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Effect of Putrescine on Inducing Symbiosis in Chickpea and Vetch Inoculated with Commercial or Indigenous Strains of *Rhizobium*

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Abstract

We investigated effect of putrescine on inducing symbiosis of strains of *Rhizobium leguminosarum* in commercial inoculants and strains isolated from local soils inoculated to roots of chickpea (*Cicer arietinum* cv. Aziziye-94) or vetch (*Vicia sativa* cv. Karaelci). In the growth medium of the plants inoculated with the strains of *R. leguminosarum*, putrescine (10 μM) was applied on the 10th day after planting. Growth parameters and symbiotic characteristics were determined 45 days after the inoculations. Nodule number, dry weight of nodules, roots and shoots, and nitrogen and chlorophyll contents significantly varied between putrescine-treated and control plants. The putrescine treatment increased the dry weight accumulation of nodules and shoots of both plants studied by more than 40%. Total nitrogen and chlorophyll contents also were significantly enhanced in response to putrescine treatment. Further, the stimulatory effect of putrescine was not strain related (commercial inoculants and strains isolated from local soils). In conclusion, the trials under controlled environmental conditions showed that the 10 μM putrescine application in plant growth medium significantly caused an increase in symbiotic parameters of chickpea and vetch inoculated with both commercial inoculants and indigenous strains of *R. leguminosarum*.

Keywords: *Cicer arietinum*, chlorophyll, nitrogen, polyamine, *Rhizobium*, symbiosis, *Vicia sativa*

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1. Introduction

Polyamines such as putrescine, spermidine and spermine are low-molecular polyvalent cations that contain two or more amino groups. They are ubiquitous compounds and are involved in myriad of processes including the regulation of protein and RNA synthesis (Tabor and Tabor, 1991; Salisbury and Ross, 1992), and initiation and control of cell division (Serafinin-Fracassini, 1991; Kao, 1997). Polyamines have been implicated as vital modulators in a variety of growth, physiological and developmental processes in plants (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990). They are not plant hormones, because they are too abundant, but they might be considered as plant growth regulators or merely one of several kinds of metabolites needed for certain developmental processes (Evans and Malmberg, 1989). Putrescine is known to modulate a variety of plant growth and development processes (Alexieva, 1994) including formation and effectiveness of different nitrogen-fixing systems (Vassileva and Ignatov, 2002; Wisniewski and Brewin, 2000).

Rhizobium, a soil bacterium, can interact with roots of legume plants to form symbiotic root nodules (Brewin, 1991; Hardi et al., 1998). Relatively high concentrations of the diamine putrescine were observed in root or stem nodules of many leguminous plants, and generally putrescine levels in root nodules are five to ten times higher then those in other plant organs (Fujihara et al., 1994). On the other hand, low concentrations of exogenous putrescine positively affected symbiotic parameters and viability of the bacteroids (Ozawa and Tsuji, 1993; Vassileva and Ignatov, 1999a). Exposure of plants to higher putrescine concentrations seriously impaired nodule functions (Vassileva and Ignatov, 1999a) and peribacteroid membrane transport processes (Whitehead et al., 2001). Chickpea (*Cicer arietinum* L.) and vetch (*Vicia sativa* L.) are widely produced crops that fix nitrogen in symbiosis with *R. leguminosarum* bacteria (Hardarson and Danso, 1993). *Rhizobium* species producing nodules on chickpea are generally specific only to this species and inoculation with effective strains is advised in soil with no or few bacteria (Rupela and Saxena, 1987; Somasegaran et al., 1988). It is difficult to cultivate such plants in soils containing non-effective *Rhizobium* strains (Somasegaran et al., 1988). In the present study, we investigated the effect of putrescine on nodulation and some growth parameters of two important legume plants.

2. Materials and Methods

Plant materials and bacterial strains

Chickpea (*Cicer arietinum* L. cv. Aziziye-94) and vetch (*Vicia sativa* L. cv.

Karaelci) seeds were obtained from the Agricultural Faculty of Atatürk University, Turkey. A standard culture in Peat and IFO-14778 strains (commercial inoculants) of *R. leguminosarum* were obtained from Institute of Soil and Fertilizer, Ankara, Turkey, and the Institute of Fermentation, Ojaka, Japan, respectively. The strains HF3, HF28, HF161, HF177 and HF204 of *R. leguminosarum* were isolated from wild chickpea and vetch plants (*C. anatolicum* and *V. cracca*) growing on local soils in the regions on cool high altitudes of Erzurum, Eastern Anatolia (2000–2500 m, 29°55'N and 41°16'E). Also, the symbiotic performance and success of the strains studied in chickpea plants were shown by Kantar et al. (2003).

