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Full Length Research Paper

Genotypic variability for tolerance to salinity and phosphorus deficiency among N_2-dependent recombinant inbred lines of Common Bean (*Phaseolus vulgaris*)

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Common bean (*Phaseolus vulgaris* L.) is often subject to various environmental constraints including soil salinity and phosphorus deficiency as major limitations for the yield of most grain legumes, especially when the plant growth depends upon N_2 fixation. In order to assess the genetic variation for tolerance to moderate salinity and phosphorus deficiency and identify the related morphological, physiological and genetic traits, 37 common bean recombinant inbred lines (RILs) were inoculated with *Rhizobium tropici* CIAT899, and grown in a glasshouse with 25 mM NaCl or 75 µmol P plant^{-1} week^{-1}, compared to optimal nutrient solution in hydroaeroponic culture system. Large genotypic variation in tolerance to P deficiency and salt was found with some RILs being tolerant to both constraints. By contrast some of the RILs showed tolerance to only one constraint while the most sensitive to salinity were also sensitive to P-deficiency. By using 18 microsatellite primer-pairs with six most contrasting RILs, 4 alleles were found to discriminate among the RILs. It is concluded that these genotypes and the microsatellites primers can be used to identify genes involved in salinity and P deficiency tolerance of N_2-dependent legume.

Key words: *Phaseolus vulgaris*, genotypic variability, microsatellites, phosphorus deficiency, rhizobia; salinity, symbiosis.

INTRODUCTION

The limitation of symbiotic nitrogen fixation (SNF) by environmental constraints, especially salinity and phosphorus deficiency, restricts the extension of the legume cultivation and the development of a sustainable agriculture. In salty zones the enhancement of legume productivity requires the development of salt-tolerant symbioses. This approach implies the genetic improvement of both partners, although it is generally accepted that the rhizobial microsymbiont is more tolerant than the legume macrosymbiont (Singleton and

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Bohlool, 1983). Exploration of the host variability in salt response would permit not only to identify some tolerant species and lines, but also to determine useful criteria for genetic improvement of salt tolerance.

A large genetic variability in salt tolerance was found among legume species and lines (James et al., 1993). The tree legumes, such as *Prosopis* and *Acacia* spp., were highly tolerant to salinity (Rhodes and Felker, 1988). By contrast, grain legumes have generally been considered either sensitive or moderately tolerant to salinity (Lauchli, 1984). Common bean, chickpea, and pea were the most sensitive legumes (Soussi et al., 1999), whereas soybean was the most tolerant one (Delgado et al., 1994). There are fewer studies on intra-specific variability in salt tolerance. Among 19 lines of common bean cultivated for 13 days on a nutrient solution supplemented with 0, 40 or 80 mM NaCl, variability was observed for height, dry matter of leaves, stems and roots (Yamamouchi et al., 1997). Variability in sensitivity to salinity was also found among beans grown in Tunisia, a local line showing a sensitive index (SI) of -21% versus -46% for Gabriella (Slama, 1986). However, in both studies, the differences between lines at early development-stage did not maintain in later stages. Although salinity affects the photosynthetic capacity of leaves, the legume growth was found to be more affected by salt when its nitrogen nutrition depended upon N_2 than upon mineral nitrogen (Delgado et al., 1994). Generally, nodular activity was less affected by salt than nodulation (Singleton and Bohlool, 1983). Thus, the infection process seems to be the most sensitive to salt (Velagaleti et al., 1990).

Phosphorous is a primary factor limiting the production of bean in many parts of the world, particularly in acidified or calcareous soils in tropical or Mediterranean zones. Thus, low soil-P availability is a primary constraint to agricultural 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 (Lynch and Beebe, 1995). The P deficiency affects particularly the rhizobial symbiosis. Nodules are a strong sink for P with three times higher P concentrations than other organs (Vadez et al., 1996). Consequently, legumes have higher P requirements than non symbiotic plants (Israel, 1987). Moreover, SNF in common bean is affected by P deficiency more than in other legumes (Ribet and Drevon, 1995).

The identification and use of genotypes tolerant to mineral deficiencies and/or toxicities are essential for reducing production costs and dependence of farmers on soil amendment inputs (Singh et al., 2003). In order to increase common bean production it may be possible to improve its SNF potential (Bliss, 1993) and expression under P deficiency (Vadez et al., 1999). Research directed towards increasing SNF has emphasized improving both the plant genotype (Miranda and Bliss, 1991) and the rhizobial strain components of the symbiosis (Kipe-Nolt et al., 1993). There is evidence for genotypic variability in traits associated with N_2 fixation in common bean (Park and Buttery, 1989) including under deficient P supplies (Vadez et al., 1999). High common bean SNF under P deficiency was reported to be related to nodule number (Pereira et al., 1989), nodule mass (Kipe-Nolt et al., 1993) and early nodulation (Chaverra and Graham, 1992), nodule P concentration (Vadez et al., 1996) and P use efficiency for SNF (Vadez and Drevon, 2001). Thus it has been attempted to increase bean productivity by selecting lines able to fix adequate N_2 under low P availability (Vadez et al., 1999).

