

Preincubation of *Rhizobium leguminosarum* bv. *phaseoli* with Jasmonate and Genistein Signal Molecules Increases Bean (*Phaseolus vulgaris* L.) Nodulation, Nitrogen Fixation and Biomass Production

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ABSTRACT

The objective of this research work was to study the effect of *Rhizobium leguminosarum* pre-induced with genistein (G) and methyl jasmonate (MJ) on nodulation, nitrogen fixation and early growth of the common bean (*Phaseolus vulgaris* L.). Four day-old seedlings were transferred to pots, containing sterilized sand and turface (2:1 v:v) mixture and, receiving Hoagland's nutrient solution, without nitrogen. The plants were grown in a growth chamber using a randomized complete block design with six replications. The rhizobia were cultured in TY medium and incubated with G, and/or MJ. The incubated rhizobia were used after 24 hours to inoculate the bean seedlings. The results showed that both G and MJ enhanced the nodule number, nitrogen content per plant, and plant dry matter. Genistein and MJ, when applied together to cultures, showed synergistic effects on all the characters studied. The results of this study indicated that MJ alone, or in combination with G, can be used to promote bean nodulation, nitrogen fixation and early plant growth.

Key words: Genistein, Jasmonates, Nitrogen fixation, Nodulation, *Phaseolus vulgaris*, *Rhizobium leguminosarum* bv. *Phaseoli*.

INTRODUCTION

Nitrogen fertilizer has now become a major environmental problem and a health hazard (Addiscott, 1996; Zahran, 1999) while symbiotic N₂ fixation offers a sustainable alternative. The common bean is a nitrogen fixing legume and one of the world's most important sources of dietary protein (Hantngston *et al.*, 1986). In spite of its ability to fix nitrogen, the addition of nitrogen fertilizers is usually recommended for bean production (Piha and Munns, 1987) since the common bean appears to be inferior to other grain legumes in nodulation and N₂ fixation

(Richardson *et al.*, 1988; Bandyopadhyay *et al.*, 1996; Graham, 1981). However, it is generally thought that nitrogen fixation in beans is not genetically inferior (Franco and Munns, 1981), rather that host variety and *Rhizobium* strain incompatibilities contribute to the frequently unsatisfactory response of beans to inoculation under field conditions (Graham, 1981).

Like other legume crops, there have been many investigations of ways to improve symbiotic nitrogen fixation by beans, leading to yield improvement. However, the bean symbiotic N₂ fixation system seems unable to provide sufficient nitrogen, while

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Rhizobium inoculants are seldom used to reduce bean dependence on combined nitrogen since the response of bean to inoculation has proved too variable (Graham, 1981). The use of higher inoculation rates, modified inoculant carriers, and strains selected for competitiveness in nodulation have all failed to overcome this problem (Montealegre *et al.*, 1995). There have been a few reports of increased seed yield following rhizobial inoculation (e.g. Park and Buttery, 1989); however, many other reports suggest that inoculation with commercial strains, compared to an uninoculated control, fails to increase the seed yield (Weiser *et al.*, 1985; Richardson *et al.*, 1988; Chavera and Graham, 1992). An increase in the rhizospheric concentration of *nod* gene inducers through exogenous application has been reported for some legumes, but not for beans (Bandyopadhyay *et al.*, 1996; Pan *et al.*, 1998).

Among the rhizospheric factors potentially influencing nitrogen fixation by symbiotic systems, plant molecular signals involved in plant-rhizobia interactions are promising. It is reported that the presence of appropriate flavonoids in root exudates is an important contributing factor in nodule formation (Richardson *et al.*, 1988). The nodulation status (nodule number and nodule weight) of peas is reported to be improved by the addition of the flavonoid naringenin (Bandyopadhyay *et al.*, 1996). Several studies have documented the use of flavonoid inducer molecules as a tool in enhancing nodulation and nitrogen fixation (Davis and Johnston, 1990; Bandyopadhyay *et al.*, 1996; Pan and Smith, 1998). Isoflavonoid application to the rooting medium or to the culture medium has been demonstrated to increase nitrogen fixation, biomass production and grain yield. For example, when genistein was added to the rooting medium of soybeans, the nodule number, nodule weight, and nitrogen concentration of the plants all increased. Also, in soybeans, the inoculation of seeds with *Bradyrhizobium japonicum* pre-incubated with genistein increased nodulation at low root zone temperatures (Zhang and Smith, 1995).

