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Methyl Jasmonate, Alone or in Combination with Luteolin and Sinorhizobium Meliloti Accelerates Nodulation and Nitrogenase Activity of Alfalfa at Suboptimal Root Zone Temperature

[1]O. Younesi and [2]A. Moradi

[1]University of Tehran, Iran
[2]Department of Agronomy and Plant Breeding, Faculty of Agriculture, Yasouj University, Yasouj, Iran

Abstract: Jasmonic acid (JA) is a plant hormone produced via the octadecanoid pathway from its precursor, linolenic acid. Jasmonates are involved in plant wound responses and defense against insects and fungal elicitors. They can also act as signal molecules in the *Sinorhizobium* -alfalfa symbiosis. Pre-incubation of *Sinorhizobium meliloti* inocula with luteolin (Lu), an effective inducer of nodulation genes in this species enhances alfalfa nodulation, nitrogen fixation and yield under low root zone temperature (RZT). Since jasmonates are also able to induce nodulation genes and cause the production of lipo-chitooligosaccharides (LCOs) by *S. meliloti*, we conducted a greenhouse experiment, to determine whether pre-incubation of *S. meliloti* with methyl jasmonate (MeJA) alone or in combination with luteolin, prior to inoculation, would at least partially overcome the negative effects of low root zone temperature (RZT) on alfalfa nodulation, nitrogen fixation and plant growth. Two RZT regimes (25 and 17°C) and four inducer molecule treatments [control, Lu (10 mM), MeJA (50 mM) and Lu + MeJA (10 mM+50 mM)] were used in the study. Our results indicate that low RZT redused nodulation, nitrogenase activity and plant growth. Both Lu and MeJA, alone or in combination, enhanced alfafa nodulation, nitrogenase activity and growth at suboptimum RZTs. This study showed that MeJA, alone or in combination with Lu, can used to partially overcome the low RZT induced inhibitory effects on nodulation and nitrogen fixation.

Key words: Alfalfa · Luteolin Methyl jasmonate · Nodulation · *Sinorhizobium meliloti*

INTRODUCTION

Leguminous plants have the ability to enter into symbiosis with bacteria, collectively known as rhizobia, to produce root and sometimes stem nodules. Within these nodules rhizobia fix atmospheric dinitrogen into ammonia, which is then utilized by the plant [1-2].

In areas with suboptimal root zone temperatures (RZTs) early in the growing season, nitrogen fixation by legume crops is a major limitation to crop yield [3] as nitrogen fixation is more sensitive than dry weight to RZT [4]. Low RZTs delay the onset of nitrogen fixation in legumes [5-6]. The infection and early nodule development processes are the most sensitive to suboptimal RZTs [7-3-8].

In exudates and extracts from legume roots, flavones and isoflavones have been identified as the inducing molecules for *(Brady)rhizobium* nodulation genes [9]. Luteolin is also one of the flavonoids inducing nodulation genes in the bacterium *S. meliloti* [10]. It is released from alfalfa imbibing seeds; it increases growth rate of *S. meliloti* [11] and thus plays an important role in nodule formation [12]. Appropriate flavonoids in root secretions are important factors in root nodule formation [13]. Low temperature decreases both the biosynthesis and excretion of luteolin from plant roots into the rhizosphere.

Jasmonic acid (JA) and its derivatives, collectively known as jasmonates, are naturally occurring signal compounds in plants. They are involved in plant growth and development and responses to biotic and abiotic stresses [14, 15, 16, 17]. Jasmonates play a central role

Corresponding Author: O. Younesi, University of Tehran, Iran.

in the plant wound responses [18]. Besides its roles in-planta, JA can also act as an important signaling molecule in rhizobia-legume symbioses. It has recently shown that jasmonates induce transcription of nodulation genes in *B. japonicum* and that pre-incubation of *B. japonicum* cells with jasmonates enhances nodulation and nitrogen flxation of soybean plants at both optimal and sub-optimal root zone temperature conditions [19].