Plant growth

Chickpea and vetch seeds were surface-sterilized with 95% ethanol for 5 min and 3% H_2O_2 for 5 min, and then rinsed six times with sterile water (Vincent, 1970). The seeds were germinated in Petri dishes containing sterile water and double filter papers for 5 days at 25°C in a growth chamber. Then, 4 intact seedlings obtained were aseptically transplanted to 1-l plastic pots containing sand (washed 10 times with sterile water and then autoclaved). The plants were supplied with a nitrogen-free solution every other day (Broughton and Dilworth, 1971). *R. leguminosarum* strains were cultured and maintained on yeast extract-mannitol (YEM) agar at 29°C (Vincent, 1970). A 1-ml bacterial suspension of *R. leguminosarum* strains containing approximately 10^8 viable cells was added in each growth pot containing the intact seedlings. For inoculation of chickpea seedlings, IFO-14778, Peat culture, HF3 and HF177 strains were used.

Inoculation of vetch seedlings was performed with Peat culture, HF28, HF161 and HF204 strains. 10 μM putrescine (Sigma Chem. Com.) was applied to each growth medium at equal volumes (250 ml) on the 10th day after planting since nodulation in the plants generally begins on 12–15 days (Somesagaran and Hoben, 1985). The plants were grown under controlled conditions (25/22°C day/night, photoperiod of 16 h under light intensity of 550 $\mu mol\ m^{-2}\ s^{-1}$) in a growth chamber. Height of plants was measured and plants were harvested on the 45th day after planting since the highest nodulation stage in the plants was generally on 45–50 days (Somesagaran and Hoben, 1985). Nodules, roots and shoots were removed to determine their dry weights.

Nitrogen content

Shoots of plants were dried for 24 h at 80°C in a forced-air oven. They were ground and milled to determine the N content by Kjeldahl analysis (Vincent, 1970). Total amount of N was used as the criterion for nitrogen-fixing capacity of plants.

Chlorophyll content

Chlorophyll (Chl) was extracted with 80% acetone from fresh leaves after sedimentation by centrifugation at 6,000 rpm for 5 min. Absorbance of the extracts was measured spectrophotometrically at 645 and 663 nm (Arnon, 1949). Jaspars's formula (1965) was used for the estimation of total Chl content.

Statistical analysis

The data obtained as an average of four repetitions were analyzed according to the Student's t test for independent two samples at the 0.05 level of confidence using SPSS 11.0 for Microsoft Windows.

3. Results

In chickpea (*C. arietinum*) inoculated with strains of *R. leguminosarum* in commercial inoculants (Peat and IFO-14778) and indigenous strains (HF3 and HF177) isolated from local soils, the results of 10 µM putrescine treatment are presented in Table 1. Treatment with putrescine increased nodule dry weight and nodule number, compared to the control. In the plants inoculated with strain HF177, for example, putrescine increased dry matter accumulation and number of nodules by 69% and 54% over controls, respectively. Also, after inoculation with Peat culture, the stimulation of dry weight and number of nodules was increased by 60% and 50% over controls, respectively. Putrescine treatment also significantly ($P<0.05$) increased dry weights of root and shoot in the chickpea plants, but the effect was greater with shoot dry weight than root dry weight. The shoot dry weights in the plants inoculated with strains HF177, HF3 and Peat culture were increased by 38%, 49% and 53% by putrescine treatment, although the root dry weight in the same plants were increased by 32%, 21% and 46%, respectively, compared to that of control. On the other hand, putrescine applied in growth medium of vetch plants caused an increase on dry weight and number of nodules, compared with the control plants (Table 2). After the 10 µM putrescine treatment, in vetch inoculated with strains HF161 and HF204, nodule dry weights were higher by 58% and 65% than that of control, respectively. In the same plants, stimulation of nodule number was higher 41% and 53% than that of control, respectively. In the both plants, plant height was not significantly ($P<0.05$) affected by putrescine treatment and strain difference.

Table 1. Effect of 10 µM putrescine treatment on dry weights of nodule and plant, nodule number and plant height in chickpea (*Cicer arietinum* L. cv. Aziziye-94) plants inoculated with commercial inoculants and indigenous strains of *R. leguminosarum*. Putrescine was applied to each pot on the 10th day, and the plants were harvested on the 45th day after planting.