Methyl jasmonate (MJ) is a volatile compound emitted by plant leaves and involved in inter-plant communication, inducing proteinase inhibitor gene expression in neighboring plants (Farmer and Ryan, 1990). However, at sub-micromolar concentrations, it can promote root growth (Tung *et al.*, 1996). The application of JA to leaves also decreases the expression of nuclear and chloroplast genes involved in photosynthesis (Creelman and Mullet, 1997). The central role of jasmonates in plant responses to herbivores and defense mechanisms has been fully investigated. The addition of MJ stimulates the *de novo* transcription of genes that are known to be involved in plant chemical defense mechanisms (Gundlach *et al.*, 1992). However, with regard to rhizobia, it has been shown that the *nod* genes are inducible by JA and MJ, as well as by flavonoid inducers (Rosas *et al.*, 1998). Some synergistic effects have been observed when two signal inducer molecules were used to pre-incubate cultures of rhizobia subsequently used as inocula. For example, the combination of hesperetin and naringenin resulted in a better induction of *nod* gene activities than either of them alone (Begum *et al.*, 2001).

There is no published work regarding nodulation, N₂ fixation and the resultant growth of bean plants when inoculated with rhizobia pre-incubated with inducer molecules. The work reported here was therefore conducted to evaluate the response of bean plants to *Rhizobium leguminosarum* bv. *phaseoli* pre-induced with genistein and/or methyl jasmonate.

MATERIALS AND METHODS

Seeds of white bean (*Phaseolus vulgaris* L.) cultivar Obeto were surface-sterilized using 40% bleach for 1.5 minutes (Montealegre *et al.*, 1995) and then placed in vermiculite filled plastic trays of 27×50×6 cm, for germination. Two of the four day-old seedlings were transferred to 1-L pots, containing 950 mL of sterilized sand and surface

(2:1 v:v) mixture. The experiment was structured following a Randomized Complete Block Design (RCBD) with six replications and two samplings. The plants were grown in a growth chamber at 25°C temperature, with a 16/8 h photoperiod and 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ light intensity. Throughout the growth period, each pot received 40 mL of Hoagland's nutrient solution without nitrogen, (Hoagland and Arnon, 1950) four times a week; the plants were also watered with distilled water if the growth medium appeared dry enough to warrant this.

Rhizobium leguminosarum bv. *phaseoli* strain 127K105 (Nitragin, Inc. Milwaukee, USA) was cultured in TY medium and, after 48 hours of shaking at 26°C, the bacterial culture was divided into four flasks, each flask representing one treatment. The inoculant treatments were as follows: 1) Control, *R. leguminosarum* bv. *phaseoli* without added inducer, 2) *R. leguminosarum* bv. *phaseoli* induced with genistein (20 μM), 3) *R. leguminosarum* bv. *phaseoli* induced with MJ (50 μM), and 4) *R. leguminosarum* bv. *phaseoli* induced with both genistein (20 μM) and MJ (50 μM). The 50 μM concentration was selected for MJ on the basis of reported β -galactosidase activity of rhizobial strains containing a reporter gene attached to a *nod* gene promoter *Rhizobium leguminosarum* in one case (Rosas *et al.*, 1998) and *Bradyrhizobium japonicum* strain 532C in a second case (Mabood, personal communication). After 24 hours of further shaking, the inoculants were diluted to an optical density of A_{600} 0.2 using sterile distilled water (Ultraspex 4300 Pro UV/Visible Spectrophotometer). An OD_{600} value of 0.08 indicates approximately 10^8 cells mL^{-1} (Bhuvaneshwari *et al.*, 1980). Each seedling received 1 mL of inoculant applied to its base. The plants were seven days old when inoculated.

Leaf nitrogen status was measured indirectly using a SPAD (Soil Plant Analysis Device) leaf greenness meter (SPAD-502, Minolta, Japan). The SPAD data were collected seven days before harvesting. At the end of the experiment, when the plants were thirty eight days old, data were collected for

the following variables: leaf number, leaf area, shoot and root dry weight, nodule number and weight. The nodule and shoot nitrogen concentrations of the plants were measured using an NC 2500 Elemental Analyzer (CE Instrument Inc., Italy). Nitrogen content was derived by multiplying the dry weight by the nitrogen concentration. Shoot and nodule nitrogen content data are given on a per plant basis. The data obtained were statistically analyzed using CoStat software, and comparisons of means were conducted using an ANOVA protected LSD ($p < 0.05$) test. This experiment was repeated twice. The results were similar for both experiments, and the data from the second experiment are given as an example.

