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Genotypic variability for tolerance to salinity of N$_2$-fixing common bean (*Phaseolus vulgaris*)

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Abstract – The common bean (*Phaseolus vulgaris* L.) is often subject to various environmental constraints in the soil. One of these constraints is salinity which is a major limitation for grain legumes yield, especially when the plant growth depends upon N$_2$ fixation. In order to confirm the variability of the response to moderate salinity, and identify the morphological and physiological criteria associated with tolerance, 9 common bean lines (BAT477, Coco blanc, DOR585, ABA16, NAG310, Flamingo, BRB17, Candide and Striker) were inoculated with *Rhizobium tropici* CIAT899, and grown in a glasshouse with or without 25 mM NaCl on sterilized sand. A genotypic variation in tolerance to salt was found: ABA16 and BRB17 were the most sensitive lines whereas BAT477 and Flamingo were the most tolerant. The estimated parameters of growth and symbiotic nitrogen fixation showed that (i) some lines which exhibited vigorous growth under the control treatment were greatly affected by salt treatment, (ii) the relative tolerance of some lines to salt seems to depend on the ability to maintain an adequate leaf area and to develop an abundant and efficient nodular system.

Phaseolus vulgaris / salinity / N$_2$ fixation / genotypic variability

Résumé – Variabilité génotypique de la tolérance au sel chez le haricot (*Phaseolus vulgaris*), en condition de fixation symbiotique de N$_2$. Le haricot (*Phaseolus vulgaris* L.) est souvent soumis dans le sol à diverses contraintes environnementales. L'une des ces contraintes est la salinité qui représente une limitation majeure du rendement des légumineuses à graines, particulièrement quand la croissance des plantes dépend de la fixation symbiotique de l'azote. Pour confirmer la variabilité de la réponse à une salinité modérée et identifier les critères morphologiques et physiologiques associés à la tolérance, 9 lignées de haricot (BAT477, Coco blanc, DOR585, ABA16, NAG310, Flamingo, BRB17, Candide et Striker) sont inoculées avec une souche efficiente *Rhizobium tropici* CIAT899, et cultivées sur sable stérile sous serre vitrée en absence ou en présence de 25 mM NaCl. Une variation génotypique dans la tolérance au sel a été observée : ABA16 et BRB17 sont les lignées les plus sensibles alors que BAT477 et Flamingo sont les plus tolérantes. Les paramètres estimés relatifs à la croissance et à la fixation symbiotique de l'azote montrent que (i) certaines lignées exprimant une croissance vigoureuse en milieu témoin sont sévèrement affectées par le traitement salin, (ii) la relative tolérance au sel d'autres lignées repose particulièrement sur leur aptitude à maintenir une importante surface foliaire et à développer un système nodulaire abondant et efficace.

Phaseolus vulgaris / salinité / fixation symbiotique de N$_2$ / variabilité génotypique

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1. INTRODUCTION

The limitation of symbiotic nitrogen fixation (SNF) by environmental constraints, especially salinity, restricts the development of a sustainable agriculture, and the extension of this legume cultivation, particularly in Mediterranean areas. Salinity affects the initiation, development and function of nodules, as well as the photosynthetic capacity of leaves, though SNF was found to be more affected by salt than plant growth [6, 10, 25, 40]. Generally, nodular activity is less affected by salt than nodulation [2, 13, 30]. Thus, the infection process seems to be the most sensitive to salt [33, 41]. Nevertheless, a large genetic variability in salt tolerance was found among legume species and lines [6, 18].

The enhancement of legume productivity in salty zones requires the development of salt-tolerant symbiosis. This approach implies the genetic improvement of the two partners of symbiosis. However, it is generally admitted that the *Rhizobium* is relatively more tolerant to salinity than their macrosymbiont [30]. The latter seems to constitute the most determinant factor for the symbiosis efficiency [2, 19].

Exploration of the variability in salt response would permit us not only to identify some tolerant species and lines, but also to determine useful criteria for genetic improvement of salt tolerance. Several works carried out on interspecific variability showed that the tree legumes, such as *Prosopis* and *Acacia* spp., are highly tolerant to salinity [20]. Grain legumes have generally been considered either sensitive or moderately tolerant to salinity [17]. Common bean, chickpea, and pea were the most sensitive legumes [3, 32], whereas soybean was the most tolerant one [3, 6].

