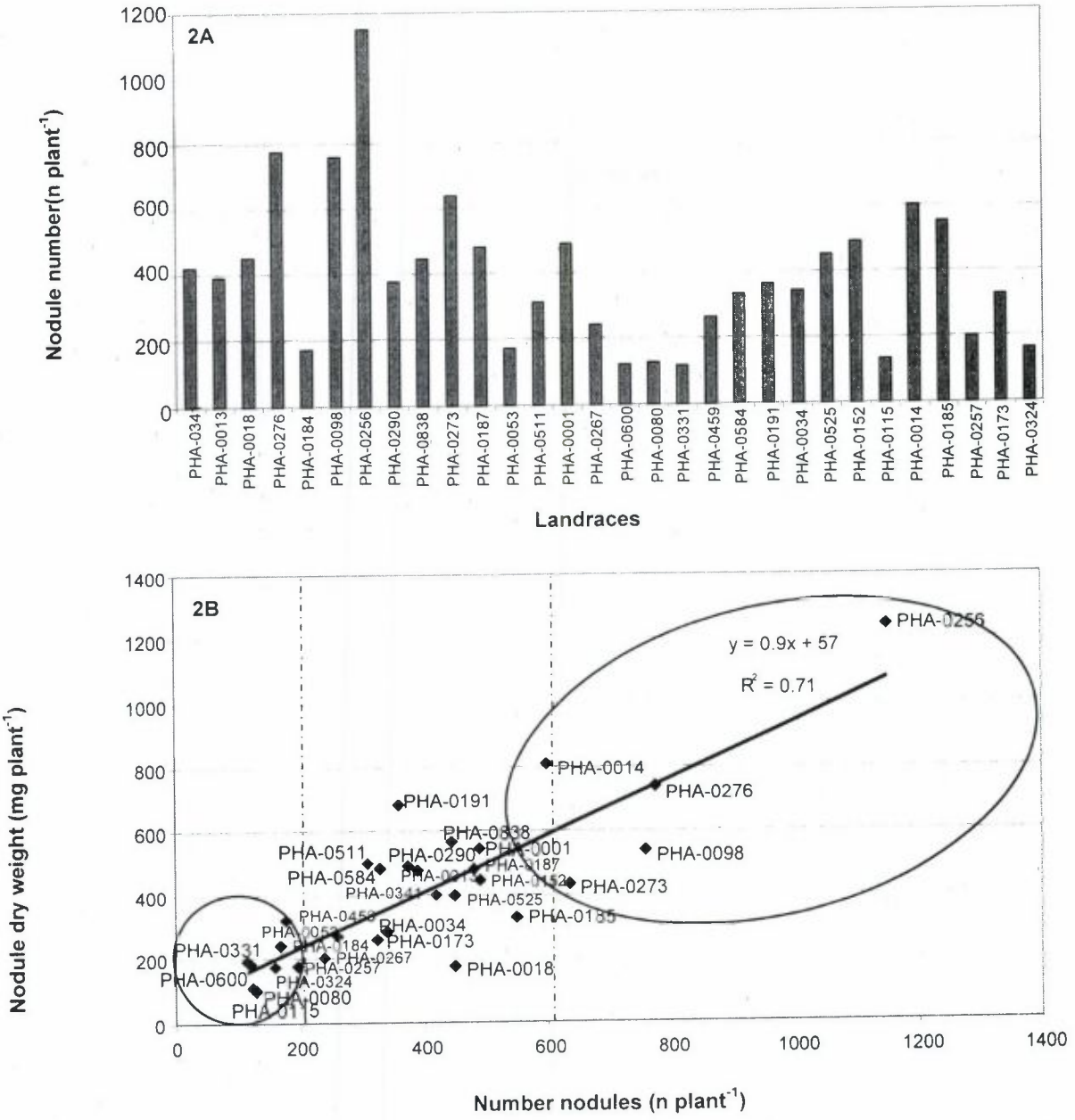

Figure 2. Number of nodules per plant (2A) and relation between nodule growth (mg dry weight plant^{-1}) and nodulation number (2B) of 30 landraces in the preliminary screening experiment in hydroaeroponic growth under glasshouse. Data are individual values for accession. Plant harvested at 45 days after sowing.

number. In contrast, PHA-0276 and PHA-0013 had nodules significantly smaller than the previous landraces.

