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ORIGINAL PAPER

Phenotypic plasticity with respect to salt stress response by *Lotus glaber*: the role of its AM fungal and rhizobial symbionts

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Abstract Our hypothesis is that *Lotus glaber* (a glycophytic species, highly tolerant to saline–alkaline soils) displays a plastic root phenotypic response to soil salinity that may be influenced by mycorrhizal and rhizobial microorganisms. Uninoculated plants and plants colonised by *Glomus intraradices* or *Mesorhizobium loti* were exposed to either 150 or 0 mM NaCl. General plant growth and root architectural parameters (morphology and topology) were measured and phenotypic plasticity determined at the end of the salt treatment period. Two genotypes differing in their salt tolerance capacity were used in this study. *G. intraradices* and *M. loti* reduced the total biomass of non-salinised, sensitive plants, but they did not affect that of corresponding tolerant ones. Root morphology of sensitive plants was greatly affected by salinity, whereas mycorrhiza establishment counteracted salinity effects. Under both saline conditions, the external link length and the internal link length of mycorrhizal salt-sensitive plants were higher than those of uninoculated control and rhizobial treatments. The topological trend (TT) was strongly influenced by genotype × symbiosis interaction. Under non-saline conditions, nodulated root systems of the sensitive plant genotype had a more herringbone architecture than corresponding uninoculated ones. At 150 mM NaCl, nodulated root systems of tolerant plants were more dichotomous and those of the corresponding sensitive genotype more herringbone in architecture. Notwithstanding the absence of a link between TTs and variations in plant growth, it is possible to predict a dissimilar adaptation of plants with different TTs. Root colonisation by either symbiotic microorganisms reduced the level of root phenotypic plasticity in the sensitive plant genotype. We conclude that root plasticity could be part of the general mechanism of *L. glaber* salt tolerance only in the case of non-symbiotic plants.

Keywords Glomus intraradices · Mesorhizobium loti · Phenotypic plasticity · Salt stress · Lotus glaber

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Introduction

In heterogeneous environments, plant genotypes respond to environmental constraints by morphological and physiological adjustments, which contribute to the survival and propagation of the species. These changes in phenotypic expression in response to environmental influence are known as phenotypic plasticity (Schlichting 2002; West-Eberhard 1989), which is considered an attribute of the genotype. It is ge-

nerally accepted that the wide distribution of a given plant species may be due to a high phenotypic plasticity or a high genetic variability among individuals (Bradshaw 1965). *Lotus glaber* Mill (narrow-leaf trefoil), a glycophytic, perennial legume from Europe, is currently being intensively studied due to the occurrence of genotypes highly tolerant to saline–alkaline soils in different populations of the Salado River basin (9×10^6 ha). Soils in this region are characterised by a patchy structure of salt accumulation, which makes the installation of traditional legume pastures like alfalfa or clover less efficient (Montes 1988). Although several authors have attempted to explain the physiological mechanisms of salt tolerance in *L. glaber*, the issue is far from being unravelled (Mendoza et al. 2005; Sannazzaro et al. 2006, 2007; Teakle et al. 2006, 2007). *L. glaber* plasticity of reproductive and vegetative attributes has been reported under different experimental conditions, and it is thought to contribute to *L. glaber* colonisation of heterogeneous environments of the Flooding Pampa (Stofella et al. 1998; Kade et al. 2003). However, there is a lack of information regarding phenotypic plasticity of *L. glaber* responses to salt stress. Our main hypothesis is that *L. glaber* displays a plastic phenotypic response to soil salinity stress levels and that such a plasticity plays a role in the *L. glaber* general mechanism of salt tolerance. Studies on adaptive phenotypic plasticity have focused mainly on the morphological traits of aerial plant organs, and the abovementioned works are not the exception. However, since roots are the primary organ sensing salt stress conditions in soil, it would be relevant to focus also on the phenotypic plasticity of this organ.

Previous work has indicated that *L. glaber* roots are able to establish symbiotic relationships with soil microorganisms. In lowlands of the Salado River basin, this legume was found to be associated with arbuscular mycorrhizal (AM) fungi (Sannazzaro et al. 2004; Mendoza et al. 2005), particularly with the AM species *Glomus intraradices*, which seems to be well adapted to saline environments (Aliasgharzadeh et al. 2001; Landwehr et al. 2002). An isolate of this fungal species favoured growth of a tolerant and of a sensitive genotype of *L. glaber* under saline conditions (Sannazzaro et al. 2006, 2007). AM plants showed higher net growth, shoot/root and K^+/Na^+ ratios and protein concentrations than controls. In turn, some studies have focused on the association of this legume with the nitrogen-fixing bacteria *Mesorhizobium loti* (e.g. Fulchieri et al. 2001; Iribarne et al. 1998), although none of them addressed the effect of these microorganisms on the productivity of *L. glaber* under saline conditions. Since AM fungi and rhizobial bacteria have been shown to diminish the detrimental effects of salinity (Gupta and Krishnamurthy 1996; Ruiz-Lozano et al. 1996; Ruiz-Lozano and Azcon 2000; Al-Karaki and Hammad 2001; Feng et al. 2002), testing the hypothesis that these microorganisms influence the phenotypic plasticity of roots becomes relevant.

Root architecture has been directly related to plant productivity (Lynch 1995) since it can provide clues about resource cost and transport and exploration efficiency, especially under limiting edaphic conditions. The architecture of a root system can be analysed according to several measurable variables: Topology, root length and branch density are the most important ones (Fitter 1987; Bouma et al. 2001). The theory underlying a topological classification of a root system relies on the consideration that this is comprised of external links (root segments between a meristem and a branch junction) and internal links (a segment between two junctions), which may be used to define a topological index, representing attributes of the root system in terms of acquisition and storage of soil resources or anchorage. These topological indexes vary between set limits, their minimum being associated with a "dichotomous" root branching system and their maximum, with a "herringbone" pattern (Fitter 1987). "Dichotomous" root systems have a relatively lower construction cost than "herringbone" roots and are also more efficient in terms of transport than the latter due to a shorter pathlength (Fitter 1987; Fitter and Stickland 1991). In contrast, the "herringbone" structure is more efficient in habitats where soil resources are scarce, since it minimises intra-root competition for nutrients.

Functionality variations have been found between tap and lateral root types with regard to nutrient and ion uptake (Waisel and Eshel 1992), suggesting that root architecture may account for differences in the contents of these elements. In turn, there is evidence that root architecture and functionality can be altered by interactions with AM fungi (Schellenbaum et al. 1991; Šmilauerová and Šmilauer 2002; Cruz et al. 2004), salinity (Poljakoff-Mayber 1975, 1988; Esechie et al. 2002; An et al. 2003; Alshammary et al. 2004) and rhizobial bacteria (Barea et al. 1996; Tricot et al. 1997).

