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Original Research Article

Effects of uredine and two flavonoids on nodulation and nitrogen fixation of common bean (*Phaseolus vulgaris* L.) under conditions of osmotic stress

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K e y w o r d s

Flavonoids, uredine, *Phaseolus vulgaris*, *Rhizobium etli*, osmotic stress, nitrogen fixation

A B S T R A C T

The effect of pre-stimulating *Rhizobium etli* with the two flavonoids, naringenin and hespertin, and with uredine on nodulation and nitrogen fixation of common bean (*Phaseolus vulgaris* L.) plants subjected to low, medium and high osmotic stress was investigated under greenhouse conditions. The addition of naringenin or uredine to *R. etli* prior to use as inoculum enhanced common bean nodulation, growth and nitrogen fixation under low and moderate osmotic stresses while both compounds were not effective when plants were subjected to high osmotic stress. The most effective concentrations of naringenin and uredine that alleviated the inhibitory effect of osmotic stress were 15 μM and 10 μM, respectively. Pre-treatment of inocula with hespertin, on the other hand, either did not influence (at low concentration) or reduced (at high concentrations) nodulation and nitrogen fixation even in the absence of stress. The results of this study indicated that naringenin and uredine may be used to improve nodulation and nitrogen fixation in common bean under low and moderate osmotic stress conditions.

Introduction

The common bean (*Phaseolus vulgaris*), an important legume crop worldwide and a primary source of dietary protein is reported to forms nitrogen fixing root nodules with a wide diversity of rhizobia including six species *of Rhizobium* and one *Sinorhizobium* (Amarger *et al.,* 1994; Eardly *et al.,* 1995; Herrera-Cervera *et al.,* 2000; Diouf *et al.,* 2000; and Rodriguez-Navarro *et al.,* 2000). Nodule formation involves several complex interactions between rhizobia and the host plant.

In this symbiotic system, rhizobia attach to and enter the root hairs of the host plant and move into the root cortex via a tube-like infection thread. Simultaneously, the cortical cells are induced to divide forming the nodule primordium which eventually differentiates into a mature nodule (Mylona *et al.,* 1995; Cohn *et al.,* 1998). The coordination of these events involves an intensive exchange of signal molecules between the host plant and rhizobia. The first apparent exchange of signals is the

secretion of specific flavonoid compounds by the host plant (Peters and Vermas, 1990). These compounds activate the expression of the *nod* genes in rhizobia (Burn *et al.,* 1987) which in turn produce the rhizobial nod factors. The nod factors act as the primary morphogenic signals for nodulation (Banfalvi *et al.,* 1988), although other plant factors including plant hormones (Zaat *et al.,* 1989; Fang and Hirsch, 1998) and uridine (Smit *et al.,* 1995) have been shown to participate actively in nodule development.

In nature both nodulation and nitrogen fixation are sensitive to abiotic, environmental, factors such as soil texture, temperature, pH, moisture and salinity (Hungria and Stacey, 1997, Zahran, 1999). Because common bean is frequently grown on drought-prone soils, osmotic stress represents an important environmental constraint which decreases its nodulation and nodule function (Rachid and Sinclair, 1998; Bouhmouch *et al.,* 2001). While all stages of nodule development are affected by environmental stresses, early processes including exudation of flavonoid from roots and induction of *nod* genes appear to be the most sensitive (Richardson *et al.,* 1988, Dusha *et al.,* 1989, Zhang *et al.,* 1995). Environmental stresses have been shown to inhibit flavonoid production as well as subsequent nodulation and nitrogen fixation (Kapulnik *et al.,* 1987, Appelbaum, 1990, Cho and Harper, 1991, Zhang and Smith, 1994, Pan and Smith, 1998). Moreover, the addition of flavonoids to the inoculant or the rhizosphere has been reported to improve nodulation and nitrogen fixation in soybean, pea and lentils. Such treatments have been also found to alleviate (at least partially) the inhibitory effects of environmental stresses on their nodulation (Zhang and Smith, 1995, Begum *et al.,* 2001, Novak *et al.,* 2002, Lira Junior *et al.,* 2004). However, to date there have been no work as to whether flavonoids improve nodulation and nitrogen fixation of common bean plant under osmotic stress. In the present investigation the effect of addition of two flavonoids; naringenin and hesperetin to rhizobial inoculant on nodulation and nitrogen fixation in common bean has been evaluated under greenhouse conditions. The effectiveness of these compounds on the nodulation response of plants subjected to osmotic stress was also determined. Since uridine has been reported to stimulate cortical cell division during early stages of nodule development (Smit *et al.,* 1995) and appears to play an important role in the establishment of the symbiosis, its effect on nodulation was also investigated.

