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ORIGINAL PAPER

Agrobacterium rhizogenes transformed soybean roots differ in their nodulation and nitrogen fixation response to genistein and salt stress

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Abstract We evaluated response differences of normal and transformed (so-called 'hairy') roots of soybean (*Glycine max* L. (Merr.), cv L17) to the Nod-factor inducing isoflavone genistein and salinity by quantifying growth, nodulation, nitrogen fixation and biochemical changes. Composite soybean plants were generated using *Agrobacterium rhizogenes*-mediated transformation of non-nodulating mutant *nod139* (*GmNFR5α* minus) with complementing *A. rhizogenes* K599 carrying the wild-type *GmNFR5α* gene under control of the constitutive CaMV 35S promoter. We used genetic complementation for nodulation ability as only nodulated roots were scored. After hairy root emergence, primary roots were removed and composite plants were inoculated with *Bradyrhizobium japonicum* (strain CB1809) pre-induced with 10 μM genistein and watered with NaCl (0, 25, 50 and 100 mM). There were significant differences between hairy roots and natural roots in their responses to salt stress and genistein application. In addition, there were noticeable nodulation and nitrogen fixation differences. Composite plants had better growth, more root volume and chlorophyll as well as more nodules and higher nitrogenase activity (acetylene reduction) compared with natural roots. Decreased lipid peroxidation, proline accumulation and catalase/peroxidase activities were found in 'hairy' roots under salinity stress. Genistein significantly increased nodulation and nitrogen fixation and improved roots and shoot growth. Although genistein alleviated lipid peroxidation under salinity stress, it had no significant effect on the activity of antioxidant enzymes. In general, composite plants were more competitive in growth, nodulation and nitrogen fixation than normal non-transgenic even under salinity stress conditions.

Keywords *Agrobacterium* · Composite plants · Genistein · Hairy roots · Salt stress

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Introduction

The process of biological nitrogen fixation is of great agronomic interest and an alternative to fossil fuel produced nitrogen fertilizer. It contributes at least half of the annual amount of nitrogen fixed in soil ecosystems (Jensen et al. 2012). Symbiosis between soil-living bacteria, collectively called rhizobia, with more than one hundred agriculturally important legumes results in atmospheric N_2 being reduced into ammonium, and then glutamine which the plant is able to use (Ferguson et al. 2010). Effective symbiotic nitrogen fixation can replace, or at least minimise the need for applied agricultural nitrogen fertilizer, providing a sustainable conduit for the delivery of nitrogen into the earth's biosphere (Caetano-Anollés and Gresshoff 1991; Graham and Vance 2003).

Symbiotic nodulation and subsequent nitrogen fixation are sensitive to numerous environmental stresses (Serraj 2002). Salinity as one of the most important abiotic stresses is a serious threat to agriculture in arid and semiarid regions. Salinity affects legume development, nodulation

and symbiotic nitrogen fixation. Infection of root hairs by rhizobia and subsequent nodule development (El-Hamdaoui et al. 2003) are particularly sensitive to salinity (Rao et al. 2002). Furthermore, several studies attributed salt-mediated inhibition of nitrogen fixing activity to a reduction of nodule respiration and to a decrease of cytosolic proteins, including leghemoglobin production (Ikeda et al. 1992; Delgado et al. 1994). Reduction of photosynthetic activity by salt can also reduce nitrogen fixation (Georgiev and Atkias 1993). Therefore, understanding how symbiotic nitrogen fixation is affected under severe environmental conditions is particularly important both for agriculture and the preservation of the environment.

Legumes have the ability to form nitrogen-fixing root nodules. Among them soybean (*Glycine max* L. Merr) is the most widely cultivated legume (Ferguson and Gresshoff 2009) and forms determinate type nodules in a symbiosis with *Bradyrhizobium japonicum* and *Rhizobium fredii*. This symbiotic relationship is initiated when the rhizobia sense complex cocktails of sugars, flavones or isoflavones, which are perceived as *nod*-gene inducers in '*Rhizobium*' bacteria. Flavonoids induce expression of the bacterial nodulation (Nod) genes that encode proteins involved in the synthesis and secretion of lipo-oligo-saccharides (Nod factors; Spaink 2000). Perception of Nod-factor requires a dimeric receptor protein (Nod Factor LysM type Receptor) made up of *GmNFR1* and *GmNFR5* in soybean (Indrasumunar et al. 2010, 2011; Lee et al. 2011) and a complex downstream signaling cascade controlled by the plant (Ferguson et al. 2010; Reid et al. 2011). The combined processes of infection and cell division lead to the formation of nitrogen-fixing nodules. For soybean, the isoflavones genistein and daidzein released by plant roots induce the expression of common nodulation genes (*nodYABC*) of the bacterium (Kosslak et al. 1987; Sanjuan et al. 1992; Loh et al. 2002) and also the bacterial host-specific genes (such as *nodZ* and *nodFE*). Little is known about the membrane transport involved in flavonoid secretion from legume roots (Sugiyama et al. 2008). Nonetheless, in response to signals from the plant, the bacteria synthesize lipochito-oligosaccharide Nod factors that deform root hairs, elicit host biochemical changes and initiate host cell division (Fisher and Long 1992). Daidzein and genistein are the major signal components of soybean root extracts (Kosslak et al. 1987, 1990). Studies reported by Zhang et al. (1996), Zhang and Smith (1997) and others (Bandyopadhyay et al. 1996; Pan and Smith 1998) showed that pre-incubation of *B. japonicum* with genistein increased nodule number and nitrogen fixation in legume plants.

Procedures for obtaining transgenic roots have been developed using *A. rhizogenes*, a soil pathogen which elicits adventitious, genetically transformed roots (Beach and Gresshoff 1988; Stiller et al. 1997; Martirani et al. 1999; Kereszt et al. 2007). This leads to the production of so-called "composite plants" comprising a transgenic hairy root system attached to non-transformed shoots. Legume composite plants with a transformed root but untransformed shoot can be nodulated by rhizobia (Beach and Gresshoff 1988). Although transformation of roots using *A. rhizogenes* leads to morphologically normal roots, the question arises as to whether such roots in interacting symbiotically with rhizobia could produce more nodules or fix more atmospheric nitrogen. To address this question, *A. rhizogenes* K599-transformed roots were used for assessment of nodulation and nitrogen fixation, and compared with non transformed roots. Furthermore, although, a lot of work has been done on induction of transformed hairy roots from various plants of different genera, there are no reports on nodulation and nitrogen fixation of soybean composite plants under conditions of salt stress interacted by genistein, so these comparative aspects is the novelty of this study.