Correlation between shoot growth and nodulation

Plant growth depended upon SNF since N was the limiting factor in this screening experiment. Therefore, to assess the efficiency in utilization of the rhizobial symbiosis among landraces, we searched for relation of shoot growth as a function of nodulation, either mass or number (Figs. 6A or 6B). According to the regression models shown in Fig. 6A, two groups of landraces could be distinguished: (i) the first group with PHA-0034, PHA-0053, PHA-0184, PHA-0267, PHA-0276, was characterized by a significantly higher shoot/nodule mass ratio of 0.02 shoot dry weight nodule dry weight^{-1}, (ii) the second group with PHA-0013, PHA-0014, PHA-0018, PHA-0290, PHA-0341, PHA-0838, Linex and Moggette, with a mean shoot/nodule mass ratio of 0.01 shoot dry weight nodule dry weight^{-1}.

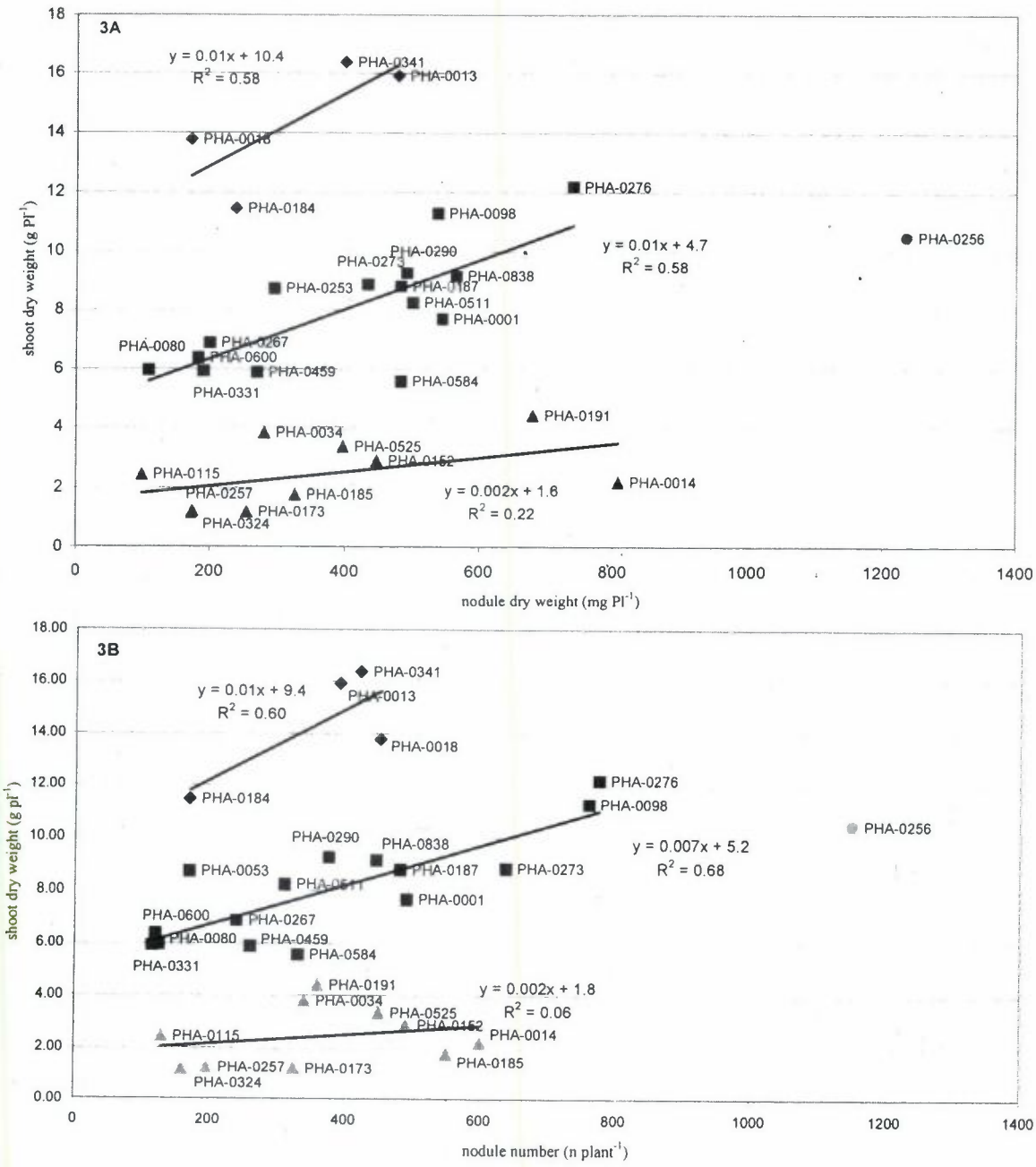

Figure 3. Relation and coefficient of regression between shoot growth (g dry weight plant^{-1}) and nodule biomass (mg dry weight plant^{-1}) (3A) or nodule number (3B) of 30 landraces in the preliminary screening experiment in hydroaeroponic growth under glasshouse. Data are individual values for accession. Plant harvested at 45 days after sowing.

This dependence of growth upon nodulation is confirmed by the Fig. 6B which shows significant correlations between shoot mass and nodule number. Exceptions though were PHA-0276 and PHA-0184 associated with high nodulation or contrastingly, PHA-0013, PHA-0014, PHA-0290 and PHA-0838 with a low nodulation.

4. Discussion

In this work a large genotypic variability for SNF was revealed within the common bean European germplasm, as illustrated by the contrast between PHA-0013, PHA-0014, PHA-0290 and PHA-0838 having a high SNF, versus PHA-0276, PHA-0267, PHA-0018 and PHA-0053, having a significantly lower SNF (Figs. 1 and 2). Landraces PHA-0184, PHA-0290, and PHA-0341 with growth habit of type III or IV had higher SNF than the type I commercial varieties Linex and Moggette. While this is often true (Graham, 1981; Nleya et al., 2002), notable exceptions were reported before, especially within the snap bean cultivars (Bliss, 1993; Vadez et al., 1999). Interestingly,

