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South African Journal of Botany 79 (2012) 9 – 18

SOUTH AFRICAN JOURNAL OF BOTANY

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Morphological and physiological response of soybean treated with the microsymbiont *Bradyrhizobium japonicum* pre-incubated with genistein

A. Dolatabadian [a], S.A.M.M. Sanavy [a,*], F. Ghanati [b], P.M. Gresshoff [c]

[a] *Agronomy Department, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran*
[b] *Plant Biology Department, Faculty of Basic Science, Tarbiat Modares University, Tehran, Iran*
[c] *ARC Centre of Excellence for Integrative Legume Research, the University of Queensland, Brisbane, Australia*

Received 1 September 2011; received in revised form 26 October 2011; accepted 6 November 2011

Abstract

Genistein, a major root-secreted isoflavone of soybean (*Glycine max* (L.) Merr), is critical for the legume-*Bradyrhizobium* symbiosis as it induces several bacterial *nod*-gene systems. An experiment with soybean grown under salt stress was conducted to evaluate the effect of exogenous genistein addition to the *Bradyrhizobium* culture medium on subsequent nodulation, nitrogen fixation and selected plant physiological attributes. Five day-old plants (in pots) were inoculated with a liquid *B. japonicum* broth culture and irrigated with B&D solution containing either 0, 25, 50 and 100 mM NaCl. Four weeks after inoculation, maximum photochemical efficiency of PSII (Fv/Fm), photosynthetic rate, stomatal conductance, and transpiration rate were measured. Number of nodules per plant and apparent nitrogen fixation (as acetylene reduction activity) were determined. Salt stress decreased nodule number/plant and nitrogenase activity/plant and induced large changes of both photosynthetic parameters and antioxidant enzyme activity, compared to the control, genistein reversed the effect in each level of salinity tested. Moreover, pre-treatment of the microsymbiont with genistein enhanced maximum photochemical efficiency, photosynthetic rate, stomatal conductance and transpiration rate, while the enzymatic activities of catalase, superoxide dismutase and peroxidase in leaves and roots were not affected. It can be concluded that preincubation of the *B. japonicum* inoculant with genistein probably contributed towards growth in soybean via enhancement of nodulation and nitrogen fixation under both normal and salt stress conditions.

Keywords: Antioxidant enzymes; Development; Isoflavone; Photosynthetic parameters; Salinity; Symbiosis

1. Introduction

Soybean (*Glycine max* L. Merr.) is the most important legume crop in the world (Ferguson and Gresshoff, 2009), offering high-quality protein (about 40% of seed) and oil (about 20% of seed), and increasing the input of combined nitrogen as well as carbon into the soil. An integrated interaction between the soil bacterium *Bradyrhizobium japonicum* and its plant host results in the formation of nitrogen fixing root nodules (Ferguson et al., 2010). The symbiosis benefits both partners as the prokaryotic partner receives carbohydrate in the form of sucrose-derived malate (Udvardi et al., 1988), and the symbiotic bacteria provide the plant with nitrogenous compounds. When in symbiotic association with *B. japonicum*, soybean plants can fix up to 200 kg ha^{-1} yr^{-1} of nitrogen (Smith and Hume, 1987), reducing the need for expensive and potentially environmentally damaging nitrogen fertilizer (Zhang et al., 2002; Sutton et al., 2011).

In general, legume plants exude into their rhizosphere complex cocktails of sugars, flavones or isoflavones, which are perceived as *nod*-gene inducers in 'Rhizobium' bacteria. Several *nod*-genes collaborate to synthesize lipo-oligo-saccharides

* Corresponding author. Tel.: +98 21 44196522 23, +98 21 44194911 4; fax: +98 21 44196524.
E-mail address: Modaresa@modares.ac.ir (S.A.M.M. Sanavy).

