Article

The Low Root Zone Temperature Effects on Nitrogen Fixation, Growth, and Antioxidant Responses of Lentil Inoculated with *Rhizobium leguminosarum*

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The plant molecular signals that induce legume-*Rhizobium* symbiosis are potentially an important source of plant growth promotion in agricultural systems. A study was conducted to evaluate the effects of low root zone temperature (RZT) on nitrogen fixation, antioxidant response, and growth of the lentil plant (*Lens culinaris* L.), which harbor isoflavonoids that may exert positive impacts on human health, inoculated with *R. leguminosarum* bv. viciae pre-cultured with additional plant-to-rhizobia signal compounds (inducers), naringenin (NA), methyl-jasmonate (MJ), and NA+MJ treatment, under greenhouse conditions. Two RZT levels, 20 and 14°C, were maintained through daily irrigations until the plants were harvested 25-30 days after transplantation. The addition of inducer compounds increased the dry weight and leaf greenness of plants under both temperature regimes. Photosynthesis levels were increased significantly ($p<0.05$) by approximately 12.3% when compared to the group without inducer treatment (control). Nitrogen fixation per plant increased with increasing numbers of nodules. The fixed nitrogen per plant in the NA and NA+MJ treatment groups increased by 18.2 and 20.4% as compared to the controls. No interaction between inducer and temperature factors was detected. Increasing low RZT levels significantly ($p<0.05$) reduced antioxidant activity, proline content, ascorbate peroxidase, and glutathione reductase contents in the plants treated with induced rhizobia as compared to the controls. Phenol content was recorded as higher at a temperature of 14 than at 20°C, and was significantly ($p<0.05$) increased in the inducer treatments. These results, collectively, suggest that the pre-incubation of bacterial cells with plant-to-bacteria signal compounds could enhance lentil growth, photosynthetic rates, and nitrogen fixation, and could also alleviate low RZT stress.

Key words: *antioxidant, growth, lentil, low root zone temperature, nitrogen fixation, nodulation*

The legumes-the soybean, pea, and lentil-have proven to be of value in the nutraceutical industry, as they contain chemical compounds that are thought to have health-promoting, disease-preventing or medicinal properties; chief among these compounds are the isoflavones [Messina and Messina, 1994]. Legume-rhizobia symbiosis, the principal source of biological atmospheric nitrogen fixation for agricultural systems, is known to be sensitive to a number of environmental factors. Low root zone temperature (RZT) is a particularly important factor for legume production in short season areas such as Eastern Canada [Whigham and Minor, 1978]. The growth of plants dependent on nitrogen fixation is hampered more profoundly by low soil temperatures than is the case with plants receiving mineral nitrogen [Abberton *et al.*, 1998]. Low RZT also inhibits inter-organismal signaling between the two symbiotic partners. It has been shown that low RZTs inhibit the biosynthesis and rhizosecretion of plant-to-bacteria signal molecules (for example genistein (GE)) from soybean roots, which are necessary for the induction of the *nod* genes of *B. japonicum* [Zhang and Smith, 1997]. Low RZTs also inhibit the induction of bacterial nodulation genes required for the biosynthesis of bacteria-to-plant signaling molecules, lipo-chitooligosaccarides (LCOs), the so-called Nod factors [Zhang *et al.*, 1995]. The disruption of Nod factor production/excretion at low

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temperature incubations has been previously reported in *R. leguminosarum* bv. trifolii [McKay and Djordjevic, 1993], and in *B. japonicum* [Duzan *et al.*, 2005]. Low RZTs delay the onset of nodulation [Pan and Smith, 1998] and reduce the rate of subsequent nodule growth, resulting in effects on small final nodule size [de Lira Juniora *et al.*, 2005]. The presence of appropriate flavonoids in root exudates is a critical factor in nodule formation [Richardson *et al.*, 1988] and dictate rhizobia-legume specificity. The nodulation status of the pea (*Pisum sativum* L.) was most profoundly improved via the addition of the flavonoid naringenin (NA) [Bandyopadhyay *et al.*, 1996], possibly via the induction of *nod* gene expression in *R. leguminosarum*. In the soybean - *B. japonicum* symbiosis, Kosslak *et al.* [1987] reported that GE was the most effective inducer for the expression of the *nod*YABC operon of *B. japonicum*. Exogenous application of GE results in the short-circuiting of plant-bacterium signaling, and has been confirmed as an effective means to mitigate the adverse effects of low RZT on nodulation and nitrogen fixation [Zhang and Smith, 1997]. 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. Additionally, this beneficial effect of GE increased with decreasing RZTs [Zhang *et al.*, 1995].