Materials and methods

Plant material

Seeds of common bean (*Phaseolus vulgaris* L.) were surface sterilized by immersion in 75% ethanol for 30 seconds followed by rinsing in sterile distilled water and then immersion in 10% commercial bleach for 20 min. The seeds were washed three times with sterile distilled water and germinated in small trays on moistened filter paper. Young, 3–4 days old seedlings were individually transferred to Magenta jars containing sterile vermiculite embedded in nitrogen free medium prepared according to Broughton and Dilworth (1971). Seedlings in jars were allowed to acclimatize for 24h in the greenhouse (10–12 h of normal light, 25–30^{o}C day / 15–20^{o}C night temperature and 30–40% relative humidity) before being used for experiments.

Osmotic stress adjustment

Young seedlings in Magenta jars filled with sterile vermiculite were embedded in and irrigated with the nitrogen free medium

either alone (control) or supplemented with the required amounts of polyethylene glycol (PEG) 6000 prepared as described by Michel and Kaufman (1973) so as to have water potential levels of -2, -4 and -6bars. These levels of water potential represented low (-2 bars), medium (-4 bars) and high (-6 bars) osmotic stress.

Rhizobial inoculum

Highly efficient nitrogen fixing *Rhizobium etli* strain previously isolated from the soils of Jordan (Tamimi and Young, 2004) was selected for this study. Bacteria were grown on a rotary shaker for 48h at 28^{o}C in TY broth either alone (control) or in broth supplemented with 5–20 μM of the signal compound; naringenin, hesperetin or uridine (Sigma). Following incubation, cell suspensions were pelleted in sterile centrifuge tube at 4000 rpm for 10 min, washed once with sterile distilled water and re-suspended to an A_{600} of 0.08 (approximately 10^8 cells/ml) (Bhuvaneswari *et al.,* 1980) and used as an inoculum.

Nodulation and nitrogen fixation tests

Two days after acclimatization of plants in the greenhouse, 1ml of naringenin induced, hesperetin or uridine induced *R. etli* cell suspension prepared as described earlier, was applied onto the roots of individual non stressed and osmotically stressed plants. Control plants were inoculated with 1 ml of the inoculum prepared from cell suspension without pre-treatment with these signal compounds. Five plants were used per treatment (experiment) and each experiment was repeated 3 times. All plants were kept in the greenhouse in a randomized block design and irrigated with the nitrogen deficient medium either alone or supplemented with the appropriate concentration of PEG 6000 that maintains its water potential at the desired level. Thirty days after inoculation, nodule number, nodule dry weight, shoot dry weight and nodule leghaemoglobin content (estimated by the method of Wilson and Reisenauer (1963) were determined.

Results and Discussion

The data in Figure 1 showed that pre-treatment of *R. etli* with narengenin and uredine increased mean nodule numbers formed per inoculated plant although naringenin treatment appears to be more effective in stimulating nodulation. In both treatments, nodule numbers increased with increasing concentrations of the signal compound and the stimulation was the highest at 10 μM for uredine and 15 μM for naringenin. At these concentrations bean plants inoculated with naringenin or uredine induced *R. etli* developed approximately three and two times the number of nodules, respectively, compared to the number elicited by untreated bacteria. Both of these compounds, however, decreased nodule numbers at higher concentrations. On the other hand, nodule numbers on plants that received *R. etli* pre-incubated with 5-10 μM hespertin did not differ from those that received un-induced bacteria. At higher concentrations, however, hespertin treatment resulted in a marked inhibition of nodulation.

Nodule dry mass also increased by treatment of *R.etli* with uredine or naringenine but was either unaffected or reduced by hesperetin treatment (Fig. 2). Nodule weight of plants inoculated with *R. etli* pre-treated with 10 μM uredine and 15 μM naringenin increased by 40% and 75%, respectively, compared to plants inoculated with un-induced inoculum. Hespertin treatment had no significant effect on nodule dry mass at 5–10 μM but sharply reduced nodule dry weight at higher concentrations.