Strains	Nodule dry weight (mg/plant)	Nodule number (per plant)	Root dry weight (mg/plant)	Shoot dry weight (mg/plant)	Plant dry weight (mg/plant)	Plant height (cm/plant)
IFO-14778	8.6	22	303.3	405.7	717.6	66.2
IFO-14778+Put.	12.3*	28*	345.3*	466.1*	823.7*	65.5
HF3	8.31	19	188.5	281.6	478.4	66.4
HF3+Put.	12.4*	29*	228.3*	420.8*	661.5*	64.2
Peat	7.81	18	200.6	285.7	494.1	66.4
Peat+Put.	12.5*	27*	292.4*	436.6*	741.5*	62.2
HF177	5.64	13	214.7	373.5	593.8	67.5
HF177+Put.	9.53*	20*	283.5*	516.6*	809.6*	70.5

Put.-Putrescine. *Significant according to the Student's t test for two independent samples ($P \leq 0.05$).

Table 2. Effect of 10 µM putrescine treatment on dry weights of nodule and plant, nodule number and plant height in vetch (*Vicia sativa* L. cv. Karaelci) plants inoculated with commercial inoculants and indigenous strains of *R. leguminosarum*. Putrescine was applied to each pot on the 10th day, and the plants were harvested on the 45th day after planting.

Strains	Nodule dry weight (mg/plant)	Nodule number (per plant)	Root dry weight (mg/plant)	Shoot dry weight (mg/plant)	Plant dry weight (mg/plant)	Plant height (cm/plant)
Peat	5.46	22	49.42	51.50	106.4	100.5
Peat+Put.	7.88*	30*	62.79*	93.72*	164.4*	99.3
HF161	2.16	17	47.90	88.62	138.7	106.4
HF161+Put.	3.41*	24*	64.63*	142.7*	210.7*	110.5
HF204	1.93	15	57.12	85.29	144.3	113
HF204+Put.	3.18*	23*	69.34*	151.46*	224.0*	118
HF28	3.17	21	61.76	134.4	199.3	100.8
HF28+Put.	4.36*	29*	71.74*	163.2*	239.3*	103.5

Put.-Putrescine. *Significant according to the Student's t test for two independent samples ($P \leq 0.05$).

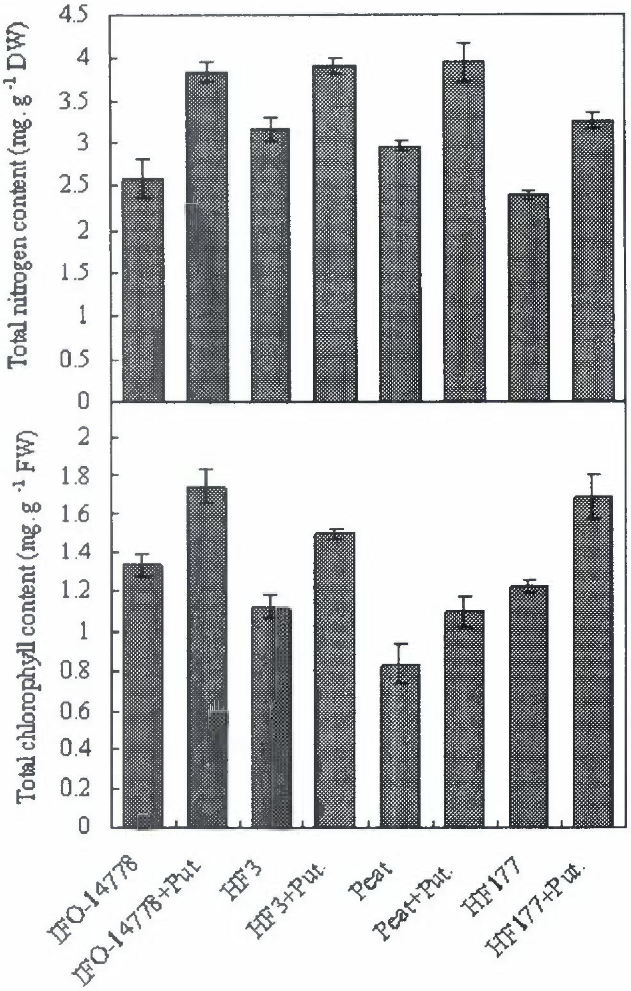

Figure 1. Effect of 10 µM putrescine on total N and chlorophyll contents in chickpea (*Cicer arietinum* L. cv. Aziziye-94) shoots inoculated with various *R. leguminosarum* strains. Putrescine was applied to each pot on the 10th day, and total N and chlorophyll contents in the shoots were determined on the 45th day after planting. Values are means ± S.E. FW-fresh weight; DW-dry weight; Put.-Putrescine.