Studies carried out on intraspecific variability in salt responses are few especially within bean species. Among 19 lines of common bean cultivated for 13 days on a nutrient solution supplemented with 0, 40 and 80 mM NaCl, variability was observed for height, number and dry matter of leaves, stems and roots [38]. Variability in sensitivity to salt stress was also found among beans grown in Tunisia, a local line showing a sensitivity index (SI) of –21% versus –46% for Gabriella [31]. However, in both studies, the performance of lines under salt stress which was established in early stages of development was not maintained in later stages. The aim of the present work was to explore the genotypic variability in salt tolerance in 9 common bean lines and to investigate the effects of NaCl on the behavior of common bean lines: growth of the whole plant, leaf area, nodulation and nitrogen accumulation in different tissues.

2. MATERIALS AND METHODS

2.1. Culture conditions

The common bean lines used in this study were Coco blanc that is extensively cultivated in the Mediterranean basin, Candide (Klauss, France), Striker (supplied by C. Lluch, University of Granada, Spain), ABA16, BAT477, DOR585, NAG310, Flamingo and BRB17 (supplied by M. Trabelsi, ESA Mateur, Tunisia, from a collection initially supplied by B. Voyssest from CIAT, Colombia). Experiments were carried out in a glasshouse in 1 L pots filled with sterilized sand. Bean seeds were surface-sterilized in 3% (w/v) calcium hypochlorite for 15 min, moistened with sterilized water, and inoculated with 1 ml of liquid inoculant containing approximately 10^9 bacteria of *Rhizobium tropici* CIAT899. The seedlings were irrigated with the following N-free nutrient solution: KH_2PO_4 (0.36 mM), $CaCl_2$ (1.65 mM), $MgSO_4$ (1 mM), K_2SO_4 (0.7 mM), H_3BO_3 (4 µM), $MnSO_4$ (4 µM), $ZnSO_4$ (1 µM), $CuSO_4$ (1 µM), $CoCl_2$ (0.12 µM), $NaMoO_4$ (0.12 µM), FeEDTA (40 µM). In addition, plants received 2 mM urea as starter-N supply during the first 2 weeks, i.e. before nodule emergence.

When the first trifoliate leaf appeared, about 21 days after sowing (DAS), plants were distributed into two plots: the first one was irrigated with the above nutrient solution (control) and the second was watered with the same solution supplemented with 25 mM NaCl. There were ten replicates for each line and each treatment. Plants were irrigated every two days with 100 ml per pot leading to a light out-flow. The day/night temperatures and relative humidities were 25/20 ± 5 °C, 65/85 ± 5%, respectively.

2.2. Measured parameters

Two harvests were made: (i) at the beginning of treatment (21 days after sowing, DAS) and (ii) at the flowering stage, 45 DAS. Plants were separated into leaves, stems, roots and nodules. Leaf area was measured using an Area Meter (LI-COR model LI-3000A).

After desiccation at 65 °C during 72 h, the dry weight of organs as well as the total number of nodules were determined. The tissue content in total nitrogen was determined by the Kjeldahl procedure. The symbiotic nitrogen fixation (SNF) was estimated as the difference between N quantities (mmol·plant^{-1}) at 45 and 21 DAS.

2.3. Parameters of result analysis

The mean relative growth rate, i.e. the rate of increase in total dry weight per unit of plant dry weight, was calculated according to the following expression [15]:

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1), \text{ RGR in mg·mg}^{-1}\cdot\text{d}^{-1});$$

with W, total plant weight (mg), and t, the time (days); the subscripts 1 and 2, initial and final harvest.

The net assimilation rate, i.e. the rate of dry matter production per unit of leaf area, calculated according to the following expression:

$$NAR = (W_2 - W_1) / (t_2 - t_1) \times [(LA_2 - LA_1) / (\text{Ln } LA_2 - \text{Ln } LA_1)] \text{ in mg DW·cm}^{-2}\cdot\text{d}^{-1};$$

with LA, the leaf area (cm^2); subscripts 1 and 2 denote respectively initial and final harvest.