Recent research findings have shown that certain non-flavonoid molecules, most notably jasmonate (JA) and MJ also induce the expression of *nod* genes, such as in the strains *R. leguminosarum* and *B. japonicum* [Rosas *et al.*, 1998]. JA is a fatty-acid hormone that is generated via the octadecanoid pathway [Vick and Zimmerman, 1984; Creelman and Mullet, 1997] from its precursor, linolenic acid. JA is involved in plant wound responses and is also relevant to defense against insects and fungal elicitors [Farmer *et al.*, 1998; Ryan, 2000]. JA and MJ are also involved in salinity stress; for example, pretreatments with JA and MJ diminished the inhibitory effect of high salinity concentrations on growth and photosynthesis in barley, pea and rice [Moon *et al.*, 1997; Lee 2008]. These research findings suggest that, in addition to its roles *in-planta*, JA can also function as an important signaling molecule in rhizobia-legume symbioses for alleviating low RZT constraints.

The principal objective of this study was to determine the effects of low RZT on the growth, nitrogen fixation, and antioxidant responses of lentil inoculated with *R. leguminosarum* bv. viciae pre-incubated with plant-to-rhizobia signal compounds.

Materials and Methods

Plant material and cultivation conditions. The experiments were conducted on February 2008 in the greenhouse of Jeonnam ARES, in Jeonnam, Korea. Seeds of the lentil medicinal plant (*Lens culinaris* L. Bo-11), which evidences anticancer and antioxidant activity [Dueñas *et al.*, 2006], were surface-sterilized for 3 min in 2% sodium hypochlorite and then rinsed 5 times in distilled water [Bhuvaneawari *et al.*, 1980]. The seeds were germinated and grown in sterilized vermiculite in trays. 5-8 days after seeding, one seedling was transplanted into each ϕ 11 cm pot, containing 1,000 mL of a mixture of sterilized sand and Turface (3:1, v/v). Three days after transplantation, the healthy seedlings were inoculated with the induced *R. leguminosarum* bv. viciae for lentils, as described below. The plants were watered daily with half-strength modified nitrogen-free Hoagland's solution [Hoagland and Arnon, 1950], 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 a nitrogen-free nutrient solution. In order to provide controlled RZTs, the pots were placed in plastic containers (68×42 cm and 15 cm deep) with the bottom of the pots sealed to the tank; water at the desired temperatures (14 and 20°C for lentil plants) was circulated through each tank using temperature-adjusted compressors and water pumps. In order to allow for excess water drainage from the pots, a hole was drilled in the tank bottom below each pot. The temperature in the greenhouse was maintained at 25±2°C with a relative humidity of 75% and a 16 h photoperiod created via the use of supplemental lighting from high-pressure sodium lamps. All plants were harvested 25 days for the lentils after transplantation. The experiment was structured in accordance with a randomized complete block design (RCBD) with four replications.

Induction and inoculation. *R. leguminosarum* bv. viciae 175G106 for lentils was cultured for 48 h at 28°C, on an orbital shaker at 150 rpm in Tryptone Yeast (TY) medium [Vincent, 1970]. The cultured bacterial broth was divided into four sterilized flasks, each flask representing one treatment, and then each inducer, 15 mM Naringenin (NA, for *R leguminosarum* bv. viciae) and 50 mM MJ or a combination thereof (15 mM NA+ 50 mM MJ) was added 3 days after transplantation [Rosas *et al.*, 1998]. After 24 h of induction and incubation, the cells were adjusted to a concentration of $1.0×10^9$ cells/mL, based on optical density, and 1 mL of inoculums was applied to each seedling.