Luteolin, however, is expensive and reducing the cost of commercial inoculant production by pre-incubation of inoculants with MeJA is an alternative; it is an effective inducer of nod genes of *S. meliloti* [19], is substantially cheaper than luteolin and does not have negative effects on *S. meliloti* cells, also a property of Luteolin. In addition, MeJA promotes lateral root initiation and growth at sub-micromolar concentrations (less than 10^8 M. Tung *et al*., 1996). The hypothesis for the current experiment was that nodulation, nitrogen fixation and subsequent dry matter accumulation by alfalfa plants may be increased at low RZTs when plants are inoculated with rhizobia pre-induced with Luteolin and MeJA alone or in combination together.

MATERIALS AND METHODS

The experiment was conducted in the soil microbiology laboratory and greenhouse of College of Agriculture, Tehran University, Iran. Seeds of alfalfa (Bami cultivar) were obtained from The Seeds and Plant Improvement Institute, Karaj, Iran. Seeds of the selected cultivar were sterilized (2% sodiumhypochlorite containing 4 mL^{-1} Tween 20) for 7–10 min then washed several times with distilled water [20]. Then seeds placed in sterilized trays of 27×50 cm and 6 cm deep, filled with vermiculite and allowed to germinate. Four-day-old seedlings were transferred to 1 litre pots, containing 950 ml of sterilized sand and turface (2:1 v: v) mixture. The plants were grown in a growth chamber (Conviron Inc., Winnipeg, Manitoba) with day/night temperatures of 25°C and a 16:8 h photoperiod.

In order to provide controlled RZTs the pots were placed in plastic tanks (68 ×42 cm and 15 cm deep) with the bottom of the pots sealed to the tank; water at the desired temperatures (17 and 25°C) was circulated in each tank using temperature adjusted compressors and water pumps. In order to allow excess water drainage from the pots, a hole was drilled in the tank bottom below each pot. After transplanting, the plants were acclimatized in respective controlled temperature tanks for 24 h prior to inoculation with rhizobial cells. Plants were inoculated with *S. meliloti*, strain 1021, as described below. Nitrogen free Hoagland's solution [21] was used as a nutrient solution for the plants. Each pot received 40 ml of modified Hoagland's solution four times a week. If plants required watering at other times, double distilled water was applied. The water and Hoagland's solutions were temperature adjusted prior to application.

Inoculant Preparation: *S. meliloti*, strain 1021 was cultured in yeast extract mannitol broth [22] for 7 days and sub- cultured for 5 days in 2 L flasks shaken at 125 rpm at 25°C. When the sub-cultures reached the mid log phase, distilled water was used to dilute the inoculums to an OD_{620} of 0.08 (equivalent to 10^8 cells mL 1) [20]. The subculture was divided into two flasks, each flask representing one treatment. Filter sterilized commercial luteolin was added to the subculture to a final concentration of 10 m in a 2 L flask and incubated at 30°C without shaking for 48 h [23].

Inoculum was prepared by culturing *S. meliloti*, strain 1021 in 500 mL flasks containing 100–200 mL yeast extract mannitol (YEM) culture medium [22]. The cultures were shaken at 150 rpm at 28 °C for 3 days, after which these cultures were transferred to 4 L ?asks containing 2 L of media and shaken for a further 5 days, as already described. Stock solutions of filter sterilized Luteolin and methyl jamonate (methyl 3-oxo-2-[2-pentenyl]cyclo-pentaneacetic acid, 95% purity, Sigma–Aldrich) were prepared in dimethyl sulfoxide and added to the subcultures so that the final Lu and MeJA concentrations were 10 and 50 mM, respectively. No inducer was added to the control flasks (containing bacterial culture only). The induced sub-cultures were shaken for another 24 h, as described previously. The subculture was diluted to an OD of 0.1 (10^8 cells L^{-1}) before applying to seedling. Each seedling received 1 ml of inoculant applied at the stem base. The inoculants were temperature adjusted before application to the temperature treatments. The plants were six days old when inoculated.