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doi:10.1016/j.sajb.2011.11.001

(Nod Factors), decorated in strain-specific fashion, to induce two parallel developmental pathways, namely root epidermal or root hair infection as well as cortical and pericycle cell division (Mathews et al., 1989a, 1989b). Perception of Nod-factor requires a dimeric receptor protein (the Nod factor receptor) made up of NFR1 and NFR5 in soybean (Indrasumunar et al., 2010, 2011) and a complex downstream signaling cascade controlled by the plant (Ferguson et al., 2010; Reid et al., 2011). Invading rhizobia penetrate the new cells of the nascent nodule, finally entering a closer symbiotic relationship in inverted plant membrane-bound vesicles called 'symbiosomes'. Here carbon and nitrogen compounds are exchanged. The combined processes lead to the formation of lumps or nodules along the root in which nitrogen fixation takes place.

Specifically 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) and also the bacterial host specific genes (such as *nodZ* and *nodFE*; Horvath et al., 1986). Almost nothing 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 (LCO) nod factors that deform root hairs and initiate host cell differentiation (Fisher and Long, 1992). The major lipochito-oligosaccharide for soybean nodulation consists of five β-1, 4-linked N-acetylglucosamines with various modifications (such as methyl-fucose) of the reducing and non-reducing ends, in a species-specific manner that plays a key role in determining the host specificity of the rhizobia (Supanjani et al., 2006). Daidzein and genistein are the major signal components of soybean root extracts (Kosslak et al., 1987, 1990; Sutherland et al., 1990), though daidzein has less *nod* gene-inducing ability than genistein (Mathews et al., 1989b; Sutherland et al., 1990). These substances are operative at very low concentrations (10^{-6} – 10^{-7} M) and stimulate bacterial *nod* gene expression within minutes (Ali et al., 2001). The isoflavone genistein also has antifungal activity (Rivera-Vargas et al., 1993), is the precursor to the phytoalexin kievitone produced by *Phaseolus vulgaris* (Garcia-Arenal et al., 1978) and is involved in the pathway leading to the glyceollin response of soybean cells (Graham and Graham, 2000).

Studies reported by Zhang et al. (1996), Zhang and Smith (1997) and several other studies (Bandyopadhyay et al., 1996; Pan and Smith, 1998) showed that pre-incubation of *B. japonicum* with genistein increased nodule number and nitrogen fixation.

The genistein was reported to inhibit binding of the auxin transport inhibitor naphthyl pthalamic acid (NPA) in zucchini (*Cucurbita pepo*) hypocotyl segments (Jacobs and Rubery, 1988). In legumes, flavonoid compounds were found to accumulate in cells near the site of nodule initiation. It was suggested that this leads to localized auxin accumulation, resulting in nodule organogenesis (Mathesius et al., 2000).

All stages of the soybean nitrogen fixation symbiosis are inhibited by suboptimal conditions such as drought, temperature, acidity and salinity. *Rhizobium* growth is sensitive to high osmotic pressures. So salinity can affects microbial activity via hyperosmotic stress and depress symbiotic performance (Rao et al., 2002; Rout and Shaw, 2001). On the other hand salt stress limits plant productivity through diminished photosynthetic efficiency, carbon metabolism, leaf-chlorophyll content (Seeman and Critchley, 1985) as well as nitrogen fixation in legumes (Delgado et al., 1994; Ferri et al., 2000; Soussi et al., 1999). Salt stress (Miransari and Smith, 2007) mainly hinders the early events, more related to the physiology of the root hair such as its growth, diameter, structure and curling (Miransari et al., 2006).

Since genistein plays an important role as a signal molecule in the early stages of symbiosis establishment between soybean and *B. japonicum*, we were interested in whether salinity stress disrupt signalling, and whether pre incubation of *B. japonicum* with genistein could increase soybean nodulation and nitrogen fixation. The objective of this study was to evaluate soybean responses to salinity stress and genistein pre-treated *B. japonicum* inocula in terms of nodulation, nitrogen fixation and selected physiological and biochemical parameters.