The aims of the present study were: (1) to compare plasticity of diverse architectural root traits with respect to salt-stress response between two *L. glaber* genotypes differing in salt tolerance and (2) to evaluate the influence of arbuscular mycorrhizal and rhizobial symbionts on root system plasticity.

Materials and methods

Biological material

Inoculum of *G. intraradices* (BAFC 3108) was multiplied in 1-l pot cultures with soil–perlite (1:3 *v*/*v*) and *Sorghum halepense* (L.) Pers. (=*Andropogon halepensis* Brot.) as host during 4 months and consisted of 1 g of root fragments with no less than 70% of their root length colonised. *M. loti* strain B733 was grown in YEM, and each plant was inoculated with 10^9 CFU.

Experiments were performed using *L. glaber* stem cuttings derived from individuals recovered from saline lowlands. Differences in sensitivity to saline stress among 80 *L. glaber* individuals had been previously determined according to their survival time when subjected to 300 mM NaCl (Paz et al. 2005). Two genotypes differing in their salt tolerance capacity (from now on termed "tolerant" and "sensitive" genotypes) were chosen for this study and were vegetatively propagated according to Mujica and Rumi (1998). Seven-day-old rooted cuttings were transferred to pots containing 250 ml of a sterilised vermiculite–perlite mix (1:1 *v/v*) and inoculated with the corresponding microsymbiont. Control plants received an equal amount of autoclaved inoculum.

Plant growth conditions

Plants were grown in a greenhouse, with a 16/8 h photoperiod at 25°C/21°C (day/night) and 55/75±5% relative humidity. Light intensity (230 μmol m^{-2} s^{-1}) was provided by daylight and Grolux fluorescent lamps (F 40W). Plants were irrigated with half-strength Hoagland's nutrient solution with the following N and P concentrations: (1) control plants: 10 mM $Ca(NO_3)_2$, 10 mM KNO_3, 2 mM KH_2PO_4; (2) AM plants: 10 mM $Ca(NO_3)_2$, 10 mM KNO_3, 1 mM KH_2PO_4 and (3) nodulated plants: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM KH_2PO_4. For the saline treatment, 100 mM NaCl was added to the irrigation solution during 30 days and 150 mM NaCl for an additional 30-day period. Nutrient solutions with or without salt were applied to plants by capillarity and renewed every 2 days. Previous work performed in our laboratory has shown that this method prevents soil electrical conductivity oscillations during the experiment.

Measurements and analytical determinations

Plants were harvested and divided into shoots and roots. Fresh and dry weight of shoots and roots were determined. Assessment of root colonisation was performed according to McConigle et al. (1990). Mycorrhizal dependency was calculated as follows: MD% = (dry weight of mycorrhizal plant − dry weight of non-mycorrhizal plant) 100/dry weight of mycorrhizal plant, for a given NaCl level (Plenchette et al. 1983).

The morphological parameters of root systems considered were root length, external and internal link length and number and type of roots, from which total root branching frequency (number root tips/centimetre of root length), specific root length (centimetre of root length/gram fresh weight), and the contribution to the total root length by each type of root were calculated. Root parameters proposed by Fitter (1987) to characterise root topology were measured: magnitude (μ): number of exterior links, altitude (a): number of links in the longest unique path from the base link to an exterior link and total external pathlength (P_e): sum of the number of links in all paths from all external links on the base link. Based on these parameters, two topological indexes were used to estimate the tendency of root system architecture to one of the two extremes of topological structure, either herringbone or dichotomous pattern (Fig. 1). These indexes were:

1. Topological index (TI) = log a/log μ (Fitter 1991) and
2. Topological trend (TT; Trencia 1995), which ranges from 1 in the herringbone structure to 0 in the dichotomous one. The mathematical expression of the latter index is: $TT = (P_e0 - P_e(\min))/(P_e(\max) - P_e(\min))$ where P_e0=observed P_e and $P_e(\min)$ and $P_e(\max)$ are the possible minimum and maximum P_e values, respectively. Fitter (1987) proposed the following formulae for assessing these extreme values:

$$P_e(\max) = {}^1\!/_2\left(a_{\max}^2 + 3a_{\max} - 2\right)$$

where $a_{\max} = \mu, P_e(\min) = \mu(a_{\min}+1) - 2a_{\min} - 1$, and $a_{\min} = [\log_2(\mu - 1\ )]+2$.

To evaluate the extent of phenotypic plasticity in response to salinity, a plasticity index (PI) was calculated for each root parameter as the difference between the two means corresponding to a salt treatment divided by the maximum mean value (Valladares et al. 2000). Mean phenotypic plasticity was calculated for each genotype by averaging the PIs obtained for each variable.

Statistical analysis

Growth and root architectural data were subjected to a three-way analysis of variance using the genotype, symbiosis and all the possible interactions among these factors as variation source, except for the analysis of the contribution of each type of root where the factors included type of root, symbiosis and salinity. Means were compared by Duncan's test.

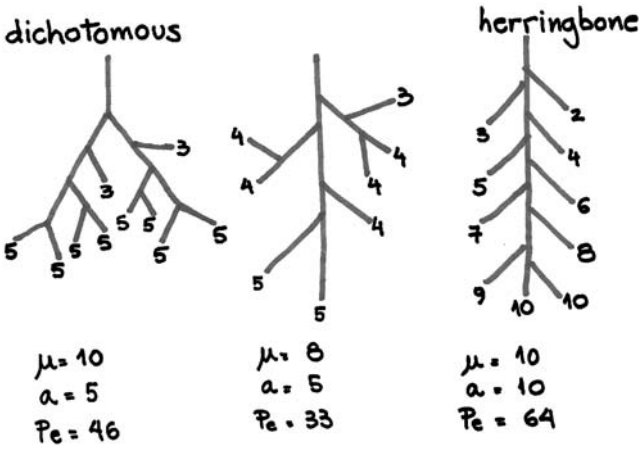

Fig. 1 Extreme topological root structures

Results

Plant growth

Levels of mycorrhizal colonisation in salt-treated plants were similar to those of control ones regardless of genotype or nodulation (Table 1). At 150 mM NaCl, mycorrhizal colonisation tended to decrease in nodulated, salt-sensitive plants. In turn, salinity reduced the number of nodules per root but only in non-mycorrhizal plants of both genotypes.