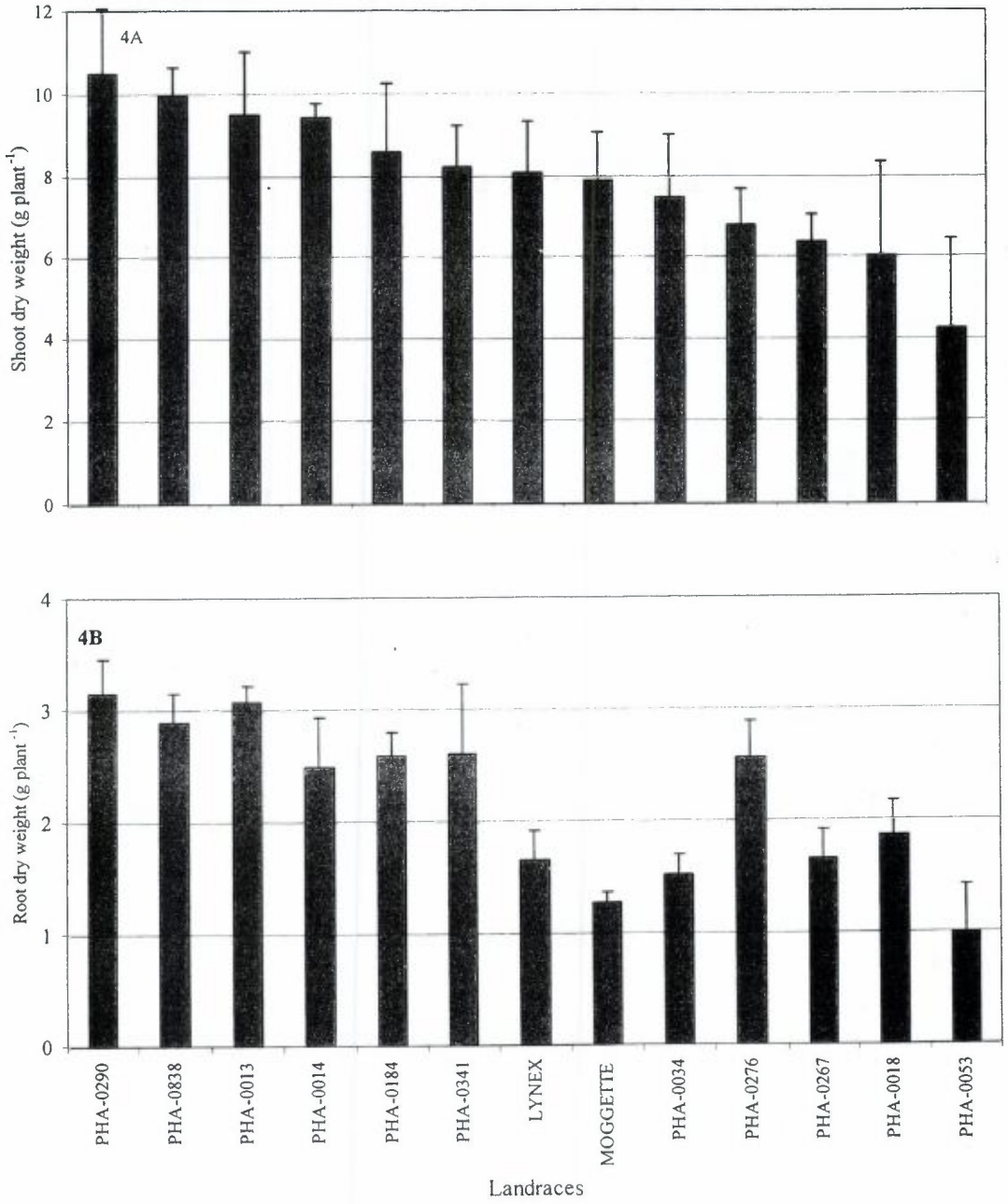

Figure 4. Shoot (g dry weight plant^{-1}) (4A) and root (g dry weight plant^{-1}) (4B) biomass of 11 landraces in the screening experiment in hydroaeroponic growth under glasshouse. Data are mean ± standard deviation of six replicates. Plants harvested at 45 days after sowing.

landraces PHA-0014 and PHA-0034, with type II and I growth habit respectively, had the highest SNF in this work. This was due to high nodule biomass for PHA-0014 and high nodule activity for PHA-0034. This finding agrees with the conclusion of Hardarson et al. (1993) and Nleya et al. (2002), that there is a substantial variability of SNF among different types. The latter might apply especially to germplasm from the Iberian peninsula, since this region was identified as a secondary center of diversity for the common bean (Rodiño, 2001; Santalla et al., 2002; Rodiño et al., 2003).

Previously, Miranda and Bliss (1991) concluded that most common bean landraces have low SNF capacity because of their original domestication as a home-garden crop. This process resulted in low selection pressure on the symbiosis with rhizobia. More recently, common bean varieties were bred without reference to improving SNF *per se*, particularly in Europe.