RESULTS AND DISCUSSION

Nodule number was increased by the application of inducer treatments to the inocula (Table 1). The genistein and MJ treatments caused more infections, or the success of a greater number of already initiated infections, leading to the formation of more nodules (Table 1). Bean plants exude several *nod* gene inducer molecules of which naringenin and genistein are the most potent. The utility of naringenin (a flavonoid) induced rhizobial cells in nodule initiation and development has been demonstrated for peas (Bandyopadhyay *et al.*, 1996).

The finding that genistein and/or MJ increased nodulation is in agreement with previous results. Zhang and Smith (1995) reported that pre-incubation of *B. japonicum* cells with genistein accelerates nodulation under controlled environment low root zone temperature (RZT) conditions. They attributed this increase in nodulation at low RZT to the alteration of the time-course of each nodulation stage. Incubation of *B. japonicum* cells with genistein enhanced the early stages of nodule formation thus accelerating the onset of nodule development. The induction of *B. japonicum* cultures with MJ also increased nodulation in soybean plants (Mabood and Smith, personal communication).

**Table 1.** Effect of *R. leguminosarum* bv. *phaseoli* induced with various inducer signal molecules on plant growth and nodulation variables of 38 days old plants of common bean (*Phaseolus vulgaris* L.).

Treatments	Leaf area (cm ² plant ⁻¹)	Leaf number Plant ⁻¹	Nodule number plant ⁻¹	Nodule dry weight g plant ⁻¹	Plant N content mg plant ⁻¹	Nitrogen increase %	Plant N Concentration mg g ⁻¹	SPAD readings	Ind. Nodule dry weight mg nodule ⁻¹	Nodule N Concentration mg g ⁻¹
Control	719.8	12.16	351 c	0.374	126.01 c	---	0.0390	37.6	1.08 a	0.0388
G	822.9	13.33	457 b	0.375	139.65 ab	10.82	0.0396	40.4	0.82 b	0.0388
MJ	706.0	13.16	480 ab	0.375	138.30 b	9.75	0.0403	41.8	0.79 b	0.0396
G+MJ	814.1	13.58	544 a	0.394	151.18 a	19.97	0.0402	41.45	0.73 b	0.0394
Difference level	ns	ns	*	ns	*	---	ns	ns	***	ns

G: Genistein, MJ: Methyl jasmonate SPAD: Chlorophyll meter

MJ is a new class of signalling molecule in rhizobia-legume symbioses. Incubation of *B. japonicum* cells with MJ induced the expression of *nod* genes, leading to higher levels of nod factor production (Mabood and Smith, personal communication). It may be that the higher levels of nod factor led to the formation of more nodules, removing a nitrogen limitation to growth. However, Souleimanov *et al.* (2002) reported that nod factors can increase plant growth directly. If this were the case, the increased growth would have increased nodule specific activity in the short run and nodule activity over the longer term. When the plants in the experiment were harvested, we noted that inducer treated plants had nodules of different sizes, some of them being as large in size as those of plants inoculated with uninduced *R. leguminosarum* cells, while others were small and smaller up to those that seemed to be in the early stages of appearance. It may be that both control and inducer-treated plants formed the same number of nodules initially, both responding to the same autoregulation of nodule number (Bhuvanewari *et al.*, 1980), and that the greater levels of nod factor produced by the pre-induced *R. leguminosarum* cells caused faster plant growth for those treatments. This greater growth would have increased N demand, leading to greater nodule specific activity. As the plants grew and the N demand grew, the autoregulation effects would eventually allow the formation of new nodules, with this occurring sooner in the plants inoculated with pre-induced inocula. This would explain nodule weights that were similar (all newly formed nodules were very small and would have contributed very little to the nodule weight per plant) but the greater nodule numbers on plants treated with induced inoculants.

Nodule dry weight was not different among the treatments, although the genistein plus methyl jasmonate treatment did produce numerical increases that were not statistically significant (Table 1). However, these increases may have been biologically meaningful as nodule dry weight was highly cor-

related with both plant dry matter and plant nitrogen content (Table 2). Conversely, as described above, the slightly increased nodule mass may have been the result of increased plant growth, rather than the cause of it, as described in the previous paragraph. In any case, the inducer treatments did cause more nodules to be formed (Table 1). The same treatments also resulted in a decrease in the average weight per nodule (Table 1), so that plants inoculated with induced rhizobial cells formed more and smaller nodules, with no significant increase in nodule dry weight.