The results in Figure 3 showed that shoot dry weight of plants receiving uredine or naringenin pre-treated *R. etli* increased significantly compared to plants receiving untreated cells while hespertin treatment did not. A 40-60% increase in shoot dry mass was obtained when *R. etli* cells were pre-treated with uredine or naringenin although naringenin treatment was more effective in improving shoot dry weight.

The data presented in Figures 4, 5 and 6 showed that bean nodule number, nodule dry weight and shoot dry matter were all reduced by osmotic stress imposed by adding different concentrations of PEG to the growth medium. Pre-treatment of bean inoculant with uredine or naringenin significantly improved nodulation and nitrogen fixation under the imposed osmotic stress. Compared to un-stressed control, nodule numbers of plants grown at -2, -4 and -6 bars were reduced by 40, 65 and 95% respectively. In plants inoculated with bacteria pre-stimulated with 10 μM uredine, the number of nodules formed was 2 times greater than those at produced by untreated cultures. Treatment with 15 μM naringenin, however, appears to offer more improvement of nodulation under osmotic stress. The number of nodules formed in plants grown at -2, -4 and -6 bars was 2, 6 and 5 times greater than those formed in controls, respectively (Fig. 4).

Pre-stimulation of rhizobia with uredine or naringenin also improved nodule dry weight (Fig. 5). When plants were grown at -2, -4 and -6 bars the total nodule dry mass per plant was reduced by 35, 60 and 90%, respectively, compared to the unstressed controls. Nodule dry mass in plants grown at -2 bars and inoculated with rhizobial culture stimulated with 10 μM uredine was 65% higher than those produced by untreated controls. At -4 bars, nodule mass was 10-15% higher than those shown by controls while at -6 bars nodule mass was not significantly improved by uredine treatment. Treatment of rhizibial culture with 15μM naringenin on the other hand, was much more efficient than uredine in alleviating the effect of osmotic stress. At -2, -4 and –6 bars nodule mass increased by 115, 250 and 200% , respectively, over those shown by untreated controls. Shoot dry matter of plants subjected to osmotic stress was similarly increased by uredine and naringenin treatments (Fig. 6). At -2, -4 and -6 bars shoot dry mass of plants inoculated with untreated rhizobial culture decreased by 25, 50 and 60%, respectively whereas dry weight of plants grown at -2 and -4 bars and inoculated with cultures pre-stimulated with 10 μM uredine and15 μM naringenin were (25, 15%), (40 and 25%) higher than controls respectively. At higher osmotic stress (-6 bars) both uredine and naringenin treatment did not improve shoot dry weight.

The effect of uredine and naringenin on nodule leghaemoglobin content, which is considered to reflect the level of nitrogen fixation, has been estimated for plants grown under osmotic stress (Fig. 7). In non-stressed plants pre-stimulation of rhizobial inoculum with 10 μM uredine or 15 μM naringenin resulted in a 15 and 25% increase in nodule leghaemoglobin content relative to untreated control, respectively. However, when untreated plants were grown at water potentials of -2, -4 and -6 bars, respective nodule leghaemoglobin content was reduced by 12, 46 and 97% compared to non stressed controls. This decrease in nodule leghaemoglobin content was significantly lowered by pre-treatment of rhizobial culture with uredine or naringenin, although naringenin treatment was more effective. Nodule leghemoglobin content in plants treated with 10 μM uredine and grown at -2 and -4 bars was approximately 15% higher than those observed in the control plants

while at -6 bars, both treated and control plants showed similar nodule leghaemoglobin content. Treatment with 15 μM naringenin, however, increased nodule leghaeoglobin content by 25 to 30% in plants grown at all water potentials employed as compared to untreated controls.

Legume crops are valued for their ability to fix atmospheric nitrogen through symbiotic association with rhizobia. In common bean, as other legume crops, nodule development is restricted by osmotic stress which is encountered in many parts of the world. There have been several investigations of ways to improve nitrogen fixation under stressful conditions (Graham, 1981; Bandyopadhyay *et al.,* 1996).