2. Material and methods

2.1. Chemical materials

Hydrogen peroxide, Ethanol 96%, perlite and vermiculite (grade 2 or 3), 10 cm diameter plastic pots Broughton and Dilworth solutions [Add 500 ml of each stock solution per liter: solution A (2 M $CaCl_2$), solution B (1 M KH_2PO_4), solution C (20 mM Fe-citrate), solution D (0.5 M $MgSO_4$, 0.5 M K_2SO_4, 2 mM $MnSO_4$, 4 mM H_3BO_4, 1 mM $ZnSO_4$, 4 mM $CuSO_4$, 0.2 mM$CoSO_4$, 0.2mMNa_2MoO_4)], *Bradyrhizobium japonicum* strain CB1809, yeast extract-mannitol broth (YMB), Genistein, NaCl, Acetone, Riboflavin, L- Methionine, EDTA, Na_2CO_3, Nitro blue tetrazolium (NBT), Sulphosalicylic acid, Guaiacol, Glacial acetic acid, Ninhydrin, Toluene, Thiobarbituric acid, Trichloroacetic acid. In this study the chemicals used were obtained from Sigma Chemical Company (N.Y., USA) or Merck Chemical Company (Deisenhofen, Deutschland).

2.2. Plant growth

Soybean seeds (*Glycine max* L. Merr.) cv. L17 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 water. Four seeds were sown in 10 cm diameter plastic pots containing autoclaved perlite and vermiculite (1:1 ratio) at depth of 1–2 cm. The pots were placed in a growth cabinet (L/D = 16/8 h, T = 28/25 °C), and watered with full strength of Broughton and Dilworth solution (Broughton and Dilworth, 1971). Throughout the growth period, each pot received 50 ml of B&D nutrient solution. Each treatment was replicated three times with four plants per pot.

2.3. Inocula preparation and inoculation

The inoculant was produced by culturing *Bradyrhizobium 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. The lipochito-oligosaccharide biosynthesis was induced by genistein over the course of 48 h. The culture appeared greyish-white when it was compared with yeast extract-mannitol broth without genistein. The five day old plants were inoculated with yeast extract-mannitol broth. One millilitre of inoculant per plant was applied by pipette onto the rooting medium and watered with B&D solution.

2.4. Salt stress induction

Once the symbiosis was well-established, the plants were subjected to salt stress by adding NaCl to the nutrition solution at 25, 50 and 100 mM. Control plants were maintained in a NaCl-free solution. Twice a week, the plants were watered with distilled water to prevent salt accumulation.

2.5. Photosynthetic parameters

Four weeks after inoculation, maximum photochemical efficiency of PSII (Fv/Fm), photosynthetic rate (P_n: μmol m^2s^{-1}), stomatal conductance (g_s: mol m^2s^{-1}) and transpiration rate (E: mmol m^2s^{-1}) were measured. Maximum photochemical efficiency was determined by a portable fluorometer (PAM-2000, H Wals GmbH, Effeltrich, Germany) connected with a leaf-clip holder and with a trifurcated fibre-optic (2010-F, Walz). Before measurement, the leaves were dark-adapted for 15 min. The maximum photochemical efficiency of PSII was determined from the ratio of variable (Fv) to maximum (Fm) fluorescence. A Li-6400 instrument (Li-Cor, Lincoln, USA) was used for measurement of photosynthetic rate, stomatal conductance and transpiration rate.

2.6. Plant measurement

Plants were removed from pots 30 days after inoculation. The roots were gently washed with water to remove all perlite and vermiculite and then nodule number, nodule weight, root volume as well as shoot and root weight were determined. Afterwards the shoot and root were detached and dried at 70 °C for 24 h to calculate dry weight. A sample of decapitated root with nodules of each plant was used for the nitrogenase (ARA) assay. Additionally, fresh samples were collected, quickly frozen in liquid nitrogen and stored in a deep-freezer (−80 °C) for enzyme assays and analytical determinations.