Analysis and measurements. Plant growth and nodulation measurements included plant height, leaf greenness (SPAD-502, Minolta, Osaka, Japan), nodule number and nodule dry weight, shoot and root dry weight [Zhang *et al.*, 1995]. The nitrogen content and

photosynthesis of plants were evaluated using, an NC 2500 Elemental Analyzer (CE Instrument Inc., Milan, Italy) and a Li-Cor 6400 (Li-Cor Inc, Lincoln, NE), respectively.

Antioxidant enzyme activity and phenol content. Proline content was determined via the method of Bates *et al.* [1973]. In brief, 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 h, and then absorbance at 520 nm was determined. Contents of proline (L-proline, Sigma) are expressed as μmol/g FW. Phenol content in the leaves was estimated in accordance with the methods described by Sadasivam and Manickam [1991]. In order to determine the levels of antioxidant enzymes, fully expanded leaves were homogenized in 50 mM phosphate buffer (pH 7.5) containing 1.0% (w/v) polyvinyl-pyrrolidone (PVP), 0.1 mM EDTA and 0.5% (v/v) Triton X-100 [Anderson, 2002]. The H_2O_2 levels of ascorbate peroxidase (APX) [Chen and Asada, 1989] and glutathione reductase (GR) [Rao *et al.*, 1996] in the leaves and roots were colorimetrically measured. The oxidation rate of ascorbate was estimated by following the reduction in absorbance at 290 nm for 3 min. Protein contents were determined in accordance with the Bradford method [Bradford, 1976], using bovine serum albumin (BSA) as a standard.

Data analysis. Data were statistically analyzed via analysis of variance using CoStat software (CoHort Software, Monterey, CA). Means comparisons were conducted via an ANOVA protected least significant difference (LSD) ($p<0.05$) test. Treatments of signal molecules causing *nod* gene induction were compared via a randomized complete block model with four replications of each treatment.

Results and Discussion

Plant growth and photosynthesis. Low root zone temperature (RZT) effects on the growth and nodulation of the lentil plant (*Lens culinaris* L.), which contains isoflavonoids that may have positive impacts on human health, on *R. leguminosarum* bv. viciae induced by the plant-to-rhizobia signal compounds, naringenin (NA) and methyl-jasmonate (MJ), are shown in Table 1. Shoot and total dry weight within the inducer-treated group were increased significantly ($p<0.05$), as compared to the without-inducer group (control). NA, MJ and a combination of NA+MJ treatments (inducer treatment) induced increases in the shoot and total dry weight by 14, 0.9, and 14% (shoot dry weight) and 14, 4.8 and 15% (total dry weight), respectively. NA and combined NA+MJ treatments increased leaf greenness and photosynthetic rate significantly ($p<0.05$), by 6.7 and 12.3%, respectively, as compared to the controls. However, no interactions were detected between the inducer and temperature factors on the biomass, photosynthetic rate, and growth of lentils. As a whole, the inducer treatments induced increases in the biomass, as compared to other inducer treatments. Similar results have been reported by Dashti *et al.* [2000] and Lee [2008] for soybean and pea plants, respectively. In general, low RZTs delay the onset of photosynthetic rate, thus exerting effects on small final

Table 1. Effect of root zone temperature on lentil growth and biomass to *R. leguminosarum* induced by *nod* gene inducers

Treatment	Dry weight (g plant^{-1})			Plant height (cm)	Leaf greenness (SPAD)	Photosynthetic rate (mmol cm^{-2} s^{-1})
	Shoot	Root	Total			
Temperatures						
20°C	0.156a	0.060a	0.216a	28.4a	35.6a	7.39a
14°C	0.084b	0.058a	0.142b	25.9b	29.5b	5.08b
Inducers						
Control	0.112b	0.053a	0.165b	26.2a	31.0b	5.79b
NA	0.128a	0.060a	0.188a	27.6a	33.1a	6.45a
MJ	0.113b	0.059a	0.173b	27.1a	33.1a	6.15ab
NA+MJ	0.128a	0.062a	0.190a	27.6a	33.1a	6.56a
Significance of factors						
Temperatures (Tem)	***	ns	***	***	***	***
Inducers (In)	**	ns	**	ns	**	**
Tem X In	ns	ns	ns	ns	ns	ns

Means separation within columns by LSD at 5% level ($p<0.05$, $n=4$). ns, *, ** and *** for the overall model, no significant difference of different at the 0.05, 0.01 and 0.001 level of probability, respectively.