It is reported that smaller nodules have greater specific nitrogenase activities than larger nodules (Lindemann and Ham, 1979) and, in our case, the increase in nodule number and associated decrease in nodule size led to an increase in the total N present in the plants (Table 1). Plants inoculated with bacteria pre-incubated with genistein and MJ had the highest plant nitrogen contents, resulting in an increase of close to 20% over the control (Table 1). The pre-induction of the rhizobial *nod* genes apparently led to more nitrogen being fixed in nodules of plants inoculated with them than with the uninduced control inoculant. Given that the seeds used in the experiment were from a relatively uniform seed lot, and were further screened for uniformity by us prior to use, the N content per seed would have varied little, so that differences in total N per plant represent differences in the amount of nitrogen fixed. Thus, treatment of the inocula with inducer compounds led to the formation of more nodules and more efficient nodules, this leading to improved N₂ fixation and improved growth. The high nodule number and N content observed in this experiment are consistent with the strong correlation observed between the number and percentage of effective nodules produced on beans (Olivera and Graham, 1990). The nodule number per plant was not correlated with the N content per plant (Table 2). This is probably because the increased N content follows from greater nodule efficiency due to the smaller nodule size.

Table 2. Correlation coefficients between different aspects of bean plants inoculated with preincubated rhizobia with genistein and methyl jasmonate treatments.

	Dry weight plant ⁻¹	Plant nitrogen content	Leaf number plant ⁻¹	Nodule number plant ⁻¹	SPAD	Leaf area plant ⁻¹	Ind. nodule dw	Nodule dw plant ⁻¹
Nodule N content	46.0**	59.0**	55.0**	29.3 ^{ns}	33.8 ^{ns}	37.2 ^{ns}	2.9 ^{ns}	92.6***
Nodule dw plant ⁻¹	79.9**	60.4**	55.0**	29.3 ^{ns}	46.8*	35.0 ^{ns}	1.1 ^{ns}	
Ind. nodule dw	-6.6 ^{ns}	-12.1 ^{ns}	-46.0*	-89.9**	-40.9*	-25.2 ^{ns}		
Leaf area plant ⁻¹	35.6 ^{ns}	22.3 ^{ns}	52.3**	37.2 ^{ns}	17.3 ^{ns}			
SPAD	26.5 ^{ns}	26.8 ^{ns}	51.4*	45.7*				
Nodule number plant ⁻¹	29.6 ^{ns}	24.0 ^{ns}	61.8**					
Leaf number plant ⁻¹	28.1 ^{ns}	32.9 ^{ns}						
Plant nitrogen content	88.8***							

In addition, a number of the nodules may have been immature for plants treated with genistein and methyl jasmonate. Thus, a larger number of smaller nodules from the out-set may have increased nodule efficiency and the total availability of N, reducing or relieving an N limitation to growth or, as described above, enhanced levels of nod factor production may have led to greater growth and the production of a second flush of nodules, still not fully formed and small at the time of harvest.

The higher amounts of nitrogen in plants inoculated with induced rhizobia probably contributed to the greater observed chlorophyll production (as measured by leaf greenness) and, although no data were collected on photosynthetic rates, these were probably increased because dry matter accumulation was increased (Figure 1). The greater leaf greenness and its known association with available N favours the “greater nodulation and relief of N limitation” explanation of improved growth. The N content of plants, across all the treatments, was highly and positively correlated with plant dry weight (Table 2). This indicates that, when inducers were not added to the applied

inocula, bean growth was N limited. Addition of inducers to the inocula increased N fixation, removing the N limitation and resulting in greater plant growth. There was a correlation between total N and dry matter, suggesting that higher N availability increased the amount of potential photosynthetic activity, leading to higher growth rates (Table 2).

There were no significant differences between the inducer treatments regarding the nitrogen concentrations of bean plants and their nodules in present study. The reason for this may have been a dilution effect (dilution of additional N by additional dry matter), since dry matter was increased by inoculation with induced rhizobia (Figure 1). However, they could also have been a result of growth driven increases in nodulation and N₂ fixation. A plant with a greater growth rate will have a greater N demand, which can result initially in increased amounts of N fixed per nodule mass (greater nodule specific activity) followed by the formation of additional nodules over the longer term. Pan and Smith (1998) observed higher nitrogen concentrations in soybean plants treated with genistein induced rhizobia but, in their ex-