2.7. Nitrogen fixation

Nitrogen fixation of roots was assayed using the nitrogenase acetylene method reduction (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 a 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 variable oxygen barrier.

2.8. Chlorophyll

Chlorophyll was extracted in 80% acetone from the leaf samples according to the method of Arnon (1949). Extracts were filtered and total chlorophyll content was determined spectrophotometrically at 645 and 663 nm, respectively. Chlorophyll content was expressed as mg g^{-1} fresh weight according to Eq. (1) (Arnon, 1949).

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

where: V = final volume; W sample weight

2.9. Antioxidant enzyme activity

Catalase 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 Abs mg^{-1} protein min^{-1}.

Superoxide dismutase activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue 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 nitroblue tetrazolium.

Peroxidase 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 Abs mg^{-1} protein min^{-1}. The activity of each enzyme was expressed on a protein basis. Protein concentration of the crude extract was measured by the method of Bradford (1976).

2.10. 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 12000 rpm 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 one 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.

2.11. 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 MDA extinction coefficient ($\varepsilon = 155\ \mu M\ cm^{-1}$) and the results were expressed in nmol MDA g^{-1} fresh weight.

2.12. Leaf water potential

The amount of pressure required to force water from the cut end of the petiole equals the leaf's water potential (Smith and Prichard, 2003). A fully expanded leaf was severed and placed in a pressure bomb with its petiole protruding through the seal. Pressure was applied to the tissue until water just appeared at the cut end of the petiole, at which time pressure was recorded.

2.13. Statistical analysis

The experiment was structured following a completely randomized design arranged in a 2×4 factorial with three replications. 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 between two factors 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 LSD at the 5% probability level.

3. Results and discussion

Various morphological and physiological factors in soybean were negatively affected by salinity stress while treatment with *B. japonicum*, pre-incubated in the presence of genistein, tended to circumvent these stress effects (Table 1). The interaction between genistein and NaCl was significant on maximum photochemical efficiency (Fv/Fm), transpiration rate, photosynthesis rate, nodule number, nodule weight, acetylene reduction activity and chlorophyll content (Table 2). On the other hand stomatal conductance, photosynthetic rate, and transpiration rate were reduced by the addition of NaCl while pre-incubation of *B. japonicum* with genistein increased these parameters when compared with the exposure of plants to NaCl alone (Table 1 and 2). Many studies have reinforced that NaCl causes plant growth inhibition by changes in photosynthetic rate (Gupta et al., 2002), stomatal conductance (Schröppel-Meier and Kaiser, 1988) and transpiration rate (Walters and Horton, 1991). Salt stress causes stomatal closure, which reduces the CO_2/O_2 ratio in leaves and inhibits CO_2 fixation. In this study, we found statistically significant differences among salinity levels for stomatal conductance. Photosynthesis and transpiration rate declined with increasing NaCl concentration due to its adverse impact on stomatal conductance this effect was more pronounced when genistein was not applied into inoculum. The highest and the lowest transpiration and photosynthesis rate were recorded in control plants inoculated with 10 μM genistein pre-incubated *B. japonicum* cells and the highest salt concentration without genistein, respectively (Table 2). Here-observed increases of stomatal conductance and transpiration rate with concomitant increased photosynthesis rate can be attributed to more nitrogen fixation and nitrogen availability due to an enhancing effect of genistein on nodulation this result is in agreement with Miransari and Smith (2008) reports.

We observed a close correlation between the photosynthetic rate and nitrogenase activity ($r^2 = 0.90^{**}$). Moreover, the increase in nitrogen fixation, satisfying plant demands, would stimulate photosynthesis. On the other hand, large portions of leaf nitrogen are associated with Rubisco, the key enzyme of CO_2 assimilation (Horton, 2000), therefore it would be expected that improvement of nodulation and nitrogen fixation can promote photosynthesis. These results showed that salinity and genistein application had a significant effect on Fv/Fm (Table 2). Fv/Fm ratio decreased with increasing salt concentration. In contrast, genistein treatment alleviated the effects of salt stress and enhanced Fv/Fm (Table 2). Alternatively, salinity induced significant changes in Fv/Fm in sorghum. According to our results, it seems that the effects of salt stress on Fv/Fm in soybean plants depend on the enhancing effects of genistein on nodulation and plant growth.