Genotype × symbiosis, genotype × salinity and symbiosis × salinity interactions were significant for shoot and total dry weights, whereas genotype × salinity and symbiosis × salinity interactions were significant for root dry weight (Table 2). In total, shoot and root dry weights under non-saline conditions were higher for the salt-sensitive genotype than for the salt-tolerant one (Fig. 2). Increasing soil salinity caused a reduction of about 60% in the weight of uninoculated and nodulated plants of the salt-sensitive genotype, whereas biomass of the salt-tolerant genotype was not affected by salt treatment. In contrast, salt treatment increased total dry weight in AM plants of both genotypes and the shoot dry weight of salt-tolerant AM plants. The reduction observed in the root dry weight of mycorrhizal and nodulated plants were not statistically significant.

Under non-saline conditions, inoculation with either of the two microsymbionts led to a reduction in total and shoot dry weights of salt-sensitive plants, but did not affect salt-tolerant ones. At 150 mM NaCl, inoculation with *G. intraradices* increased the total and shoot dry weights in both genotypes and the root dry weight of salt-sensitive plants compared to the corresponding uninoculated controls. No changes were registered in the dry weight of nodulated plants with respect to uninoculated plants under the saline conditions. Salinity increased the mycorrhizal dependency of plants in both genotypes (Fig. 3).

Root architecture

Root systems of *L. glaber* cuttings consisted of adventitious roots and lateral root branches of three orders. Total root length (TRL) was individually affected by the three factors: genotype, symbiosis and salt treatment (Table 2). Under non-saline conditions, mycorrhizal roots of the salt-sensitive genotype were longer than those of salt-tolerant plants (Fig. 4a). Salinity did not affect the total root length of uninoculated controls, but lowered the length of nodulated, salt-tolerant plants and had no effect on mycorrhizal ones. Under saline conditions, mycorrhizal roots were longer than those of uninoculated and nodulated plants of both genotypes.

External link length (ELL) and internal link length (ILL) were affected by genotype × symbiosis and symbiosis × salinity interactions (Table 2). At 0 mM NaCl, the ELL of salt-sensitive, uninoculated and AM plants and the ILL of AM plants were about twice those of the corresponding salt-tolerant ones (Fig. 4b, c). In contrast, no difference was observed between the sensitive and the tolerant genotypes in the remaining treatments. Although salt-induced decreases in ELL and ILL could be observed in several treatments, they were not statistically significant. Under both saline conditions, the ELL and the ILL of mycorrhizal salt-sensitive plants were higher than those of uninoculated control and rhizobial treatments. A similar result was obtained from the ELL of salt-tolerant plants grown at 150 mM NaCl when compared to uninoculated and nodulated plants. Mycorrhizal plants also showed higher ELL than uninoculated plants under non-saline conditions.

Specific root length (SRL) was affected by genotype × symbiosis and symbiosis × salinity interactions (Table 2). Salinity increased the SRL of uninoculated and nodulated, salt-sensitive plants, but did not affect the SRL of salt-tolerant ones (Fig. 5a). Under both saline situations, uninoculated plants of the salt-tolerant genotype showed higher specific root length than the corresponding salt-sensitive ones. Neither root symbiosis affected the SRL of salt-sensitive plants, but mycorrhizal colonisation decreased it at 0 mM NaCl. In turn, mycorrhizal colonisation reduced the SRL of salt-tolerant plants under non-saline conditions, and both symbionts had similar effects at 150 mM NaCl, although the effect was more obvious in the mycorrhizal treatment.

Table 1 Mycorrhizal length colonisation and number of nodules per root in sensitive and tolerant genotypes

Plant genotype	Salt treatment (mM)	% Mycorrhizal length colonisation		Number of nodules per plant	
		G. intraradices	*G. intraradices* + *M.* loti	*M. loti*	*G. intraradices* + *M. loti*
Salt-sensitive	0	52.1 (10.3) a	39.4 (4.4) abc	24.8 (10.2)a	32.0 (19.3) b
	150	46.3 (6.9) ab	31.0 (2.1) c	2.3 (1.8) b	31.1 (15.5) b
Salt-tolerant	0	51.8 (14.9) a	52.3 (11.0) a	8.8 (2.6) a	10.9 (4.7) a
	150	46.5 (5.7) a	43.7 (6.7) a	1.3 (1.0) b	9.3 (2.2) a

Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$).

Table 2 Significance of three-way ANOVA for the effect of genotype, symbiosis and salinity factors on all dependent variables of *L. glaber*

	Genotype	Symbiosis	Salinity	Genotype × symbiosis	Genotype x salinity	Salinity × symbiosis	Genotype × symbiosis × salinity
Total DW	**	*	*	*	**	**	ns
Shoot DW	**	**	ns	*	*	*	ns
Root DW	**	**	**	ns	*	**	ns
Total root length	*	**	**	ns	ns	ns	ns
ELL	**	**	**	**	ns	*	*
TT	ns	**	ns	**	ns	ns	ns
Total branching frequency	ns	*	ns	ns	ns	ns	ns
Specific root length	**	**	*	**	ns	**	ns
Total branching	**	ns	ns	ns	ns	ns	ns
N° nodal branches	ns	**	ns	ns	ns	ns	ns
No. first-order laterals	*	ns	*	ns	*	ns	ns
No. second-order laterals	**	*	ns	*	ns	ns	*
No. third-order laterals	**	ns	ns	ns	*	ns	ns
Contribution to total length 19	**	ns	ns	**	ns	ns	ns
Contribution to total length 30	**	ns	ns	**	*	**	ns

TDW total dry weight, *SDW* shoot dry weight, *RDW* root dry weight, *TRL* total root length, *ELL* external link length, *ILL* internal link length, *SRL* specific root length, *TNB* total number of branches, *TBF* total branching frequency, *TT* topological trend

Total root branching was modified by genotype and salinity (Table 2). At 0 mM NaCl, salt-sensitive plants had more than 100% more ramifications than salt-tolerant ones (Fig. 5b). In turn, the total number of branches in salt-sensitive plants was reduced by salinity and nodulation. In contrast, no difference was observed among treatments in the total number of root branches of salt-tolerant plants. Total branching frequency (TBF) was exclusively affected by the root symbionts (Table 2). At 0 mM NaCl, mycorrhizal sensitive plants had lower TBF than uninoculated ones (Fig. 5c), and a similar relationship was found in the tolerant plants grown at 150 mM NaCl. Nodal roots were only affected by the symbiotic factor (Table 2). Under non-saline conditions, nodulation reduced the number of nodal roots of salt-sensitive plants, but not that of salt-tolerant ones (Table 3). A similar effect was observed in salt-stressed, mycorrhizal plants of the salt-tolerant genotype.