Efforts to improve these crops and their resistance to abiotic constraints are presently underway. The diversity of legumes was studied with molecular tools, including various DNA markers that demonstrated their potential to analyze genome structure and evolution (Zurawski and Clegg, 1993). Among DNA markers, the so-called sequence-tagged microsatellite sites (STMS) or simple sequence repeat (SSR) markers, have been recognized for genotype identification (Scott et al., 2000). Micro-satellites can be profitably utilized in common bean not only for polymorphism detection and gene-tagging, but also for genotype identification and for assessment of genetic diversity. Therefore, on the basis of microsatellite markers, diverse parents can be selected. With the availability of a rich collection of microsatellite primers recently made available through collaborative efforts in common bean, and through individual efforts elsewhere, microsatellites will certainly become the markers of choice in the future for a variety of studies.

The aim of the present work was (i) to explore the genotypic variability in phosphorus deficiency and salt tolerance among 37 common bean lines of the cross of BAT477 and DOR364 and (ii) to assess the level of microsatellite-based genetic diversity among six RILs that were potentially useful in common bean breeding programmes.

MATERIALS AND METHODS

Plant and bacterial material

Thirty seven lines of *Phaseolus vulgaris* were chosen among the descent of the cross of two parental lines BAT477 and DOR364 at the International Centre of Tropical Agriculture (CIAT Cali-Colombia). BAT477 was reported to be P-efficient with a high N_2-fixation potential. These lines were referenced as BT21138-i-1-1-M-M-M, with i being equal to 1, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 22, 23, 24, 25, 26, 27, 28, 29, 32, 34, 36, 37, 38, 60, 61, 62, 64, 66, 73, 75, 83, 104, 115, 124 and 147. They are shortly denominated as such in this work.

Seeds of bean were sterilized in 2% calcium hypochlorite, washed with sterile distilled water and germinated at 28°C in soft agar containing 100 ml of Bergersen solution (Vincent, 1970). After 4 days, seedlings were inoculated by soaking during 30 min within 100 ml of inoculant containing approximately 10^8 cells ml^{-1}. The rhizobial inoculant was prepared from *Rhizobium tropici* CIAT 899

preserved in tubes at 4°C on YEM media (Vincent, 1970). Rhizobia were grown in liquid YEM solution into an erlenmeyer with agitation during 2 days at 28°C, in darkness.

Hydroaeroponic culture of nodulated beans

After inoculation, the seedlings were transferred into hydroaeroponic culture in a temperature-controlled glasshouse as described previously by Vadez et al. (1996). Seedlings were passed carefully through a pierced rubber stopper, fixed with cotton fitted around the hypocotyle, and mounted on the topper of a 0.40 x 0.20 x 0.20 m vat supporting 20 plants equally spaced. They received 20 L of the nutrient solution revised by Vadez et al. (1996), added with distilled water and intensely aerated by compressed air at a flow of 400 ml min^{-1} L^{-1} solution.

Plants were distributed into three treatments: the first one with the above nutrient solution as control, the second one with the same solution supplemented with 25 mM NaCl, and the third one with a deficient phosphorus solution corresponding to 75 µmol KH_2PO_4 $week^{-1}$ $plant^{-1}$ versus 250 µmol KH_2PO_4 $week^{-1}$ $plant^{-1}$ for the control sufficient P treatments. There were three replicates for each line and each treatment.

Relative stress susceptibility index

Plants were harvested at the flowering-early pod filling stage (R7-R8) circa 45 days after sowing (DAS) according to velocity. They were separated into shoot, nodule and root components. They were dried at 70°C for 2 days to constant weight. Each fraction was weighted. The following indices were calculated: relative stress susceptibility index R.S.S.I. = R.B.D./S.I.I. with relative biomass deficit (R.B.D.) and stress intensity index (S.I.I.) where R.B.D. $= \frac{sdw_c - sdw_s}{sdw_c}$; with sdw: mean shoot dry weight of a genotype in control (c) and stressful (s) conditions.

and S.I.I. = $\frac{SDW_c - SDW_s}{SDW_c}$ with SDW : mean shoot dry weight of all genotypes in control (c) and stressful (s) conditions. The cut-off limit at which a line could be regarded as tolerant or susceptible is RSSI= 0.5

DNA isolation

Leaves of each of six RILs named 34, 83, 104, 115, 124 and 147 were harvested separately at 3 weeks after sowing, and ground into a fine powder under liquid nitrogen. Genomic DNA was isolated using a Plant DNAZOL (Invitrogen) procedure according to Ausubel et al. (1990). Polysaccharides were selectively precipitated as described by Michaels et al. (1994). DNA concentrations were determined electrophoretically by comparison with known amounts of phage λ DNA used as standards.