Some recent publications have reported the use of flavonoid inducer molecules as a tool for enhancing nodulation and nitrogen fixation (Davis and Johnston, 1990, Pan and Smith, 1998). The application of flavonoids to the rooting medium or to the *Rhizobium* culture medium has been demonstrated to improve nodulation and nitrogen fixation in many legume plants grown under stress (Zhang and Smith, 1997; Begum *et al.,* 2001). The results of this study showed that low, medium and high osmotic stress strongly inhibited nodulation, nodule leghaemoglobin and growth of bean plants.

A significant increase in plant nodule number, nodule dry weight per plant, nodule leghaemoglobin content and shoot biomass were observed under low and medium osmotic stress following the inoculation of plants with rhizobia pre-incubated with naringenin and uredine. Naringenin concentration of 15 μM and uredine concentration of 10 μM had the largest stimulatory effect. Pre-stimulation of inocula with hespertin, on the other hand did not improve the nodulation response of non stressed or stressed plants. There are two possible explanations for the stimulative effect of naringenin and uredine. Firstly, it is possible that osmotic stress decreased the biosynthesis and excretion of these signal molecules which play an important role in the early stages of the plant-rhizobia interaction.

Secondly, *R. etli* may have become less sensitive to plant signal molecules at the imposed stress. Preincubation with naringenin and uredine possibly compensated for the shortage of these signal molecules thus improving nodulation and nitrogen fixation. It is likely that prestimulation with naringenin has activated the *R. etli nod* genes prior to inoculation The expression of *nod* genes is known to stimulate the formation of the nod factor which is responsible for inducing many of the early stages of nodule development (Kondorosi, 1992).

Apparently the activation of the nod genes prior to inoculation may have been responsible for the stimulatory effect of naringenin. While it is difficult to explain how uredine stimulated nodulation and nitrogen fixation, it seems likely that this compound has increased the sensitivity of the bacterial symbiont to flavonoids secreted by roots making it possible for the plant to form more nodules despite the possibility of flavonoids being secreted at suboptimal levels. However, it should be emphasized that this explanation is a mere speculation and only detailed work on the mechanism of uredine stimulation could reveal the exact pathway by which this compound stimulated nodulation. Nevertheless, The results of this study appear to agree with reports suggesting that pre-stimulation of rhizobia with flavonoids can to some extent stimulate nodule development in legume plants such as pea (Bandyopadhyay *et al.,* 1996) and soybean (Pan *et al.,* 1998).

Fig.1 The nodulation response of bean plants inoculated with *R. etli* pre-incubated with different concentrations of naringenin, hespertin or uredine

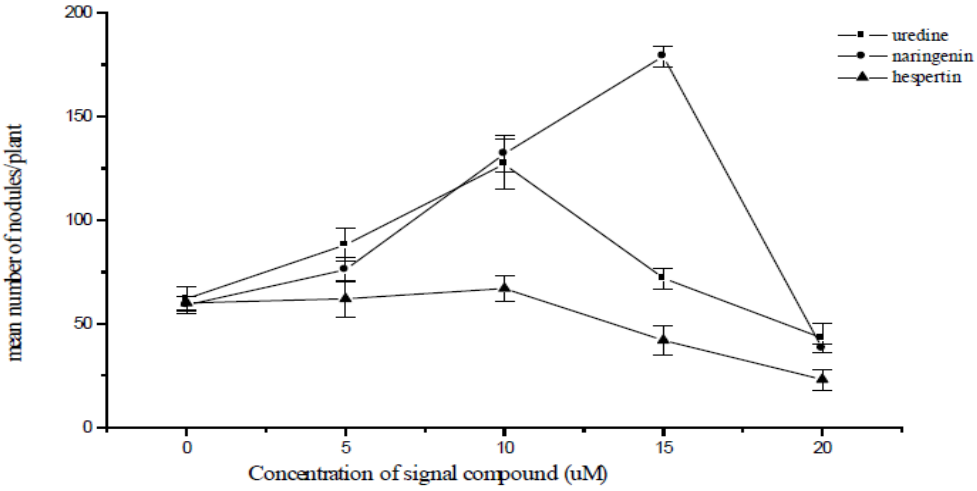


Fig.2 Effect of pre-incubating *R. etli* with different concentrations of naringenin, hespertin or uredine on nodule dry mass.