Materials and methods

Plant and bacteria materials

Two different soybean (*G. max* L. Merr.) genotypes including cultivar L17 and non-nodulating mutant *nod139* (*GmNFR5a*; in cv. Bragg background) were used throughout this study (obtained from the Seed and Plant Improvement Institute (SPII), Karaj, Iran and the ARC Centre of Excellence for Integrative Legume Research, University of Queensland, Brisbane, Australia, respectively). Cucumopine-type *Agrobacterium rhizogenes* strain K599 with *p35SGmNFR5α* and *B. japonicum* strain CB1809 were used in this work. The binary vector pCAMBIAl305.1, created by replacing the *GUS* gene fragment of pGFPGUSPlus with a *p35SGmNFR5α* (Indrasumunar et al. 2010) was introduced into *A. rhizogenes* by electro-transformation (Gene Pulser Xcell, Bio-Rad). Electroporation cuvettes 1 mm gap were placed on ice. Eppendorf of frozen electro-competent cells of *Agrobacterium* were allowed to thaw on ice. 1 mg DNA of the recombinant plasmid was mixed with 50 ml of electro-competent cells in the electroporation cuvettes on ice. The condition for electroporation was set as follow:

Choose mode T: 2.5 kV
Set resistance R: R5 (129 Ω)
Set charging voltage: 1.44 kV

The electro-competent cells containing the DNA mixture were transferred to electroporation cuvette. Pulse was given and 1 ml of liquid LB medium was added immediately, mixed gently and transferred to a 1.5 ml eppendorf tube and incubated at 28 °C for 1 h.

200 and 400 µl of transformed culture were spread on petri plates containing solid LB medium supplemented with 50 µg of rifampicin and 50 µg of kanamycin ml^{-1}, so that only transformed cells should multiply. Plates were wrapped with sealing film and kept at 28 °C for 2–3 days.

At the end of incubation colonies were picked and cultured in 5 ml liquid LB medium in 50 ml tube containing 50 µg of rifampicin and 50 µg of kanamycin ml^{-1}.

Culture tubes were kept at 28 °C on shaker in *Agrobacterium* growth room for 48 h with shaking. Transformants were confirmed through PCR.

Seed germination, bacteria culture and infection

Soybean seeds were surface-sterilized in a hydrogen peroxide/ethanol solution for 2 min (10 ml of 30 % H_2O_2 and 75 ml of 96 % ethanol filled up to 100 ml with sterile distilled water) and rinsed several times with sterile distilled water. Seeds of cultivar L17 were sown in 10 cm diameter plastic pots (four seeds in each pot) containing autoclaved perlite and vermiculite (1:1 ratio) while *nod139* seeds (150 seeds) were sown in a plastic box (30 × 50 × 30 cm) (Fig. 1a). The pots and box were placed in a humid growth chamber (L/D: 16/8 h, T: 28/25 °C, RH: 70 %), and watered with full strength of Broughton and Dilworth (1971) solution. Preparation of *Agrobacterium* was done on the same day as seed sowing. *A. rhizogenes* was grown in LB broth medium (from glycerol stock). After 24 h, a loop of bacteria was streaked onto the surface of LB plates containing the 50 µg ml^{-1} rifampicin and incubated at 28 °C for 1 day. On the next day a single colony was re-streaked onto a fresh plate containing 50 µg ml^{-1} kanamycin and incubated at 28 °C again. On the third day after seed sowing, the inoculant was produced by culturing *B. japonicum* strain CB1809 in yeast extract-mannitol broth in 250 ml flasks shaken at 150 rpm at 28 °C. After 24 h genistein was dissolved in methanol and added into yeast extract-mannitol broth to reach a final concentration of 10 µM. 5 day old L17 plantlets were inoculated with *B. japonicum* pre-incubated with or

