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Pre-incubation of *Sinorhizobium meliloti* with Luteolin, Methyl jasmonate and Genistein Affecting Alfalfa (*Medicago sativa* L.) Growth, Nodulation and Nitrogen Fixation under Salt Stress Conditions

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ABSTRACT

Salinity is among important soil stresses adversely affecting the process of nitrogen (N) fixation in leguminous plants in different parts of the world. It has been indicated that salinity can inhibit the early stages of nodulation process between bacterium and the host plant including the exchange of signal molecules (*nod* gene inducers). There has not been any research regarding the effects of *nod* gene inducers on the growth of alfalfa inoculated with *Sinorhizobium meliloti* under saline conditions. A growth chamber experiment was conducted to determine the effects of pre-incubation of *S. meliloti* with effective inducers of *nod* genes Luteolin, Methyl jasmonate and Genistein on the growth and N-fixation of two different alfalfa (*Medicago sativa* L.) cultivars (Yazdi and Hamedani) under salt stress. *Nod* gene inducers increased alfalfa growth and N fixation under normal as well as under salt stressed conditions. Yazdi cultivar showed to be more tolerant to salinity than Hamedani with a higher growth rate and N fixation. Luteolin was the most effective *nod* gene inducer on plant growth and N fixation under normal and as well under salt stressed conditions. The results suggest that pre-incubation of *S. meliloti* with effective *nod* gene inducers can improve alfalfa growth and N fixation under salinity stress.

Keywords: Alfalfa (*Medicago sativa* L.), Genistein, Luteolin, Methyl jasmonate, , *Nod* gene inducers, Salinity, *Sinorhizobium meliloti*.

INTRODUCTION

The process of symbiosis between legume and rhizobia is the most important source of biological nitrogen (N) fixation in agricultural systems (Kahindi *et al.,* 1997). Alfalfa is one of the important nitrogen extractors, in the form of nitrate because of its capability to produce many nodules for nitrogen fixation (Rasse and Smucker, 1999). *Sinorhizobium meliloti* is the bacteria developing symbiotic association with alfalfa (*Medicago sativa* L.) plants. The symbiotic process between *S. meliloti* and alfalfa is complex and includes a series of signal exchange between the plant and bacteria (Long, 1989; Brewin, 1991; Denarie, *et al.,* 1993; Rhijin *et al.,* 1995).

During the early stages of symbiosis and in response to signal molecules such as flavonoids and plant hormones, released through legume roots, a series of genes known as *nod* (nodulation) genes are expressed in bacteria, resulting in the formation of nodules (Rosas *et al.,* 1998). As a result of *nod* gene expression, through nod gene inducers, *nod* factors, lipo-chito-oligosaccharides, are produced causing root hair curling, cell division and root nodule formation. Cells of *S.*

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meliloti then invade the developing root nodule through infection threads, eventually forming nodule where they differentiate into bacteroids for nitrogen fixation (Long, 1989; Brewin, 1991; Denarie and Cullimore, 1993; Rhijin and Vanderleyden, 1995).

Jasmonic acid and Methyl jasmonate, collectively known as jasmonates, are signaling molecules in plants, play a role in response to wounding, pathogen attack, reproduction, development, metabolic regulation and abiotic stress (Devoto and Turner, 2003; Howe, 2004; Mabood *et al.,* 2005). Mabood *et al.,* (2005) expressed that Jasmonates can act as signal molecules in the early stages of legume-rhizobia symbiosis, inducing *nod* genes of *Bradyrhizobium japonicum*. Studies have shown that Genistein is also an important isoflavonoid inducing rhizobial nodulation genes in the early stages of symbiosis between soybean (*Glycine max* L.) and *B. japonicum* (Zhang *et al.,* 1996; Pan and Smith, 2000).

Nod factors produced in the presence of Genistein can support nodule growth through regulating hormonal activities (Hirsch, 1992) and cell division (Verma, 1992). Luteolin is also one of the flavonoids inducing nodulation genes in the bacterium *S. meliloti* (Hubac *et al.,* 1993). It is released from alfalfa imbibing seeds; it increases growth rate of *S. meliloti* (Hartwig *et al.,* 1991) and thus plays an important role in nodule formation (Alexander, 1985). Appropriate flavonoids in root secretions are important factors in root nodule formation (Richardson *et al.,* 1998).