Experimental Design and Data Analysis: The experiment was structured as a 4 ×2 factorial following a randomized complete block design (RCBD) with four replicates. Two factors (inducer molecules and temperatures) were studied in the experiment. The inducer molecule

treatments applied to the *S. meliloti* culture during pre-incubation were (1) 10 mM Lu, (2) 50 mM MeJA, (3) both 10 mM Lu and 50 mM MeJA and (4) no inducer molecule(s) (un-induced inoculant–control). The two temperature used I n the experiment were 17 and 25°C. The temperature treatments were started one day before inoculation and ended at harvesting. Plants were harvested one month after sowing and nodule number and dry weight, shoot and root dry weight and nitrogenase enzyme activity measured by acetylene reduction method (Herdina and Silsbury, 1990) were measured. Results were analyzed statistically by analysis of variance using the Statistical Analysis System computer package [24]. When analysis of variance showed significant treatment effects, the LSD test was applied to make comparisons among the means at the 0.05 level of significance [25].

RESULTS AND DISCUSSION

Based on the results obtained, temperature has significant effects on all the traits observed (Table 1).

Low temperature at the root zone of plants reduced the Plant height, root and shoot dry weight (Table 2). Osmotic root hydraulic conductance is a possible basis for this type of reduction in chilling sensitive plants, when they are exposed to chilling treatments [26]. In our study, the decreased amounts of dry matter (Table 2) were less than that of the nitrogen fixation. This is consistent with the finding that nitrogen fixation proce was more sensitive to RZT than dry weight [4].

Analysis of all inducer molecule treatments did not detect any differences in plant height due to the applied treatments (Table 1), as previously noted by Zhang and Smith (1996) and [27]. However, the application of luteolin, methyl jasmonate, or both inducers together caused increases in root and shoot dry weight, versus the inoculant only control ($P < 0.05$). This increase was the same at both temperatures. These results indicate a difference in response pattern between alfalfa and soybean. Soybean responds strongly to MeJA preinduction of rhizobia at low RZT, but not at optimal RZT (Mabood, unpublished data) in the same way that it responds to genistein preinduction [5], while alfalfa responds strongly and similarly at both suboptimal and optimal RZTs.

Parallel changes were also observed in other traits including nodule number and dry weight. At 25°C RZT, nodule numbers of plants ranged from 16 to 27 When the RZT was 17°C, the number of nodules per plant was reduced to 78.7% of that at 25°C (Table 2).

Plants receiving *S. meliloti* induced with luteolin (10 mM) or both compounds together (Lu 10 mM + MeJA 50 mM) had greater nodule number than the control plants (receiving uninduced cultures) at both temperatures. This could be due to an increase either in the number of infections initiated (as observed microscopically) or in the proportion of infections leading to nodule formation. In our experiments, preincubation with Lu or Lu and MeJA together prior to alfalfa inoculation activated the sinorhizobial nod genes. The expression of the sinorhizobial nod genes has been shown to stimulate production of the bacterial nod factor [28] Kondorosi, 1992). This nod factor has been identified as a lipo-oligosaccharide [29] that is able to induce many of the early events in nodule development [30] including deformation and curling of plant root hairs, the initiation of cortical cell division and induction of root nodule meristems [31]. Nodulation events started earlier at the suboptimal RZT tested, presumably because the added luteolin stimulated the production of the lipo-oligosaccharide.

The pre-incubation of S. *meliloti* with MeJA (50 mM) at 25°C RZT did not affect ($P < 0.05$) nodule number in the experiment (Table I). However, at 17°C RZT, pre- treatment of *S. meliloti* with MeJA (50 mM) increased nodule numbers in the plants. At loss of inducer, the plant nodule weights at 17°C RZT was only 69.05% of those of plants grown at 25°C RZT (Table II). All inducer treatments caused increases in the total nodule weights of alfalfa plants at optimal and suboptimal RZT.