The sensitivity index, i.e. the difference between dry matter production of plants raised in solution containing 25 mM NaCl and the control one expressed in percent of this latter, was calculated according the following expression [31]:

$$SI_{NaCl} = [100 \times (W_{NaCl} - W_{control})] / W_{control}.$$

2.4. Statistical analysis

All values are means of 10 replicates per treatment. After a two way ANOVA analysis, means were compared at the 0.05 probability level using a Tukey HSD test.

3. RESULTS

3.1. Growth and leaf area

The bean lines expressed different growth potentials on control treatment (Fig. 1): Candide, Flamingo, ABA16 and Coco blanc were significantly more productive compared to BRB17. Except for BAT477, salt significantly decreased ($p < 0.05$) the growth of all other lines. In order to rank the lines along a scale of sensitivity to salt, we calculated the sensitivity index (SI). This parameter was more negative when the line was sensitive to NaCl. According to SI values, the lines ABA16 and BRB17 were sensitive to salt treatment whereas BAT477 and Flamingo were relatively tolerant.

The accumulation of dry matter during a treatment depends upon the initial size of plants, the treatment duration and the rate of growth during treatment. Relative growth rate (RGR) eliminates differences in
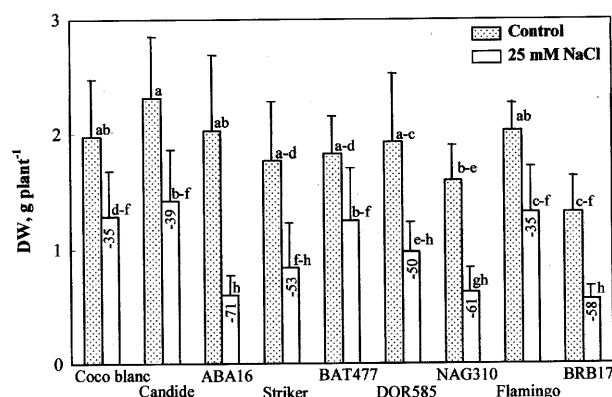

Figure 1. Effect of salt on whole plant growth (g DW·plant^{-1}) for 9 lines of common bean. Numbers in histograms correspond to sensitivity index (SI) to salt. Values are means of 10 replicates ± standard deviation. Means with the same letter are not significantly different ($p < 0.05$).

biomass production related to treatment duration and/or initial plant size (at the beginning of salt treatment). For such reasons, RGR gives a relative basis for comparison of the effect of salt on plant growth among species and genotypes [15]. Salt significantly reduced RGR ($p < 0.05$) in all lines, except for Flamingo and BAT477 (Tab. I). The sensitivity index established on the basis of relative growth rate (not shown) was perceptibly identical to the one determined on the basis of the final dry matter. Therefore, the variability of the response to salt depended more on the growth activity during the salt treatment than on the initial vigor of plants.

The RGR is a function of the net assimilation rate (NAR) and the leaf area [4]. In the present study, salt significantly reduced ($p < 0.05$) leaf area for most lines of common bean (Tab. I). Flamingo and BAT477 were not affected, whereas ABA16 was the most sensitive to salt stress. The calculated sensitivity index on the basis of the leaf area (not shown) was related to those established previously. In order to test whether the limitation of the growth resulted essentially from a reduction of photosynthetic area, we calculated the net assimilation ratio (NAR). The data in Table I show that except for Coco blanc, NAR values were significantly affected ($p < 0.05$) by salt. However, genotypic differences were less than with previous ratios. Our results suggest that the limitation of plant growth (estimated by dry matter weight at the end of treatment or RGR) was essentially attributed to the decrease in photosynthetic area. In

Table I. Changes in the mean relative growth rate (RGR in mg·mg^{-1}·d^{-1}), the net assimilation rate (NAR in mg·cm^{-2}·d^{-1}) and leaf area (LA in cm^2·plant^{-1}) with medium salinity. Values are means of 10 replicates ± standard deviation in parenthesis. For each parameter, values not followed by the same letter differed significantly at the 0.05 probability level.

