Alleviation of drought stress in the common bean (Phaseolus vulgaris L.) by co-inoculation with Paenibacillus polymyxa and Rhizobium tropici

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1. Introduction

The common bean (Phaseolus vulgaris L.) is the world’s most important legume grown for human nutrition (Velazquez et al., 1998). Brazil is the world’s top producer of Phaseolus crops, followed by Mexico. These crops comprise an important nutrient source in the Brazilian diet. However, P. vulgaris is considered a poor fixer of atmospheric N compared with other crop legumes and generally responds poorly to inoculation in the field with Rhizobium (Wolff et al., 1991).

Furthermore, legumes and their symbiotic root nodule bacteria are extremely sensitive to drought stress (Sinclair et al., 2001). The fact that N₂ fixation is more sensitive to decreasing soil water content relative to leaf gas exchange applied soil ecology 40 (2008) 182–188

A greenhouse experiment was performed to evaluate the influence of Rhizobium when co-inoculated with each of two Paenibacillus polymyxa strains, singly and in mixture on growth, nitrogen content, phytohormone levels and nodulation of the common bean (Phaseolus vulgaris L.) under three levels of drought stress. Stress was applied continuously by the control of matric potential (θₘ) through a porous cup. Bean plants cv. Tenderlake were grown in pots with Fluvic Neosol eutrophic soil under three different θₘ (S₁ = 7.0, S₂ = 70.0 and S₃ < −85 kPa). The seeds were inoculated with Rhizobium tropici (CIAT 899) and each of P. polymyxa (DSM 36) and P. polymyxa Loutit (L) singly and in mixture (CIAT 899 + DSM36 + Loutit). Co-inoculation of bean with Rhizobium and both Paenibacillus strains resulted increased plant growth, nitrogen content and nodulation compared to inoculation with Rhizobium alone. This was particularly evident at the most negative θₘ (S₃ < −85 kPa) we used. Drought stress triggered a change in phytohormonal balance, including an increase in leaf abscisic acid (ABA) content, a small decline in indole acetic acid (IAA) and gibberellic acid (GA₃) and a sharp fall in zeatin content in bean leaves. The content of endogenous Cks decreased under water stress, possibly amplifying the response of shoots to increasing ABA content. We hypothesize that co-inoculation of bean with R. tropici (CIAT 899) and P. polymyxa strains (DSM 36) and Loutit (L) mitigates some of the negative effects of drought stress on bean.

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constitutes an important constraint on N accumulation and the yield potential of legumes subjected to soil drying (Serraj et al., 1999, 2001). This sensitivity is particularly relevant in view of the facts that water is a major limiting factor in world agriculture, and that in general, most crop plants are highly sensitive to even mild dehydration stress (Mundree et al., 2002; Meneses et al., 2006). However, drought stress responses in plants are often mediated by phytohormones so it is not surprising that the level of interest in diazotrophic organisms increased when it was discovered that they can also produce phytohormones (Juszczuk et al., 2004) such as indole acetic acid (IAA) and gibberellins (GA3) in Azotobacter vinelandii and Azospirillum brasilense (Plazinski and Rolfe, 1985), and auxins, gibberellins, and cytokinins (Cks) in A. vinelandii (Gonzalez-Lopez et al., 1986).

We undertook this study as a first step to determine if co-inoculation of bean with Rhizobium and Paenibacillus could enhancenodulation, nitrogen content and/or plant growth under controlled environmental conditions and how phytohormones changed in response to drought stress.

2. Materials and methods

2.1. Soil preparation, inoculation, and planting

The experiment was conducted in a greenhouse at a temperature range of 25–30 °C with 50–70% relative humidity and photoperiod of 1100 h. Pots were filled with soil samples (0–20 cm) of Fluvic Neosol eutrophic soil (pH 6.3), which was air dried and sieved (5.0 mm). There were 14 kg of soil in each 15 l pot. Chemical and physical analyses of the soil were conducted in accordance with EMBRAPA methods (EMBRAPA, 1997).

Bean seeds (P. vulgaris L.) cv. Tenderlake (origin University of Minnesota-COL obtained from Dr. R. Brent Thomas, Geo. W. Park Seed Co., USA) were surface sterilized (Vincent, 1970) and inoculated and/or co-inoculated using the following treatments: (I1) Rhizobium tropici CIAT 899 alone; (I2) CIAT 899 + Paenibacillus polymyxa DSM 36 (origin DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); (I3) CIAT 899 + P. polymyxa Loutil (L) (origin Otago, New Zealand); (I4) a mixture of CIAT 899, DSM 36 and Loutil (L); and (I5) no inoculation (control).