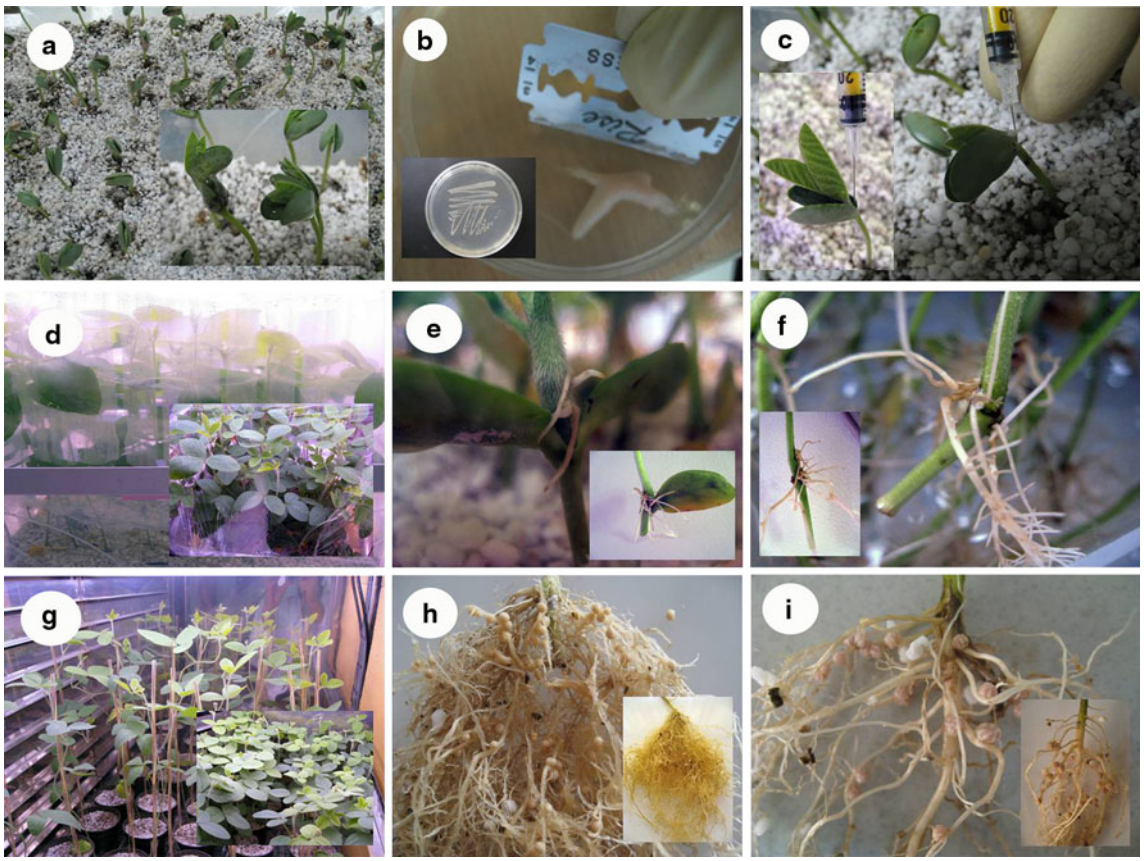

Fig. 1 Different stages of the soybean hairy root transformation. (**a**) *nod139* soybean mutants with the ideal stage for transformation: 5-day-old seedlings with unfolded cotyledons. (**b**) *A. rhizogenes* paste collected in the *lower left corner* the Petri dish and on the tip of the needle. (**c**) Stabbing of the hypocotyl close to the cotyledonary node. (**d**) *nod139* and L17 soybean plants in the germination box and pots. Note that the box was covered with a *transparent cover*. (**e**) Hairy root emergence, soybean plants 15 days after inoculation with *A. rhizogenes*. (**f**) Transformed soybean plant after the removal of the primary root. (**g**) Transfer of composite plants into new pots and inoculation with CB1809 and initiation of salt tress (**h**) Nodulation and root studies on hairy roots (**i**) Nodulation and root studies on natural roots

without genistein. One millilitre of inoculant per plant was applied by pipette onto the rooting medium. Plants were irrigated with Broughton and Dilworth solution supplemented by 0, 25, 50 and 100 mM NaCl. In parallel, 5 day old healthy *nod139* plantlets with unfolded green cotyledons were injected with *A. rhizogenes* by stabbing at the cotyledonary node with a sterile syringe needle (c.f., Kereszt et al. 2007; Indrasumunar et al. 2010, 2011). The bacteria were scraped from the plates using a razor blade and collected at the edge of the plate (Fig. 1b). A drop of bacteria was picked up with the tip of the needle and put onto the cotyledonary node site; infection was done by pushing the needle through the central part of the hypocotyl (Fig. 1c). Control plants were injected with sterile distilled water. The plantlets were covered by a plastic transparent lid and kept in humid growth chamber (L/D: 16/8 h, T: 28/25 °C, RH: 70 %), and watered with full strength of Broughton and Dilworth solution daily (Fig. 1d).

Hairy root emergence and plant cultivation

15 days after *A. rhizogenes* inoculating, numerous induced hairy roots were observed in 83 out of 150 plantlets (55.3 % induced roots) (Fig. 1e). Primary roots were removed from the plant by cutting approximately 1 cm below the cotyledonary node (Fig. 1f). Plants with induced roots were transferred into plastic pots containing fresh sterile perlite and vermiculite (Fig. 1g). Composite plants were inoculated with *B. japonicum* pre-incubated with or without genistein and watered with Broughton and Dilworth solution supplemented by 0, 25, 50 and 100 mM NaCl. Composite plants were covered with a plastic bag and kept in a growth chamber (L/D: 16/8 h, T: 28/25 °C, RH: 70 %). Plastic bags were removed to facilitate gradual acclimation of root transformed soybean plants to the environment. Covers were removed 4 days after removing primary roots.

Nodulation of non-transgenic roots and transgenic hairy roots

30 days after *B. japonicum* inoculating of hairy roots, composite and non-transgenic plants were 50 day old. At this time all plants were removed from pots (Fig. 1h, i). The roots were gently washed with water to remove all perlite and vermiculite and then nodule number, nodule weight and root volume were determined. Afterwards the shoots and roots were separated and dried at 70 °C for 24 h to calculate dry weight. Root samples with nodules of each plant were used for the acetylene reduction assay (ARA) assay. Additionally, fresh samples were collected, quickly frozen in liquid nitrogen and stored in a deep-freezer (−80 °C) for biochemical assays.

Biological nitrogen fixation

Apparent nitrogen fixation of non-transgenic and composite plants was assayed using ARA (Vessey 1994). Nodulated roots from freshly harvested plants were placed in a 600 ml bottle closed with a rubber cap. Immediately, 60 ml of air were withdrawn from the closed bottle by syringe and replaced by acetylene gas. Ethylene production was assayed in a gas chromatograph (UNICAM 4600, UK) over a short time period to prevent artefacts by closure of the acetylene induced variable oxygen barrier (Layzell and Hunt 1990).

Scanning electron microscope of nodules

Transgenic and standard nodule sections were surface coated with gold by a sputter coater (SCDOOS, Bal-Tec, Switzerland) for scanning electron microscopy analysis in a XL30 (Philips, Netherlands) low-vacuum scanning electron microscope.

Biochemical measurements

Chlorophyll

Chlorophyll was extracted in 80 % acetone from the leaf samples according to Arnon (1949). Extracts were filtered and total chlorophyll content was determined spectrophotometrically at 645 and 663 nm, respectively. Chlorophyll content was calculated according to Eq. 1 (Arnon 1949) and then data were corrected by Porra equation (Eq. 2) (Porra 2002).

$$\text{Total chloophyll} = [20.2\,(\text{D645}) + 8.02(\text{D663}) \times \text{V}/1000\,\text{W}] \quad (1)$$

where: V = final volume; W sample weight.

$$[\text{Chl}\,a + b]^{\text{Total}} = 0.895[\text{Chl}\,a + b]^{\text{Arnon}} \quad (2)$$

Antioxidant enzyme activity

Catalase (EC 1.11.1.6) activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 µl crude extract, 500 µl 10 mM H_2O_2 and 1.4 ml 25 mM potassium phosphate buffer. The decrease in absorbance was recorded at 240 nm for 1 min using a spectrophotometer (Cintra GBC, Dandenong, Victoria, Australia). Catalase activity of the extract was expressed as $\Delta\text{Absorbance}_{240}\ \text{mg}^{-1}$ protein min^{-1}.

