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Effect of *R. leguminisarum* Pre-incubated with Inducers, Naringenin and Methyl-jasmonate, on Nitrogen Fixation and the Growth of Pea at Different Salinity Levels

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ABSTRACT: The legume-rhizobia symbiosis is an important source of plant growth and nitrogen fixation for many agricultural systems. This study was conducted to investigate the effects of salinity stress on nitrogen fixation and growth of pea (*Pisum sativum* L.), which has antimutagenic activities against chemical mutagen, inoculated with *R. leguminosarum* bv. viciae cultured with additional plant-to-rhizobia signal compounds, naringenin (NA,15 uM), methyl-jasmonate (MJ, 50 uM) or both, under greenhouse conditions. Three salinity levels (0.6, 3.0 and 6.0 dS m^{-1}) were imposed at 3 days after transplanting and maintained through daily irrigations. Addition of signal compounds under non-stress and stress conditions increased dry weight, nodule numbers, leaf area and leaf greenness. The inducers increased photosynthetic rate under non-stress and stress conditions, by approximately 5-20% when compared to that of the non-induced control treatment. Under stress conditions, proline content was less in plants treated with plant-to-bacteria signals than the control, but phenol content was significantly increased, compared to that of the control. The study suggested that pre-incubation of bacterial cells with plant-to-bacteria signals could enhance pea growth, photosynthesis, nitrogen fixation and biomass under salinity stress conditions.

Key Words: pea, growth, inducers, nitrogen fixation, photosynthesis, proline

INTRODUCTION

During their lifetime, plants are exposed to various types of environmental stresses, such as salinity, drought, pH, temperature, nutrients, and pathogens. Environmental stress is the most serious problem that limits plant growth and crop productivity in agriculture[1]. Of this, salinity and drought stress have long existed, and today 20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity[2]. Plants exposed to salinity stress suffer from membrane destabilization[3], inhibition of the photosynthetic machinery[4], and general nutrient imbalance[5]. Saline conditions also limit the initial events involved in the process of symbiosis, nodule ontogenesis and the process of nitrogen fixation, by affecting survival and proliferation of rhizobia in the soil and rhizosphere[6]. Legume plants such as soybean, pea and lentil have proved to be of value in the nutraceutical industry, because it contains chemical compounds, mainly isoflavones that are thought to have health-promoting, disease-preventing or medicinal properties[31]. Most legumes are classified as salt-sensitive crop species[6]. Nodule formation by leguminous plants is a highly specialized process. It begins with a two-step signal exchange[7-8]. The first step is the release of plant-to-bacteria signal molecules, flavonoids, which induce rhizobial *nod* gene expression, and the second step is the release of bacteria-to-plant signal molecules, lipo-chitooligosaccarides (LCOs), and the so called Nod factors. The presence of appropriate flavonoids in root exudates is an important determinant of nodule formation by the host plant[9]. The role of flavonoid inducer molecules in enhancing legume nodulation

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and nitrogen fixation by rhizobia is well documented. For example, in pea (*Pisum sativum* L.) which may have positive impacts on human health, the nodulation was improved by the addition of the isoflavonoid naringenin (NA)[10], possibly through induction of *nod* gene expression of *R. leguminosarum*. Exogenous application of genistein (GE, isoflavone) results in short circuiting of the plant-bacterium signaling and has been proven as an effective mean to mitigate the adverse effects of low root zone temperature (RZT) on nodulation and nitrogen fixation in soybean[11-12]. Pre-incubation of *B. japonicum* with GE hastened the onset of nitrogen fixation and increased the number and size of the nodules and plant growth. These beneficial effects of GE can be increased with decreasing RZTs[13]. Recent research findings have demonstrated that some non-flavonoid molecules, jasmonic acid (JA) and methyl jasmonate (MJ) also induced the expression of *nod* genes of *R. melliloti* strains[14] and *B. japonicum*[15]. JA is a fatty-acid hormone produced through the octadecanoid pathway in plants[16-17] from its precursor linolenic acid. JA is involved in plant wound responses and defense against insects and fungal elicitors[16,18-19]. JA and MJ, a volatile derivative of JA, are also involved in salinity stress. For example, the application of JA and MJ diminished the inhibitory effect of high salinity on growth and photosynthesis of barley[20].

Little is known about nodulation, photosynthesis and growth of pea medicinal plants grown under salinity stress conditions, when inoculated with rhizobia pre-incubated with NA and MJ inducers. Therefore, the objective of this study was to investigate the effects of salinity stress on *R. leguminosarum* bv. viciae induced with plant-to-rhizobia signal compounds, NA and MJ, on nodulation, nitrogen fixation and the growth of pea grown under greenhouse conditions.