First-order laterals were affected by the interaction between genotype and salinity, whereas the interaction among the three factors affected the development of second-order laterals (Table 2). Salinity decreased the number of first-order laterals only in uninoculated sensitive plants, but did not affect the remaining treatments. Salinity reduced the number of second-order laterals of uninoculated, salt-sensitive plants, but had no effect on the remaining plants (Table 3). Under non-saline conditions, mycorrhizal roots showed a several-fold increase in the number of second-order laterals compared to uninoculated control and nodulated plants. Although the same trend was observed at 150 mM of NaCl, the difference was not statistically significant. Neither mycorrhizal colonization nor rhizobial symbiosis affected the number of second-order laterals of salt-sensitive plants. Third-order laterals were only affected by plant genotype, since their number in uninoculated and mycorrhizal, salt-sensitive plants was higher than that in the corresponding salt-tolerant ones regardless of the salt treatment (Table 3).

Root length in salt-sensitive plants was only affected by the interaction between symbiosis and salinity (Table 3). The inoculation with *G. intraradices* decreased the proportional length of nodal roots at both saline conditions, whereas the rhizobial symbiosis had no effect on this parameter (Table 4). First-order lateral behaviour differed from that of nodal roots: At 0 mM NaCl, symbiotic plants had proportionally longer first-order laterals than uninoculated plants, whereas no difference was observed in salt-treated plants due to the symbiotic effect. Second-order laterals were not affected by salinity nor symbiotic organisms in any of the two genotypes. The three double interactions among the type of root branching, symbiosis and salinity were found to contribute to total root length in the salt-tolerant genotype (Table 3). In this genotype, symbiotic interactions with *G. intraradices* and *M. loti* reduced the proportional length of nodal roots under both saline conditions (Table 5). In turn, the latter symbiont caused an

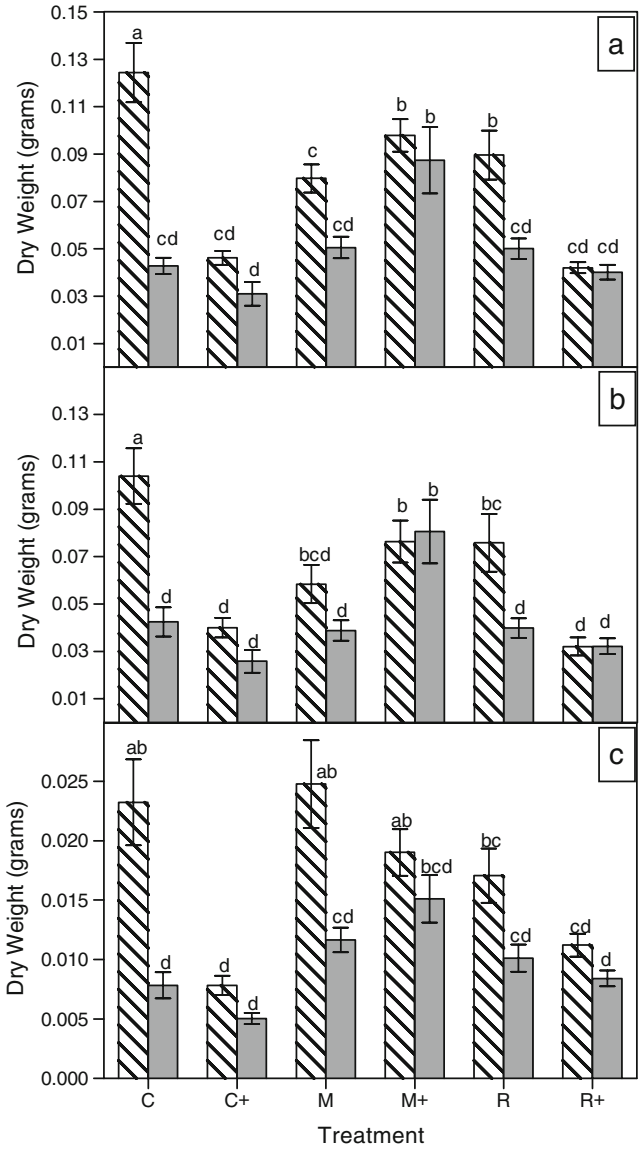

Fig. 2 Total (**a**), shoot (**b**) and root (**c**) growth of a salt-sensitive (*hatched bars*) and salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants were watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *C* uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated, *plus symbol* salinised plants. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

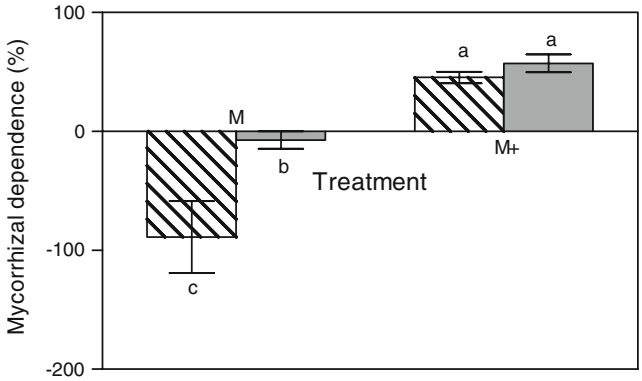

Fig. 3 Percentage of mycorrhizal dependency of a salt-sensitive (*hatched bars*) and a salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *M* mycorrhizal, *plus symbol* salinised plants. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

increase the contribution of first-order laterals to total root length at 0 mM NaCl.

The topological index (TI-log α/log μ) was not affected by any of the analysed factors and ranged between 0.70 and 0.98 (results not shown). In contrast, the TT was strongly affected by genotype × symbiosis interaction: At 0 mM NaCl, nodulated roots of the sensitive genotype showed a higher TT than those of the corresponding control (Figs. 6 and 7). On the other hand, the TT of sensitive plants in the treatment at 150 mM NaCl and *M. loti* was higher than that of the corresponding tolerant ones. The level of global phenotypic plasticity of roots was affected by a strong genotype × symbiosis interaction ($F=5.42$; $P=0.009$). Root phenotypic plasticity response to salinity was higher in salt-sensitive plants than in salt-tolerant, uninoculated ones. In turn, symbiotic root colonisation decreased the PI in the salt-sensitive genotype, but had no effect on the salt-tolerant one (Fig. 8).

Discussion

Basically, non-salinised sensitive plants decreased their total biomass when inoculated with either *G. intraradices* or *M. loti*, whereas the biomass of the corresponding tolerant plants was not significantly affected by fungal or bacterial colonisation. Such a genotypic disparity of responses to symbionts was not unexpected, since similar results had been previously obtained when analysing the response of two other *L. glaber* genotypes to colonisation by the same *G. intraradices* isolate (Sannazzaro et al. 2006). Reports of negative and neutral effects by AM fungi such as those observed in this work are common in the literature (Koide 1985; Fitter 1991; Smith and Smith 1996) and support the view that AM associations function along a mutualism–parasitism continuum. In contrast, the two genotypes increased their biomass during their interaction with *G. intraradices* under saline conditions.

