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Influence of host genotypes on growth, symbiotic performance and nitrogen assimilation in faba bean (*Vicia faba* L.) under salt stress

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Abstract

Fifteen genotypes of faba bean (*Vicia faba* L.) were inoculated with salt-tolerant *Rhizobium leguminosarum* biovar. *viciae* strain GRA19 in solution culture with 0 (control) and 75 m*M* NaCl added immediately after transplanting. Genotypes varied in their tolerance of high levels of NaCl. Physiological parameters (dry weight of shoot and root, number and dry weight of nodules) were not affected by salinity in lines VF46, VF64 and VF112. Faba bean line VF60 was sensitive to salt stress. Host tolerance appeared to be a major requisite for nodulation and N_2 fixation under salt stress. Tolerant line VF112 sustained nitrogen fixation under saline conditions. Activity of the ammonium assimilation enzymes glutamine synthetase and glutamate synthase, and soluble protein content, were reduced by salinity in all genotypes tested. Evidence presented here suggests a need to select faba bean genotypes that are tolerant to salt stress.

Abbreviations: ARA – acetylene reduction activity, NADH-GOGAT – NADH-dependent glutamate synthase, GS – glutamine synthetase.

Introduction

Infertility of soils in arid zones is often due to the presence of large quantities of salt, a problem that can be circumvented by introducing plants capable of surviving under these conditions. In addition, the use of saline water to irrigate crops in arid and semi-arid regions requires a better understanding of plant salt tolerance (Materon, 1988). However, plant types and species behave differently under stress conditions: plant response varies depending on the degree of salt stress, stage of growth, and availability, type and form of the nutrient elements in the rhizosphere.

Because legumes offer the greatest immediate prospects for increased agricultural productivity (Gibson, 1976), their resistance to salinity has been assayed and described in several reports (Abdel-Wahab and Zahran, 1981; Elsheikh and Wood, 1990; Rumbaugh et al., 1993). The mechanisms by which salinity affects plant metabolism, thereby reducing growth and development (Abdul-Kadir and Paulsen, 1982; Pessarakli et al., 1989), are still not completely understood. The interpretations that different investigators have presented in an attempt to elucidate this problem are controversial. Changes in nitrogen and nodule metabolism are commonly accepted as the most important factors contributing to the decreases in biomass when plants are grown under salt stress (Delgado et al., 1993; Rumbaugh et al., 1993; Waisel, 1989).

With problems of soil salinity on the increase worldwide, the identification of nitrogen-fixing legumes adapted to marginal saline soils deserves high priority. Using salt-sensitive soybean cultivars, Tu (1981) and Singleton and Bohlool (1984) have observed that the establishment of symbiosis was highly sensitive to salt stress. However, fully developed nodules that had formed under stress-free conditions continued to fix nitrogen when subjected to salt stress. In *Vicia faba*, salinity of the culture medium led to a drop in nitrogen fixation by affecting both the appearance of new nodule generations on the roots and the efficiency of association (Yousef and Sprent, 1983).

The need for more research on faba bean host- *Rhizobium* strain combinations capable of forming root nodules and fixing nitrogen symbiotically under salt stress is clear. In addition, the use of activity levels of several enzymes as tracers of plant salt-tolerance merits serious investigation, although other traits might also be searched for (Waisel, 1989).

It is well known that cultivars of the same species vary in their salt tolerance, and physiological testing may be helpful in assessing such variations. The aim of this study was to determine the effect of salt stress on growth, nitrogen fixation and ammonium metabolism in root nodules of fifteen genotypes of faba bean.