Superoxide dismutase (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium according to the method of Giannopolitis and Ries

(1977). The reaction mixture contained 100 µl 1 µM riboflavin, 100 µl 12 mM L-methionine, 100 µl 0.1 mM EDTA (pH 7.8), 100 µl 50 mm Na_2CO_3 (pH 10.2), 100 µl 75 µM NBT in 2.3 ml 25 mM sodium phosphate buffer (pH 6.8) and 200 µl crude enzyme extract, in a final volume of 3 ml. Glass test tubes that contained the reaction mixture were illuminated with a fluorescent lamp (120 W), while identical tubes that were not illuminated served as blanks. After illumination for 45 min, absorbance was measured at 560 nm. One unit of superoxide dismutase activity was defined as the amount of enzyme that caused 50 % inhibition of photochemical reduction of nitro blue tetrazolium.

Peroxidase (EC 1.11.1.7) activity was estimated by the method of Ghanati et al. (2002) via oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance at 470 nm was recorded spectrophotometrically for 1 min. The reaction mixture contained 100 µl crude extract, 500 µl 10 mM H_2O_2, 500 µl 28 mM guaiacol and 1.9 ml 60 mM potassium phosphate buffer (pH 6.1). Peroxidase activity of the extract was expressed as $\Delta Absorbance_{470}$ mg^{-1} protein min^{-1}.

Malondialdehyde

The level of membrane damage was determined by measuring the amount of malondialdehyde, which is the end product of lipid peroxidation according to De Vos et al. (1991). Samples were homogenized in 10 % trichloroacetic acid (w/v) and aliquots of the filtrates were heated in 0.25 % thiobarbituric acid 100 °C for 30 min. The amount of malondialdehyde in the samples was determined from the absorbance at 532 nm, followed by correction for non-specific absorbance at 600 nm using a spectrophotometer. The concentration of malondialdehyde was determined by its extinction coefficient ($\varepsilon = 155$ µM cm^{-1}) and the results were expressed in nmol malondialdehyde g^{-1} fresh weight.

Proline

Proline content of leaves and roots was determined according to method of Bates et al. (1973). Samples (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3 % w:v), and then centrifuged at 12,000*g* for 15 min. Two ml of the supernatant was added to a test tube and then two ml glacial acetic acid and two ml freshly prepared acid ninhydrin solution were added. The test tubes were incubated in a water bath for 1 h at 100 °C and then allowed to cool to room temperature. Four ml of toluene was added to the tubes and mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min, to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve, and was expressed as mg g^{-1} fresh weight.

Protein

The protein content of the crude extract was determined using bovine serum albumin (BSA, Sigma-Aldrich) as a standard, according to the method of Bradford (1976). One millilitre of Bradford solution was added to 100 µl crude extract and absorbance recorded at 595 nm for estimate of total protein content. The protein concentration was calculated from a BSA standard curve.

Statistical analysis

The experiment was structured following a completely randomized design arranged in $2 \times 2 \times 4$ factorial with three replications. Transgenic and standard roots considered as first factor, *B. japonicum* pre-incubated with or without genistein as second factor and four salinity levels as third factor were investigated. For all variables, analysis of variance (ANOVA) was performed to test for differences between salinity, genistein treatment and their interactions using the GLM procedure in SAS version 9.1. Main and interaction effects of experimental factors were determined. Where interactions were significant, we presented the results in the form of a combination of treatments and not separately or individually. The significance of differences among treatment means was compared by Duncan's multiple range test at the 5 % probability level.

Results

There were significant differences between plants with natural (non-transgenic) and transformed hairy roots (composite/complemented) for shoot and root dry weight, root volume, nodule number and nodule weight, nitrogenase, catalase, peroxidase activity as well as malondialdehyde and proline accumulation (Table 1). However, when making this comparison, one must consider the developmental history and physiological age of the analysed material. Normal seedlings develop root systems based on the embryonic tap root followed by lateral emergence from pericycle; secondary and tertiary lateral develop in a similar fashion (Han et al. 2011). A functional root system develops in balance with the shoot (Day et al. 1986 for growth analysis of soybean cv. Bragg). By contrast, *A. rhizogenes* induction of roots occurs with plants of different developmental history. Plants are older, with a

larger shoot system to act as photosynthate source and nitrogen sink, when hairy root induction has succeeded to produce roots ready for inoculation with *Bradyrhizobium*. Moreover, *Agrobacterium*-induced roots develop from different core tissue within the wounded pericycle/cambium junctions in the hypocotyl region; in other words, they are similar to adventitious roots. Thus age, plant proportions and ontogeny differ.

Having said this, it has long been assumed that 'Hairy roots' behave similar to normal roots (Martirani et al. 1999). This may be broadly correct but we detected and quantified a range of differential responses.

Genistein application had significant effect on all traits except for antioxidant enzyme activity and proline accumulation of the roots. The findings revealed that all the parameters were significantly affected by salinity stress. Significant two-way and three-way interactions were also found among all three factors (Table 1).

Shoot and root dry weight alterations

Shoot and root dry weight were affected by individual effect of root type, genistein and salinity stress and also combined effect of genistein × salinity and root × salinity (Table 1). Pre-incubation of *B. japonicum* with genistein actually increased root and shoot dry weight in both composite and non-transgenic soybean plants (Table 2).

Salinity stress significantly reduced the overall growth of plants irrespective of the type of roots. This was evident from the decline in the dry weight of roots and shoots with increasing stress. However, composite soybean plants were more resistant to salt stress than their non-transgenic counterparts and accumulated more dry matter in above or underground parts (Table 4).

Root volume alterations

Exposure of soybean plants to salinity resulted in a significant decline in root volume (Fig. 2). In addition, the effect of root type and genistein on root volume was also significant (Table 1). The root volume was obviously different for two types of root at different levels of genistein (Table 2). The combined effect of root type and genistein was more pronounced on root volume when hairy roots were treated with pre-incubated *B. japonicum* with genistein (Table 2).