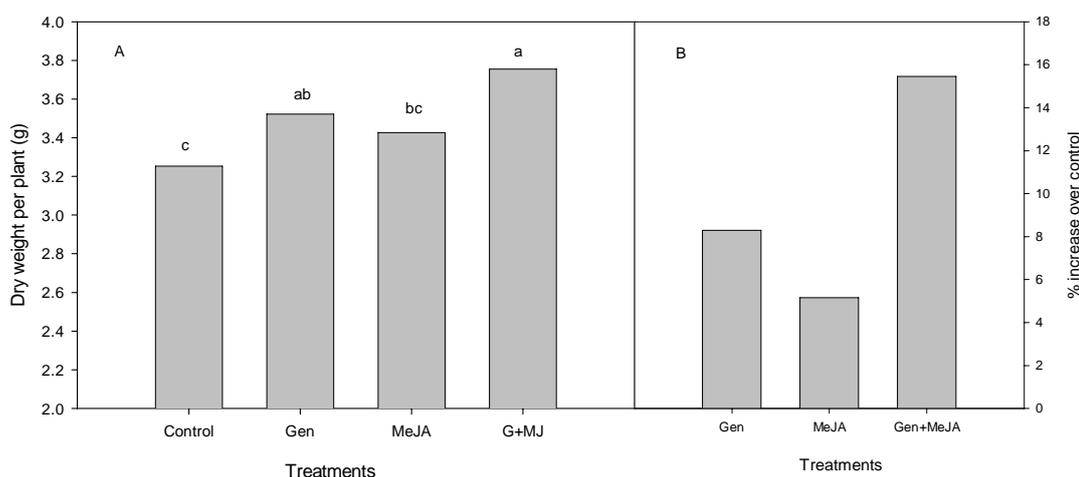


Figure 1.

- (A) Effect of *R. leguminosarum* bv. *phaseoli* induced with various inducer molecules on plant dry matter accumulation.
- (B) Percent increase in dry matter accumulation of common bean plants inoculated with *R. leguminosarum* bv. *phaseoli* induced with genistein, methyl jasmonate or both over control plants receiving *R. leguminosarum* bv. *phaseoli* only.



periments, there was very little increase in dry matter production due to the same treatments.

There was a measure of synergism between the two inducers since plants inoculated with rhizobia induced with both genistein and MJ had the highest dry matter accumulation, 14.4% greater than that of the control plants (Figure 1). Comparison of the treatments in the picture obtained from the first experiment which was in four replications gives support to these findings (Figure 2).

Following the increases in nodule number, nitrogen content of plants and dry matter production an increase in seed yield is likely, although these experiments were not allowed to continue all the way to seed development. Shoot dry matter increment, and not just absolute nodulation data, is a key variable in determining final rhizobial effectiveness on beans (Msumali and Kipe-Nolt, 2002). This has been observed in some legume species but, so far, not in beans. For example, in soybean nodulation, nitrogen content, and dry matter production have

been observed to increase following the application of genistein directly to the root media (Pan and Smith 1998). Our results indicate an increase in dry matter that is closely related to changes in plant nitrogen content (Table 2). This could be a way to increase bean dry matter production through an improvement in nodulation and nitrogen fixation. The findings reported here, therefore, are unique suggesting a possible mechanism to address the consistently reported poor nodulation of bean plants under field conditions (Smith and Hume, 1985; Weiser *et al.*, 1985; Park and Buttery, 1989; Graham and Temple, 1984). The data therefore, do not support the suggestion that the bean plant is genetically inferior in N₂ fixation ability (Piha and Munns, 1987).

Another unique aspect of our data is the positive growth response of bean plants to pre-incubation of *Rhizobium* inocula with inducer compounds are observed at a temperature of 25°C, an optimum growth and nodulation temperature for beans (Rubatzky and Yamaguchi, 1997). To our knowledge, Begum *et al.* (2001) is the most recent pub-

