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Original article

Alleviating salt stress on soybean (*Glycine max* (L.) Merr.) – *Bradyrhizobium japonicum* symbiosis, using signal molecule genistein

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ABSTRACT

For the onset of symbiosis process between soybean (*Glycine max* (L.) Merr.) and *Bradyrhizobium japonicum*, signals should be exchanged. Salinity has inhibitory effects on the symbiosis between the two partners. Hence, a greenhouse experiment was planned to: (1) determine the stressful effects of salinity on soybean and *B. japonicum* symbiosis, hypothesizing that they can inhibit the signal exchange process between the two partners, and (2) determine if the addition of genistein (a *nod* gene inducer) to *B. japonicum* (strain 532C) inocula could overcome the stressful effects of salinity on the *Bradyrhizobium* – soybean symbiosis. Three levels of salinity (control, 36 and 61 mmolar or 3.6 and 6.1 mmhos/cm) and three levels of genistein (0, 5 and 20 μM) were combined in a factorial fashion in four replicates. Soybean plants were harvested at three different times including 20, 40 and 60 days after inoculation (DAI). Genistein enhanced soybean nodulation and growth, and such effects became greater with time under high salinity levels. For example, at 60 DAI the enhancing effects of genistein on the symbiosis process in soybean was more pronounced at the highest level of salinity. The significant interaction effect between genistein 5 μM and salinity 61 mmolar may reveal the direct role of genistein 5 μM in overcoming the stressful effects of salinity on the symbiosis between *B. japonicum* and soybean, and hence, plant growth. This novel finding may be very useful to increase soybean yields in salty croplands.

1. Introduction

The relationships between plants and microbes, which are a regular and continuous feature of the biological world, have been a center of attention for many years for researchers all over the world. The benefits of such interactions have been intensely studied in recent years. Biological nitrogen fixation (BNF) is one of those interactions, in which the bacteria and the host plant provide each other with fixed atmospheric N and sugars, respectively [12]. In this symbiosis the physiological and biochemical aspects of both symbionts are involved [29].

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For the process of symbiosis and hence, BNF to begin, the exchange of signal molecules between the plant and microbes is necessary. Sensitive chemoreception systems in plants are responsible for plant reactions to signal substances, produced by microorganisms [3,5,19]. Researchers have shown that many isoflavonoids stimulate infection of plant roots by beneficial microbes and such mutualistic bacteria elicit the exudation of some classical phytoalexins [7]. For example, during the establishment of the symbioses between soybean and *Bradyrhizobium*, and between *Phaseolus* and *Rhizobium*, diadzein, genistein, and coumestrol serve as signal molecules. The transcription of nodulation genes (*nod*, *nol*) in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli* [8] are specifically induced by these three isoflavonoids [9].

The levels of flavonoids are directly effective on nodule formation and N_2 fixation [2]. Plant age and physiological conditions may determine the amount and type of the excreted signal molecule [11]. In addition, nodulation factors (Nod factors) of rhizobia are among the microbial substances perceived as signals by plants [9,20]. One example of the complexity of elicitor perception by the host plant is the soybean – *Bradyrhizobium* interaction.

The formation of isoflavonoids in legumes can be affected by abiotic elicitors. Soil stresses such as salinity, pH and low root zone temperature may partially or completely inhibit the early steps of symbiosis, including the process of signal exchange between legumes and *Bradyrhizobium* bacteria [15,16,21], and affect the bacterial growth [6] and hence, N_2 fixation [4]. High salt concentrations [10] limit nodulation in a way that is not related to the rhizobial number in the rhizosphere. Salt stress [15,21] mainly hinders the early events, more related to the physiology of the root hair such as its growth, diameter, structure, and curling [20]. On the contrary, changes in osmolarity appear to slightly affect the early events related to the rhizobial genome, such as the *nodD* and *nodABC* expression in *B. japonicum*. Hence, it seems unlikely that desiccation or salinity have direct effects on the competition between strains.