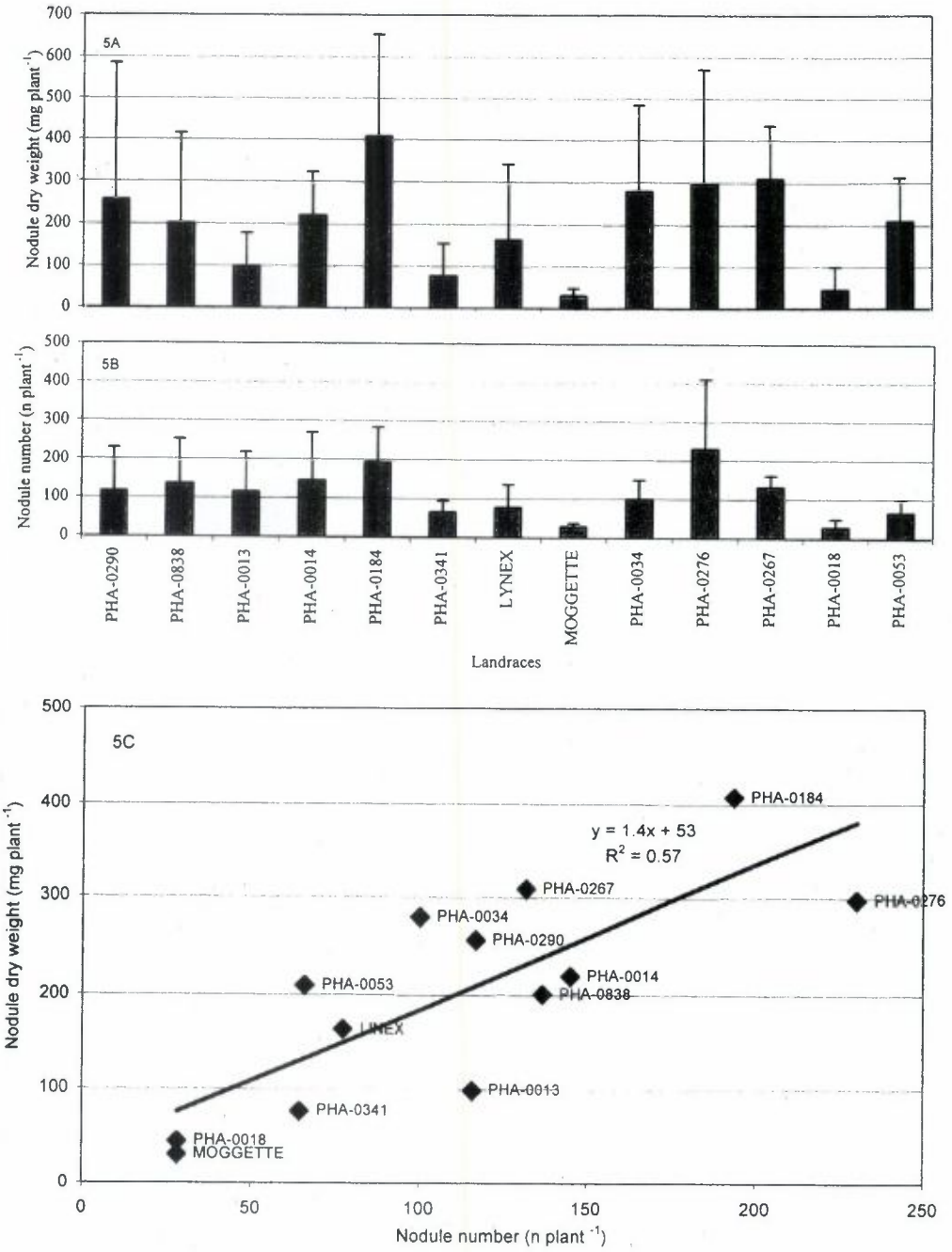

Figure 5. Nodule biomass (mg dry weight plant^{-1}) (5A) and nodule number (5B) and relation between nodule growth (mg dry weight plant^{-1}) and nodulation number (5C) of 11 landraces in the screening experiment in hydroaeroponic growth under glasshouse. Data are mean ± standard deviation of six replicates in 5A and 5B and mean values with regression coefficient and parameters in 5C. Plants harvested at 45 days after sowing.

Under glasshouse conditions the genotypic variation in SNF potential was generally due to variation in nodule number (Fig. 5A). Individual values above 500 nodules plant^{-1} confirmed the large nodulation potential of the studied germplasm. Although some landraces such as PHA-0034 or PHA-0054 may compensate their low nodule number by a large nodule growth, generally both parameters appeared to be well correlated for the studied germplasm (Fig. 5C). However, some landraces such as PHA-0013 may compensate their low nodule mass by a high nodule activity. This conclusion is illustrated in Fig. 6A where two groups of landraces differed in the slope of the linear regressions of shoot biomass as a function of the nodule biomass. Rey-Poiroux and Drevon (2003) proposed to qualify this slope as an estimate of the landrace efficiency in utilization of the rhizobial symbiosis (EUSR = slope of the