In a number of studies, application of *nod* inducing molecules as a tool to increase nodulation and N fixation has been proved (Davis and Johnston, 1990; Bandyopadhyay *et al.,* 1996; Pan *et al.,* 1998). Such environmental stresses as salinity, pH and low temperature in root zone, partially or completely inhibit the early stages of symbiosis, including signal transfer between legume plants and rhizobium (Hungria *et al.,* 1991; Ikeda, 1994; Miransari and Smith, 2007; 2008; 2009). Accordingly, the proliferation of bacteria (Brown *et al.,* 2001) and N fixation (Bilgrami, 1989) is influenced.

In legumes, salinity by sodium chloride, in the range of 50 to 100 mM, significantly limited yield by adversely affecting plant growth, root nodules bacteria, the process of symbiosis, as well as N fixation (Zahran and Sprent, 1986; Bekki *et al.,* 1987). Environmental stresses influence the early stages of symbiosis and nodulation, with flavonoids having their role in these stages and their effect as signal molecules in symbiosis establishment between legume-*rhizobium* having been studied. Mabood *et al.* (2006a) found that Jasmonates induced rhizobial *nod* genes and therefore enhanced the establishment of symbiosis process.

Poustini *et al.* (2005) studied the effects of low temperature on bean plants showing that pre-incubation of the rhizobial cells with Genistein and Methyl jasmonate increased nodulation, N fixation and growth in both optimal and suboptimal root zone temperatures. Zhang and Smith (1996) indicated that the reduction in soybean yield in low temperatures is mainly due to N limitation. Pre-incubation of *B. japonicum* with Genistein increased grain yield as well as protein. Miransari and Smith (2009) studied the effects of salinity on soybean growth and nodulation showing that pre-treating rhizobial cells with Genistein increased nodulation and growth in soybean.

Begum *et al.* (2001a) studied pea growth and nodulation under short growing season and found that plants, inoculated with preinduced *Rhizobium leguminosarum* bv. v*iceae* with Hesperetin, Narngenin, Luteolin and Apigenin carried a higher nodule number than the control plants. With respect to the significance of salinity stress affecting legume production in different parts of the world and because salinity stress usually influences the early stages of symbiosis the following objectives were tested through this experiment. (1) The effects of salinity on alfalfa growth and nodulation, and (2) to evaluate if pre-incubating *S.* meliloti with different *nod* gene inducers including Luteolin, Methyl jasmonate and Genistein can alleviate the adverse effects of salinity on alfalfa growth, nodulation and nitrogenase activity under salt stress.

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MATERIALS AND METHODS

To evaluate how pre-incubating *S. meliloti*, strain 1021 (provided by the Institute for Genetics, Department of Biology, University of Dresden, Germany) with different *nod* gene inducers, including Luteolin, Methyl jasmonate and Genistein, affect alfalfa growth, a growth chamber experiment was conducted. The experiment was arranged in three randomized blocks, with a 2×2×4 factorial design, two cultivars (Yazdi and Hamedani), two levels of salt (0 and 120 mM NaCl) and three inducers of nodulation (Luteolin, Methyl jasmonate and Genistein) along with control.

S. meliloti, strain 1021 were grown in Petri dishes at 28°C for 48 hours in Yeast Mannitol Agar (YMA) medium (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.2 g, yeast extract 0.5 g, agar 15 g plus distilled water 1,000 ml). A single colony of *S. meliloti*, strain 1021 was cultured in 4 ml yeast mannitol broth (YMB) medium (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.2 g, yeast extract 0.5 g and distilled water 1,000 ml). It was shaken at 28°C for 24 hours and then subcultured into 400 ml of fresh YMB medium and then shaken at 28°C for 24 hours. The Bacterial culture was distributed in four flasks containing 100 ml YMB medium. Each flask represented one treatment, namely; *S. meliloti* with no inducer addition (control); *S. meliloti* induced with Luteolin (10 µM); *S. meliloti* induced with Methyl jasmonate (50 µM); and *S. meliloti* induced with Genistein (10 µM).

Luteolin, Methyl jasmonate and Genistein stock solutions were prepared and then (except control) Luteolin, Methyl jasmonate and Genistein were added to the culture and shaken at 28°C for 1 day.

The related concentrations of each inducer were selected according to the results obtained by other researchers (Capela *et al.,* 2005; Mabood and Smith, 2005; Miransari and Smith, 2009).

Alfalfa cultivar seeds (Yazdi and Hamedani) (provided by the Institute of breeding and Providing Seeds and Seedlings of Karaj, Iran) were surface sterilized in sodium hypochlorite (2.5%) and then rinsed several times with distilled water. Seeds were germinated on plates containing water - agar (1%) at 28°C for 48 hours (Howieson *et al.,* 2000).