Significant differences among four different salinity levels and two genistein levels on shoot and root dry weight of soybean were observed (Table 1). Salinity decreased shoot, root and total dry weight while pre-inoculation of *B. japonicum* with genistein led to an increase of dry matter in both portions of the soybean plants (Table 1). The detrimental effects of NaCl on dry weight may be due to the direct effect on photosynthesis (Parida et al., 2003) and plant's ability to seek nutrients in the soil and transport them to growing shoots. Decrease in shoot dry weight accompanied by a decline in root dry weight is a normal growth phenomenon (Hawkins and Lewis, 1993). 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). Decreases in root and shoot mass have been reported in chickpea

Table 1
The main effects of salinity and genistein levels on stomatal conductance, root dry weight, shoot dry weight, total dry weigh, root volume, catalase, peroxidase and superoxide dismutase activity, malondialdehyde and proline accumulation (Plants harvested at 30 days after inoculation).

Treatments	level	Stomatal conductance (mol m^2s^{-1})	Root dry weight (g)	Shoot dry weight (g)	Total dry weight (g)	Root volume (cm^3)	Leaf water potential (−Mpa)	Catalase (Abs mg^{-1} protein min^{-1})		Peroxidase (Abs mg^{-1} protein min^{-1})		Superoxide dismutase (% inhibition)		Malondialdehyde (nmol MDA g^{-1} fresh weight)		Proline (mg g^{-1} fresh weight)	
								Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Genistein level	0 μM	0.02b	0.39b	0.73b	1.12b	9.00b	6.71a	143.65a	167.79a	159.25a	183.33a	1.43a	1.38a	1.75a	2.35a	0.03a	0.04a
	10 μM	0.04a	0.55a	1.44a	1.99a	12.41a	5.35b	140.74a	161.59a	157.45a	182.89a	1.37a	1.35a	1.77a	2.32a	0.03a	0.04a
LSD (5%)		0.005	0.14	0.12	0.23	1.24	0.29	5.98	16.27	3.84	2.01	0.15	0.09	0.08	0.07	0.003	0.003
Salinity level	0 mM	0.05a	0.97a	1.84a	2.82a	11.83a	3.73d	104.88c	113.71c	134.33d	152.60d	1.26b	1.04c	1.35c	1.96d	0.01c	0.02d
	25 mM	0.04b	0.52b	1.15b	1.67b	11.83a	4.51c	107.73c	124.15c	147.49c	167.62c	1.34b	1.33b	1.42c	2.15c	0.01c	0.03c
	50 mM	0.03c	0.27c	0.91c	1.19c	10.66a	7.20b	161.88b	192.56b	165.58b	199.91b	1.37b	1.34b	1.81b	2.44b	0.03b	0.05b
	100 mM	0.02d	0.10c	0.43d	0.54d	8.50b	8.70a	194.27a	228.38a	185.99a	212.30a	1.76a	1.63a	2.45a	2.77a	0.06a	0.06a
LSD (5%)		0.007	0.20	0.18	0.33	1.76	0.42	8.45	23.02	5.43	2.85	0.21	0.13	0.12	0.09	0.004	0.005
Sources of variation	Degree of freedom																
Genistein (G)	1	**	*	**	**	**	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Salinity (S)	3	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
G × S	3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Error	16	0.00	0.02	0.02	0.07	2.08	0.11	47.76	353.74	19.74	5.43	0.03	0.01	0.00	0.00	0.00	0.00
Coefficient of variations (%)		15.06	34.98	13.78	17.57	13.47	5.70	4.86	11.41	2.80	1.27	12.68	8.12	5.62	3.49	11.72	9.13

ns; not significant, * significant at $P<0.1$; **significant at $P<0.05$. Values within the each column and followed by the same letter are not different at $P<0.05$ by an ANOVA protected LSD Test

Table 2
Interaction of salinity and genistein levels on maximum photochemical efficiency, transpiration rate, photosynthetic rate, nodule number, nodule weight, nitrogenase activity, chlorophyll content and leaf water potential (Plants harvested at 30 days after inoculation).