Depending on the time of salt application (before or after bacterial inoculation) the appearance of stressful effects may be somehow different [31]. Water stress, ions imbalances and toxicity are the effects of salt stress on plant growth, influencing the photosynthesis rate and nodule activity directly. After the establishment of the symbiosis, plants fixing N_2 and plants fed with N respond similarly to salinity [15]. The toxic and osmotic effects of salinity may also adversely influence soil microorganism [4]. In addition, the rhizobia by themselves are more tolerant to salt stress than the legumes – *Rhizobium* symbiosis and hence than the nodules formation [26,33].

High levels of salinity result in little or no root hair curling and deformation, and eventually, complete inhibition of nodulation [31,34]. In the presence of NaCl usually less infection threads are formed in the root hairs [34]. Researchers have shown that the addition of the signal molecule, genistein may reduce the stressful effects of low root zone temperature on the soybean – *Bradyrhizobium* symbiosis, both under controlled environment [23,35,37] and field conditions [38,39] by increasing soybean yields [26,38]. Genistein excretion was reduced in some and not all [25] soybean cultivars under low root temperature conditions. Also Miransari et al. [20] found that addition of Nod factor; lipo-chitooligosaccharide can overcome the stressful effects of low pH on soybean root hair curling. The effects of low root zone temperatures are also through influencing the ability of *B. japonicum* cells to detect genistein, as under such conditions *B. japonicum* cells may require higher levels of genistein for *nod* gene activation [23,37].

Because soil stresses affect the early stages of soybean – *B. japonicum* symbiosis and hence nodulation, we hypothesized that the inhibitory effects of salinity are through affecting the signal exchange process between the two partners. Since to our knowledge there is only one field experiment [21] on the effects of signal molecule on the soybean – *B. japonicum* symbiosis under saline conditions, we therefore performed this experiment. The objectives were to: (1) determine the stressful effects of salinity on the soybean – *B. japonicum* symbiosis, hypothesizing that such effects are related to the inhibitory effects of salinity on the signal exchange process between the two partners, and (2) determine if the addition of genistein (a *nod* gene inducer) to *B. japonicum* (strain 532C) inocula could overcome the stressful effects of salinity on the *Bradyrhizobium* – soybean symbiosis.

2. Materials and methods

2.1. *Seedling preparation*

Seeds of the soybean (*Glycine max* [L.] Merr.) cv AC Bravor were surface sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20) and rinsed several times with distilled water [3]. The seeds were planted in plastic trays containing vermiculite. Seven-day-old seedlings at the vegetative-cotyledonary stage (unifoliate leaves unrolled sufficiently so that the edges should not be touching [12]) were transplanted into 15-cm plastic pots containing a sterilized Turface (Applied Industrial Materials Corp., Deer field, IL): sand (1:1, v/v) mixture then inoculated at 1 ml of inocula (containing 10^8 cells) per plant and maintained in the greenhouse (25 °C, 50–60% humidity).

2.2. *Inoculum production*

To produce inoculum, *B. japonicum* strain 532C [14] was cultured in yeast extract-mannitol broth [32] while shaken at 150 rpm in 500 ml flasks at room temperature. Sterile genistein solutions were used to preincubate the bacteria.

2.3. *Salinity and genistein (signal molecule) treatments*

The levels of genistein 0, 5, and 20 μM were tested [37]. Genistein was added under aseptic conditions to the cultures 24 h before inoculation. Using NaCl, preparations of a modified Hoagland's N free nutrient solution [13] were adjusted to the desired levels of salinity: control (with no NaCl) 36, and 61 mmolar (3.6 and 6.1 mmhos/cm, respectively). To prepare the Hoagland's N-free solution, instead of using $CaNO_3$ and KNO_3, 1 mM of $CaCl_2$, K_2HPO_4, and KH_2PO_4 were used [37]. These solutions were used right after inoculation (7-day plants) two to three times a week, at 100, 150, and 200 ml per