Lines	Treatments	RGR	NAR	LA
Coco blanc	Control	0.08 (0.01) a-c	0.63 (0.08) de	332 (66) a
	25 mM NaCl	0.05 (0.01) d-f	0.56 (0.08) e-g	191 (52) c-f
Candide	Control	0.07 (0.01) b-d	0.74 (0.07) bc	281 (47) b
	25 mM NaCl	0.05 (0.01) ef	0.58 (0.08) ef	179 (39) d-f
ABA16	Control	0.07 (0.02) b-d	0.65 (0.08) c-e	309 (21) ab
	25 mM NaCl	0.02 (0.01) g	0.41 (0.09) h	63 (8) h
Striker	Control	0.08 (0.01) ab	0.74 (0.11) bc	211 (56) c-e
	25 mM NaCl	0.05 (0.02) d-f	0.48 (0.1) f-h	116 (28) g
BAT477	Control	0.07 (0.01) b-d	0.63 (0.07) de	235 (52) c
	25 mM NaCl	0.05 (0.02) d-f	0.46 (0.07) gh	204 (35) c-e
DOR585	Control	0.08 (0.02) a-c	0.78 (0.08) ab	237 (45) c
	25 mM NaCl	0.05 (0.01) d-f	0.47 (0.07) f-h	165 (44) ef
NAG310	Control	0.09 (0.01) a	0.82 (0.13) ab	193 (16) c-f
	25 mM NaCl	0.05 (0.02) ef	0.47 (0.10) f-h	81 (18) h
Flamingo	Control	0.07 (0.01) b-d	0.71 (0.05) b-d	241 (27) c
	25 mM NaCl	0.05 (0.02) d-f	0.56 (0.09) e-g	230 (43) cd
BRB17	Control	0.08 (0.01) ab	0.87 (0.09) a	146 (37) fg
	25 mM NaCl	0.04 (0.01) f	0.49 (0.06) f-h	73 (14) h

addition, salt lowered photosynthetic performance for the remaining leaf area.

3.2. Nodular development and nitrogen status

Salt significantly decreased ($p < 0.05$) the nodule dry matter of all bean lines (Fig. 2), though ABA16, BRB17 and Striker were more affected than BAT477, DOR585 and Flamingo. BRB17 maintained the lowest nodular growth with or without salt. For the majority of lines, the reduction of nodule dry matter was significantly larger than that of the nodule number (Fig. 3). Thus, salt inhibited not only the nodulation, but also the nodule growth.

Salt did not significantly modify the N content in shoots and roots whatever the bean lines (Tab. II). By contrast, it generally significantly decreased ($p < 0.05$) nitrogen accumulation in nodules, except for Flamingo and ABA16. The reduction of plant growth by salinity, that was associated with the constant N content, particularly in the shoots that represent the major part of the plant, suggested that the dry matter production may be determined by the lines' capacity to fix N_2.

SNF was significantly ($p < 0.05$) decreased by salinity, except for Flamingo (Fig. 4). BAT477 was also less affected, confirming the higher salt tolerance of these two lines compared to other genotypes, particularly ABA16 and BRB17. The salt-induced decreases in SNF
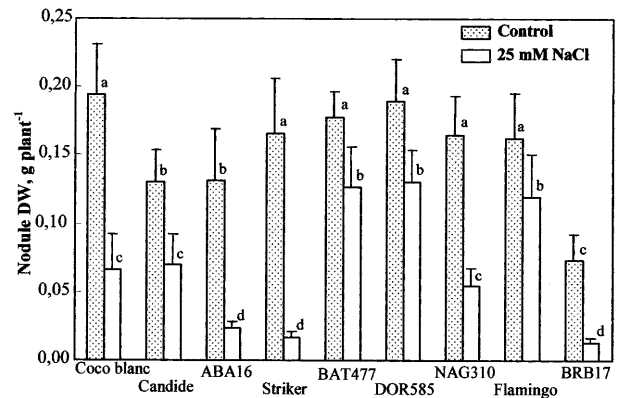

Figure 2. Effect of salt on the nodule growth (g DW·plant^{-1}) for 9 lines of common bean. Other details as in Figure 1.

were higher than those in growth for all lines, suggesting that the SNF was more sensitive to salt than the host-plant growth.