Five bean seeds were sown in each pot and then inoculated with 5 ml pot⁻¹ of liquid cultures of Rhizobium sp. (10⁶ CFU ml⁻¹) and P. polymyxa (10⁶ CFU ml⁻¹). After emergence was complete, three plants were left per pot. Hoagland and Arnon (1938) solution, without N, was applied weekly at a rate of 2 ml kg⁻¹ of soil.

2.2. Water stress application and analysis

Drought stress was applied by means of a porous cup arrangement (Bataglia, 1989). The auto-irrigation system consisted of a porous ceramic filter cup (3.5 cm in diameter and 14 cm in height) placed in the center of the pot. The porous cup and tubing were filled with distilled water. The different soil water contents were obtained by setting the vertical distances between the middle of the cups and the reservoir with a constant ψₘ that is equivalent to (15, 40 and 100 cm) to S₁ = −1.5 kPa (without stress, or control); S₂ = −4.0 kPa, and S₃ = −10.0 kPa. These represented ψₘ values at the porous cup walls and consequently of the soil water when in equilibrium (soil’s matric potential). As the plant roots absorbed water, a potential gradient developed, inducing water flow from cup to soil. For this reason, the ψₘ at treatment S₁ represents a soil ψₘ of −7.0 kPa; at treatment S₂, the soil ψₘ reached −70 kPa; and at treatment S₃, the soil ψₘ exceeded −85 kPa (Fig. 1), which was the upper limit of measurement on the tensiometer we used.

Drought stress treatments were first applied 25 days after germination (30-d-old), and every 15 days thereafter. Plants were harvested 45 days after sowing and the following parameters were evaluated: relative water content (RWC) (Barrs and Weatherley, 1962); nodule water potential was measured with a thermocouple psychrometer chamber (HR-337 Wescor, Logan, UT, USA) (Durand et al., 1987); leaf transpiration rate (E) and stomatal conductance (gs). E and gs were measured using the “steady porometer” LI COR, Mod. LI 1600 with auxiliary qantastic sensors (LI COR, Inc. SRQ 12231) coupled to the porometer (readings were taken from 0900 to 1000 h, on the abaxial side of the more recently expanded leaf from each plant). To obtain standardized leaf water potential (ψₑₑₑ) (Scholander et al., 1964) measurements, the following precautions were taken: all readings were taken from 0900 to 1000 h; leaves of the same age, located on the apical part of the plant; ψₑₑₑ readings were taken daily at 1000 h, throughout the entire drought period; and for shoot xylem sap analysis, exudates obtained by pressurization using the Scholander chamber were collected in calibrated microcapillaries and stored at −20 °C until assayed.

The ureide-N concentration was colorimetrically analyzed (Vogels and van der Drift, 1970); leghemoglobin (LHb) concentration in nodules was assayed spectrophotometrically (540 nm) using a Drabkin solution as the “blank” (Wilson and Reisenauer, 1963).
Approximately 2 g leaves of bean were harvested from each experimental treatment group and used for protein and hormone analysis. Protein concentration was determined according to Bradford (1976). Extraction and purification of abscisic acid (ABA), IAA, GA3 and cytokinins (such as zeatin) were determined according to Yurekli et al. (2001). Analyses of plant hormones were performed according to Izumi et al. (1988) and Nefedieva (2003) with some modifications. Extracts were dissolved in a small volume of methanol and used for HPLC analysis. Samples (20 μl) were injected into a reversed-phase LC18 column, connected to an HPLC pump (Cecil 1100, Cambridge, UK), and the column was eluted with a linear gradient using 20–80% methanol in 1% (w/v) aqueous acetic acid at a flow rate of 1.2 ml min⁻¹ for ABA; using 20–75% methanol in 0.4% acetic acid at a flow rate of 1.0 ml min⁻¹ for GA3 and IAA; and using 10% acetonitrile at a flow rate of 2.0 ml min⁻¹ for cytokinins. The results were compared with analytics standard from respective hormones (Fei, 2000).

Other measurements included plant height, shoot, nodule and root dry weights (65 °C for 72 h), and shoot/root ratio. Total N was determined using a Tecator 1030 auto-analyzer following the Kjeldahl method (Bremner, 1965).

### 2.3. Statistical design and analysis

The experimental design was a split–split plot arranged in four completely randomized blocks. Each block was subdivided into plots containing a different degree of water stress, i.e., S₁, S₂ and S₃. Each plot (degree of water stress) was divided into five subplots, each containing one inoculation treatment, i.e., (I₁) R. tropici CIAT 899 alone; (I₂) CIAT 899 + P. polymyxa DSM 36; (I₃) CIAT 899 + P. polymyxa Loutit (L); (I₄) a mixture of CIAT 899, DSM 36 and Loutit (L) and (I₅) no inoculation (control).