Material and methods

Plant material and growth conditions

The genotypes of *V. faba* L. used in this study are fourteen inbred lines selected from natural parents of different geographical origin, growth habitats, seed size and earliness (Cabrera and Martín, 1986), and were compared to Alborea (Semillas Pacífico SA, Sevilla, Spain), a commercial cv. of faba bean well adapted to the Mediterranean area. Seeds were surface-sterilized with ethanol and germinated at 26°C in the dark on sterile vermiculite. Seedlings, selected for uniformity, were planted separately in modified Leonard jar assemblies (two per jar) containing vermiculite and a nutrient solution (Rigaud and Puppo, 1975) that contained 2 m*M* KNO_3 to stimulate plant growth without inhibiting nodule growth and activity (Caba et al., 1990). Each seedling was inoculated with the *Rhizobium leguminosarum* biovar. *viciae* (ca. 10^9 cells mL^{-1} strain GRA19 (Hervas et al., 1991). This strain has been characterized previously (Cordovilla, 1993) as salt-tolerant. The seedlings were then covered with moist vermiculite and a layer of dry, sterile perlite. The jars were arranged randomly in a growth chamber with a 16-8 h light-dark cycle, 23-16°C day-night temperature, relative humidity 55-75% and photosynthetic photon flux density (400-700 nm) of 450 μmol m^{-2} s^{-1}, supplied by Sylvania Cool-White Lifeline fluorescent lamps (F96T12-CW-VHO, Sylvania Ltd, Quebec, Canada) and incandescent lamps (30% fluorescent wattage).

Salt treatments

Two concentrations of salt (0 and 75 m*M*) in the form of NaCl were added to the growth medium immediately after transplanting. These concentrations were maintained until harvesting.

Harvest

The various genotypes, which all flowered at different times, were harvested at the beginning of flowering period. We compared all plants at the same physiological growth stage, when nodule functioning was highest, independent of days in culture. Six replicates were included per harvest. The plants were uprooted and their root systems thoroughly rinsed with distilled water. Nodules of similar size and appearance were carefully detached, rinsed with distilled water, dried with filter paper, weighed and kept on ice. Shoot, root and nodule dry weights were recorded after 24 h at 70°C. Four plants per treatment were used to determine nodule dry weight.

Nitrogen fixation assay

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction in the entire root systems of six plants as well as on small nodulated root portions of the remaining plants as recommended by Herdina and Silsbury (1990). Nodulated root portions (1 g root plus nodules) and root systems were placed in vials and sealed with serum caps. Ten percent of the internal atmosphere was replaced with acetylene, and 0.2 mL aliquots were taken after 5, 10 and 15 min incubation and analyzed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a flame ionization detector and a 3 mm (internal diameter) × 2 m (long), Poropak-R-type column (80-100 mesh) according to Ligero et al. (1991).

Preparation of cell-free extracts

Nodule homogenates for determining glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT) and protein were prepared according to a slightly modified version of the technique of Groat and Vance (1981). Sample (1 g fresh weight) were homogenized on ice with acid-washed quartz sand and 12 mL (ice-cold) of an extraction medium containing 100 m*M* maleic acid-KOH, pH 6.8, 100 m*M* sucrose, 2% (v/v) 2-mercaptoethanol and 15% (v/v) ethylene glycol, plus 0.5 g polyvinyl polypyrrolidone. The homogenate was filtered through 4 layers of cheesecloth and the nodule debris removed the filtrate was centrifuged at 3500

Table 1. Dry matter accumulation (g plant^{-1}) in shoots and roots of different lines of *Vicia faba* inoculated with *Rhizobium leguminosarum* GRA19 and treated with 0 and 75m*M* NaCl. For each line, the number in brackets refers to flowering date, in days after sowing

Genotype	NaCl (m*M*)			
	0	75	0	75
	Shoot dry weight		Root dry weight	
VF4(36)	3.78	1.85	1.34	0.75
VF6(49)	4.79	2.53	1.90	0.88
VFI5(31)	2.46	1.66	0.77	0.52
VFI6(55)	3.73	1.05	1.37	0.53
VFI7(56)	4.50	1.74	1.51	0.78
VF27(42)	2.08	1.15	0.55	0.50
VF38(42)	3.70	1.09	1.09	0.46
VF46(58)	2.99	2.82	1.52	1.18
VF47(55)	6.60	4.19	2.25	1.33
VF60(57)	9.13	1.75	3.94	0.73
VF61(54)	4.58	1.83	1.87	0.67
VF64(32)	2.19	1.60	0.91	0.56
VF83(47)	4.43	2.06	2.05	1.19
VF112(27)	1.43	1.04	0.59	0.43
Alborea (40)	3.29	1.01	1.30	0.59
LSD (p=0.05)	1.32		0.69	
F statistics				
Plant genotype (A)	19.15**		30.03**	
NaCl concentration (B)	234.47**		235.09**	
Interaction (A,B)	8.74**		15.60**	

**$p \leq 0.01$.

g at 2°C for 8 min. The resulting supernatant was centrifuged once more at 30,000 *g* for 20 min, which produced a clear solution of host cell cytoplasm and its organelles; this was used to measure enzyme activity.