Table 2. Effect of root zone temperature on lentil nodulation, N content and fixed N to *R. leguminosarum* induced by *nod* gene inducers

Treatment	Nodule number (plant⁻¹)	N content(mg g⁻¹)		Fixed N (mg plant⁻¹)
		Shoot	Root	
Temperatures				
20°C	60.4b	3.038a	3.49b	0.683a
14°C	65.2a	2.318b	3.98a	0.426b
Inducers				
Control	58.4b	2.59a	3.68a	0.499b
NA	67.5a	2.74a	3.76a	0.590a
MJ	59.5b	2.65a	3.67a	0.530b
NA+MJ	65.9a	2.74a	3.84a	0.601a
Significance of factors				
Temperatures (Tem)	**	***	***	***
Inducers (In)	**	ns	ns	**
Tem X In	ns	ns	ns	ns

Means separation within columns by LSD at 5% level ($p<0.05$, $n=4$). ns, *, ** and *** for the overall model, no significant difference of different at the 0.05, 0.01 and 0.001 level of probability, respectively.

biomass [Zhang *et al.*, 1995]. The presence of *nod* gene inducers under low RZT stress conditions is a critical factor in plant growth and photosynthetic rates, and dictates rhizobia-legume specificity.

Nodulation and nitrogen fixation. Low RZTs generally delay the onset of nodulation [Pan and Smith, 1998] and reduce the rate of subsequent nodule growth, resulting in effects on small final nodule size [de Lira Juniora *et al.*, 2005]. Lentil plants inoculated with *R. leguminosarum* induced by *nod* gene inducers generated a greater number of nodules at low RZT, as compared to those inoculated with non-induced rhizobia under greenhouse conditions (Table 2). The nodule number in the NA and combined NA+MJ treatments was increased by 15.6 and 12.8%, but MJ treatment was not increased as compared to the controls. These findings indicate that NA in root exudates, present as stimulatory flavones, was an important contributing factor to nodule formation induced by *nod* gene inducers. These results show that the nodulation of the pea plant was increased as the result of the addition of the isoflavonoid naringenin [Bandyopadhyay *et al.*, 1996; Lee 2008]. MJ has been shown to inhibit plant growth and photosynthesis [Gross and Parthier, 1994], but little is currently known regarding the number of nodules of the lentil plant induced by MJ. The nodule number of lentils grown under low RZT stress was increased by approximately 7.9% as compared to the controls. Nitrogen fixation per plant was increased with increasing nodule numbers. The fixed nitrogen per plant in the NA and NA+MJ treatment groups increased by 18.2 and 20.4%, respectively, as compared to the controls. However, no interaction between the inducer
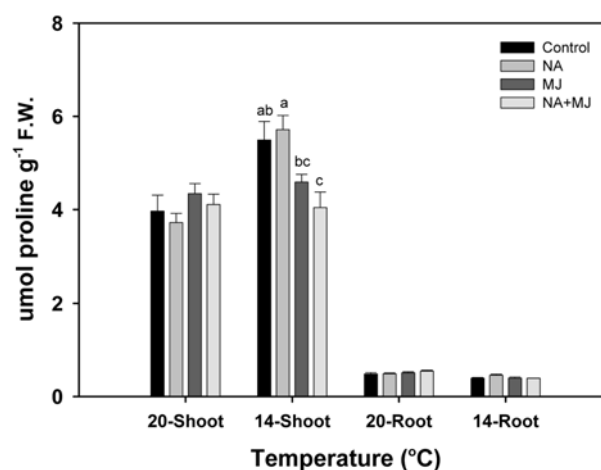

Fig. 1. Low RZT effect on the proline content to *R. leguminosarum* induced by *nod* gene inducers. Means within bars mean±SE ($n=4$) and ANOVA ($p<0.05$).

and temperature factors was found in the context of nitrogen fixation and nodulation. Under stress conditions, the effects of MJ increased with increases in low RZT; furthermore, combined NA+MJ treatments also exerted synergistic effects. This result shows that lentil plants treated with inducers could be overcome by low RZT stress.