Nodulated plants of both chickpea and vetch plants treated with 10 µM putrescine showed significant ($P<0.05$) increases in N content of the plant shoot, in comparison with the controls (Figs. 1 and 2). In chickpea plants inoculated with strains HF3, Peat culture, HF177 and IFO-14778, the putrescine treatment stimulated N content by 24%, 33%, 36% and 49% over controls, respectively (Fig. 1). Also, in vetch plants inoculated with strains HF28, Peat culture,

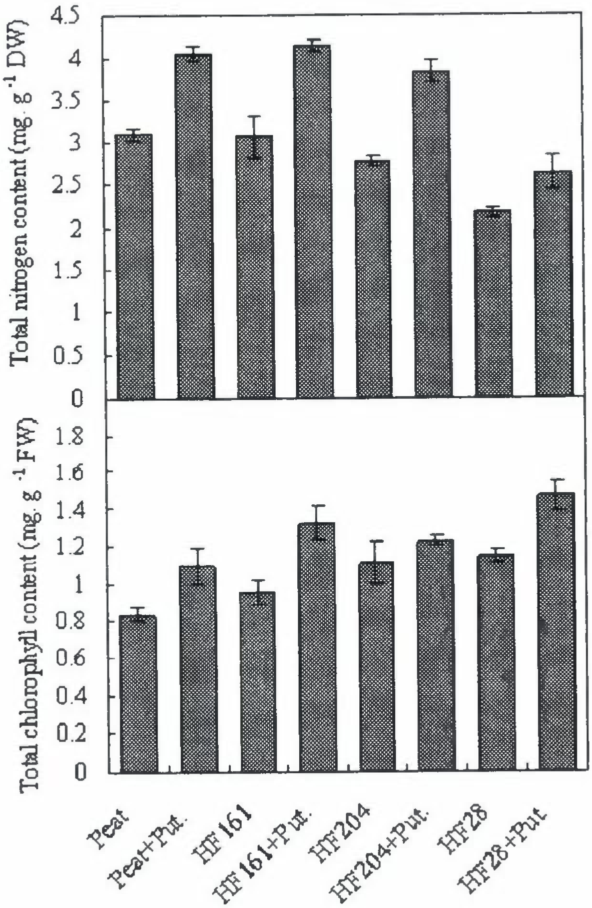

Figure 2. Effect of 10 µM putrescine on total N and chlorophyll contents in vetch (*Vicia sativa* L. cv. Karaelci) shoots inoculated with various *R. leguminosarum* strains. Putrescine was applied to each pot on the 10th day, and total N and chlorophyll contents in the shoots were determined on the 45th day after planting. Values are means ± S.E. FW-fresh weight; DW-dry weight; Put.-Putrescine.

HF161 and HF204, the treatment caused increases in 22%, 31%, 35% and 38%, respectively (Fig. 2).

Total Chl content of the leaves in the both plants inoculated with strains of *R. leguminosarum* in the commercial inoculants and the indigenous strains was increased by application of 10 µM putrescine in growth medium. The stimulatory effect of the putrescine treatment on Chl content of chickpea plants inoculated with strains IFO-14778, Peat culture, HF3 and HF177 was 31%, 32%, 33% and 38% over control, respectively (Fig. 1). In vetch plants inoculated with strains HF204, HF28, HF161 and Peat culture, however, its effect was 11%, 28%, 28% and 30%, respectively (Fig. 2).

4. Discussion

Exogenous application of putrescine can promote root formation, root growth (Jarvis et al., 1983; Geneve and Kester, 1991) and initiation of lateral root formation (Wen-Quan, 1989). Its applications can also induce symbiosis or nodulation in a few legume plants (Vassileva and Ignatov, 1999a; Wisniewski and Brewin, 2000; Whitehead et al., 2001). In our preliminary experiments it was observed that high concentrations of putrescine such as 100 and 250 μM caused a significant decrease in nodulation and growth of both chickpea and vetch plants (data not shown). A lower putrescine concentration (10 μM) was therefore used in this work. Some researchers also observed a similar result in applications of putrescine at low concentrations (Vassileva and Ignatov, 1999a; Whitehead et al., 2001). Vassileva et al. (1998) reported that putrescine at 10 μM was the most effective in stimulating bacteria and colony formation. In this study, however, the effect of 10 μM putrescine on symbiotic efficiency of chickpea and vetch (*C. arietinum* and *V. sativa*) inoculated with either strains of *R. leguminosarum* in the commercial inoculants or the indigenous strains isolated from local soils was investigated.