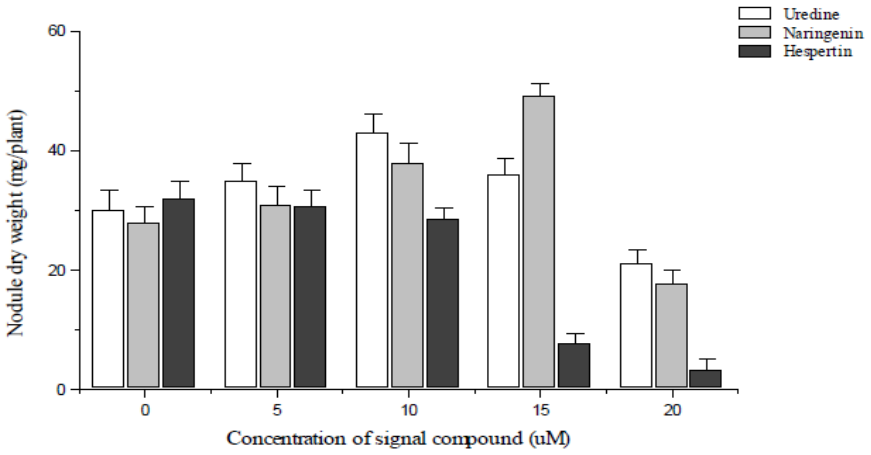


Fig.3 Growth of bean plants, expressed in terms of shoot dry mass, inoculated with *R. etli* pre-incubated with different concentrations of naringenin, hespertin or uredine

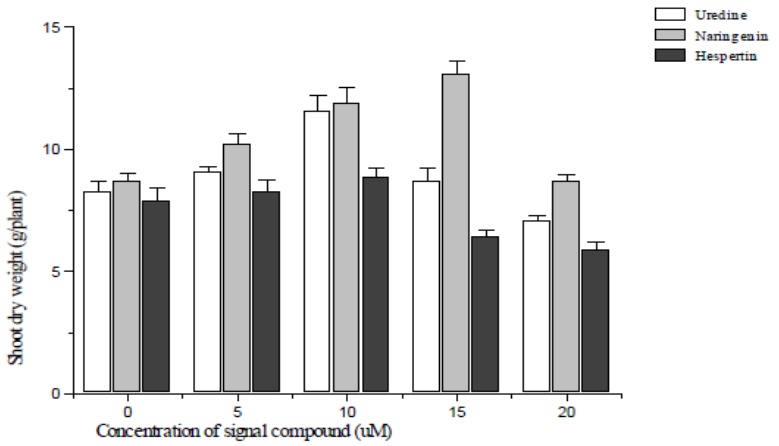


Fig.4 Effect of osmotic stress on the nodulation response of bean plants inoculated with *R.etli* pre-treated with 15 µM naringenin or 10 µM uredine

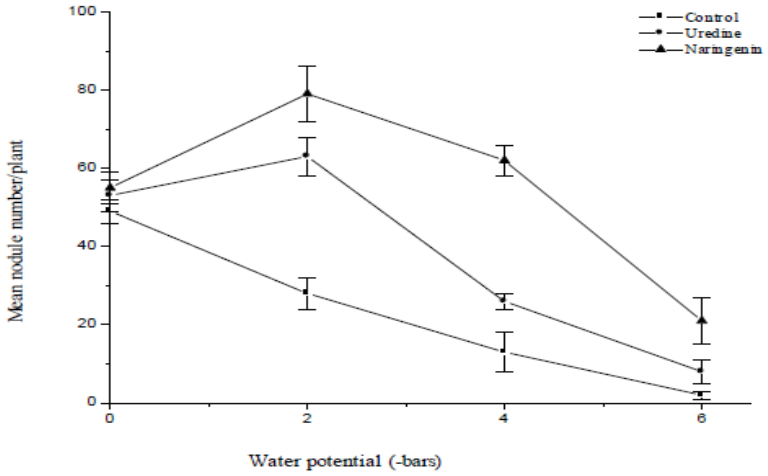


Fig.5 The effects of low (-2 bars), medium (-4 bars) or high (-6 bars) osmotic stress on the dry weight of nodules produced in bean plants inoculated with *R. etli* pre-treated with 15 µM naringenin or 10 µM uredine.