Interestingly, the total biomass of tolerant plants observed in the mycorrhizal plus salt treatment was even higher than that of the corresponding controls without salt. The fact that a similar effect was observed in comparable experiments using *Prosopis hassleri* inoculated with *G. intraradices* (Scambato, unpublished results) suggests that *G. intraradices* might confer a similar halophytic behaviour on host plants. Although

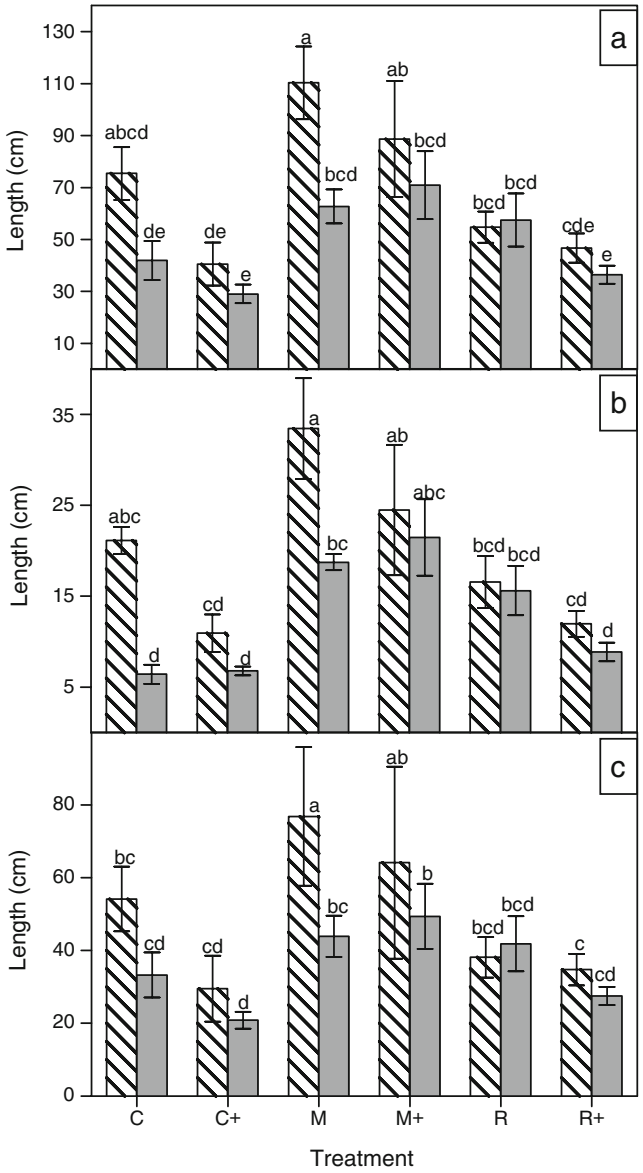

Fig. 4 Total root length (**a**) and external (**b**) and internal (**c**) root length of a salt-sensitive (*hatched bars*) and a salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *C* uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated, *plus symbol* salinised plants. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

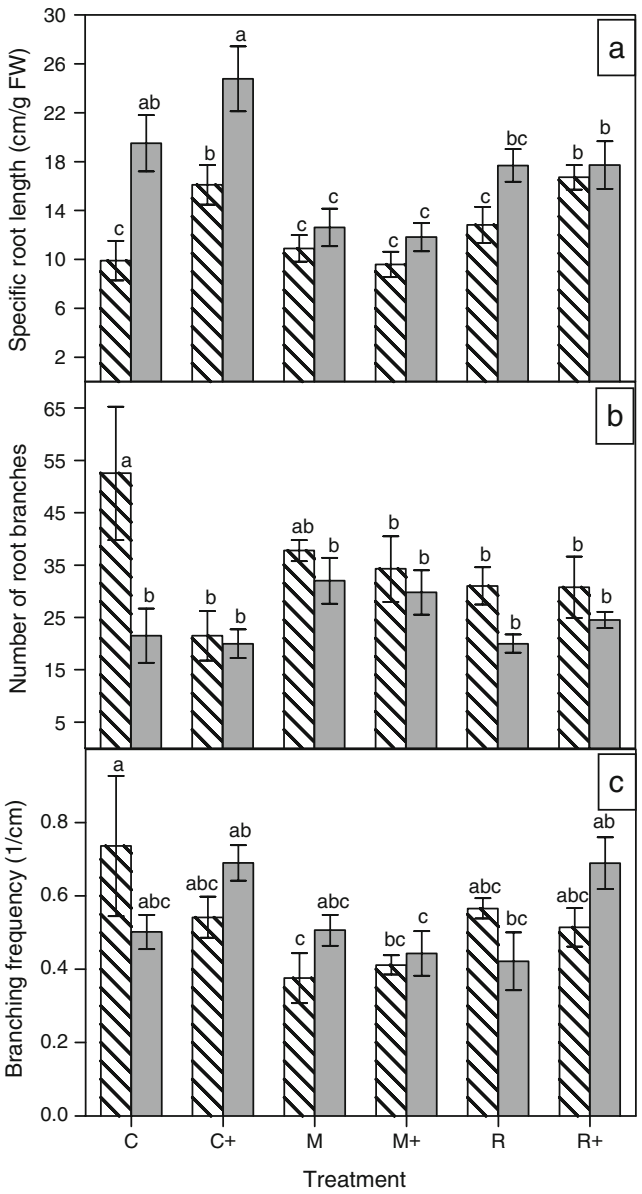

Fig. 5 Specific root length (**a**), total number of root branches (**b**) and total branching frequency (**c**) of a salt-sensitive (*hatched bars*) and a salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *C* uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated, *plus symbol* salinised plants. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

the *M. loti* strain used in this work was selected for its infectivity and ability to grow in culture media supplemented with 150 mM NaCl (own unpublished results), this strain was far from effective in improving plant growth of salt-stressed plants. It is important to note that neither P nor N was limiting in the experimental conditions used. Therefore, differences among treatments should not be ascribed to differential nutrient absorption.

