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

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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

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(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