Microsatellite primer characterization

Eighteen primer pairs of microsatellite loci developed by L'taief et al. (2008) were tested by PCR with template DNA from a core set of 6 *P. vulgaris* L. accessions. The PCR reaction was carried out in a 25 µl final volume containing 10 ng of genomic DNA, 2 µM of each of the forward and reverse primers, respectively, 10 x reaction buffer, 1.5 mM $MgCl_2$, 250 µM total dNTP and 1 unit of Taq DNA polymerase. The temperature cycling profile involved an initial denaturation step of 2 min at 96°C. This was followed by 35 cycles at 96°C for 20 s, an annealing phase at 55°C for 23 s, elongation at 72°C for 20 s and a final elongation period at 72°C for 5 min. Between 4 and 12 µl of the reaction mix were electrophoresed on 1.8% agarose gels to check for successful amplification.

RESULTS

Growth

The bean lines expressed different growth potentials in the control treatment with 34, 37, 60, 62, 64 and 75 being significantly more productive than 3, 6, 13, 25, 26, 61 and 66 (Table 1). Regarding the growth under P-deficiency, the susceptibility index (Figures 1A, B, C) showed that the lines most tolerant to P-deficiency were 22, 7, 9, 13, 12, 61, 5, 28, 36, 3, 6, 66 and to a lesser extent 64, 75, 147, 14, 115, 10, 23, 104, 32, 25, 26, while the most susceptible lines were 62, 73, 38, 34, 27, 60, 37, 83. On the other hand, the tolerance to salinity did not follow the same trend as for P-deficiency and shoot dry weight was less affected by P-deficiency as compared to salinity. The most susceptible line to salinity were 62, 60, 1, 83, 29, 124, 147, 13 while lines 25, 6, 11, 36, 66 were the most tolerant. Root growth was less affected by both P-deficiency and salt than the shoot, however, the most affected by P-deficiency were lines 73 and 14 and while lines 14 and 62 showed the highest root dry weight decrease under saline conditions. Regarding shoot to root ratio, only line 32 may be classified as susceptible to salinity with the RSSI higher than 1. Interestingly the RILs 66, 6, 3, 36, 28, 5, 61, 9, 7, 22 and 26 combined tolerance to moderate salinity and P deficiency. By contrast, the lines 60, 62 and 83 were sensitive to both P deficiency and salinity. Tolerance to both stress, did not seem to be related to root to shoot ratio. Indeed, the less affected ratios were associated to the most sensitive lines.

Nodulation

The phosphorus deficiency and salt decreased the nodule dry weight of all lines except line 66 for P-deficiency and line 3 for salt stress (Figures 2A and B). However, RILs 32, 62, 34, 27, 60, 83, 124, 24, 75, 147, 14, 13, 5, 36 and 66 were more affected than RILs 38, 4, 32 and 3, the later showing an increase of NDW due to salt. For the majority of lines, the salt-induced decrease in nodule growth was significantly larger than that of the nodule number (Figure 2). In addition, the nodule growth was more sensitive to P-deficiency than to salinity. Regarding the number of nodules, lines 25, 36 and 66 were the less affected under P-deficient conditions, for the remaining lines, the nodule number was greatly affected. Besides, the less affected lines by salt stress were 73, 38, 4, 64, 7 and 66. Line 25 combined tolerance

Table 1. Variation of the shoot dry weight (g DW.plant^{-1}), root dry weight (g DW.plant^{-1}), nodule dry weight (g DW.plant^{-1}) and nodule number in 37 common bean lines, in control condition. Results are means ± SD at level of 0.05 of 3 replicates per line. Plants harvested at 45 DAS.