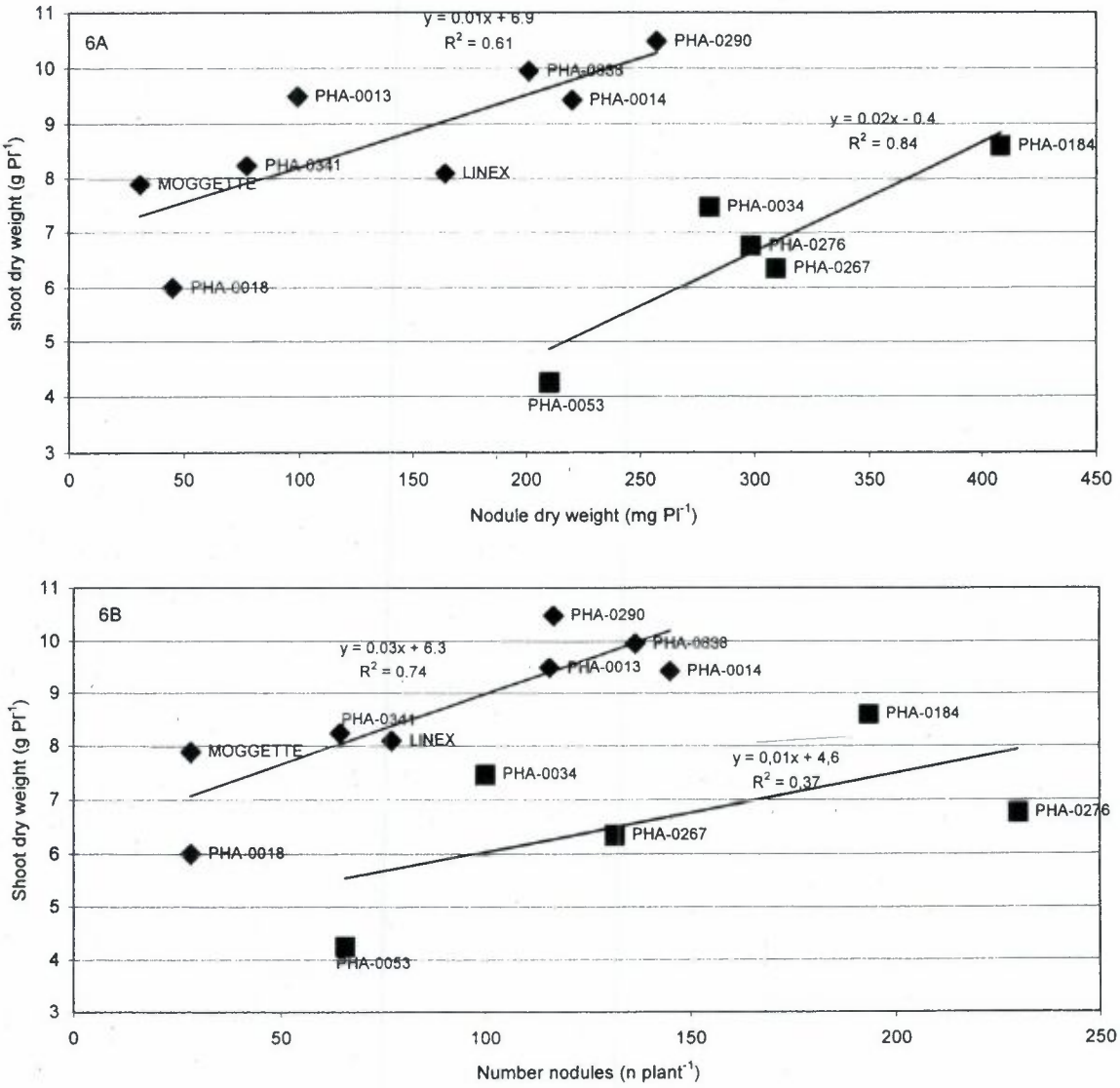

Figure 6. Relation and coefficient of regression between shoot growth (g dry weight plant^{-1}) and nodule biomass (mg dry weight plant^{-1}) (6A) or nodule number (6B) of 11 landraces in the screening experiment in hydroaeroponic growth under glasshouse. Data are mean values of 11 landraces in the screening experiment in hydroaeroponic growth under glasshouse with regression coefficient and parameters. Plants harvested at 45 days after sowing.

regression of the shoot growth as a function of nodulation). Thus, the highest slope corresponds to the highest efficiency for growth, i.e. a given increase in nodule mass resulted in the highest increase in plant growth. A large variation of the EUSR is suggested in Fig. 3A where three groups of landraces could be distinguished.

In conclusion, genetic variability for the SNF and the N_2-dependent growth has been found in this work in white kidney and canellini common bean market classes from the Iberian peninsula. Therefore, we agree with Tsai et al. (1993) that it would be possible to select bean cultivars for both high SNF and yield through plant breeding. A significant potential exists for further improvement of the capacity to sustain these interactions with rhizobia.

Therefore, breeding for improved N_2 fixation must be incorporated into a bean improvement program as a standard practice with a priority equivalent to other objectives such as yield improvement, suitable plant type, disease and insect resistance, adaptability and seed quality. In addition, more research of common bean landraces capable of expressing a high SNF is recommended under controlled environmental conditions with the hydroaeroponic system since the latter optimizes their nodulation capacity.

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