MATERIALS AND METHODS

Plant cultivation and conditions

The experiment was conducted on February 2008 in the greenhouse of Jeonnam ARES, Jeonnam, Korea. Seeds of pea medicinal plant (*Pisum sativum* L.) cultivar Delta-11 that contained bioactive compounds, which has antimutagenic activities against chemical mutagen[30], were surface-sterilized in 2% sodium hypochlorite for 3 min and then rinsed five times with distilled water[21]. The seeds were germinated and grown in sterilized vermiculite in trays. Seven days after seeding, one seedling was transplanted into each 10 cm pot, containing 400 mL of sterilized sand and turface (3:1, *v:v*) mixture. The healthy seedlings were inoculated with induced *R. leguminosarum* bv. viciae as described below. Plants were watered daily with a half strength modified nitrogen-free Hoagland's solutions[22], in which the $Ca(NO_3)_2$ and KNO_3 were replaced with 0.5 mM $CaCl_2$, 0.5 mM K_2HPO_4, and 0.5 mM KH_2PO_4 to provide nitrogen free nutrient solution. Salinity levels were adjusted to 0.6 dS m^{-1}, 3.0 dS m^{-1} and 6.0 dS m^{-1} using NaCl and the solution irrigated starting at three days after inoculation. Control plants were watered with the above half strength of N-free Hoagland's solution without salinity. The temperature in the greenhouse was maintained at 25 ±2°C with a relative humidity of 75% and a 16 hr photoperiod created by using supplemental lighting from high-pressure sodium lamps. All plants were harvested 25 days after transplanting. The experiment was structured following a randomized complete block design (RCBD) with four replications.

Induction and inoculation

R. leguminosarum bv. viciae 175G106 (Nitragin Inc., Milwakee, WI, USA) was cultured at 28ºC, on an orbital shaker at 150 rpm, for 48 hr in Tryptone Yeast (TY) medium[23]. The cultured bacterial broth was divided into four sterilized flasks, each flask representing one treatment. The bacterial cultures were induced with 15 uM NA, 50 uM MJ or their combination (15 uM NA + 50 uM MJ), while the control cultures were not induced with any type of inducer molecules. The cultures were then shaken for another 24 hr as described above. The 50 µM concentration was selected for MJ based on the reported β-galactosidase activity of rhizobial strains containing a reporter gene attached to a *nod* gene promoter-*R. leguminosarum*[14]. Begum et al.[24] reported that the best concentration of naringenin (NA) for the induction of *R. leguminosarum* bv. viciae nodulation genes was 10-15 µM. After 24 hr of induction and incubation, the cells were adjusted to 1.0 x 10^9 cells mL^{-1}, based on optical density, and 1 mL of inoculums was applied at the

base of each seedling at 3 days after transplanting.

Analysis and measurements

At the end of the experiment, data were collected on plant height, leaf greenness (SPAD-502, Minolta, Japan), leaf area (Delta-T Devices, Cambridge, UK), nodule number and nodule dry weight, shoot and root dry weight[13]. The nitrogen content and photosynthesis of plants were measured using an NC 2500 Elemental Analyzer (CE Instrument Inc., Italy) and Li-Cor 6400 (Li-Cor Inc, USA), respectively.

Proline and phenol content

Proline was extracted and its concentration was determined by the method of Bates et al.[25]. Briefly, 0.5 g of leaf and root tissue were homogenized with 3% sulfosalicylic acid and filtered through Whatman No. 2 filter paper. The filtered extracts were treated with acetic acid and acid ninhydrin, boiled for 1 hr and then absorbance at 520 nm was determined. Contents of proline (L-Proline, sigma) are expressed as μmol g^{-1} FW. Phenol content in the leaves was estimated according to the procedures of Mahadevan and Sridhar[29].

Data analysis

Data were collected and analyzed statistically by analysis of variance using CoStat software (CoHort softwate, Monterey, USA). Mean comparisons were conducted using an ANOVA protected least significant difference (LSD) ($P<0.05$) test.