Enzyme assays

Glutamine synthetase (EC 6.3.1.2) was determined by the hydroxamate synthetase assay, adapted from Farnden and Robertson (1980) and Kaiser and Lewis (1984). Assays were optimized for the amount of enzyme to give a linear reaction at least within 30 min. Two blanks without enzyme and without L-glutamate were also analyzed.

NADH-glutamate synthase (EC 1.4.1.14) activity was determined spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986), always within 2 h of extraction. The volume of the extract was adjusted for each enzyme. Two controls (without a-ketoglutarate and without glutamine) were used to correct for endogenous NADH oxidation. The decrease in absorbance (linear at least 12 min) was recorded for 10 min with a Beckman DU-70 spectrophotometer.

Protein determination

The soluble proteins in tissue extracts were determined by Bradford's method (1976) with bovine serum albumin (Merk, fraction V) as the standard.

Statistical design and analysis

The experimental layout was a randomized block design. All values are means of 6 replicates per treatment. All results were subjected to a two-way analysis of variance with a least significant difference (LSD)

Table 2. Total nodule number plant^{-1} and nodule dry weight (g plant $^{-1}$) of different lines of *Vicia faba* inoculated with *Rhizobium leguminosarum* GRA19 and treated with 0 and 75 m*M* NaCl

Genotype	NaCl (m*M*)			
	0	75	0	75
	Total nodule number		Total nodule weight	
VF4	283	316	0.30	0.22
VF6	278	249	0.38	0.21
VF15	174	152	0.14	0.09
VF16	723	181	0.51	0.13
VF17	389	388	0.53	0.24
VF27	232	131	0.20	0.09
VF38	147	146	0.23	0.12
VF46	356	309	0.26	0.27
VF47	217	187	0.37	0.26
VF60	393	143	0.93	0.26
VF61	405	395	0.39	0.20
VF64	199	226	0.17	0.13
VF83	496	436	0.43	0.24
VF112	232	227	0.16	0.14
Alborea	250	141	0.22	0.09
LSD (p=0.05) 52	52		0.08	
F statistics				
Plant genotype (A)	45.66**		48.26**	
NaCl concentration (B)	100.44**		317.50**	
Interaction (A,B)	22.66**		22.68**	

**$p \leq 0.01$.

test between means. Sources of variance (NaCl concentration or plant genotype) have been compared using Duncan's test.

Results

When plants grew without saline treatment, marked variability was observed between lines in symbiotic performance. The data for dry weight of shoots and roots (Table 1), nodulation (Table 2), nitrogenase activity (Table 3) and ammonium assimilation in nodules (Table 4) indicate marked variability in the efficiency of N_2 fixation.

Growth response to applied NaCl treatment varied with genotype, as shown by shoot and root dry weight (Table 1). Both shoots and roots were significantly reduced (p<0.05) in most genotypes, except for VF15, VF27, VF46, VF64 and VF112 in which shoot dry weight was less than 3 g per plant. In lines VF4 and VF38 only shoot growth was significantly inhibited. Maximal reductions in shoot dry weight caused by salinity were 81%, 72% and 71% in lines VF60, VF16 and VF38, respectively.