Five germinated seeds were planted in pots each containing sterile vermiculite, under growth chamber conditions, with light cycle of 16 (light) and 8 (dark) hours, along with day and night temperatures of 28 and 15°C, respectively. Each germinated seed was inoculated with 1 ml of fresh culture of *S. meliloti*, strain 1021, pre-incubated with the three different inducers (Beck *et al.,* 1993).

Salt treatment was applied at sowing (Miransari and Smith, 2009). Salty Hoagland's solution (Hoagland and Arnon, 1950), with no nitrate, containing 120 mM of NaCl (S_2) and control (S_1) were employed. Salinity concentration was selected based on salt tolerance threshold and maximum salt tolerance of alfalfa (Shannon, 1984; Fougere *et al.,* 1991). Plants were harvested one month after sowing (Miransari and Smith, 2009) 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. Data analysis was performed using MSTATC statistical software and comparison of means was performed with Duncan's multiple range test.

RESULTS

Effects of Cultivar on Nodulation and Plant Growth

Analysis of variance indicated that there existed significant differences between shoot and root dry weights, nodule number ($P< 0.01$), and dry weight ($P< 0.05$) for different alfalfa cultivars. Yazdi cultivar resulted in

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higher nodule number and dry weight, as well as higher shoot and root dry weights than Hamedani cultivar (Table 1).

Effects of Inducers on Nodulation and Plant Growth

Analysis of variance indicated that there was a significant interaction between salt and inducers on nodule number and dry weight and on shoot and root dry weights ($P< 0.01$). The results revealed that salinity decreased shoot and root dry weights and also nodule number as well as dry weight. Accordingly, although salinity adversely affected nodulation and plant growth, plants inoculated with rhizobium, pre-incubated with inducers, had higher nodule number and dry weight as well as shoot and root dry weights in both S_1 and S_2 treatments ($P< 0.01$) relative to control (Figures 1 and 2). The percentage of increase was higher in S_2 treatment than in S_1 and as well in Luteolin treatment in comparison with the other related treatments (Table 2).

Nitrogenase Activity (Acetylene Reduction)

Analysis of variance showed that there was a significant interaction between salt and cultivars on nitrogenase activity ($P<$

Table 1. Mean comparison of nodule number and dry weight, and shoot and root dry weights in alfalfa cultivars.

Cultivars	Nodule number	Nodule dry weight (mg plant^{-1})	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})
Yazdi	15^{a}	1.383^{a}	15.44^{a}	6.09^{a}
Hamedani	14^{b}	1.288^{b}	14.89^{b}	5.76^{b}

Means followed by different letters in each column, are statistically different using Duncan's method.

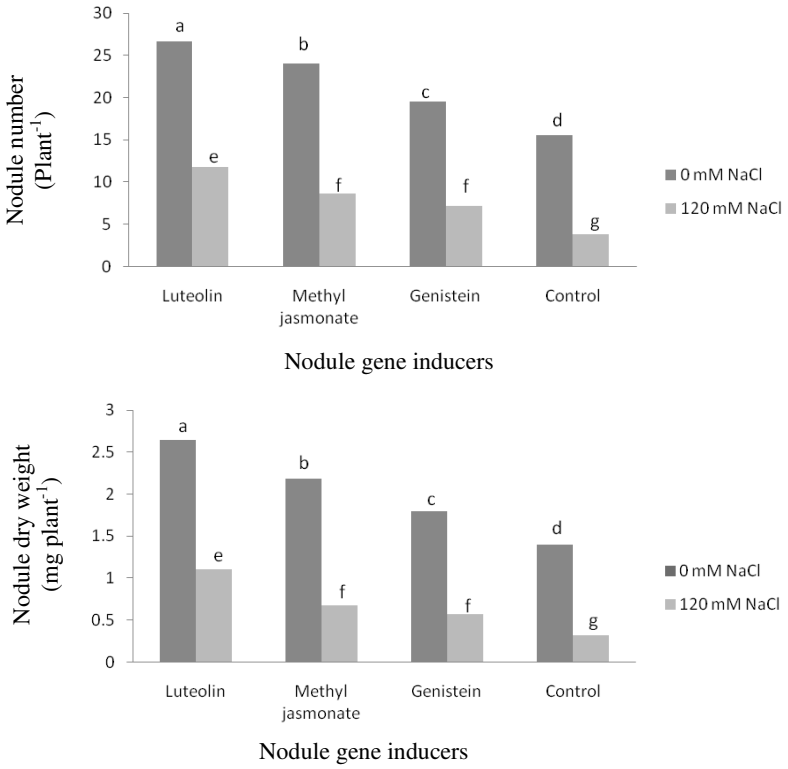

Figure 1. Nodule number and dry weight as affected by salt and inducer treatments. Histograms with different letters are statistically different as by Duncan's test.