Plant biomass was lower in the salt-tolerant genotype than in the salt-sensitive one. This result is in agreement with our previous reports (Sannazzaro et al. 2006, 2007) for other sensitive and tolerant *L. glaber* genotypes in which higher Na^+ and proline (a good indicator of stress perception) contents were found in the sensitive with respect to the tolerant genotype. Thus, although the salt tolerance mechanism in *Lotus* species has not been yet elucidated itself, one could

Table 3 Percentage of contribution to the total root length by nodal and lateral roots in the sensitive *L. glaber* genotype

	Salt-sensitive				Salt-tolerant			
	Nodal	First-order	Second-order	Third-order	Nodal	First-order	Second-order	Third-order
C	3.2 a	17.5 a	1.3 a	0.7 a	2.9 ab	13.2 ab	0.9 d	1.0 b
C+	4 ab	14.3 b	1.5 bcd	0.2 a	3.5 a	9.9 b	1.4 d	0.3 b
M	3.0 ab	16.8 a	12.3 abcd	0.4 a	2.2 ab	11.4 b	7.6 abc	0.6 b
M+	2.0 ab	19.3 ab	7.8 abcd	1.1 a	1.9 b	11.7 ab	6.6 bcd	1.7 b
R	1.8 b	15.5 a	4.8 bcd	0.7 b	2.3 b	13.7 b	4.6 bcd	0.7 b
R+	2.6 ab	17.3 ab	2.3 bcd	0 b	1.9 ab	11.6 ab	1.5 d	0 b

Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. Average data for at least four replicates. Means with the same letter are not significantly different ($P<0.05$).
C uninoculated, *M* mycorrhizal, *R* rhizobial

speculate that a faster growth could lead to higher Na^+ accumulation in growing plant organs and, therefore, to a shorter survival time in the sensitive genotype.

As far as phenotypic plasticity is concerned, PIs in *L. glaber* plants were comparable to those found for aboveground traits in *Mosla* species grown at varying levels of water status (Guan et al. 2004). Our results supported part of the first hypothesis raised in "Introduction", since they showed that phenotypic plasticity to salt stress response does exist for most of the traits analysed herein. When confronted with salt stress, uninoculated sensitive plants were globally more plastic than non-AM tolerant ones, suggesting that adjusting root traits is a more important adaptive mechanism in this genotype than in the tolerant one. Interestingly, salt-treated sensitive plants adjusted their root parameters (RDW, TRL, ELL, ILL, SRL and TB) to levels close to those displayed by tolerant roots, suggesting that in this latter genotype, measured traits would have a more suitable magnitude to minimise salt stress impact. Despite existing evidence supporting the genetic origin of morphological variation in field populations of *L. glaber* (Kade et al. 2003), our results suggest that root plasticity could also be related genetically determined and underline the need to assess phenotypic plasticity of *L. glaber* response to saline status among salt-contrasting field populations of this species. We can conclude that plasticity in root development under salt stress conditions may be one of the key traits for sensitive *L. glaber* genotypes to adapt to saline environments.

In addition, we have observed that phenotypic plasticity varied with the presence of mycorrhizal or rhizobial symbionts in the roots, which supports the second hypothesis proposed herein. These microsymbionts reduced the level of root phenotypic plasticity in the sensitive genotype, but these changes in phenotypic plasticity could not be linked to salt stress alleviation of symbiotic *L. glaber* plants, since mycorrhizal colonisation resulted in a general improvement of plant biomass in salt-stressed plants, whereas rhizobial colonisation did not. Thus, the second part of the first hypothesis, suggesting that root plasticity would be part of the general mechanism of *L. glaber* salt tolerance, should only be accepted in the case of non-symbiotic plants.

This work, as well as previous works (Sannazzaro et al. 2006), has shown that under saline conditions, AM *L. glaber* plants had larger root systems than non-mycorrhizal ones.

Table 4 Number of nodal and lateral roots of different orders in the sensitive and tolerant *L. glaber* genotypes

Treatment	Nodal	First-order	Second-order	Third-order
C	41 b	37 bc	20 cd	2.08 e
C+	41 ab	45 ab	12 de	1.40 e
M	26 cd	59 a	14 cd	1.50 e
M+	26 cd	48 ab	21 cd	6.19 e
R	28 bc	64 a	8 d	0.04 e
R+	29 bc	61 a	10 d	0 e

Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. Average data for at least four replicates. Means with the same letter are not significantly different ($P<0.05$).
C uninoculated, *M* mycorrhizal, *R* rhizobial

Table 5 Percentage of contribution to the total root length by nodal and lateral roots of different orders in the tolerant *L. glaber* genotype

Treatment	Nodal	First-order	Second-order	Third-order
C	61 a	35 cd	4 e	0.00 e
C+	56 a	42 bc	2 e	0.00 e
M	30 cd	47 bc	23 de	0.00 e
M+	30 cd	59 bc	11 e	0.04 e
R	25 cd	63 a	11 e	0.04 e
R+	47 bcd	49 bc	5 e	0.00 e

Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. Average data for at least four replicates. Means with the same letter are not significantly different ($P<0.05$).
C uninoculated, *M* mycorrhizal, *R* rhizobial

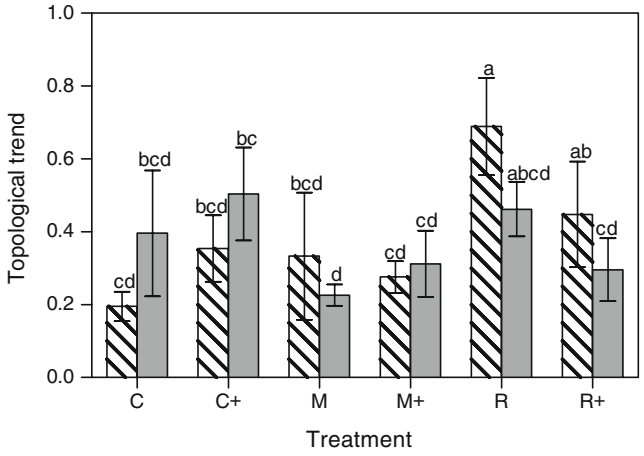

Fig. 6 Topological trend of a salt-sensitive (*hatched bars*) and a salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *C* uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated, *plus symbol* salinised plants. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

However, total root mass is not enough to effectively describe many root functions involved in plant–soil relationships. RDW, TRL, TB and SRL levels observed in salt-stressed, salt-sensitive AM plants were quite different from those observed in salinised, uninoculated plants and similar to those of uninoculated and mycorrhizal plants grown without salt. These results may be interpreted as a reversion of salinity effects on root morphology by the fungus. The mechanisms underlying this effect are not clear, but plant and fungal hormones could be involved (Barker and Tagu 2000; Hause et al. 2007). In fact, we have observed changes in zeatine and acid insoluble ash concentrations in *L. glaber* tap roots colonised by the same *G. intraradices* isolate (unpublished results).