Figure 5 shows that Candide, Coco blanc, NAG310, BRB17, Striker and ABA16 were more affected by salt for nodule growth and for SNF. The data for these lines were very close to the regression line, indicating that the inhibition of SNF by salt constraint was particularly linked to the decrease in the nodular growth. By contrast,
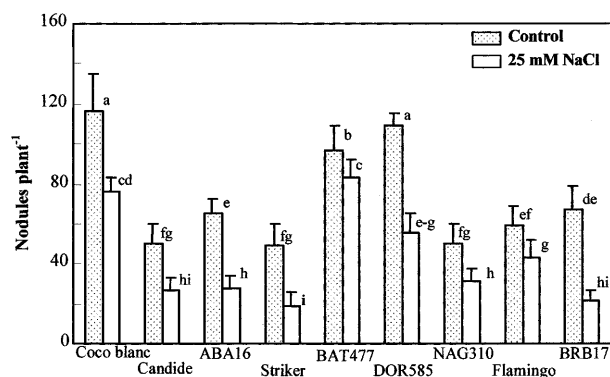
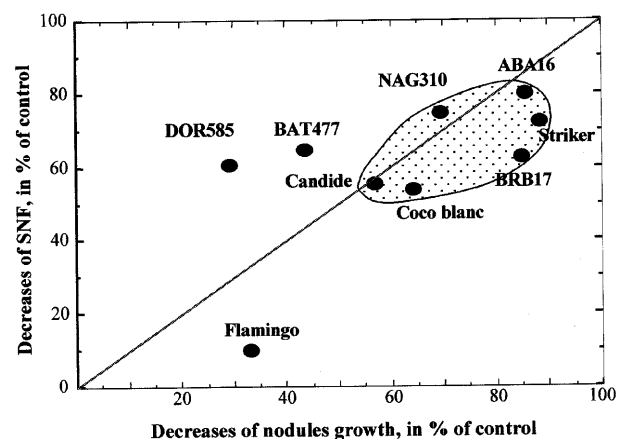**Figure 3.** Effect of salt on the nodule number (nodules·plant^{-1}). Other details as in Figure 1.

Figure 5. Relationship between decreases of SNF and the inhibition of the nodular growth in plants cultivated on 25 mM NaCl. The two parameters were expressed in percent of those of plants cultivated under control solution. The points situated on the diagonal correspond to lines expressing similar inhibition of SNF and the nodule growth.

Figure 4. Changes in symbiotic nitrogen fixation SNF (mmol·plant^{-1}) with medium salinity. Numbers in histograms correspond to sensitivity index established on the basis of the SNF. $SI_{NaCl} = [100 \times (SNF_{NaCl} - SNF_{control})]/SNF_{control}$; SNF, symbiotic nitrogen fixation estimated as difference between N quantities (mmol·plant^{-1}) at 45 and 21 DAS. Other details as in Figure 1.

SNF activity of the beans were affected at low levels of salt (25 mM NaCl). Nevertheless, a genotypic variability in response to salt has been found at the vegetative development stage. The lines BAT477 and Flamingo were less affected by salt treatment but BRB17 and ABA16 were particularly sensitive and suffered a severe leaf chlorosis from the first days of treatment, followed by a necrosis and leaf drying.

The same value of sensitivity index on the basis of plant growth (estimated by plant dry weight at the end of treatment or by RGR), or leaf expansion or also the N$_2$-fixing potentialities, show an interdependence of the three parameters. Indeed, several studies showed that activities of the nitrogenase and nitrate reductase were positively correlated with the photosynthesis [21, 35]. Thus, such favorable conditions for photosynthesis, like an increase in CO$_2$ concentration, light intensity or leaf area, increased SNF [7, 14, 36].