Lines	Nodule number	Nodule dry weight	Shoot dry weight	Root dry weight
1	204.50±7.78	0.46±0.05	7.72±3.57	2.04±0.50
3	107.00±72.17	0.20±0.19	2.05±0.61	0.61±0.21
4	143.33±56.86	0.39±0.23	6.11±0.82	1.38±0.13
5	95.00±49.24	0.18±0.06	4.97±1.01	1.13±0.44
6	131.67±135.31	0.28±0.21	4.12±1.52	0.94±0.40
7	98.33±62.52	0.24±0.14	5.75±2.51	1.31±0.09
9	207.00±77.83	0.32±0.10	4.87±2.76	1.42±0.52
10	171.3377.88	0.34±0.25	6.18±1.95	1.51±0.61
11	171.67±40.72	0.36±0.11	6.15±3.76	1.23±0.48
12	358.67±32.02	0.54±0.23	6.03±1.70	1.80±0.50
13	235.00±80.47	0.38±0.07	3.95±2.12	1.00±0.13
14	474.67±393.35	0.71±0.25	5.38±0.67	1.96±0.83
22	189.00±79.83	0.35±0.06	5.20±2.07	1.23±0.17
23	210.00±134.54	0.42±0.23	7.27±4.77	1.84±0.47
24	298.67±44.56	0.67±0.43	6.42±3.57	1.56±0.43
25	122.33±122.13	0.17±0.17	4.45±0.62	1.31±0.27
26	218.33±140.21	0.28±0.14	4.66±2.00	1.49±0.47
27	172.67±90.78	0.31±0.07	6.87±1.03	1.44±0.35
28	162.33±32.19	0.27±0.03	3.64±1.54	0.96±0.24
29	230.00±43.59	0.49±0.36	7.13±4.15	1.47±0.34
32	156.00±29.46	0.30±0.10	5.35±5.21	1.11±0.93
34	329.67±119.50	0.89±0.47	8.22±1.79	1.85±0.41
36	110.67±66.68	0.14±0.09	6.05±0.98	1.35±0.15
37	340.00±140.00	0.81±0.27	8.22±3.62	1.50±0.67
38	210.00±138.92	0.45±0.26	5.42±1.44	1.08±0.25
60	154.00±101.53	0.44±0.43	10.05±3.02	1.78±0.75
61	97.33±89.05	0.23±0.11	3.97±3.67	0.94±0.56
62	218.00±104.75	0.42±0.17	10.14±7.43	2.24±1.33
64	133.00±123.01	0.33±0.27	9.21±3.48	1.76±0.56
66	106.67±66.58	0.17±0.09	3.10±1.37	0.80±0.21
73	121.00±24.76	0.28±0.13	4.18±2.94	1.09±0.56
75	178.67±25.01	0.46±0.23	8.42±1.78	1.65±0.26
83	158.67±48.01	0.51±0.21	7.52±0.94	1.89±0.21
104	333.33±130.51	0.48±0.27	8.28±0.18	1.50±0.47
115	316.67±175.59	0.57±0.37	9.17±0.38	2.15±0.33
124	213.33±130.51	0.49±0.40	8.50±0.36	1.85±0.22
147	250.00±173.49	0.40±0.25	5.44±1.52	1.36±0.22

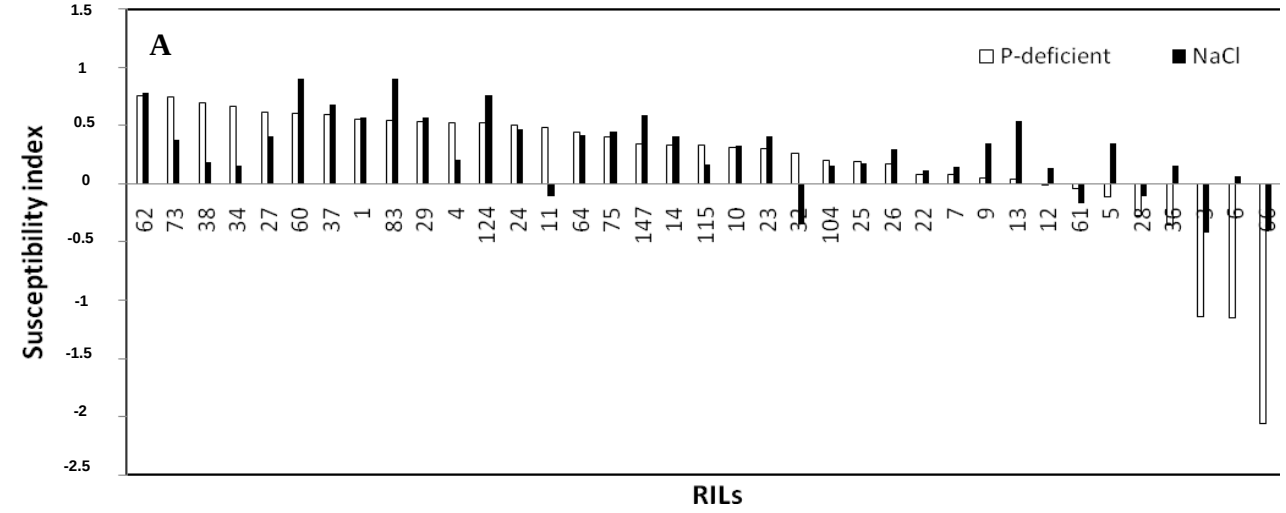
of nodulation to moderate salinity and P deficiency.