Temperature had negative significant effects on nitrogenase enzyme activity (Table 1). In the present study, an increase in nitrogenase enzyme activity was observed following the inoculation of plants with rhizobia pre-incubated with inducer. The pre-induction may have relieved a shortage of signal molecules involved in plant-bacteria interactions at the early stages of symbiosis establishment. Earlier work on soybean indicated that genistein in plant tissues may be reduced under low RZT conditions [32]. Low RZTs also decrease both the biosynthesis of genistein and the excretion of geinistein from plant root cells into the soil rhizosphere [33-39]. We found that, for alfalfa, all nitrogenase activity and nodule related traits were reduced when RZT decreased from 25 to 17°C. This may indicate that the production and/or excretion of signal molecules by alfalfa plant roots is retarded by low RZT. At least one of these molecules could be luteolin, as preincubation of the *S. meliloti* cultures used as inocula increased the levels of variables including nodule dry weight, nodule numbers and dry matter produced plant^{-1}. The nitrogenase enzyme activity

Table 1: Analysis of variance for the traits investigated in alfalfa plants inoculated with *S. meliloti* preincubated with or without inducer molecules at two levels of root zone temperature

Treatments	Nodule number	Nodule dry weight	Root dry weight	Plant height	Shoot dry weight	Nitrogenase activity
Temperatures	**	**	**	**	**	**
Inducers	*	*	*	ns	**	**
Interactions	*	**	**	ns	*	**

**, *: significant at, 1 and 5 percent probability respectively; ns: not significant

Table 2: Nodulation and Growth parameters of alfalfa plants inoculated with *S. meliloti* preincubated with or without inducer molecules at two levels of root zone temperature

Treatments	Inducers	Nodule number	Nodule dry weight (mg plant $^{-1}$)	Plant height (cm)	Root dry weight (mg plant $^{-1}$)	Shoot dry weight (mg plant $^{-1}$)	Nitrogenase activity (mmolC_2H_4 plant $^{-1}$)
17°C	Lu 10 µM	17.3c	1.54c	15.3 b	11.34c	21.6c	1.83b
	MeJA 50 µM	14.4c	1.33d	15 b	10.75c	19.13d	1.66c
	Ge + MeJA	23.7ab	1.62c	15.8 b	14.53b	23.84c	1.9b
	Control (inoculant only)	8d	0. 87e	14.6b	7.38	15.41e	1.27d
25°C	Lu 10 µM	21b	1.88b	20.3 a	15.5b	25.5b	2.16a
	MeJA 50 µM	16.5c	1.67c	21.3 a	15.45b	25.73b	1.96b
	Ge + MeJA	27.6a	2.12a	22.2 a	18.32a	28.42a	2.23a
	Control (inoculant only)	16c	1.26d	20 a	10.8c	19.66d	1.76c

Means followed by the same letter within a column are not significantly different at $p<0.05$, as determined by Duncan's Multiple Range test

was highest when both inducer compounds together was used for pre- incubation of bacteria in both temperatures 17 and 25°C (Table II). At control plants (without inducer), the nitrogenase enzyme activity at 17°C was 72.16% of plants at 25°C RZT, whereas with lu (10 mM), MeJA (50 mM) or both compounds together (Lu 10 mM + MeJA 50 mM), this increased to 84.72%,84.69% and 85.2% of plants at 25°C RZT.

In summary, this experiment describing the role of MeJA pre-incubated *S. meliloti*, alone or in combination with luteolin, on alfalfa plant nodulation and yield under low temperature condition. The role of luteolin in this capacity has previously been documented. MeJA is far cheaper than luteolin and is not damaging to *S. meliloti* cells. It can be considered as an alternative to the luteolin-based technology. MeJA can be incorporated into *S. meliloti* inoculants used in regions of the world where spring soil temperatures are cool. These inoculants will be superior in performance to those containing uninduced *S. meliloti* cells and less expensive and/or dif?cult to produce than those containing luteolin.

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