Table 1 ANOVA significance levels for the main and interaction effects of root type, genistein and salt stress

Parameters		Sources of variation								
		Root	Genistein	Salinity	Root × genistein	Genistein × salinity	Root × salinity	Root × genistein × Salinity	Error	C.V
Shoot dry weight		**	**	**	**	ns	**	ns	0.02	14.55
Root dry weight		**	**	**	**	ns	**	ns	0.01	12.84
Root volume		**	**	**	*	ns	ns	ns	2.93	11.04
Nodule number		**	**	**	**	ns	ns	**	22.37	8.03
Nodule weight		**	**	**	ns	**	**	**	0.00	2.51
Nitrogenase activity		**	**	**	ns	**	ns	ns	1.52	11.03
Chlorophyll		ns	**	**	**	**	**	ns	0.02	7.15
Catalase activity	Leaf	**	ns	**	ns	ns	**	ns	40.82	4.74
	Root	**	ns	**	ns	ns	**	ns	190.39	8.81
Peroxidase activity	Leaf	**	ns	**	ns	ns	ns	ns	35.11	3.85
	Root	*	ns	**	ns	ns	ns	ns	61.23	4.33
Superoxide dismutase activity	Leaf	ns	ns	**	ns	ns	ns	ns	0.02	9.95
	Root	ns	ns	**	ns	ns	ns	ns	0.01	9.36
Malondialdehyde	Leaf	**	**	**	**	ns	**	ns	0.00	5.08
	Root	**	**	**	**	**	**	ns	0.00	4.19
Proline	Leaf	**	**	**	**	ns	**	*	0.00	13.97
	Root	**	ns	**	*	ns	*	ns	0.00	7.73
Protein	Leaf	ns	**	**	ns	ns	ns	ns	0.02	7.58
	Root	ns	**	**	ns	**	ns	ns	0.00	8.79

*, ** and ns significance at P level of 0.05, 0.01 and no significant, respectively

Table 2 Significant two-way interaction between root type and genistein

Root × genistein		Shoot dry weight (g)	Root dry weight (g)	Root volume (cm^3)	Chlorophyll (mg g^{-1}FW)	Malondialdehyde (nmol MDA g^{-1}FW)		Proline (mg g^{-1}FW)
						Leaf	Root	Root
Natural roots	Genistein 0 μM	0.73c	0.39d	9.00d	1.72d	1.75a	2.35a	0.04a
	Genistein 10 μM	1.44b	0.55c	12.41c	2.04c	1.77a	2.32a	0.04a
Hairy roots	Genistein 0 μM	0.63c	1.23b	17.50b	2.74b	1.80a	1.79b	0.03b
	Genistein 10 μM	1.90a	1.64a	23.16a	3.16a	1.37b	1.38c	0.03b

Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

Nodule number and weight

Nodule number and nodule weight were significantly increased through the addition of genistein while they were found to decrease with increasing levels of salinity (Table 5). The data indicated that nodule number and nodule weight were more increased in hairy roots than non-transgenic roots (Table 5). The highest nodule number and nodule weight were observed in hairy roots treated with pre-incubated *B. japonicum* with 10 μM genistein under zero stress conditions (Table 5). Although induced nodules on hairy roots were smaller than nodules on non-transgenic roots, increase in nodule weight per plant was due to increase in nodule number by genistein application.

Nitrogenase activity

Hairy roots showed significantly higher nitrogenase activity as compared to natural roots (Fig. 3). Two-way interaction between genistein application and salinity stress is shown in Table 3. Exposing the nodulation process to salinity resulted in a sharp reduction in nitrogenase activity. In other words, nitrogenase activity has decreased proportionally in relation to salt concentration.

Genistein application increased nitrogenase activity either in control or in each level of salinity compared to lack of genistein (Table 3). The highest nitrogenase activity was observed in inoculated plants with pre-incubated *B. japonicum* with genistein and 0 or 25 mM NaCl.

Chlorophyll alterations

Leaf chlorophyll content increased significantly in composite plants compared to non-transgenic plants. In addition, chlorophyll content enhanced as a result of genistein application in both non-transgenic and composite plants (Table 2). Chlorophyll content was reduced dramatically in all the soybean plants as a result of increasing salinity. Saline stress led to the yellowing of leaves, which ultimately resulted in significant damage to the chlorophyll pigments. Chlorophyll content improved in salt-stressed plants on account of genistein application (Table 3).

Antioxidant enzymes activity

Catalase activity either in roots or in shoots was affected by salinity stress and root type (Table 1). There was no significant difference between natural roots and hairy roots as

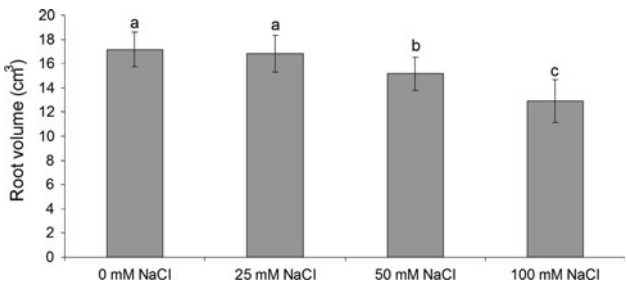

Fig. 2 Main effect of salt stress on root volume. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

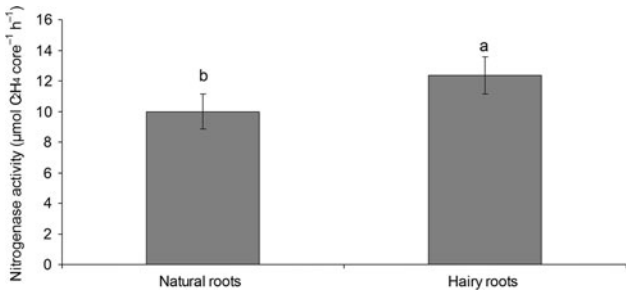

Fig. 3 Main effect of root type on nitrogenase activity. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