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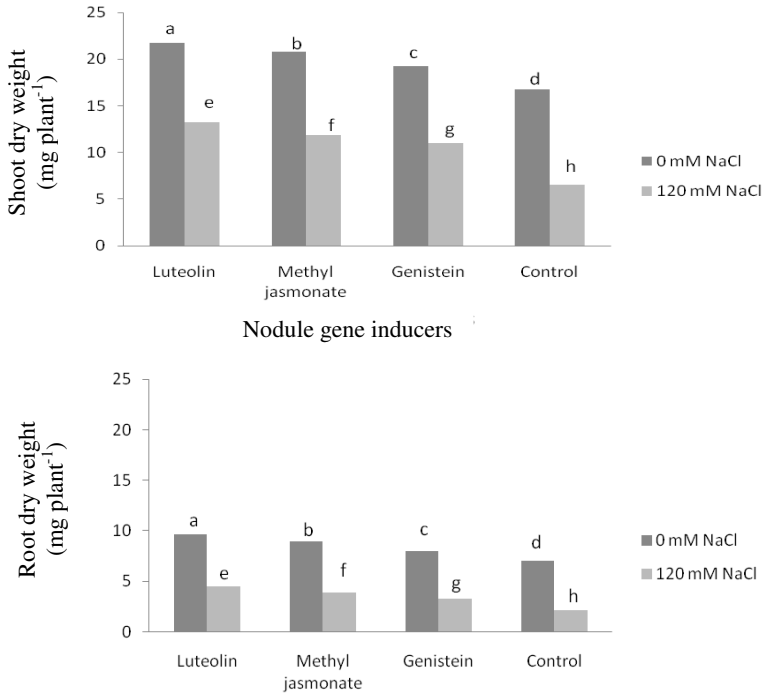

Figure 2. Shoot and root dry weight as affected by as well as salt and inducer treatment. Histograms with different letters are statistically different as by Duncan's test.

0.05). Salinity decreased nitrogenase activity in both cultivars; however Yazdi cultivar exhibited higher nitrogenase activity than Hamedani cultivar (Figure 3). However, inoculation of plants with rhizobium, pre-incubated with inducers, resulted in higher nitrogenase activity in both S_1 and S_2 treatments ($P< 0.01$) relative to the control plants (Figure 3). Accordingly, the percentage increase was higher in S_2 treatment than in S_1 and in Luteolin and Methyl jasmonate treatment as in comparison with the other related treatments (Table 2).

DISCUSSION

The results finally indicate that application of inducers increased nodulation and plant growth under stressed and non-stressed conditions relative to control. Salinity can

Table 2. Effect of different nod gene inducers on the percentage of increase in nodulation and plant growth at 0 and 120 mM NaCl in comparison with control. Means of cultivars Yazdi and Hamedani.

	Nodule number (%)		Nodule dry weight (%)		Shoot dry weight (%)		Root dry weight (%)		Nitrogenase activity (%)	
Inducers	S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2
Luteolin	72.63^{aB}	217.8^{aA}	90.73^{aB}	280^{aA}	29.72^{aB}	102.8^{aA}	38.12^{aB}	116.2^{aA}	140.1^{aB}	184.7^{aA}
MJ	55.55^{aB}	132.2^{bA}	57.43^{bB}	129.7^{abA}	24.08^{bB}	82.17^{bA}	27.67^{bB}	85.77^{bA}	74.83^{bB}	140^{bA}
Genistein	26.56^{bB}	88.33^{bA}	29.87^{bB}	91.39^{bA}	14.61^{cB}	68.49^{cA}	13.94^{cB}	53.97^{cA}	23.5^{cB}	99.05^{cA}

S_1: Salinity treatment control; S_2: 120 mM NaCl, MJ: Methyl jasmonate.
Means followed by different letters are significantly different using Duncan's method.
Capital letters compare levels of salt, while small ones compare different inducers within each stress level.