N fixation ability in legume plants was generally related to nodule number and the dry weights of nodule and plant (Chandra and Pareek, 1984; Vassileva and Ignatov, 1999a). Therefore, in this study, specific attention was focused on the dry weights of nodule and the plant, nodule number, plant height, and total N and Chl contents of putrescine-treated and control plants. Treatment with 10 μM putrescine caused a significant increase in nodule development (dry weight and number of nodules) and some growth parameters (dry weights of root and shoot) of both plants studied. Increased nodule number related positively to the dry weights of the nodules on chickpea and vetch (Tables 1 and 2). Vassileva and Ignatov (1999a) also observed similar results with *Galega orientalis* inoculated with *R. galegae*. They reported that putrescine was the most effective for increasing the number and biomass of nodules of *Galega orientalis-R. galegae* with respect to the other polyamines.

Further, it was shown that the attachment ability of bacteria was enhanced from 2 to 3-fold in the presence of putrescine (Vassileva et al., 1998). Our results are in good agreement with the above-mentioned results. The increases in dry weight can be explained by the higher metabolic activity of nodules and plants as suggested by Vassileva and Ignatov (2002). It can be concluded that 10 μM putrescine can improve nodulation, not only in *Galega orientalis* with *R. galegae* but also other legume plants such as chickpea and vetch inoculated with different strains of *R. leguminosarum*. The present study also shows that indigenous strains of *R. leguminosarum* can form nodules on the roots of the both plants studied (*C. arietinum* and *V. sativa*). Even on nodule production capacity, indigenous strains HF177 for chickpea and HF204 for vetch were more effective than strains of *R. leguminosarum* in commercial inoculants (Tables 1 and 2). This result may show that the *R. leguminosarum* strains obtained from wild legume plants can be used instead of the strains of *R. leguminosarum* in commercial inoculants in agriculture of the legume plants.

Total N accumulated in legume plants is one of the best parameters to measure N fixation under experimental conditions (Danielle et al., 1987). Therefore, after 10 μM putrescine treatment, total N content of shoots was also determined as a criterion of nitrogen-fixing capacity of both plants. The treatment with putrescine led to a stimulatory effect on total N content of inoculated chickpea and vetch plants inoculated with *R. leguminosarum* strains (Figs. 1 and 2). Total N content increased by *Rhizobium* bacteria in legume plants could be attributed to higher N fixation and a greater flux of energy to nitrogenease (Houwaard, 1980; Salisbury and Ross, 1992). It can be concluded that 10 μM putrescine treatment increased total amount of N in the plant shoots possibly by increasing N fixation in root nodules. The additional ammonium supply by putrescine containing N may also make a contribution to the increase of total N content observed in the plants treated with the low putrescine level because very low ammonium concentrations allow nodules to mobilize nitrogen reserves for amino acid synthesis (Vassileva and Ignatov, 2002).

In this study, treatment with 10 μM putrescine to roots of the plants studied significantly enhanced total Chl content in leaves of both plants inoculated with strains of *R. leguminosarum* in the commercial inoculants or the indigenous rhizobia. The increase of total Chl content was also generally related to the increases in both total N content and the growth parameters tested in the same plants. It was proposed that putrescine could positively affect photosynthetic activity (Beigbeder et al., 1995) and retard Chl loss and senescence (Kakkar and Nagar, 1996; Lee et al., 1997; Subhan and Murthy, 2001). Zheleva et al. (1994) also demonstrated that the leaves of pea treated with some polyamines showed a 50% increase in the Chl content. In addition, bean plants grown in soil amended with polyamine-producing *Streptomyces griseoluteus* had higher level of Chl than that in the control treatment, and the increase in levels of the pigment was related to the growth promotion (Nassar et al., 2003).

In conclusion, our trials under controlled environment conditions showed that the application of 10 μM putrescine in plant growth medium caused an increase in symbiotic activity of chickpea and vetch inoculated with strains of *R. leguminosarum* in commercial inoculants and indigenous strains isolated from local soils. An improvement was therefore observed in nodulation and growth promotion of these plants. Further, the strains of *R. leguminosarum* obtained the wild chickpea and vetch could form nodules on the domesticated chickpea and vetch, sometimes even more effective than commercial inoculants. The prominent stimulatory effect of putrescine on both plants was not dependent on the type of strains (commercial inoculants or indigenous rhizobia) used for inoculation. Thus, exogenous putrescine treatments at low concentrations may be useful to improve the symbiotic capability or to increase the yield of legume plants.

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