The number of nodules per plant in the absence of salt stress varied widely between different pure lines of *V. faba* (Table 2), from 147 nodules (VF38) to 723 nodules per plant (VF16). Salinity significantly reduced (p<0.05) the total number of nodules per plant in lines VF16 (75%), VF27 (43%), VF60 (64%) and cv. Alborea (44%). Total nodule weight per plant was more sensitive to salinity than nodule number, and significant decreases were seen in many lines, including some in which the number of nodules was unaffected (e.g. VF38 and VF61). However, the pure lines showing the greatest inhibition in nodule dry weight per plant (75% in VF16, 55% in VF27, and 72% in VF60) were also those in which the number of nodules per plant was markedly reduced, as noted above. The nodule dry weight showed a high correlation with shoot and

Table 3. Acetylene reduction activity (ARA) per weight of nodule (μmol C_2H_4 g^{-1} and ARA per $plant^{-1}$) (μmol C_2H_4 $plant^{-1}$ h^{-1}) in different lines of *Vicia faba* inoculated with *Rhizobium leguminosarum* GRA19 and treated with 0 and 75 m*M* NaCl

Genotype	NaCl (m*M*)			
	0	75	0	75
	Per unit nodule weight		Per plant	
VF4	22.50	4.24	6.67	0.93
VF6	21.01	4.30	7.96	0.14
VF15	21.05	5.78	3.04	0.40
VF16	17.17	2.61	9.67	0.65
VF17	26.40	10.18	13.77	0.89
VF27	19.61	1.49	4.05	0.18
VF38	27.11	2.82	6.46	0.77
VF46	12.36	3.56	3.13	0.93
VF47	16.45	1.83	5.97	0.48
VF60	21.32	5.34	19.92	1.08
VF61	23.50	2.21	9.05	0.29
VF64	28.95	1.14	5.01	0.27
VF83	14.59	2.52	6.28	0.80
VF112	14.77	16.73	2.38	1.51
Alborea	30.18	1.62	6.60	1.62
LSD (p=0.05)	3.57			
F statistics				
Plant genotype (A)	21.48**		29.50**	
NaCl Concentration (B)	1494.71		951.49**	
Interaction (A,B)	12.43		30.28**	

**$p \leq 0.01$.

root dry weight (Fig. 1), which is an indication of the connection between the nodule and plant growth. Average individual nodule weights decreased with salinity, as reflected by the smaller nodules formed at higher salt concentrations. In lines VF15, VF46, VF64 and VF112, salinity had no effect on the number or dry weight of nodules per plant.

Both total and specific nitrogenase (acetylene reduction) activity (ARA; Table 3) were severely depressed by salinity, except in VF112, which showed no change in comparison with control plants. Nitrogenase activity per unit weight in control plants varied from 12 μmol C_2H_4 g^{-1} h^{-1} (VF46) to 30 μmol C_2H_4 g^{-1} h^{-1} (cv. Alborea). For ARA per plant, in control plants, the highest value (20 μmol C_2H_4 $plant^{-1}$ h^{-1}) was found in line VF60.

Ammonium assimilation activity was determined in nodule cytosol (Table 4). Activity of GS was 2.4- (VF47) to 8-fold (VF17) higher than NADH-GOGAT, and both activities were significantly decreased ($p<0.05$) by salinity. In lines VF6, VF15, VF17 and VF112, GS was more markedly affected by salt stress than NADH-GOGAT. The reduction in GS ranged from 28% (VF15) to 81% (VF6), with severe decreases in lines VF83 (71%) and VF112 (74%). However, GS activity in cv. Alborea decreased by only 7%. Genotypic variability in NADH-GOGAT activity was greater than for GS activity, and showed a different pattern of changes in response to salinity. The most markedly affected lines were VF6, VF47 and VF83 (on average, 74% decrease), and the least affected line was VF15 (9%).

Soluble protein content of the nodules (Table 5) varied little between the genotypes studied here. Salinity significantly ($p<0.05$) decreased soluble protein content in all lines except VF15. The reduction varied from 16% (lines VF27 and VF64) to 52% (line VF6).