Our results showed that the net assimilation rate was involved in the reduction of plant growth by salt. NAR represents the combined physiological processes of photosynthesis and respiration. Salinity can inhibit photosynthesis in several ways: reduction of leaf chlorophyll content [39], increase in stomatal resistance, inhibition in vivo of the Rubisco by high concentrations of Cl$^-$ in chloroplasts, feedback inhibition of carbon metabolism as the result of reduced growth [23, 24] or a combination of these factors [4]. However, not only a decrease in photosynthesis, but also an increase in maintenance respiration could effectively lower NAR [4]. Indeed, it has

the decrease in SNF for BAT477 and DOR585 was also due to a decrease in nodule N$_2$-fixing activity. Flamingo expressed the lowest decreases in nodule growth and SNF.

4. DISCUSSION AND CONCLUSION

In this study, nine common bean (*Phaseolus vulgaris* L.) lines showed sensitivity to salt stress, particularly for the symbiotic nitrogen fixation process. The growth and
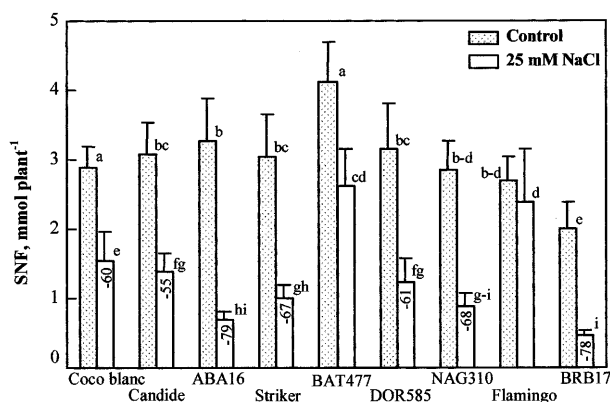
Table II. Nitrogen content of leaves, stems, roots and nodules of bean lines ($\mu mol \cdot g^{-1}$ DW). Values are means of ten replicates ± standard deviation in parenthesis. Values within a column not followed by the same letter differed significantly at the 0.05 probability level.

Lines	Treatments	Leaves	Stems	Roots	Nodules
Coco blanc	Control	2299 (210) ab	1264 (277) ab	1371 (122) b	5042 (734) a
	25 mM NaCl	2312 (241) b-f	1142 (285) a-d	1347 (102) b	3533 (321) fg
Candide	Control	1788 (468) d-h	1065 (259) a-e	1780 (263) a	4145 (485) c-e
	25 mM NaCl	2043 (238) b-f	939 (114) c-e	1348(159) b	3304 (362) g
ABA16	Control	2268 (133) ab	981 (83) b-e	1389 (177) b	4350 (411) b-d
	25 mM NaCl	2213 (180) a-c	1169 (252) a-d	1630 (199) a	3682 (398) e-g
Striker	Control	2335 (319) ab	1199 (177) a-c	1345 (143) b	4679 (620) a-c
	25 mM NaCl	2065 (299) b-e	1172 (261) a-d	1605 (199) a	3308 (500) g
BAT477	Control	2025 (278) b-f	1067 (98) a-e	1393 (127) b	4829 (157) ab
	25 mM NaCl	1834 (60) d-h	1070 (153) a-e	1364 (79) b	3714 (286) e-g
DOR585	Control	1654 (184) gh	1068 (316) a-e	1281 (80) bc	5152 (394) a
	25 mM NaCl	1760 (262) h	850 (164) e	1258 (155) bc	4154 (461) c-e
NAG310	Control	2439 (371) a	1044 (204) b-e	1002 (216) d	5197 (304) a
	25 mM NaCl	2132 (210) a-d	870 (86) de	987 (193) d	3939 (407) d-f
Flamingo	Control	1961 (192) b-g	1162 (166) a-e	1260 (105) bc	4948 (672) a
	25 mM NaCl	1734 (237) e-h	1362 (319) a	1254 (150) bc	4332 (366) b-d
BRB17	Control	1690 (392) f-h	778 (129) e	1106 (131) cd	4418 (346) b-d
	25 mM NaCl	1882 (421) c-g	838 (135) e	1278 (229) bc	3669 (501) e-g

been reported that 80% of the reduction in carbon assimilation in *Xanthium* sp. could be accounted for by a reduction in photosynthesis and 20–25% was the result of increased maintenance respiration [22].