Table 3 Significant two-way interaction between genistein and salt stress

Genistein × salinity		Nitrogenase activity (μmol C_2H_4 h^{-1})	Chlorophyll (mg g^{-1}FW)	Root malondialdehyde (nmol MDA g^{-1}FW)	Root protein (mg g^{-1}FW)
Genistein 0 μM	0 mM	8.46cd	2.61c	1.77de	1.21a
	25 mM	7.62de	1.98d	1.86cd	0.95bc
	50 mM	7.22de	1.56e	2.17b	0.88c
	100 mM	6.69e	1.37e	2.49a	0.70d
Genistein 10 μM	0 mM	18.77a	3.73a	1.59f	1.23a
	25 mM	18.56a	3.43b	1.75e	1.23a
	50 mM	12.48b	2.57c	1.93c	1.00b
	100 mM	9.55c	2.08d	2.12b	0.94bc

Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

Table 4 Significant two-way interaction between root type and salt stress

Root × salinity		Shoot dry weight (g)	Root dry weight (g)	Root volume (cm^3)	Chlorophyll (mg g^{-1}FW)	Catalase activity (Abs mg^{-1} protein min^{-1})		Malondialdehyde (nmol MDA g^{-1}FW)		Proline (mg g^{-1}FW)
						Leaf	Root	Leaf	Root	Root
Natural roots	0 mM	1.60b	0.97d	11.83d	3.06b	104.88d	113.70d	1.48cd	1.96d	0.02de
	25 mM	1.15cd	0.52e	11.83d	2.53c	107.73d	124.15d	1.57c	2.15c	0.03c
	50 mM	0.91e	0.27f	10.66d	1.85e	161.88c	192.56b	1.81b	2.44b	0.05b
	100 mM	0.43f	0.10g	8.50e	1.57f	194.27a	228.37a	2.45a	2.77a	0.06a
Hairy roots	0 mM	1.84a	1.68a	22.50a	3.28a	107.49d	114.22d	1.35e	1.40g	0.02e
	25 mM	1.31c	1.51b	21.83a	2.88b	109.17d	126.01d	1.42de	1.46g	0.02d
	50 mM	1.20c	1.35c	19.66b	2.28d	111.68d	148.35c	1.57c	1.65f	0.04c
	100 mM	0.97de	1.20c	17.33c	1.88e	180.58b	204.50b	1.72b	1.84e	0.05b

Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

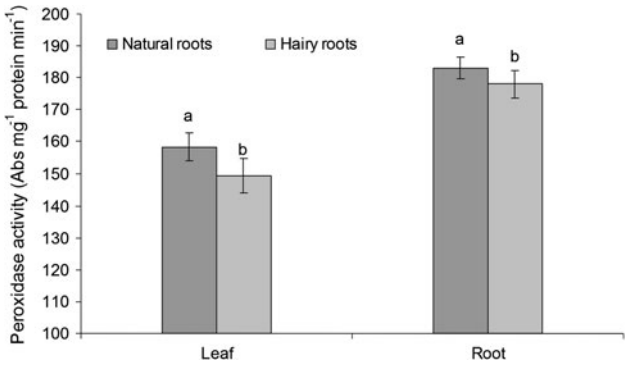

Fig. 4 Main effect of root type on peroxidase activity in soybean roots and leaves. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

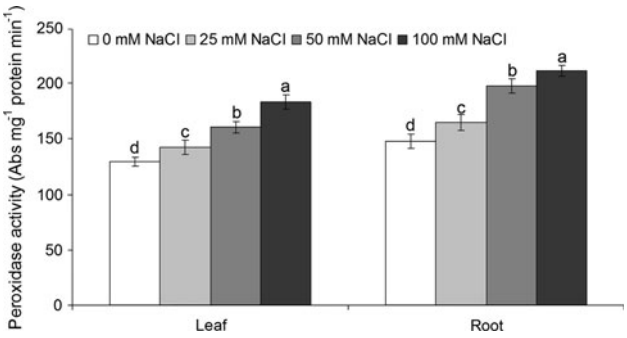

Fig. 5 Main effect of salt stress on peroxidase activity in soybean roots and leaves. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

regard root and shoot catalase activity under mild salinity stress (25 mM NaCl) whiles under medium and severe salinity stress (50 and 100 mM NaCl) natural roots had higher activity than hairy roots in both part of the plants (Table 4). In general, salinity increased catalase activity in roots and shoots however this increase was more pronounced in roots (Table 4). Peroxidase activity was affected by root type and salt stress in roots and shoots as the same (Table 1). Peroxidase activity in roots or leaves of non-transgenic plants was significantly higher than composite plants (Fig. 4). As expected, peroxidase activity increased with increasing salinity level (Fig. 5). There was another story about superoxide dismutase activity. Activity of this enzyme has just affected by salt stress and increased with increasing of salt concentration (Fig. 6).

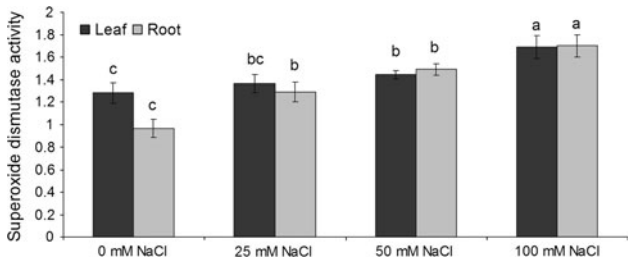

Fig. 6 Main effect of salt stress on superoxide dismutase activity in soybean roots and leaves. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

Malondialdehyde production

Malondialdehyde accumulated in roots and leaves of soybean plants exposed to salt stress. Malondialdehyde production was not affected by genistein in roots and leaves of non-transgenic plants, while in composite plants lipid peroxidation was significantly alleviated by genistein (Table 2). Genistein reduced malondialdehyde content in stressed plants (Table 3). The results showed that malondialdehyde accumulation due to salt stress, in roots and leaves of non-transgenic plants was considerably higher than composite plants (Table 4). In other words composite plants were more tolerant against salinity.

Proline accumulation

Genistein application had not significant effect on root proline accumulation in the natural or in the hairy roots; however proline accumulation in hairy roots was less than natural roots (Table 2). Although salt stress significantly increased proline accumulation in both type of roots, this increase was more noticeable in natural roots (Table 4). Three-way interaction analysis showed that the lowest proline accumulation was related to hairy roots treated with pre-incubated *B. japonicum* with genistein (Table 5).