RESULTS AND DISCUSSION

Nodulation and nitrogen content

The salinity effects on plant growth and nodulation of pea plant (*Pisum sativum* L.) on *R. leguminosarum* bv. viciae induced by plant-to-rhizobia signal compounds are shown in Table 1. NA and combination of NA+MJ treatments (hereafter, both treatments) at 0.6 dS m^{-1} (non-salinity) increased nodule number by 7.3% and 10.0%, respectively, compared with the control (without inducers). These results show that NA in root exudates present as stimulatory isoflavones was an important contributing factor to the nodule formation as the induced by *nod* gene inducers. The NA+MJ treatment at 3.0 dS m^{-1} salinity was increased by 8.0%, but MJ treatment slightly decreased as compared with the control. These indicate that MJ can inhibit plant growth and photosynthesis[26-27] but its mechanisms is unclear until now. However, MJ and NA+MJ treatments at 6.0 dS m^{-1} salinity increased nodule number by 14.8-16.3% compared to control. Nitrogen content also was increased by *nod* gene inducers. Nitrogen content was increased with increasing nodule number. This means that an increase of nitrogen content can increase nitrogen-fixation of legume plants. The fixed nitrogen per plant of NA, MJ and NA+MJ treatment at 0.6 and 3.0 dS m^{-1} salinity levels was increased by 8.4-8.9%, 3.3-4.1% and 3.1-5.1%, respectively, compared to the control. At 6.0 dS m^{-1} salinity, MJ and NA+MJ treatments increased the amount of fixed nitrogen per plant by 6.4% and 6.4%, respectively, compared to the control. This showed that MJ effects under stress conditions

Table 1. Effect of salinity on pea nodulation to *R. leguminosarum* induced by nod gene signal molecules

Inducers	0.6 dS m^{-1}				3.0 dS m^{-1}				6.0 dS m^{-1}			
	Nodule number ($plant^{-1}$)	N content (mg kg^{-1})		Fixed N (mg $plant^{-1}$)	Nodule number ($plant^{-1}$)	N content (mg kg^{-1})		Fixed N (mg $plant^{-1}$)	Nodule number ($plant^{-1}$)	N content (mg kg^{-1})		Fixed N (mg $plant^{-1}$)
		Shoot	Root			Shoot	Root			Shoot	Root	
Control	219	27.9	34.3	21.2	262	27.4	33.4	19.3	202	25.1	29.3	14.1
NA	235	29.9	34.0	23.2	274	28.6	33.2	20.3	218	26.6	30.4	14.9
MJ	220	28.9	34.9	21.9	260	27.9	32.4	20.1	232	26.3	29.7	15.0
NA+MJ	241	29.2	34.2	23.1	283	27.3	33.9	20.0	235	25.6	30.0	15.0
$LSD_{0.05}$	16.0	1.59	2.18	1.55	17.7	1.11	1.88	1.25	28.0	1.86	1.58	0.81

Means separation within columns by LSD at 5% level (p=0.05, n=4)

increases with increasing salinity levels and NA+MJ treatments also showed synergistic effects. This result indicates that induced pea plants could overcome high salinity during nodulation and nitrogen fixation.

Plant growth

The plant growth and yield are summarized in Table 2. Shoot and root dry weight, leaf area, and leaf greenness of pea plants were significantly decreased with increasing salinity levels. At 3.0 dS m^{-1} salinity level, the decrease in shoot and dry matter weight compared to the control (0.6 dS m^{-1}) was 5.3 and 6.4%, respectively while at 6.0 dS m^{-1} salinity level, the decrease was 3.0 and 14.7%. At the salinity level of 0.6 dS m^{-1}, the use of the inducers NA, MJ and NA+MJ resulted in an increase of 3.9, 0.4 and 4.3%, respectively, compared to the control. This means that *R. leguminosarum* bv. viciae induced by plant-to-rhizobia signal compounds is more effective and can accelerate the growth of plant or biomass. Leaf area and leaf greenness (SPAD) of pea plants were increased at 0.6 and 3 dS m^{-1}, and the variation of salinity and inducers was also significantly different, but plant height was not significantly increased. At the highest salinity level of 6.0 dS m^{-1}, the combined MJ+NA treatment was more effective than the control. Similar results have been reported by Bandeoglu et al.[28] for lentil plants. The photosynthetic rate was increased by *R. leguminosarum* strain induced by the inducers NA and the combined MJ+NA at 0.6 dS m^{-1} treatment but the effect was reversed using MJ. This result confirmed the findings of Tsonev et al.[20] and other researches[26-27] who reported that the use of MJ generally decreased photosynthesis, plant height and leaf area under non-salinity stress. At 3.0 dS m^{-1}, the photosynthetic rate increase over the control was 7.6 and 12.3%, respectively while at 6.0 dS m^{-1} salinity, the increase was 15.4 and 0.5%, respectively. This means that the *nod* gene inducted by MJ treatment can cause an increase in photosynthesis to overcome saline toxicity under high salinity levels.