Microsatellite polymorphism

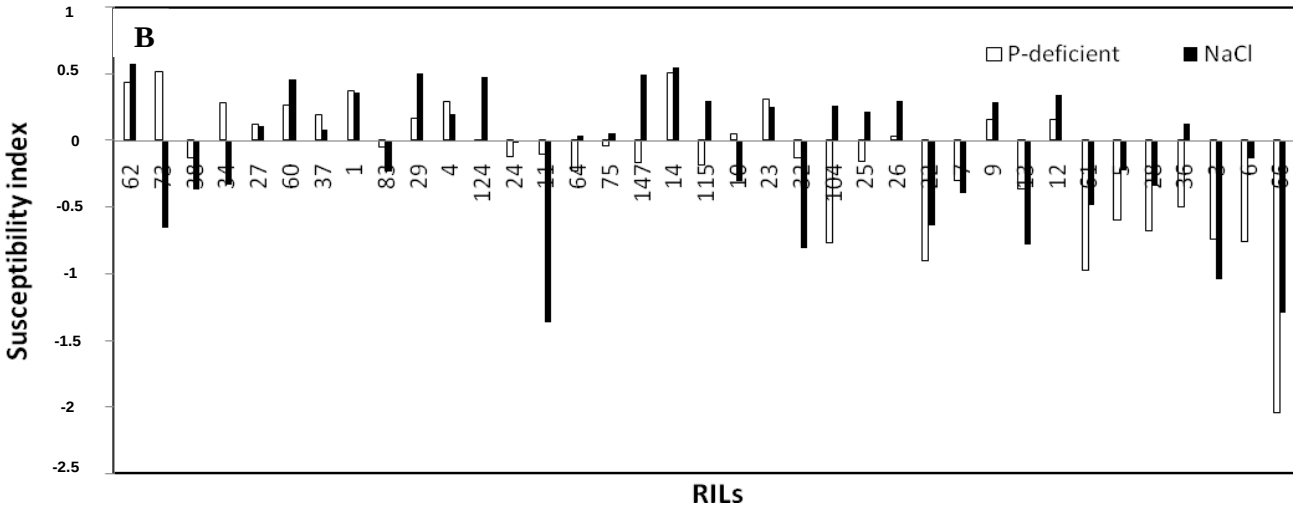
18 primer pairs were tested by PCR with template DNA from a core set of 6 *P. vulgaris* L. RILs contrasting in their tolerance to P deficiency and salinity. Sixteen primer pairs were polymorphic at an intra-specific level (Figure 3). These primer pairs (88.9%) revealed single bands in the expected size range. They were therefore considered promising candidates for *P. vulgaris* L. genotyping. The two remaining primer pairs produced multilocus patterns.

For the 18 candidate loci, the analyses were reproduced with DNA from each of the 6 RILs and PCR products separated on polyacrylamide gels. Again, 16 primer pairs amplified only one allele in each accession, suggesting a high level of homozygosity (Figure 3). The numbers of alleles revealed by these 18 markers ranged from 2 to 4. Loci, which were monomorphic in the test set, and generally carried a relatively small number of repeat units.