Each variable we studied was subjected to analysis of variance (ANOVA) (Steel and Torrie, 1960) using Statistica soft (Stat Soft, Inc, Tulsa, USA). Treatment means were separated using Tukey’s HDS test (P < 0.05).

### 3. Results and discussion

#### 3.1. The effects of drought stress on physiological processes

The watering/drought system we used included two very useful characteristics including accuracy for watering (sub-irrigation system) and imposition of controlled and constant

### Table 1 – Drought stress effects in nodule (ψₙod) and leaf water potential (ψₙeaf), shoot-to-root ratio of dry weight (shoot/root DW), relative water content (RWC) and leaf soluble protein in common bean (P. vulgaris L. cv. Tenderlake)

<table>
<thead>
<tr>
<th>Drought stress levels (ψₓₛod) (kPa)</th>
<th>ψₙod (MPa)</th>
<th>ψₙeaf (MPa)</th>
<th>RWC (% w/w)</th>
<th>Leaf soluble protein (mg protein g FW⁻¹)</th>
<th>Shoot/root DW (g DW g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>−0.42 a</td>
<td>−0.48 a</td>
<td>80.26 a</td>
<td>15.2 a</td>
<td>2.60 a</td>
</tr>
<tr>
<td>−70.0</td>
<td>−0.74 b</td>
<td>−0.89 b</td>
<td>68.98 b</td>
<td>12.9 b</td>
<td>2.34 ab</td>
</tr>
<tr>
<td>−85.0</td>
<td>−0.92 c</td>
<td>−1.00 c</td>
<td>57.25 c</td>
<td>11.6 b</td>
<td>2.11 b</td>
</tr>
<tr>
<td>% CV plot</td>
<td>6.4</td>
<td>5.5</td>
<td>13.0</td>
<td>8.5</td>
<td>13.4</td>
</tr>
</tbody>
</table>

FM is fresh matter; CVₓplot is variation’s coefficient experimental plot; in column, means (20 replicates) followed by the same letter (lower (co-inoculation) and capital (drought stress) letters) not differ statistically (P = 0.05) according to Tukey’s HSD.

**Fig. 2** – Co-inoculation effects of *Paenibacillus polymyxa* (DSM 36 and Loutit (L) strains) with *Rhizobium tropici* (CIAT 899 strain) on leaf transpiration rate (A), and stomatal conductance (B) of bean’s plants (*P. vulgaris* L. cv. Tenderlake) grown under drought stress different. The CV is variation’s coefficient and means (four replicates) followed by the same letter (lower (co-inoculation) and capital (drought stress) letters) not differ statistically (P ≤ 0.05) according to Tukey’s HSD.
drought stress as watering is controlled by demand (auto-irrigation). This resulted in better and simultaneous control for matric potentials ($\psi_m$) and watering (irrigation system).

Drought stress had a significant effect on each of these yield variables in bean RWC (Table 1). RWC was 80.26% without stress ($S_1$) and 57.25% with stress ($S_3$) during the most negative $\psi_m$. Measurements of $\psi_{nod}$ were conducted on detached and bisected bean nodules from the upper part of the roots. In the Wescor chamber, 45–60 min was necessary to reach equilibrium. Values for $\psi_{nod}$ were plotted against $\psi_{leaf}$. The linear relation ($R^2 = 0.98$) between these two potentials allowed us to estimate the $\psi_{nod}$ from the values determined with leaves, in the range of $-0.42$ to $-0.92$ MPa.

The $\psi_{leaf}$ that the plants were permitted to reach was not extremely low throughout the experiment, through the period of stress had been relatively long, reaching values of $-1.00$ MPa. Studies conducted with common bean cv. Carioca (Taller and Wong, 1989) presented a $\psi_{leaf}$ with a magnitude similar to that observed in this experiment, while (González et al., 1995) detected a $\psi_{leaf}$ of $-1.2$ MPa with soybean. It is evident that the comparison between minimum values of the $\psi_{leaf}$ among different studies is of little significance because $\psi_{leaf}$ is affected by several factors such as climate, soil, and the plant itself. In any case, the existing difference found between the $\psi_{leaf}$ values of the stressed and control plants is of fundamental importance in indicating to what extent $\psi_{leaf}$ fell in the stressed plants.