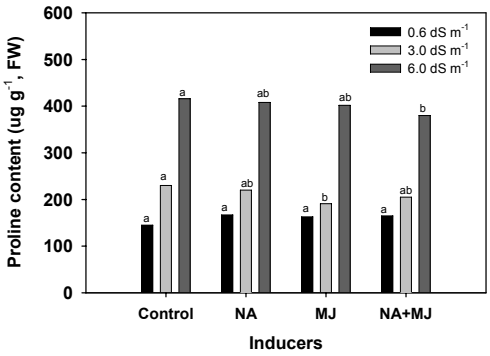
Proline content and phenol content

Figure 1 revealed that salinity levels and inducers significantly influenced the proline contents of the shoot but not the roots of the pea plants. The *R. leguminosarum* strain induced by NA, MJ and NA+MJ of the proline content in the shoot decreased by 4.3, 16.9 and 10.9% at 3.0 dS m^{-1} and 1.9, 3.3 and 8.6% at 6.0 dS m^{-1} compared to the control 0.6 dS m^{-1},

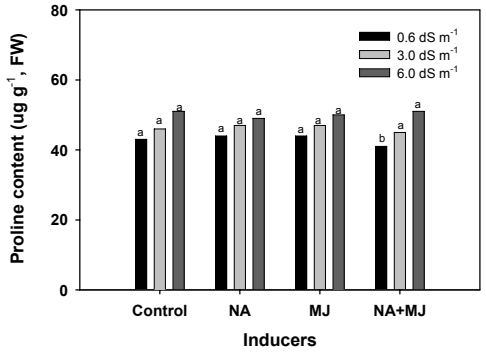
Table 2. Effect of salinity on pea growth to *R. leguminosarum* induced by *nod* gene signal molecules

Salinity levels	Inducers	Dry weight (g $plant^{-1}$)		Leaf area (cm^2 $plant^{-1}$)	Leaf greenness (SPAD)	Photosynthetic rate (umol CO_2 cm^{-2} s^{-1})	Plant height (cm)
		Shoot	Root				
0.6 dS m^{-1}	Control	0.488	0.218	49.0	35.6	13.5	13.3
	NA	0.507	0.240	52.0	36.4	14.2	13.5
	MJ	0.490	0.219	50.7	36.8	12.9	13.1
	NA+MJ	0.509	0.241	53.0	37.2	14.3	13.8
	$LSD_{0.05}$	0.02	0.03	4.0	1.17	0.71	1.74
3.0 dS m^{-1}	Control	0.462	0.204	46.7	35.6	10.5	13.5
	NA	0.470	0.207	49.0	35.4	11.5	13.8
	MJ	0.466	0.217	49.7	35.3	11.3	13.5
	NA+MJ	0.472	0.205	50.7	36.5	11.8	13.4
	$LSD_{0.05}$	0.03	0.02	4.0	0.92	1.07	1.38
6.0 dS m^{-1}	Control	0.342	0.186	37.5	23.1	5.2	12.6
	NA	0.350	0.188	37.7	23.5	5.1	12.3
	MJ	0.353	0.193	40.0	25.7	6.0	12.3
	NA+MJ	0.357	0.197	38.7	25.6	5.2	130
	$LSD_{0.05}$	0.04	0.02	3.6	1.10	0.70	1.62

Means separation within columns by LSD at 5% level (p=0.05, n=4)



(A) Leaf



(B) Root

Fig. 1. Salinity effects on the proline content (ug g^{-1}, FW; A: leaves, B: roots) to *R. leguminosarum* induced by *nod* gene inducers. Means within bars are mean ±S.E. (*n*=3) and ANOVA (P=0.05)

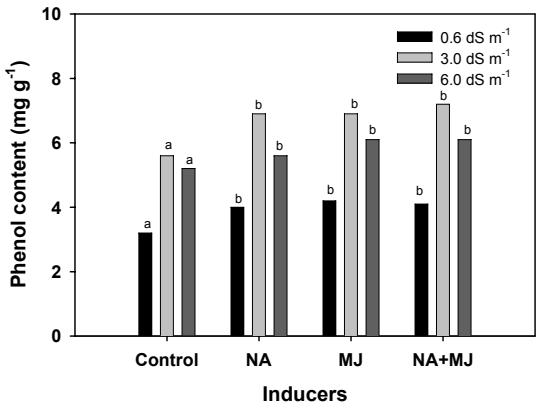


Fig. 2. Effect of different treatments on phenol content (amg g^{-1}, FW) in pea leaves. Means within bars are mean ±S.E. (*n*=3) and ANOVA (P=0.05)

respectively. On the other hand, salinity-caused stress was affected by *nod* gene inducers. The highest phenol content was recorded at 3.0 dS m^{-1} and lowest 0.6 dS m^{-1} treatments (Figure 2). *R. leguminosarum* strain induced by NA, MJ and NA+MJ can increase phenol content of pea leaves as compared to control treatment.

CONCLUSIONS

Generally, plants reacted to salinity by a reduction in growth, but there was a difference in the degree of response. The study have shown that pre-incubation of *R. leguminosarum* bv. viceae with NA and MJ signal molecules can increase dry weight, nodule numbers, leaf area, leaf greenness and photosynthetic rate of pea plants under salinity stress. These inducers can decrease proline contents in tissue under salinity stress, when compared to plants inoculated with uninduced control inocula.

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