(A) Shoot dry weight



(B) Root dry weight



(C) Shoot to root ratio

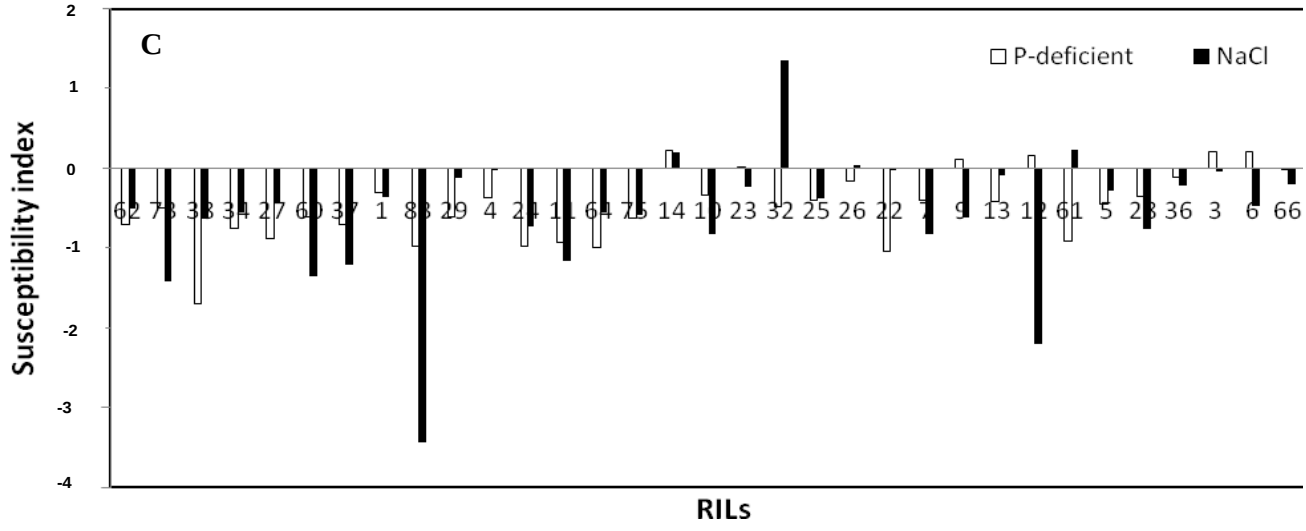
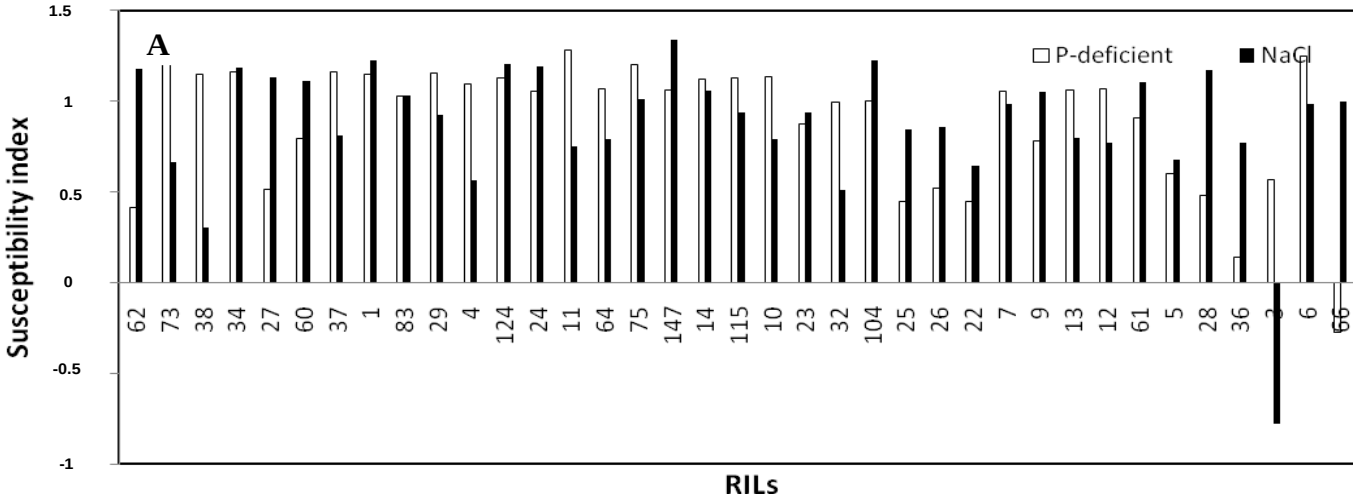


Figure 1. Variation of the relative stress susceptibility index (RSSI) in 37 common bean lines in response to salt, and phosphorus deficiency for (A) shoot dry weight (g DW.plant^{-1}), (B) root dry weight (g DW.plant^{-1}) and (C) shoot to root ratio.