Table 4. Glutamine synthetase (GS) [μmol γ-glutamyl-hydroxamate h^{-1} (g FW)$^{-1}$] and glutamate synthase (GOGAT) [μmol $NADH_{ox}$ h^{-1} (g FW)$^{-1}$] activities of different lines of *Vicia faba* inoculated with *Rhizobium leguminosarum* GRA19 and treated with 0 and 75 m*M* NaCl

Genotype	NaCl (m*M*)			
	0	75	0	75
	GS		NADH-GOGAT	
VF4	240	112	8.0	3.0
VF6	244	47	65.0	16.4
VF15	234	168	59.0	53.8
VF16	143	86	29.2	10.4
VF17	216	59	26.6	7.8
VF27	228	128	48.8	16.9
VF38	140	62	20.4	5.6
VF46	159	72	22.8	9.4
VF47	193	100	79.6	24.8
VF60	158	86	47.1	19.5
VF617	211	75	54.3	12.7
VF64	297	198	56.0	22.2
VF83	257	73	56.5	12.9
VF122	246	64	61.3	32.9
Alborea	267	248	69.5	33.4
LSD (p=0.05)	14.3		2.0	
F statistics				
Plant genotype (A)	218.60**		1130.98*	
NaCl concentration (B)	3950.69**		12103.21**	
Interaction (A,B)	57.00**		282.74**	

**$p \leq 0.01$.

Discussion

The results presented here for different physiological parameters clearly show different responses to NaCl in fifteen genotypes of faba bean. Genetic variation for tolerance in the gene pool of a crop species is of prime importance for the improvement of salt tolerance through selection and breeding. Interspecific, intra-specific and intra-cultivar variation for tolerance provides scope for breeding and selection (Ashraf and McNeilly, 1992).

With regard to the physiological parameters of shoot growth, number and weight of nodules, salinity did not cause significant reductions in genotypes VF46, VF64 and VF112 of *V. faba*, which are characterized by slow growth. Reductions in plant growth and dry matter accumulation have been observed at low salinity levels in *Vicia faba* and *Phaseolus vulgaris* (Abdel Ghaffar et al., 1982), *Glycine wightii* (Wilson, 1970), *Glycine max* (Grattan and Maas, 1988) and *Vigna radiata* (Hafeez et al., 1988). However, the plant's genotype is highly important in salt tolerance, as Velagaleti and Marsh (1989) have shown for *Glycine max*. Both fresh and dry weight could be used as the criterion for assessing relative salt tolerance (Aslam et al., 1993). Growth in *Phaseolus vulgaris*, a legume that is highly sensitive to salinity was inhibited by 77% at a concentration of 50 m*M* NaCl, and by 91% at a concentration of 150 m*M* (Seeman and Critchley, 1985). Cotton, considered a tolerant nonhalophyte plant (Greenway and Munns, 1980), showed reductions of growth of 27% with 50 m*M* NaCl, and 58% with 250 m*M* (Brugnoli and Lauter, 1991). Comparison of these percent inhibitions with the figures we obtained in *V. faba* lines VF46, VF64 and VF112 suggest that these genotypes can be considered tolerant to high levels (75 m*M* NaCl) of salinity, however the other inbred lines and cv. Alborea did not show that tolerance.

Table 5. Soluble protein content [mg (g FW^{-1}] in nodules of different lines of *Vicia faba* inoculated with *Rizobium leguminosarum* GRA19 and treated with 0 and 75 m*M* NaCl

Genotype	NaCl (m*M*)	
	0	75
VF4	12.44	7.60
VF6	13.23	6.32
VF15	10.15	10.89
VF16	10.08	8.30
VF17	11.33	7.26
VF27	12.22	10.24
VF38	11.82	9.26
VF46	10.52	8.94
VF47	12.32	8.32
VF60	10.58	7.29
VF61	10.36	6.93
VF64	12.48	10.45
VF83	11.83	7.42
VF112	10.24	7.36
Alborea	10.89	8.90
LSD (p=0.05)	1.39	
F statistics		
Plant genotype (A)	5.61**	
NaCl concentration (B)	271.91**	
Interaction (A,B)	6.15**	