Genistein level	Salinity level (mM)	Fv/Fm	Transpiration rate (mmol m^2s^{-1})	Photosynthetic rate (μmol m^2s^{-1})	Nodule number	Nodule weight (g)	Nitrogenase activity (μmol C_2H_4 $core^{-1}$ h^{-1})	Chlorophyll (mg g^{-1} fresh weight)
(0 μM)	0	656.33e	1.48c	14.45 d	42.00 d	0.24b	7.47e	2.91c
	25	644.66f	1.35de	12.52e	36.00e	0.23c	6.59f	2.33 d
	50	632.00 g	1.28e	10.85ef	24.66f	0.16 d	6.22 fg	1.54 g
	100	613.00 h	0.99f	10.60f	16.66 g	0.09e	5.76 g	1.39 g
(10 μM)	0	716.66a	1.86a	25.50a	74.66a	0.35a	18.16a	3.65a
	25	687.33b	1.58b	20.13b	68.33b	0.35a	17.29b	3.42b
	50	677.33c	1.53bc	18.33bc	47.00c	0.24b	10.28c	2.15e
	100	664.66 d	1.44 cd	16.78c	43.66 cd	0.24bc	8.10 d	1.76f
LSD (5%)		8.00	0.10	1.81	4.92	0.007	0.62	0.17
Sources of variation	Degree of freedom							
Genistein	1	**	**	**	**	**	**	**
Salinity	3	**	**	**	**	**	**	**
Genistein × Salinity	3	*	*	**	*	**	**	**
Error	16	21.37	0.00	1.09	8.08	0.00	0.12	0.01
Coefficient of variations (%)		0.69	4.10	6.47	6.44	1.89	3.59	4.28

* Significant at $P<0.1$; **significant at $P<0.05$, Values within the each column and followed by the same letter are not different at $P<0.05$ by an ANOVA protected LSD Test.

plants (Singh et al., 2001). We observed that 10 μM genistein significantly increased shoot and root dry weight relative to the control. In addition, at all levels of salinity, genistein resulted in higher nodule number and nodule weight compared with lack of genistein in stressed plants (Table 2). Indicating that genistein pre-inoculation caused activation and hence more efficiency of nodule bacteria to nitrogen fixation and hence, increased soybean growth. It has been reported that genistein stimulated plant growth (e.g., greater leaf area) through activation of *nod* genes in interacting *B. japonicum* (Zhang and Smith, 1995).

Root volume was reduced in response to salinity (Table 1). Significant difference was observed when NaCl concentration was increased from 50 mM to 100 mM. The inhibition of root growth in term of root volume can be attributed to the inhibition of mitosis, reduced synthesis of cell wall components, damage to the Golgi apparatus and changes in polysaccharide metabolism (Berkelaar and Beverley, 2000). Our findings are consistent with the findings of Siroka et al. (2004) who have reported that salinity decreases root cell development. The effect of genistein on root volume was also significant (Table 1). One possible explanation is that root volume increases with genistein because more photosynthate is transferred to the roots as a result of increased nitrogen fixation and photosynthesis. Moreover, this could be due to the Nod factor production by the inoculum. Pre-incubating *B. japonicum* promotes expression of the *nod* gene (Kosslak et al., 1987) and, thus the production of Nod factor, which can regulate plant morphogenesis at very low concentrations (Spaink, 1996).