(A) Nodule dry weight



(B) Nodule number

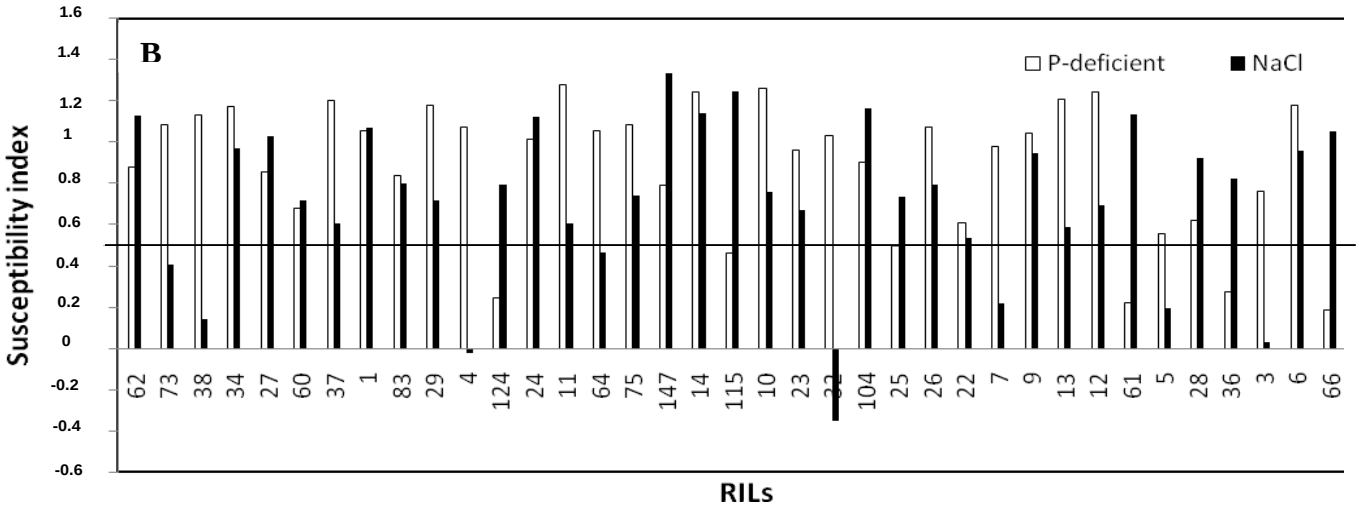


Figure 2. Variation of the relative stress susceptibility index (RSSI) in 37 common bean lines in response to salt, and phosphorus deficiency, for (A) nodule dry weight (g DW.plant^{-1}) and (B) nodule number.

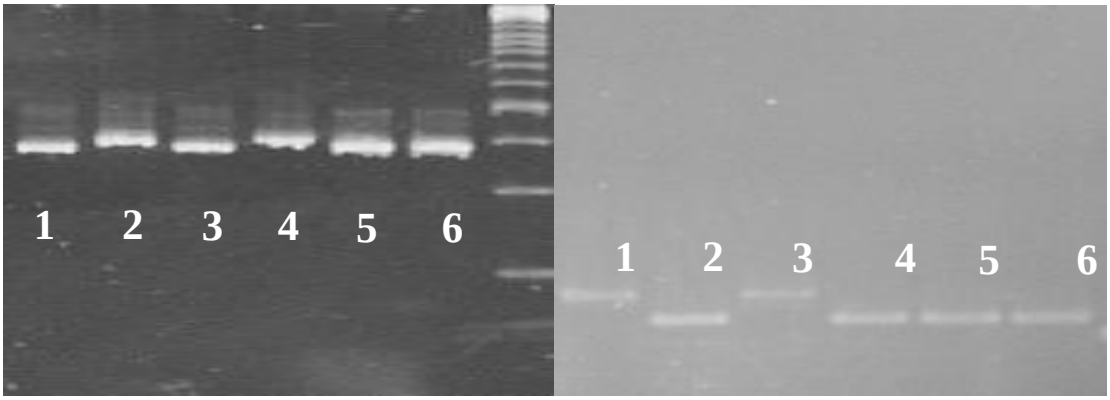


Figure 3. Representative sample of amplification profiles in six common beans RILs (34, 83, 104, 115, 124 and 147) with microsatellite primer pairs.

DISCUSSION

Effect of salinity on plant growth and nodulation

This work shows genotypic variation in tolerance to salinity for nodulation of common bean (*P. vulgaris* L.) recombinant inbred lines, and the subsequent N_2-dependent growth. The salt-induced decrease in nodule number (Figure 1C) agrees with previous studies of Zahran and Sprent (1986) showing salt inhibition of root hair infection that was attributed to Ca^{2+} deficiency. In addition, our results show that the nodule growth was decreased by salt, in agreement with previous reports in soybean (Delgado et al., 1994), pea and faba-bean (Delgado et al., 1994). Although, an increase in individual nodule growth, which would partially compensate for the decrease in nodule number, has been reported by Soussi et al. (1999). Sheng and Harper (1997) concluded that leaves regulate the number of nodules in soybean plants, and several studies showed that the nitrogenase and nitrate reductase activities were positively correlated with the photosynthesis (Vogel and Dawson, 1991). Such favourable conditions for photosynthesis, like an increase in CO_2 concentration, light intensity or leaf area, were associated with increased SNF (Hardy et al., 1977). Salinity can inhibit photosynthesis by decreasing leaf chlorophyll content (Younis et al., 1993), increasing stomatal resistance, inhibiting *in vivo* Rubisco by high concentrations of Cl^- in chloroplasts, and feedback inhibition of carbon metabolism as the result of reduced growth (Seeman and Critchley, 1985) or increase in maintenance respiration (Cramer et al., 1990).