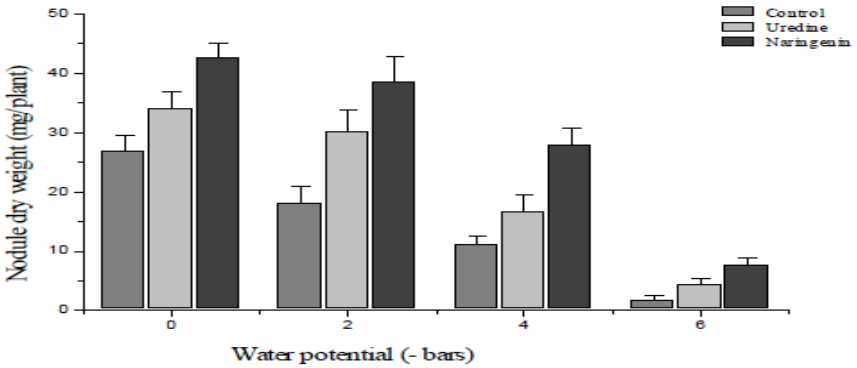


Fig.6 The effects of low (-2 bars), medium (-4 bars) or high (-6 bars) osmotic stress on the dry weight of bean plants inoculated with *R. etli* pre-treated with 15 μM naringenin or 10 μM uredine

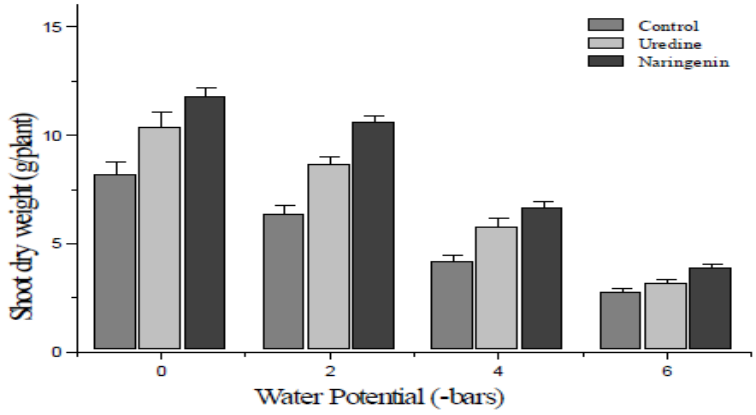
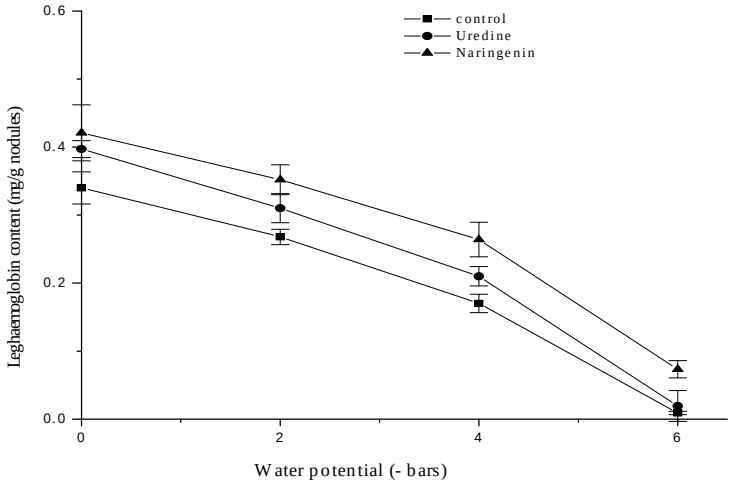


Fig.7 The effects of low (-2 bars), medium (-4 bars) or high (-6 bars) osmotic stress on legheamoglobin content of nodules formed in bean plants inoculated with *R. etli* pre-treated with 15 μM naringenin or 10 μM uredine



However, this study showed for the first time that pre-stimulation of rhizobia with uredine is almost as effective as flavonoids in stimulating nodulation and nitrogen fixation. In conclusion, the results of the present investigation document the potential role of naringenin and uredine in enhancing nodulation and nitrogen fixation in bean plant under both optimal and osmotic stress conditions.

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