Nodule initiation in the legume-*Rhizobium* symbiosis involves a complex interaction between host root, rhizobial strain, and the environment. In this study, we showed that salt treatments significantly reduced nodule number and nodule weight per plant while inoculated soybean plants with genistein pre-incubated *B. japonicum* cells improved soybean nodulation (Table 2). Reduced nodule formation could have been due to the adverse effects of salinity on nodule initiation. In addition, nodule weight decreased under salinity stress it seems that it is because of less photosynthate transport to the roots. The adverse effects of salinity on the legume-*Bradyrhizobium* symbiosis is through affecting rhizobial survival and growth, hindering the infection process, suppressing nodule function, and reducing plant ability to photosynthesis (Elsheikh and Wood, 1995). Our results also show that pre-incubation of *B. japonicum* with 10 μM genistein increased nodule number and nodule weight per plant. This could be due to an increase either in the number of infections initiated or in the proportion of infections leading to nodule formation (Zhang and Smith, 1995). The significant interaction effects also verify that genistein plays a direct role in overcoming of salinity. Many studies have been reported that genistein addition stimulates the production of lipochito-oligosaccharide under normal and stressful conditions (Lerouge et al., 1990; Zhang and Smith, 1995) which in turn elicits root hair deformation, cortical cell division and nodulin gene expression in soybean roots (Spaink, 1996). Faster formation of nodules in soybean plants and increased nodule weight following genistein treatment were also found by Pan et al. (1998).

The effects of genistein on the establishment of soybean–*Rhizobium* symbiosis (Eckardt, 2006), increased the nodule numbers and nodule weight hence resulted in the acceleration of C_2H_2 reduction under salt stress conditions. This showed that the effect of salinity on soybean nodulation and nitrogen fixation is less when plants were treated with pre-incubated *B. japonicum* with genistein. Acetylene reduction assays for nitrogenase activity showed that the maximum and the minimum C_2H_4 concentration were determined from the treated plants with pre-incubated *B. japonicum* with 10 μM genistein under no stress condition and treated plants with 100 mM NaCl without genistein application, respectively (Table 2). It would seem that increase of C_2H_2 reduction can be due to an increase in nodule number as well as nodule weight affected by genistein. Decreased ability of nodules to reduce C_2H_2 under salinity has been well-documented for other legumes (Ferri et al., 2000). The inhibition of acetylene reduction by salt stress may be due to a limitation of oxygen diffusion in nodules or due to toxic effects of Na or Cl accumulation (Serraj et al., 1998). On the other hand, reduction of photosynthetic activity by salt can also reduce nitrogen fixation.

In this experiment there are two possible explanations for the stimulative effects of genistein on nitrogen fixation improvement under conditions of salt stress. First, since genistein has been identified as a major inducer of *nod* genes in *B. japonicum* (Kosslak et al., 1987), genistein could have increased the infection rate by endogenous *B. japonicum*, resulting in increased soybean nodulation and nitrogen fixation. Second, increased C_2H_2 reduction could be partially due to other functions of genistein such as role of genistein in overcoming adverse effect of salinity (Miransari and Smith, 2009). Our results imply that increases of nitrogen fixation would lead to more pronounced nodulation by the addition of genistein. Consistently, increase in nitrogen fixation would correspond to the dilution of *nod* gene inducers from the root exudates and *nod* factors formed by bacteria.

Salinity significantly increased the leaf water potential while genistein decreased. It is well-known that, salt stress reduces root hydraulic conductivity resulting in decreased water flow from roots to shoot. This decrease in water flow due to salt stress may cause a lowering in leaf water content that would result in stomatal closure in order to maintain their water status (Robinson et al., 1997). Reduction in leaf water potential because of genistein application into inocula could be due to this probability that genistein can seriously change the photosynthetic carbon metabolism, leaf chlorophyll content, photosynthetic efficiency as well as root growth, through improvement of symbiosis relationships, nodulation and nitrogen fixation. Furthermore, the decrease in leaf water potential may be due to the accumulation of osmolytes, which are direct products of photosynthesis (Monneveux and Belhassen, 1996).