The decrease in branching in root systems of uninoculated, salt-sensitive plants due to salt treatment, without significant changes in their total root length (compared to control roots), suggests that the inhibitory effect of salinity on the induction of new laterals could be compensated for either by a higher meristematic activity or by cell expansion of existing branches. Although the specific effect of salinity on apical root meristems of *Lotus* species has not been studied so far, nuclear damage and vacuolar or mitotic abnormalities in root meristematic cells following exposure to NaCl salinity have been observed in several plant species (Huang and van Steveninck 1990; Richardson et al. 2001; Radić et al. 2005). Therefore, it is possible that cell expansion was responsible for maintaining similar root lengths in the salt-treated, salt-sensitive *L. glaber* plants. For cell enlargement to occur, it is necessary to reach a critical turgor pressure, among other factors. Turgor pressure may have been achieved in salt-sensitive roots through proline accumulation, since a several-fold high concentration of this osmosolute was observed in salt-stressed *L. glaber* roots and the concentration observed in the salt-sensitive genotype was higher than that observed in the tolerant one (Sannazzaro et al. 2007). Thus, the interpretation made for other plant species on SRL increases, as an adjustment of absorptive root surface to improve water uptake as a consequence of the osmotic stress imposed by salinity can also be applied to salt-stressed, salt-sensitive *L. glaber* plants (Eissenstat 1992; Cornelissen et al. 2003; Reich et al. 1998; Wright and Westoby 1999).

Colonisation by AM fungi reduced the SRL of roots. A similar effect was also noted by Eissenstat et al. (1993), Peng et al. (1993) and Berta et al. (1995) for sour orange, Volkamer lemon, and *Prunus cerasifera* L. However, the architectural response of AM root systems to mycorrhizal colonisation is not uniform, since Pregitzer et al. (2002) reported reduction and increase in the SRL of AM *Liriodendron tulipifera* and *Acer saccharum*, respectively. Although root diameter has not been measured in this study, we have assumed that under saline conditions, mycorrhizal roots were thicker than those of uninoculated controls since SRL is often used as a surrogate for diameter (Hodge 2004). *M. loti* also reduced the SRL in the salt-tolerant genotype treated with salt, but to a lower extent than *G. intraradices*. As far as we know, there are no reports addressing the influence of rhizobial bacteria on the SRL with which our results may be compared. In 1996, Barea et al. observed that the establishment of a symbiotic interaction between a genetically modified *Rhizobium meliloti* strain and mycorrhizal alfalfa plants induced changes in root morphology compared to the effect produced by the wild-type strain. Unfortunately, these authors did not show the difference in root architecture between plants in symbiosis with the bacteria and those without symbionts.

Root length is a better predictor of nutrient uptake than root biomass. Under saline conditions, mycorrhizal roots were longer than those of uninoculated controls. Such an increase in the TRL was achieved by the extension of both internal and external links, which resulted in a reduction of the TBF in the tolerant genotype. This result is in agreement with increased root lengths registered in poplar and *Vitis vinifera* root systems (Schellenbaum et al. 1991; Hooker et al. 1992). However, it contrasts with the lack of difference in the total root length between AM and non-mycorrhizal carob plants (*Ceratonia siliqua*, Cruz et al. 2004) and with the shorter adventitious root system of mycorrhizal *Allium porrum* (Berta et al. 1990). Longer roots may have significant value for *L. glaber* growing in environments where, in addition to the salinity problem, the availability of relative immobile ions, such as phosphate, is low (Powell 1974), a condition commonly found in the Salado River Basin.

The pattern of contribution to the TRL by each root type was clearly different in both genotypes. Among the variations detected, changes in nodal and first-order laterals should be