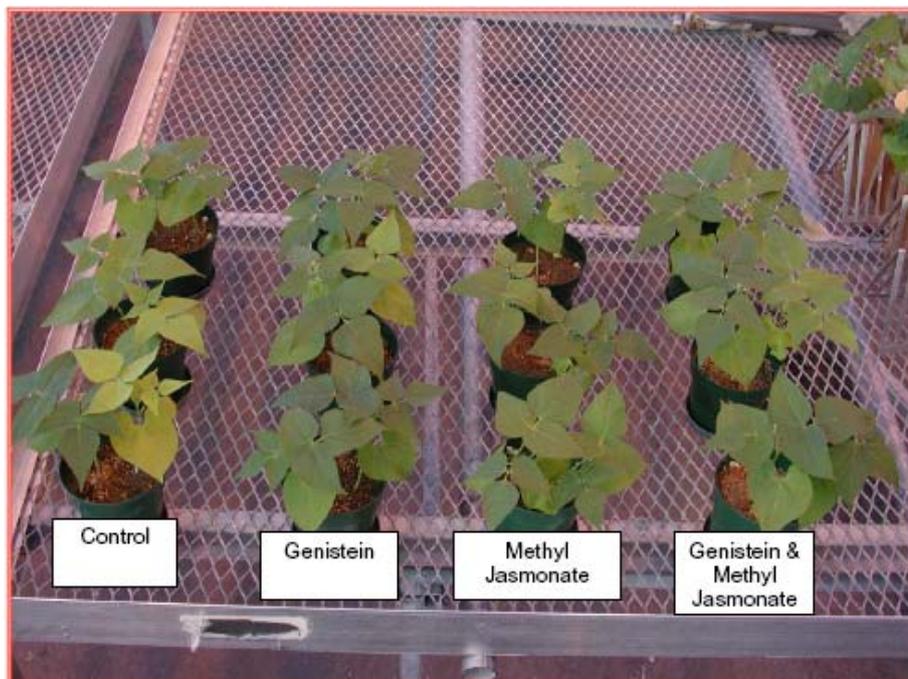


Figure 2. Picture of treated plants in experiment 1. The inducer treatments are indicated in the text boxes below each row of plants.

lication in this area. They reported some increases in nodule number and dry matter accumulation in field peas and lentils, but mainly at a lower temperature of 17°C, when *Rhizobium leguminosarum* pIJ1477, and *Rhizobium leguminosarum* bv. *trifolii* 5280 inoculants were pre-incubated with the flavonoid hesperetin. Zhang and Smith (1995) reported a higher dry matter production by soybean plants in response to the addition of genistein to *Bradyrhizobium japonicum* inoculants at stressfully low root zone temperatures, but not at optimal root zone temperatures. Here, we report a 14.4% increase in dry matter production with consistent increases in nodulation and nitrogen content suggesting that this approach could be utilized in developing a relevant technology to improve yield in bean production. It could also be promising to consider this concept as an area of investigation for yield improvement in other legume crops.

In conclusion, the data presented here indicate that bean plants may not be inferior in nodulation and nitrogen fixation potential. Higher dry matter production, up to 14.4% over the control plants, can be achieved through enhancement of the signal exchange between the rhizobia (*R. leguminosarum* bv. *phaseoli*) and the host plant (bean). Genistein and MJ both induce rhizobial *nod* genes, leading to LCO production, when rhizobial cells are incubated with them prior to the inoculation. It is clear that higher dry matter can be produced by plants inoculated with rhizobia treated with inducer molecules. Hence these results document the potential role of genistein and MJ in enhancing bean nodulation, nitrogen fixation and early plant growth.

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پیش نهفتگی رایزوبیوم لگومینوزاروم با مولکولهای سیگنالی متیل جاسمونات و
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(*Phaseolus vulgaris* L.) را افزایش میدهد

ک. پوستینی، ف. معبود و د. ل. اسمیت

چکیده

هدف این پژوهش مطالعه اثر پیش القاء رایزوبیوم لگومینوزاروم با جنیستین (G) و متیل جاسمونات (MJ) روی گره بندی، تثبیت نیتروژن و رشد اولیه لوبیا (*Phaseolus vulgaris*) بود. گیاهچه های چهار روزه به گلدانهای حاوی مخلوط ماسه و surface استریل شده با نسبت حجمی ۲ و ۱ منتقل و در طول دوره رشد محلول غذایی هوگلدن بدون نیتروژن دریافت کردند. گیاهان در یک اتاق رشد با استفاده از طرح بلوکهای کامل تصادفی با شش تکرار رویش یافتند. ریزوبیوم در محیط TY کشت شده و پس از القا با G و یا MJ به مدت ۲۴ ساعت برای تلقیح بوته های لوبیا مورد استفاده قرار گرفت. نتایج نشان داد که هر دو ماده G و MJ تعداد گره، محتوای نیتروژن در بوته و ماده خشک بوته را افزایش دادند. G و MJ وقتی با هم به محیط کشت اضافه شدند روی تمامی صفات اثر سینرژیستی داشتند. نتایج این مطالعه نشان داد که MJ به تنهایی یا همراه با G میتواند برای افزایش گره بندی، تثبیت نیتروژن و رشد اولیه لوبیا مورد استفاده قرار گیرد.