The results presented in this study show that salt reduces RGR in most bean lines. This effect was more related to a reduction of leaf expansion than to a decrease of the intrinsic assimilation capacity (NAR). Indeed, RGR and leaf area discriminate more efficiently lines of bean. In studies with a halophyte [29] and a non-halophyte [5], it was observed that LAR (leaf area ratio), but not NAR was affected by salinity. These results indicate that the leaf is limiting light interception and that the photosynthetic-assimilatory machinery is not limiting RGR. Inversely, in salt-stressed barley, it was found that NAR of salt-stressed plants was highly correlated with RGR but not with LAR [4] suggesting that the restrictions imposed by NaCl on the assimilatory machinery were more important than the reduction of leaf area as causes of growth inhibition. Reduction of growth of some *Medicago* subjected to salt was related to a decrease in leaf area rather than in assimilation efficiency [1].

The reduction of nodule numbers by salt in the final harvest compared to control plants suggested that salinity inhibited the initiation and the development of the second generation of nodulation (nodules produced in later stages) since salt stress was applied after nodulation, at the stage of the first trifoliate leaf, 21 DAS. According to

some studies, salt inhibition of root hair infection was due to mineral deficiency. Thus, it has been reported that Ca^{2+} increases root growth, root-hair emergence and the subsequent number of infection sites [41]. Our results are in agreement with previous studies. In soybean, the development of new nodules and nitrogen fixation by the existing nodules were greatly inhibited by salinity, with a resulting decline in plant nitrogen content [37]. In *Vicia faba*, the salinity decreased nitrogen fixation by affecting both the appearance of new nodule generations on the roots and the efficiency of the association [40]. More recent data show that leaves autoregulate the number of nodules in soybean plants [11, 28].

In addition, our results show that the nodule growth was also affected by salt. Similar behavior has also been reported in soybean [6, 12, 34], pea, faba-bean and common bean [6]. It has been proposed that inhibition of the photosynthesis in plants subjected to salt lead to a restriction of photosynthate transport towards nodules and reduction of the size of these organs. However, a stimulation of the nodule growth has been reported in other studies [32, 40]. This behavior would ensure a partial compensation for the reduction in the number and the efficiency of nodules.

For most studied lines, the similar level of reductions in the amount of fixed N and in the nodule growth suggests that SNF was limited mainly by the nodular development. However, for lines DOR585 and BAT477, the lower decrease in nodulation than in SNF show that salt

inhibited nodule efficiency in these lines. Indeed, it was shown previously that the NaCl supply inhibited nitrogenase activity and respiration of nodules, by decreasing the nodule O$_2$ supply [26] through decrease in the nodular conductance to the O$_2$ [9]. Indeed, an increase of the oxygen pressure in the rhizospheric environment of a bean nodulated root permitted the suppression of the inhibitory effect of salt on the acetylene-reducing activity of the nitrogenase [27]. Other studies show that the supply of nodules in photosynthates is a determining factor of the nodular activity in plants subjected to salt [2, 16]. It was concluded that adequate carbon supply could be important: (i) to maintain a resistance to entrance of toxic ions and oxygen, (ii) to maintain nitrogenase-linked respiration for N$_2$ fixation and (iii) to provide osmotica as protection against plasmolysis.

In conclusion, this work showed the existence of a variability among common bean lines in the response to salt stress: ABA16 and BRB17 were the most salt-sensitive lines whereas BAT477 and Flamingo were relatively tolerant. Several parameters such as RGR, leaf area, mass of nodules and fixed N$_2$ significantly discriminate common bean lines submitted to salt. The relative tolerance of some lines seems to depend on their ability to maintain an adequate leaf area insuring an important carbon supply permitting the development of an abundant and efficient nodular system, which in turn determines an important rate of nitrogen fixation and permits the plants to conserve their growth potentialities. The same behaviour has been observed in *Glycine max-Bradyrhizobium japonicum* symbiosis [8]. More work is needed to search at the nodules level, the physiological mechanisms involved in the lines differences in their sensitivity, and to explore a larger number of lines for more tolerant parents. At the application level, ours results suggest that the crosses between Coco blanc, extensively cultivated in the Mediterranean basin, and BAT477 or Flamingo might permit to select a descendant which would be more salt tolerant and adapted to Mediterranean market.

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