Protein content

There was no significant difference between natural roots and hairy roots in case of leaf or root protein content (Table 1). Salt stress led to decrease in protein content (Fig. 7) while genistein application had positive effect and improved protein content (Fig. 8) especially under salt stress conditions (Table 3).

Discussion

The increase in shoot and root dry weight could be due to increased Nod factor production by the inoculum. Pre-incubating *B. japonicum* promotes expression of the common *nod* genes and, thus, the production of Nod factor, which can regulate plant morphogenesis (Spaink 1996). In addition, higher nodule number and nodule weights due to

Table 5 Significant three-way interaction between root type, genistein application and salt stress

Root type	Genistein (μM)	Salinity (mM)	Nodule number	Nodule weight (g)	Leaf proline (mg g^{-1}FW)
Natural roots	0	0	42.00fg	0.24gh	0.01f
		25	36.00gh	0.23h	0.01ef
		50	24.66i	0.16j	0.03bc
		100	16.66j	0.09k	0.06a
	10	0	74.66cd	0.35cd	0.01ef
		25	68.33de	0.35d	0.01ef
		50	47.00f	0.24gh	0.03bc
		100	43.66fg	0.24gh	0.06a
Hairy roots	0	0	74.33cd	0.33e	0.02de
		25	63.00e	0.32f	0.02d
		50	46.66f	0.25g	0.03c
		100	32.33hi	0.19i	0.04b
	10	0	102.00a	0.45a	0.01f
		25	98.33a	0.41b	0.01f
		50	90.00b	0.36c	0.01f
		100	82.00c	0.35de	0.01f

Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

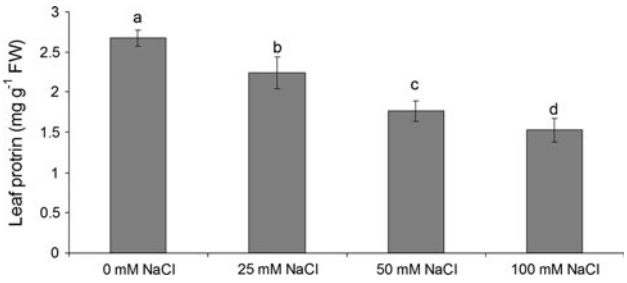

Fig. 7 Main effect of salt stress on soybean leaf protein content. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

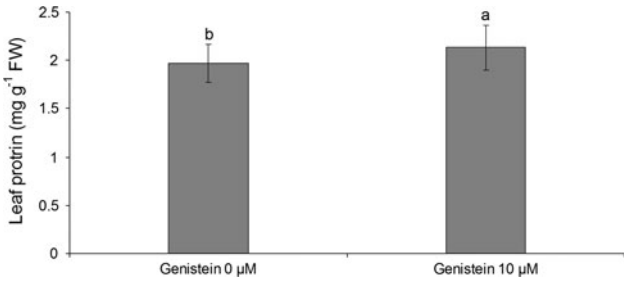

Fig. 8 Main effect of genistein on soybean leaf protein content. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by DMRT

genistein may increase nitrogen fixation resulting in higher shoot and root dry weights. These results are in accordance with the results of other researchers (Zhang and Smith 1996), who also found that genistein treatment increased dry matter accumulation of soybean. A decrease in shoot dry weight accompanied by a decline in root dry weight, i.e., altered root: shoot ratio, due to salt stress is a normal growth phenomenon (Hawkins and Lewis 1993). The detrimental effects of salinity on dry weight have been assigned to a direct inhibition of photosynthesis (Parida et al. 2003). According to Cheeseman (1988), salinity stress imposes additional energy requirements on plant cells and diverts metabolic carbon to storage pools so that less carbon is available for growth. On the other hand, effect of salinity on plant growth may result from impairment of supply of photosynthetic assimilates (Kraus and Weis 1991) and cell expansion in leaves can be inhibited by salt stress (Chartzoulakis and Klapaki 2000).

The inhibition of root growth in terms of root volume can be attributed to the inhibition of mitosis, reduced synthesis of cell wall components and changes in polysaccharide metabolism (Berkelaar and Beverley 2000). Our results are in agreement with the findings of Siroka et al. (2004) who have reported that salinity decreases root cell development. Furthermore, pre-incubating *B. japonicum* promotes expression of the *nod* gene (Kosslak et al. 1987) and, thus the production of Nod factor, which can increase root growth (Spaink 1996). Moreover, hairy roots typically show a distinct phenotype, fast growth, enhanced lateral branching roots, low doubling time (Beach and Gresshoff 1988) and plagiotropic root growth, presumably because of altered auxin metabolism (Liu et al. 2002). These characteristics of hairy roots offer additional advantages such as increase in root surface and volume which gives a greater contact between root and soil for water and nutrient uptake and better contact with Rhizobia which can hasten nodule formation and nitrogen fixation.

The increase in nodule number due to genistein application may have resulted from an increase either in number of infections initiated or in the proportion of infections progressing to nodule formation. Genistein causes expression of bacterial *nod* genes that produce bacterial Nod factor (Kondorosi 1992; Loh et al. 2002). The results obtained from this study are in agreement with Zhang and Smith (1997) who found that pre-incubation of *B. japonicum* with genistein increased nodule number and accelerated the onset of nitrogen fixation.

Decreased ability of nodules to reduce C_2H_2 under salinity has been well-documented for other legumes (Ferri et al. 2000). Our results suggest that genistein application under salinity stress could partly offset the inhibition of nitrogenase activity. The inhibition of nitrogenase activity by salt stress may be a consequence of the decrease in malate content in the nodules and it could be offset by an increase in the mean nodule weight (Soussi et al. 1999). Among nodular metabolic processes, reductions of bacteroid respiration, leghemoglobin production and alterations in the oxygen-diffusion barrier (Serraj et al. 1998) by salt stress have been reported as important factors that contribute to a decrease in nitrogen fixation (Delgado et al. 1994). Furthermore, it seems likely that some of this increase in nitrogenase activity was due to earlier nitrogen fixation hastened by genistein application, with the remainder due to the increased plant nodule numbers in the early vegetative growth stages. As mentioned before, hairy roots had the highest root volume, nodule number and nodule weight therefore have helped in more activity of nitrogenase enzyme.