Saline stress led to the yellowing of leaves, which ultimately resulted in significant damage to the chlorophyll pigments. Similar results have been reported for other legumes (Al-Khanjari et al., 2002). Leaf chlorophyll content was significantly reduced in

soybean plants either with or without genistein in bacterial growth medium. The inhibitory effects of salt on chlorophylls could be due to suppression of specific enzymes responsible for the synthesis of chlorophyll. Alternatively, the decrease in chlorophyll may be attributed to increased chlorophyllase activity (Sudhakar et al., 1997).

Chlorophyll content was increased on account of genistein application under conditions of salt stress (Table 2). 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. Genistein as a signal molecule stimulates lipochito-oligosaccharide production by the bacterial symbiont (Lerouge et al., 1990) and improve legume–*Rhizobium* interactions during nodule formation and development.

Malondialdehyde content can serve as an indicator of the rate of oxidative processes in cells. The effects of NaCl and genistein on malondialdehyde content are presented in Table 1. Genistein application had no significant effect on malondialdehyde accumulation in roots and leaves. In contrast, salinity stress increased malondialdehyde whether in leaf tissue or root. Malondialdehyde results indicated that, lipid peroxidation started to increase at 50 mM NaCl in leaves. In other words, there was no significant difference between control and 25 mM NaCl treatments. Our results indicated that root cells were more sensitive in response to salt stress because malondialdehyde content in roots was significantly higher than in leaves (Table 1). Accumulation of malondialdehyde was reported in a number of salt-sensitive plants (Gehlot et al., 2003; Luna et al., 2002; Mittova et al., 2002).

In this study, proline content increased in NaCl treated plants while it was not affected by genistein (Table 1). In leaves, proline accumulation was initiated at 50 and 100 mM NaCl stress as compared to control and 25 mM NaCl treatments. Root proline content increased at 25 mM NaCl. Proline accumulates in plant tissues under saline stress and this substance is suspected of contributing to osmotic adjustment (Delauney and Verma, 1993). Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Rudolph et al., 1986; Santoro et al., 1992). Proline may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995), and as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).

Activities of antioxidant enzymes in roots and shoots exhibited a similar change. That is to say, genistein application had not significant effect on enzyme activity but obvious differences existed due to salinity stress (Table 1). Specifically, peaks of activities of antioxidant enzymes in roots and shoots appeared at the highest NaCl concentration. Activities of catalase and peroxidase in the two parts of plant showed a similar trend, but the activity of superoxide dismutase in leaves was significantly greater than roots (Table 1). Additionally, the increase in superoxide dismutase activity in roots arose earlier. In other words, superoxide dismutase activity in roots is more sensitive than shoots. As mentioned previously, the peak of antioxidant enzyme activity in roots and shoots appeared at the highest salinity level. It indicates that reactive oxygen species scavenging system was activated efficiently and removed reactive oxygen species. Hernandez et al. (2001) suggested that salt stress can cause oxidative stress and enhance the antioxidant capacity in the leaf apoplast of pea cultivars. 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). Even under optimal conditions many metabolic processes produce reactive oxygen species. 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 defense 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 in the case of salt-tolerant cotton (Meloni et al., 2003), rice (Fadzilla et al., 1997) and wheat (Meneguzzo et al., 1999).

4. Conclusion

Overall, the results reported here show that salinity significantly reduced photosynthetic parameters, shoot and root dry weights, root volume, nodule number, nodule weight, nitrogenase activity, and chlorophyll content while antioxidant enzyme activity, proline and malondialdehyde content increased. Additionally, the results indicate that genistein was able to increase photosynthesis, nodulation and nitrogen fixation and subsequently soybean growth under saline and non-saline conditions. However, antioxidant enzymes activity was not affected by genistein application. In conclusion, our results indicate that pre-incubation of *B. japonicum* with genistein improves nodulation and nitrogen fixation of soybean subjected to salt stress and it would be recommended to use under saline soils to attain desirable symbiosis and soybean production.

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Edited by AK Cowan