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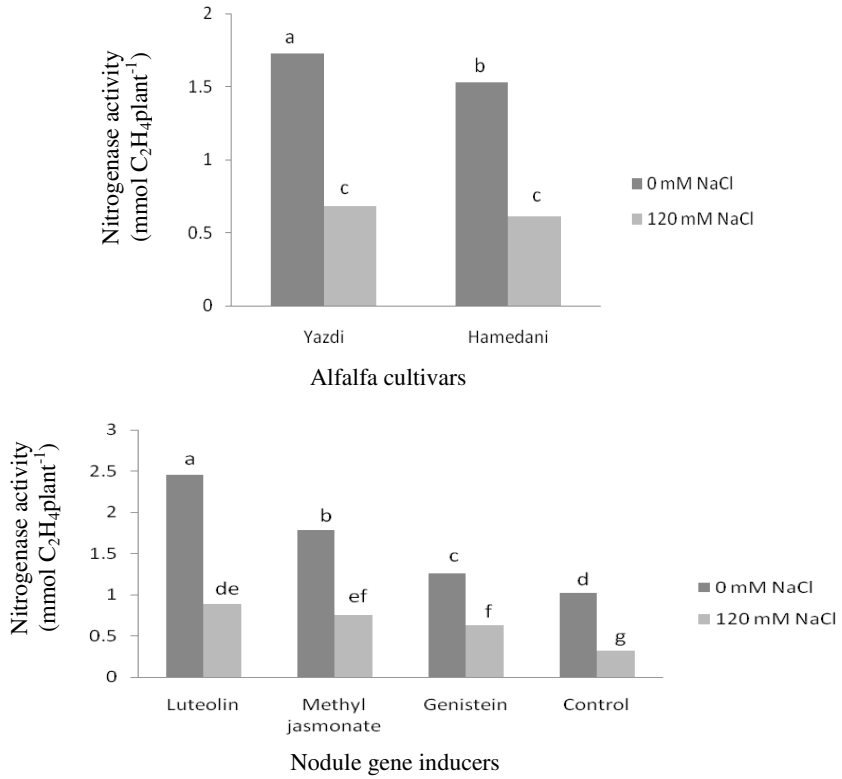

Figure 3. Nitrogenase activity as affected by salt and cultivar and salt and inducer treatments. Histograms with different letters are statistically different by Duncan's test.

adversely affect the early stages of symbiosis (Hungria *et al.,* 1991; Miransari and Smith, 2007; 2009). Most of these inhibitions are associated with such root hair morphology and physiologies, as root hair growth, diameter, structure as well as curling (Miransari *et al.,* 2006). Salt stress limits the translocation of photosynthates to the roots and thus reduces nodulation and plant growth (Miransari and Smith, 2007).

Flavonoids produced by legume plants result in the induction of rhizobial nodule genes required for infection, supporting bacterial movement toward the root, and increasing the rate of growth of bacterial cells, thus affecting root nodulation. (Phillips and Tsai, 1992). *Nod* gene inducers have resulted in enhanced nodulation in different legume host plants including pea (Ahlawat *et al.,* 1998; Bandyopadhyay *et al.,* 1996; Lira *et al.,* 2003; Novak *et al.,* 2002) bean (Poustini *et al.,* 2005, 2007) and soybean (Zhang and Smith, 1995; Pan *et al.,* 1998; Belkheir *et al.,* 2000).

There has previously been some interesting research work carried out according to the following by Professor Donald Smith and his research team on soybean regarding the use of *nod* gene inducers for the incubation of *B. japonicum* under stress. Pre-treatment of *B. japonicum* with *nod* gene inducers which resulted in an increase in plant growth and development. Genistein and Jasmonates resulted in higher dry matter than the control under different conditions including under suboptimal root zone temperature. Increased dry matter can be resulted by enhanced N fixation as the *nod* gene inducers can increase nodule number as well as dry weight (Zhang and Smith 1995; 1997; Mabood and Smith; 2005; 2006b). Pan and Smith (2000) found that incubation of *B. japonicum* with 40 µM Genistein increased root dry weight in soybean.

Begum *et al.* (2001a, b) reported that pre-treatment of *R. leguminosarum* bv. *viceae* cells with appropriate flavonoid molecules

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including Apigenin, Daidzein, Genistein, Hesperetin, Kaempferol, Luteolin, Naringenin and Rutin increased pea and lentil dry matter, subjected to sub optimal root zone temperatures, under greenhouse as well as field conditions. Poustini *et al.,* (2007) used pretreated *Rhizobium leguminosarum bv. phaseoli* with Genistein and Methyl jasmonate to inoculate bean plants and found increase in nodule number, N content and plant dry matter. According to Miransari and Smith (2009), inoculation of soybean seeds with *B. japonicum*, pretreated with the *nod* gene inducer, Genistein, increased nodule number and dry weight, N fixation and hence shoot dry weight under salinity.