Effects of salinity on chlorophyll content have been reported for other legumes (Al-Khanjari et al. 2002). Since nitrogen is a critical component of chlorophyll, and without sufficient quantities of this element, chlorophyll cannot be formed (Tucker 2004); legume–*Rhizobium* symbiosis plays an important role in nitrogen supplementation in these plants. Conversely, genistein as a signal molecule stimulates lipochito-oligosaccharide production by the bacterial symbiont (Lérougé et al. 1990) and improve legume–*Rhizobium* interactions during nodule formation and development. Based on these results, increase in chlorophyll by genistein may be due to improving nodulation and nitrogen fixation under salinity conditions. The inhibitory effects of salt on chlorophylls could be due to suppression of specific enzymes responsible for the synthesis of chlorophyll (Strogonove et al. 1970), an effect that depended on the biological processes and development stages of the plant and also on the type and concentration of the salts. Moreover, the decrease in chlorophyll may be attributed to increased chlorophyllase activity (Sudhakar et al. 1997). The lower reduction of chlorophyll content in composite plants (Table 4) might have been responsible for the higher nitrogen fixation and more available nitrogen in them because of more roots and active nodules production.

Many studies (Hernandez et al. 2001; Zhu 2001; Bor et al. 2003) have reported that salinity stress causes oxidative stress and enhances reactive oxygen species generation in plant tissues. However, even under optimal conditions many metabolic processes produce reactive oxygen species. Superoxide dismutase is an antioxidant enzyme with the ability to repair oxidation damage caused by reactive oxygen species. Thus, superoxide dismutase is considered a key enzyme for maintaining normal physiological conditions and coping with oxidative stress in the regulation of intracellular levels of reactive oxygen species (Mittler 2002). Also peroxidase is widely distributed in higher plants where it is involved in various processes, including lignification, auxin metabolism, salt tolerance and heavy metal stress (Passardi et al. 2005). Catalase, which is involved in the

degradation of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Mittler 2002). Plants possess efficient systems for scavenging reactive oxygen species that protect them from destructive oxidative reactions (Foyer et al. 1994). As part of this system, antioxidant enzymes are key elements in defence mechanisms. Many changes have been observed in the activities of antioxidant enzymes in plants under salt stress. The activity of antioxidant enzymes has been reported to increase under saline conditions (Meneguzzo et al. 1999; Meloni et al. 2003).

We found that genistein reduced malondialdehyde content in stressed plants; possibly genistein protected soybean plants from stress-induced membrane damage. In plant cells, lipid peroxidation leads to membrane permeability and loss of integrity, and ultimately to solute leakage and cellular damage (Bor et al. 2003). During salt stress, low levels of lipid peroxides have been related to the increased antioxidant capacity of salt tolerant/resistant species or cultivars (Ruiz et al. 2005; Radic et al. 2006), whereas high lipid peroxide levels were associated with salt-sensitivity (Masood et al. 2006; Koca et al. 2007). Accordingly, *Agrobacterium-rhizogenes*-transformed hairy roots were more tolerant to salt stress than those of non-transgenic.

Proline accumulation in response to environmental stresses has been considered as an adaptive trait concerned with stress tolerance (Rhodes and Hanson 1993). Proline may be acting as a compatible solute in osmotic adjustment (Perez-Alfocea et al. 1993). It may act as an enzyme protectant, stabilizes membranes and cellular structures during stress conditions, detoxifies free radicals and affects solubility of various proteins by interacting with their hydrophobic residues (Hong et al. 2000). The increase in the proline content under stress condition is due to breakdown of proline-rich protein or *de novo* synthesis (Tewari and Singh 1991). In the present study, natural roots accumulated high level of proline under salt stress as compared to hairy roots. Thus salt stress had less effect on disturbing of osmotic adjustment in hairy roots; this could be caused by an expanded root system in composite plants, which make them capable to absorb water quickly.

Changes in protein content are one of the results of salt stress in plant cells. One of the mechanisms affected by salt stress in plants was protein synthesis. It is known that soluble protein content is an important indicator of physiological status of plants. Salinity reduces both RNA amounts due to changes in cytoplasmic RNAaz activity and DNA levels as a result of disruption of synthesis mechanism. Yurekli et al. (2004) reported that total soluble protein content significantly decreased in salt sensitive *Phaseolus vulgaris*. We found that genistein application has led to increase in nitrogenase activity therefore protein content enhancement can be attributed to hastened nitrogen fixation.

Conclusion

The most interesting result of the study was that significant differences were found between natural roots and transformed hairy roots. Composite plants had extremely higher root volume, root dry weight, nodule number and nitrogenase activity. High root volume indicates that a plant can permeate a large volume of soil or that it has a high proportion of thick roots. Theoretically, such a plant would have more contact with soil and have extended rhizosphere so this advantage can help legumes to better contact with rhizobia and hasten nodulation. On the other hand, hairy roots had lower catalase and peroxidase activity under salinity stress, represents that less oxidative injuries were happened in this plants so that low malondialdehyde content or low proline accumulation confirm this saying. The genistein used in this experiment increased chlorophyll content, root volume, nodule number and nodule weight as well as nitrogenase activity. Plant growth was promoted by incubation of *B. japonicum* cells with genistein, but salt stress decreased dry matter accumulation, nodulation and nitrogen fixation. Whiles antioxidant enzyme activity, malondialdehyde content and proline accumulation increased due to salt stress. Incubation of *B. japonicum* cells with genistein could partially over come the inhibition of salinity on nodulation, nitrogenase activity and decrease lipid peroxidation. Generally, hairy roots obtained by infection of plants with *A. rhizogenes*, offers a promising system for better symbiosis and growth. The hairy roots are unique in their numerous sub-branches and fast growth so that these characteristics can be used as an advantage for better symbiosis especially under stress conditions. Although hairy roots are used only as scientific tools and there is no attempt to take advantage of their characteristics in crop production, changing the root architecture toward hairy roots must certainly be very substantial because it might be possible to increase nutrients uptake and water from the soil.

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