Effect of phosphorus deficiency on plant growth and nodulation

The decrease in bean dry weight and nodulation under P deficiency (Figure 1) agrees with the large intraspecific variability for the response to P deficiency previously observed by Vadez and Drevon (2001). This was associated with a higher RDW/SDW ratio in all lines in agreement with the studies of Araujo and Teixeira (2000) showing that the plants with such higher ratio are more tolerant to P deficiency. Thus, the preferential development of the roots under P deficiency confers a better aptitude to explore the soil and to mobilize the P resources. The higher inhibition of nodule growth than nodule number (Figure 2) shows that the nodule is the most sensitive organ to the P deficiency. This conclusion is substantiated by smaller nodules under P deficiency than under salinity (Figure 2). Similarly in soybean, P deficiency decreased the nodule biomass by 86% and the nodule number by 74% (Ribet and Drevon, 1995) in agreement with other studies on the specific P requirement for nodulation (Marschner, 1995). This suggests that the nodule growth requires more P than the nodule initiation. Thus the later would be controlled by a feedback mechanism through a mediator related to the N status of the legume (Wall, 2000). And the infection would be mostly dependent on the rhizospheric multiplication of the rhizobia around to root-hairs. The majority of the rhizobia have an optimal growth in P concentrations from 0.06 to 0.50 µM although some cannot grow in concentrations lower than 1 µM (O'Hara et al., 1988).

The nodular growth would be modulated by the availability of P in the tissues (Wall, 2000). The higher P requirement for nodule growth may be explained by the requirement for plasmic membrane synthesis, in particular for the mitochondrias and the symbiosome in the infected cells. The competition for photosynthates between the root and the nodules might also be involved since the phosphorus deficiency more severely affected the distribution of biomass between nodules and root than between shoot and root for N_2-dependent soybean, (Cassman et al., 1980). This would be related to decreases in the number of leaves per plant and the unit leaf area as consequences of P deficiency inhibiting the leaf emergence and expansion.

Microsatellite polymorphism

With the 18 microsatellite primer-pairs to amplify common bean genomic DNA, only one single band was generally produced, suggesting that the tested plants are all homozygous at the respective loci. The number of heterozygotes below 10% in our work (Figure 3) was similar to other self-pollinating species such as soybean, where no single heterozygous plant was found among 43 investigated genotypes (Akkaya et al., 1992), and *Arabidopsis thaliana*, where natural populations were characterized by fixed microsatellite alleles in a homozygous state (Innan et al., 1997). Restricted polymorphism and low heterozygosity are expected in self-pollinating species, and heterozygote deficiency at microsatellite loci was also observed in selfing animals (Jarne et al., 1994).

Sixteen STMS primer-pairs proved to be informative at an intra-specific level in *P. vulgaris*, with two markers showing the maximum of four alleles in a homozygous state in six tested accessions (Figure 3). In general, the length of the repeat unit in the cloned plasmid was positively correlated to the number of alleles, has been observed before (Smulders et al., 1997). The large size differences of microsatellite alleles will allow to map the majority of markers on the easy-to-use agarose gels. Because of their co-dominant nature, STMS markers will be easily transferable between populations segregating for different traits. They will therefore complement existing mapping approaches based on RAPDs, microsatellite-primed PCR products, AFLPs and other dominant markers in crosses segregating for e.g. salt resistance (Tohme et al., 1996; Blair et al., 2003).

Conclusion

In conclusion, our work under controlled conditions indicates that the tolerance of growth and nodulation to the osmotic constraint and to the phosphorus deficiency vary among recombinant inbred lines of the cross of DOR364 and BAT477. It provides contrasting RILs that can be used as tools to investigate whether this tolerance may contribute to the adaptation of legume growth to low input cropping systems of the Mediterranean basin where indeed these RILs can grow and fructify (M Trabelsi, pers. com.). These contrasting RILs can also be used to address whether the tolerance contributes to increase the availability of soil P as a result of proton efflux linked with SNF, and its benefit for the subsequent cereal. Also they are used for the research of physiological mechanisms and genes involved in the tolerance of symbiotic nitrogen fixation to salinity and P deficiency. The high levels of polymorphism of these bean microsatellites recommend these new markers for genetic and physical mapping and molecular characterization of *Phaseolus* species.

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