Leaf transpiration rate ($E$) and stomatal conductance ($g_s$) decreased with the increase in stress, especially at the most negative $\psi_m$ ($S_3$). Significant interactions in $E$ and $g_s$ were detected with the different bacterial strains (Fig. 2). In this study, there was a high significant correlation between $g_s$ and $E$ ($r = 0.91^{**}$) as these variables responded to decreased $\psi_{leaf}$.

### 3.2. The effects of drought stress on nodulation and plant growth

There was a significant difference in bean growth between the levels of drought stress as well as between different bacterial strains (Fig. 3). Nodule number, nodule dry matter, growth, and shoot dry matter accumulation of legumes were all reduced by low drought stress levels. Similar observations have been reported for *P. vulgaris* (Ramos et al., 2003), *Vigna unguiculata* (Figueiredo et al., 2001), *Clitoria ternatea* (Diniz et al.,...
2002), and **Glycine max** (Serraj et al., 1999). Variation among cultivars within legume species in N₂ fixation sensitivity to water deficit (Taller and Wong, 1989; González et al., 1995; Figueiredo et al., 1999; Sinclair et al., 2001) indicates that the tolerance trait found in some genotypes may be useful in breeding programs for N₂ fixation drought tolerance in legumes.

Plants co-inoculated with CIAT 899 + DSM 36 + Loutit [mixture strains *Paenibacillus* (*P. polymyxa* + *P. polymyxa* Loutit (L)) + *R. tropici*] showed greater growth, shoot dry matter accumulation, nodule number and nodule dry matter than those co-inoculated with CIAT 899 + DSM 36 (*R. tropici* + *P. polymyxa*) and CIAT 899 + Loutit (*R. tropici* + *P. polymyxa* Loutit (L)) or inoculated with the CIAT 899 (*R. tropici* alone) (Fig. 3A, C and D).

There were no significant differences in plant height within bacterial treatments, but plants treated with bacteria were taller than noninoculated controls (Fig. 3B). However, plants co-inoculated with a mixture of all strains showed increases of 16, 25 and 45%, respectively, under drought stress levels (S₁), (S₂) and (S₃) compared to treatments CIAT 899 + DSM 36 (*R. tropici* + *P. polymyxa*) and CIAT 899 + Loutit (*R. tropici* + *P. polymyxa* Loutit (L)) in which *P. polymyxa* strains were added separately with *Rhizobium*. Though not statistically significant according to the conservative Tukey’s test, these observations suggest a direct role for the PGPRs on plant growth, especially a mixture of *P. polymyxa* and *Rhizobium* strains.

The shoot nitrogen content was significant (P < 0.05) in relation to the strains showing that inoculated treatment with CIAT 899 + DSM36 + Loutit (L) (treatment I₄) was superior. It did change significantly after bacterial treatment, indicating that any inoculated treatment was superior compared to the non inoculated control (treatment I₅) (Fig. 4). However, the shoot nitrogen content did not present significant interaction to the stress (data not shown). This suggests that although the dry matter production of stressed plants was low (Fig. 3A), the nutrient concentration in the shoot was high. The data agree with most of the related research by Viets (1972) on the effects of water deficiency on the availability of nutrient.

Davies and Zhang (1991) also applied water stress in common bean using a porous cup apparatus similar to ours and observed a decrease in the total soluble protein of nodules. This observation is in agreement with our findings (Table 1), however, this parameter was not affected by bacterial inoculation. In contrast, LHb and ureide-N concentration (Fig. 5) were significantly altered by both stress and bacterial treatment.

An alternative measure of nitrogen is based on the concentration of ureide-N, and it can be clearly observed that

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**Fig. 4** – Co-inoculation effects of *P. polymyxa* (DSM 36 and Loutit (L) strains) with *R. tropici* (CIAT 899 strain) on nitrogen concentration of bean’s plants (*P. vulgaris* L. cv. Tenderlake). The inoculation’s treatments: I₁, CIAT 899 alone; I₂, CIAT 899 + DSM 36; I₃, CIAT 899 + Loutit (L); I₄, CIAT 899 + DSM 36 + Loutit (L); and I₅, no inoculation. The CV is variation’s coefficient and means (12 replicates) followed by the same letter not differ statistically (P < 0.05) according to Tukey’s HSD.

**Fig. 5** – Co-inoculation effects of *P. polymyxa* (DSM 36 and Loutit (L) strains) with *R. tropici* (CIAT 899 strain) in ureide (A) and leghemoglobin (B) concentrations, respectively, on xylem sap and nodule tissues of bean’s plants (*P. vulgaris* L. cv. Tenderlake) grown under drought stress different. The CV is variation’s coefficient and means (four replicates) followed by the same letter (lower (co-inoculation) and capital (drought stress) letters) not differ statistically (P < 0.05) according to Tukey’s HSD.
stomata; thus, a reduction in cytokinin supply as soil dries
in the formation of these two compounds (Cowan et al., 1999).

tions: cytokinins share, at least in part, a common biosynthetic
with ABA may be the result of metabolic interac-
to the same time zeatin content decreased in our study and
content dropped sharply (Table 2).