Antioxidant activity and phenol content. The legume-*Rhizobium* symbiosis is the single most important source of biologically fixed nitrogen in agricultural systems. However, it is complex, and very sensitive to environmental effects, including RZT, Salinity and pH stress. The mechanism by which this factor inhibits the symbiosis is not well understood. Salt-stressed plants are generally

Table 3. Activities of ascorbate peroxidase and glutathione reductase in leaves and roots of lentil subjected to 14 and 20°C RZT

Antioxidant	Temperature	Inducers							
		Leaf				Root			
		Control	NA	MJ	NA+MJ	Control	NA	MJ	NA+MJ
		(nmole min^{-1} mg^{-1} protein)							
APX	20	135±14a	142±12a	133±15a	130±15a	287±18a	277±27a	265±13a	274±14a
	14	356±20a	315±16b	290±25b	286±22b	526±42a	555±41a	489±33a	494±39a
GR	20	10.2±1.1a	10.9±0.8a	9.8±1.0a	9.9±1.2a	23.0±1.5a	21.3±1.6a	22.8±1.5a	20.7±1.6a
	14	45.2±2.6a	44.6±2.8a	42.7±2.2a	42.1±2.2a	63.0±2.2a	59.1±3.0a	59.7±3.7a	57.9±3.2a

Treatment means are with ±S.E. of four replications (n=4). Means separation within columns by LSD at 5% level (p<0.05, n=4).
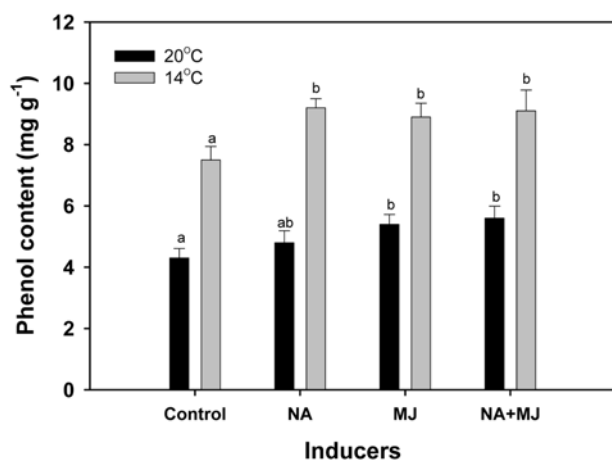

Fig. 2. Low RZT effect on the phenol content in lentil leaves to *R. leguminosarum* induced by *nod* gene inducers. Means within bars are mean±SE (n=4) and ANOVA (p<0.05).

known to accumulate a variety of organic compounds such as glucose, proline, etc. in the cell membrane for osmoregulation to occur, thus enabling growth by protecting enzyme activity [Dashti *et al*. 2000]. However, in order to understand the responses of antioxidant enzymes against low RZT levels, lentil plants were treated with *R. leguminosarum* strain and with inducers. The results are shown in Fig. 1 and Table 3. Increasing low RZT levels significantly (p<0.05) reduced enzyme activity in proline content, APX and GR, as compared to the controls. More significant differences were observed at 14 than 20. In particular, the APX of NA, MJ and combined NA+MJ treatments at 14 were reduced by 12, 18, and 19% in the leaves, respectively. GR activity responded to low RZT stress treatments in the same fashion as did APX activity. Although APX performs a crucial function in the conversion of H_2O_2 to water, GR is also an essential enzyme in this reaction, and performs a function in maintaining the redox states of ascorbate and glutathione [Chen and Asada, 1989; Rao *et al*., 1996].

Similar results have been reported by Dashti *et al*. [2000] for the soybean plant and Lee *et al*. [2008] for pea plants. The low RZT effect on the phenol contents of lentil leaves to *R. leguminosarum* induced by *nod* gene inducers are shown in Fig. 2. Phenol content was detected at higher levels at 14 than at 20. Inducer treatments caused significant (p<0.05) increases in the phenol content, as compared to the controls. In conclusion, the results of this study demonstrate that the inoculation of low RZT-stressed plants with *R. leguminosarum* plus inducers can enhance lentil nodulation, photosynthesis, and yield under low RZT stress conditions, and may also possibly alleviate low RZT stress.

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