**$p \leq 0.01$.

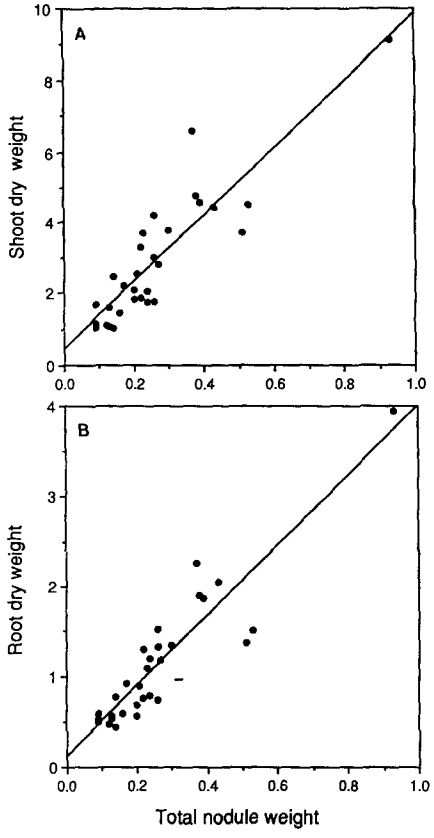

Fig. 1. Relationship between total nodule weight and shoot dry weight (**A**) or root dry weight (**B**) of different lines of *Vicia faba* inoculated with *Rhizobium leguminosarum* GRA19 and treated with 0 and 75 m*M* NaCl. (A) y=0.022+0.213x, with a significant correlation coefficient of 0.878 ($p \leq 0.01$). (B) y=0.413+9.440x, with a significant correlation coeficient of 0.897 ($p<0.01$).

Variability in N_2 fixation potential between cultivars within species has been reported for faba bean (Caba et al., 1993), soybean (Burias and Planchon, 1990), bean (Wolyn et al., 1989), alfalfa (Groat et al., 1984) and also for *V. faba* in this study. Nitrogenase activity in *V. faba* root nodules was adversely affected by high levels of salinity in all genotypes except line VF112. In this line, nodulation was also not affected by salt stress (Table 2).

The interaction between *Rhizobium* strain and cultivar can influence plant (dry weight and N_2 fixation (Mytton et al., 1988), but problems with the inoculant strain can be minimized if a strain of rhizobia adapted to saline soils is used. *Rhizobium leguminosarum* GRA19 is tolerant in the field (Cordovilla, 1993) and tolerance of the host to salt stress appears to be a major requisite for nodulation and fixation under salt stress. In faba bean, however, N_2 fixation is more sensitive to salt than plant growth, as was also found for chickpea (*Cicer arietinum*) (Elsheikh and Wood, 1990). However, Hafeez et al. (1988) reported that in *Vigna radiata*, salt stress had no effect on nitrogen fixation. Earlier studies have described a correlation between the inhibition of nitrogen fixation and leghemoglobin content during stress (Becana et al., 1986; Guerin et al., 1990). In all the genotypes we tested, whitish, irregular nodules were found in variable numbers. Similar nodules were described by Yousef and Sprent (1983) in other *V. faba* genotypes.

According to Groat and Vance (1981), treatments that reduce N_2 fixation lower NADH-GOGAT activity. Enzymes involved in ammonium metabolism (GS and NADH-GOGAT) were inhibited by salt in all genotypes studied here. Our results are in agreement with the findings of Bourgeais-Chaillou et al. (1992), who reported that NaCl reduced GS and NADH-GOGAT

in soybean. In a study of green bean, Pessarakli et al. (1989) found that salt stress inhibited ammonium metabolism and protein synthesis. Under conditions of salt stress, protein concentrations decreased markedly in all genotypes assayed, similar results have been described for other legumes (Bourgeais-Chaillou et al., 1992; Pessarakli et al., 1989). The decrease may have been due to proteolysis (von Mothes, 1956) or inhibition of protein synthesis (Kahane and Poljakoff-Mayber, 1968).

The results of our experiment indicate that *V. faba* is characterized by ample genotypic diversity, a feature necessary to develop cultivars with superior tolerance to salinity. Of the genotypes we studied, the most salt-tolerant were the slowest-growing ones. In genotype FV112, salt stress had no significant effect on dry matter accumulation or nitrogen fixation. Enzymes involved in ammonium assimilation in root nodules were sensitive to salt, and thus should not be considered reliable criteria for selection in programs aimed at the development of salt tolerance in faba bean cultivars.

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