3.3. The effects of drought stress on phytohormone content

Phytohormone content were unaffected by bacterial inocula-
tion but did show a significant response to different levels of
drought stress (Table 2): the results indicate that when \( \psi_{\text{leaf}} \)
decreased, there was a trend for increasing abscisic acid
content in the leaves at the most negative \( \psi_{\text{w}} \). Similarly,
stomatal conductance was found to decrease as a function of
decreasing \( \psi_{\text{leaf}} \). An increase in ABA content in plants,
especially in the roots, can be related to soil water content
(Davies and Zhang, 1991; Gomes et al., 2004). When the plants
go through a period of drought stress, ABA biosynthesis
increases in the roots; the increased ABA is translocated to the
shoot via the xylem and can cause stomatal closure. Although
ABA can be translocated from the shoot to the roots via the
phloem, experiments involving blockage of the phloem
vessels demonstrated that the roots can synthesize ABA. This
hormone in turn influences water conductivity and root
growth (Gomes et al., 2004).

In this study, a positive correlation \( (r = 0.81) \) between
\( \psi_{\text{leaf}} \times \text{ABA} \) and \( (r = 0.78) \) between \( \psi_{\text{leaf}} \times \text{gs} \).
In general, our results indicate that the four phytohormones we studied
in common bean responded differently to water stress. As water
stress increased, leaf ABA levels also increased, indole acetic
acid and gibberellic acid declined moderately and Cks (zeatin)
content dropped sharply (Table 2).

Cytokinins such as zeatin are often considered ABA
antagonists (Cowan et al., 1999). ABA content increased and
at the same time zeatin content decreased in our study and
those described by Thimann (1992). The antagonism between
cytokinins and ABA may be the result of metabolic interac-
tions: cytokinins share, at least in part, a common biosynthetic
origin with ABA, clearly creating the potential for antagonism
in the formation of these two compounds (Cowan et al., 1999).
A high cytokinin content can override the effects of ABA on
stomata; thus, a reduction in cytokinin supply as soil dries
might amplify shoot response to an increasing content of ABA
(Davies and Zhang, 1991).

The IAA content in the bean leaves showed a slight decline
during the water stress period. In epidermal peels of Vicia faba
(Dunleavy and Ladley, 1995), IAA stimulated stomatal opening;
when both IAA and ABA were present. However, the stomatal aperture was dependent on the relative concentra-
tion of each. Several reports have suggested that the balance
of phytohormones, particularly that between auxin and
cytokinins, is part of the nodulation stimulus, but it is not
obvious \( \text{a priori} \) in which direction the balance is shifted. The
appropriate IAA/ cytokinin balance is essential for plant
development, however not all effects of cytokinins and
auxins in plant development are dependant on this balance
(Pospisilová, 2003).

\( \text{GA}_3 \) content of bean plants subjected to stress level \( S_2 \)
(Table 2) showed a significant difference based on Tukey’s test.
The treatment-stressed \( S_2 \) showed a slight decline to 5.18%
compared to treatment \( S_1 \) (without stress); however, at the
most negative \( \psi_{\text{m}} \) (\( S_3 \)), \( \text{GA}_3 \) content declined to 20.86%. \( \text{GA}_3 \)
composition and content are usually connected with plant
growth and development. In relation to environmental effects,
irradiation and low temperature induced changes in GA
metabolism (Hedden, 1999), but little is known about changes
induced by stress.

4. Conclusions

Co-inoculation of common bean with \( R. \) tropici and \( P. \)
polympyx strains resulted in greater growth compared to
inoculation with Rhizobium alone. At the more negative \( \psi_{\text{m}} \)
(\( S_3 < -85 \) kPa), plants co-inoculated with Rhizobium and both
Paenibacillus strains mixture CIAT 899 + DSM 36 + Loutit
exhibited greater nodulation (number and biomass) and
nitrogen content compared to drought-stressed plants
inoculated with only Rhizobium. Drought stress triggers a
change in hormonal balance, including an increase in \( \text{ABA} \)
content, a small decline in IAA and \( \text{GA}_3 \) and a sharp fall in
zeatin content in the leaves of the common bean. The
content of endogenous Cks mostly decreased under drought
stress, amplifying the response of shoots to increasing \( \text{ABA} \) content.

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