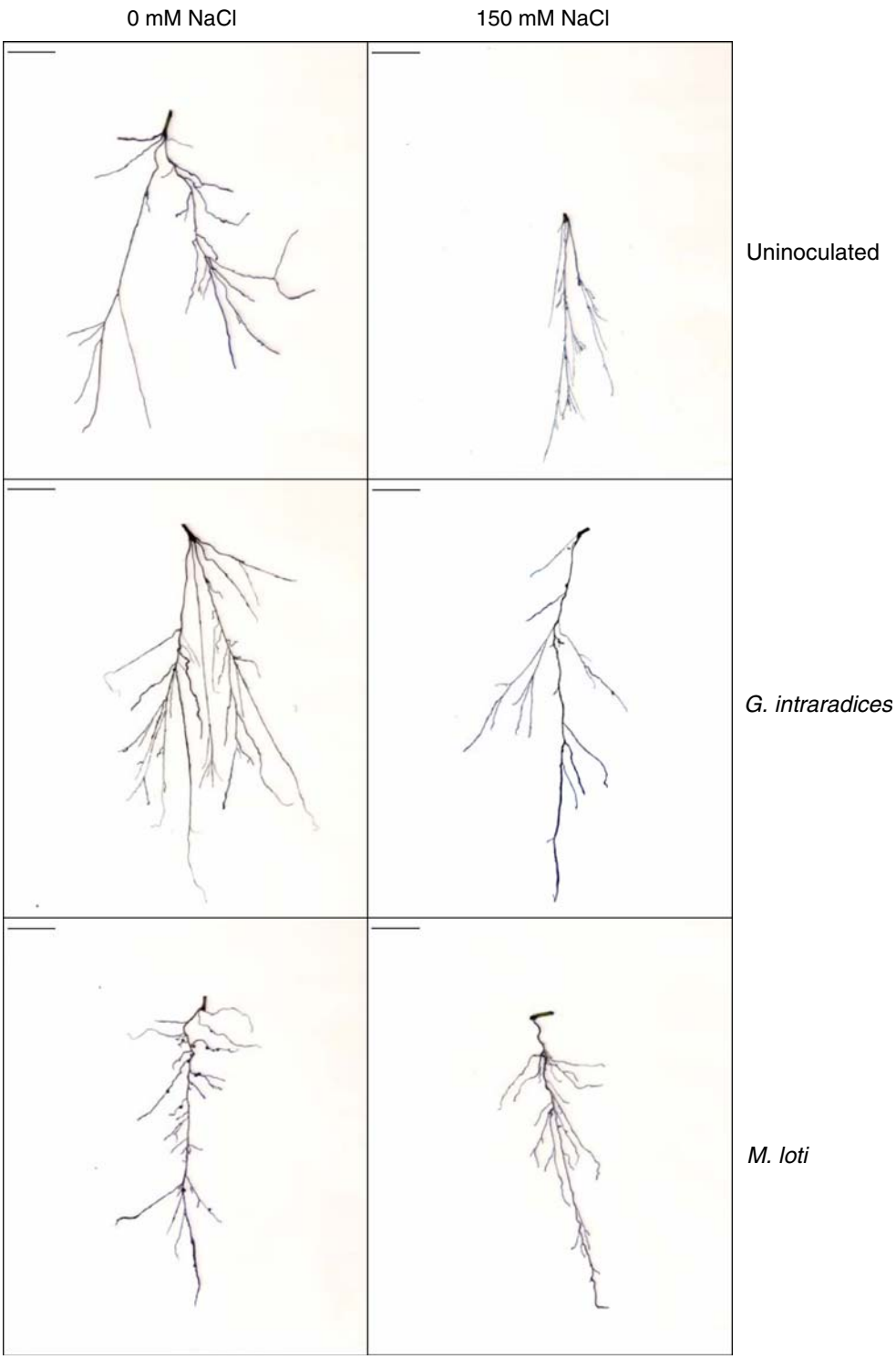

Fig. 7 Scanning of one representative root per treatment

more relevant for plant nutrition since they contributed to total root length to a greater extent. Under salt stress conditions, nodal and first-order laterals greatly contributed to the TRL in uninoculated, salt-sensitive plants, whereas in the corresponding symbiotic root systems, the main contribution shifted to first-order laterals. Inversely, the main contribution by nodal roots in salt-stressed, salt-tolerant plants without symbionts is equally assumed by nodal and first-order laterals in symbiotic plants. Therefore, it is possible that first-order laterals are the key components of symbiotic root response to high salinity levels in the soil. Variations in the number of second- and third-order laterals observed in the salt-tolerant genotype should not influence general plant growth due to their low incidence on total root length.

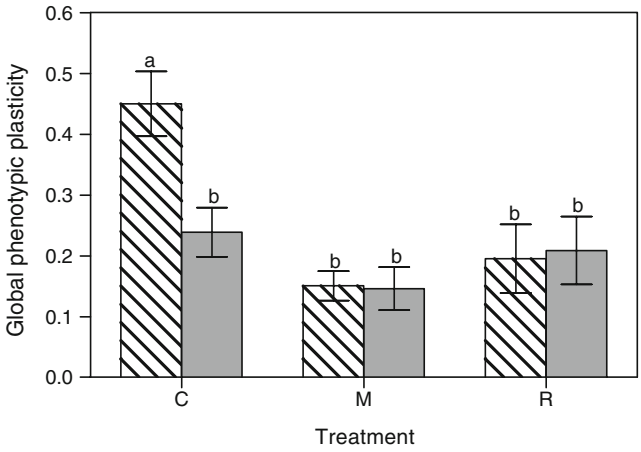

Fig. 8 Global phenotypic plasticity with respect to salt stress response of a salt-sensitive (*hatched bars*) and a salt-tolerant genotype (*grey bars*) of *L. glaber*. Seven-day-old plants watered with nutrient solution containing 100 mM NaCl during 1 month and 150 mM NaCl during another 30 days. *C* uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated. Average data for at least four replicates. Means (±SE) with the same letter are not significantly different ($P<0.05$)

The result showing higher mycorrhizal dependency in salt-tolerant plants is not surprising, since a similar relationship was found in our previous work with another set of sensitive and tolerant *L. glaber* genotypes (Sannazzaro et al. 2006). It has been proposed that MD is correlated with the thickness of a root system, that is, with its SRL (Baylis 1970; Graham and Syvertsen 1985). However, *L. glaber* genotypes displayed the opposite situation: Tolerant plants, which had higher MD, also showed higher SRL than sensitive ones.

It has been proposed that root topology (together with the overall size of a root system and the geometry of orientation and morphology of individual roots) is an important component of nutrient uptake capacity (Fitter 1987; Fitter et al. 1991; Berntson 1994; Nielsen et al. 1994) and anchorage (Ennos 1992). However, diverse indexes used to describe root topology poorly correlate within one another (Berntson 1997). Moreover, in general, there is a clear tendency for the estimates of topology to change with plant size (Glimskär 2000). These facts make results on root topology from authors using different topological indexes difficult to compare. In the present work, root topology was not affected by salinity or interactions with an AM fungus. The later observation concords with that reported by Cruz et al. (2004) who found no colonisation effect by *G. intraradices* on root topology of carob (*C. siliqua* L.) but contrasts with that of Hetrick et al. (1988) who showed that mycorrhizal fungi affect root topology of *Andropogon gerardii* in ways apparently not involving phosphorus nutrition.

Under non-saline conditions, nodulated roots of the salt-sensitive *L. glaber* genotype were more herringbone (featured a more ordered structure with root branching restricted to main axis) than corresponding uninoculated plants (more branched with many lateral roots of multiple orders). A similar, but not statistically significant, trend has been observed under saline conditions. Other results have shown a clearly higher TT value in nodulated plants, compared to mycorrhizal salt-sensitive ones, under both saline conditions. The increased TTs in nodulated plants were related to the reduction in the number of second- and third-order laterals. As far as we know, this is the first report on the detection of a rhizobial effect on root topology. Moreover, information on the effects of other plant-growth-promoting bacteria on root architecture is scarce. One of the few published reports (Gamalero et al. 2004) showed that the total root length, surface area and volume in tomato and cucumber roots increased with two strains of a PGPR *P. fluorescens* (92rk and P190r). In turn, the finding that *L. glaber* genotype influenced the TT of salinised nodulated plants was also unexpected. This index, which has been formerly used in the study of root architecture in trees (Trencia 1995; Martínez-Sánchez et al. 2003) shows that at 150 mM NaCl, root systems of rhizobial tolerant plants were more "dichotomous" and those of the corresponding sensitive genotype more "herringbone". An important aspect of this work that should not be overlooked is the fact that *L. glaber* was described as having a primary root system (Kirkbride 1994), whereas *L. glaber* stem cuttings developed adventitious root systems. Hence, precaution must be taken in the extrapolation of these results to seed-borne *L. glaber* plants.

Notwithstanding the absence of results linking observed TTs and variations in plant growth, it is possible to predict a dissimilar adaptation of plants with different TTs. In a scenario of homogeneous and non-limiting nutrient distribution, mycorrhizal (more dichotomous) root systems would be more advantageous than nodulated (more herringbone) root systems due to their lower construction cost (Fitter 1987; Fitter and Stickland 1991; Fitter 2002), reduced overlapping depletion zones between neighbouring roots and higher exploratory capacity per unit of root biomass. Even if the nutrient content in soil were low or patchily distributed, mycorrhizal roots would also be more advantageous since extraradical hyphae increase the root absorptive capacity (Graham and Syvertsen 1985; Koide and Kabir 2000). Alternatively, nodulated (more herringbone) root systems would be favoured where water is limiting (Fitter 1985, 1987). Changes in root system architecture due to nutritional and water content differences are well documented (Fitter and Stickland 1991; Sorgonà and Cacco 2002; Doussan et al. 2003), and hence, interaction among symbionts, genotypes and edaphic conditions on root architecture should be expected in the field. Further research is needed in order to fully understand how root architecture accounts for the variability in adaptation to stressing soil conditions observed among *L. glaber* genotypes as well as among different *Lotus* species (Schachtman and Kelman 1991; Mendoza 2001; Teakle et al. 2007).

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