The results of this research work also indicated that the *nod* gene inducers resulted in higher root and shoot dry weight, nodule number and dry weight as well as in nitrogenase enzyme activity, under stressed and non-stressed conditions relative to the control. Yazdi cultivar as compared with Hamadani cultivar had higher shoot and root dry weight, as well as higher nodule number and dry weight under salinity conditions. Yazdi cultivar probably uses some such mechanisms as absorption of a lower level of sodium and chloride ions, production of osmolyets and allocation of salt to its cellular vacuoles, increasing plant resistance to salinity. It may also be able to excrete higher levels of signal molecules under salinity resulting in higher root nodulation.

Luteolin was the most effective signal molecule under salinity. Plants inoculated with pre-treated rhizobial cells with Luteolin exhibited higher nodulation, nitrogenase activity, N fixation and dry matter accumulation in both normal and salt stress conditions than plants pre-treated with rhizobial cells from Methyl jasmonate and Genistein. It is likely that the biochemical structure of Luteolin is much less affected by salt ions relative to Methyl jasmoante and Genistein. In addition, it is probably a more affective *nod* gene inducer activating the nodulation genes in *S. meliloti*. It can also be due the fact that alfalfa produces Luteolin, inducing the *nod* genes in *S. meliloti* (Hartwig *et al.,* 1990), while Genistein produced by soybean inhibits such induction (Hirsch *et al.,* 2001). Signal molecules act completely specifically in the induction of rhizobium *nod* genes. This is the reason for an indication of the specificity of legume-rhizobium symbiosis (Long, 2001).

CONCLUSIONS

The results, which to the best of the authors' knowledge have not been previously investigated, indicate the effectiveness of the signal molecule Luteolin in the alleviation of salt stress on alfalfa growth and nodulation. Also the differences between different cultivars indicated that Yazdi cultivar was a more resistant cultivar to salinity than Hamedani. Pre-incubation of *S. meliloti* with, especially, Luteolin can inhibit the adverse effects of salinity on alfalfa growth and nodulation.

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اثر پیش تیمار باکتری ریزوبیوم *Sinorhizobium meliloti* با جنیستین، متیل جاسمونات و لوتئولین بر رشد، گره بندی و تثبیت نیتروژن گیاه یونجه تحت شرایط تنش شوری

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چکیده

شوری یکی از مهمترین تنش های محیطی در نقاط مختلف جهان است که تثبیت نیتروژن در گیاهان لگومینوز را تحت تأثیر قرار می دهد. شوری می تواند مراحل اولیه همزیستی بین باکتری ریزوبیوم و گیاه میزبان شامل تبادل سیگنال های مولکولی (ترکیبات القاء کننده های ژن های تولید گره) را بازداری کند. براساس اطلاعات، بررسی بر روی اثرات ترکیبات القاء کننده های ژن های تولید گره در رشد گیاهان یونجه تلقیح شده با *Sinorhizobium meliloti* تحت شرایط تنش شوری انجام نشده است. به منظور تعیین اثر پیش تیمار *Sinorhizobium meliloti* با ترکیبات القاء کننده ژن های تولید گره (لوتئولین، متیل جاسمونات و جنیستین) بر روی رشد و تثبیت نیتروژن گیاه یونجه (رقم یزدی و همدانی) تحت تنش شوری آزمایشی در شرایط اتاقک رشد انجام شد. نتایج نشان داد که ترکیبات القاء کننده ژن های تولید گره سبب افزایش رشد و تثبیت نیتروژن گیاه یونجه هم در شرایط بدون تنش شوری و هم در شرایط تنش شوری می شوند. رقم یزدی دارای رشد بهتر و تثبیت نیتروژن بیشتری نسبت به رقم همدانی و در نتیجه دارای مقاومت بیشتری نسبت به تنش شوری بود. لوتئولین یکی از مؤثرترین مولکول های سیگنال دهنده گیاه-باکتری در رشد و تثبیت نیتروژن گیاه هم در شرایط بدون تنش و هم در شرایط تنش شوری بود. نتایج نشان داد که پیش تیمار *Sinorhizobium meliloti* با ترکیبات القاء کننده ژن های تولید گره می تواند سبب بهبود رشد و تثبیت نیتروژن گیاه یونجه تحت شرایط تنش شوری شود

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