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Root Growth, Respiration and β-Glucosidase Activity in Maize (*Zea mays*) and Common Bean (*Phaseolus vulgaris*) Inoculated with *Azospirillum brasilense*

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Abstract

As *Azospirillum* is shown to improve plant growth, the influence of inoculation on plant metabolism in maize and common bean seedlings has been assessed. Respiration rates have been measured and changes in β-glucosidase (which may be involved in phytohormone release from conjugates) activity detected. A trend of inoculum concentration-dependent increased respiration rates was detected in inoculated maize seeds in Petri dishes. A similar phenomenon was observed for common bean seedlings grown in an hydroponic system, when growth promotion and enhanced respiration rates occurred after inoculation with *Azospirillum* at 10^6 cfu/ml. In the hydroponic growth system, *Azospirillum* inoculation at 10^6 cfu/ml, promoted root fresh weight as well as shoot fresh and dry weight of maize seedlings. Treatment with *Azospirillum* reduced the Km and Vmax values of β-glucosidase activity, in crude extracts from maize root tips. *In vitro* inoculation of detached root segments caused reduction in β-glucosidase kinetic values. In this case, changes in Km and Vmax were already observed after 5 h and increased up to 24 h. This effect was promoted when inoculum concentration was increased. Reduction in

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Vmax and Km may indicate lower total activity of β-glucosidase, but higher affinity to the substrate of specific β-glucosidases. Analysis of *Zm-p60.1* gene expression, coding for the maize hormones involved in β-glucosidase deglycosilation, did not indicate changes in the transcript level following inoculation with *Azospirillum*.

Keywords: *Azospirillum brasilense*, *Zea mays*, *Phaseolus vulgaris*, plant growth promoting rhizobacteria (PGPR), β-glucosidase, respiration

1. Introduction

The free living N_2-fixing rhizobacteria of the genus *Azospirillum* live in close association with plants and may promote plant growth and yield under appropriate agronomic conditions (Okon and Vanderleyden, 1997). Mechanisms involved in the positive effects of *Azospirillum* on plant growth have not been totally elucidated.

Positive effects on root development, with a concomitant improvement of mineral and water uptake by inoculated roots has been previously observed (Okon and Vanderleyden, 1997). It has been proposed that plant growth substances (auxins, gibberellins and cytokinins), produced by the bacterium, cause changes in hormone levels in the root tissue. However, this claim has not been demonstrated *in vivo*. *Azospirillum* inoculation affected the levels of free indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) (Fallik et al., 1994) and gibberellic acid (GA_3) (Fulchieri et al., 1993). *Azospirillum* also altered the specific activities of enzymes involved in the tricarboxylic acid and glycolysis pathways in roots of maize and other plants (Fallik et al., 1994). In legumes (alfalfa, common bean), *Azospirillum* increased the quantity of flavonoids which promote nod gene expression in *Rhizobium* (Burdman et al., 1996; Volpin et al., 1996).

β-glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) catalyze the hydrolysis of glycosidic linkages in aryl and alkyl β-glycosides and cellobiose, and occur in all living organisms. Almost all known β-glucosidases have a subunit molecular weight of 55 to 65 kDa, acidic pH for optimal activity (pH 5–6) and an absolute requirement for a β-glucoside as substrate. β-glucosidases from widely different sources show remarkable similarity in substrate specificity for glycone (glucose) and some nonphysiological aglicones (e.g., nitrophenols and umbelliferone), although they may have widely different physiological glucosidic substrates with different aglicone moieties. In general, β-glucosidases from different orders and kingdoms appear to differ in their specificity for the aglycone (an aryl or alkyl group) linked to the glucosyl group by a β-glucosidic bond (Esen, 1993). Plant β-glucosidases

participate in defense mechanisms against pathogens by releasing compounds such as thiocyanates, coumarins, hydroxamic acid and terpens. In addition, they catalyze the hydrolysis of non-active conjugates, such as glycosides of flavonoids (Hartwig and Phillips, 1991) and phytohormones (auxins, gibberellins, cytokinins and abcisic acid) releasing their active aglycones (Campos et al., 1993).

It appears that the presence of *Azospirillum* in the rhizosphere elicits or activates the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus releasing active compounds in their active forms. The purpose of this work was to characterize the effects of *A. brasilense* on the development of maize and common bean seedlings, and to investigate the changes in β-glucosidase activity which might be linked to increased availability of free phytohormones.

2. Materials and Methods

Inoculum preparation and plant material

Azospirillum brasilense Cd (ATCC 29729) was grown on liquid malate minimal medium (Okon et al., 1976) for 24 h at 30°C, with shaking (150 rpm). Cells were washed, harvested by centrifugation (1000 g, 10 min, twice) and resuspended in 0.1 M phosphate buffer (pH 6.8) for inoculation in the hydroponic systems.

Seeds of *Zea mays* cv. Jubilee (sweet corn) and *Phaseolus vulgaris* cv. Bulgarian were cordially supplied by Hazera Co. (Israel) and Gedera Seeds Co. (Israel), respectively. They were surface sterilized for 2 min in 95% ethanol, then for 1 min in 1% sodium hypochlorite and were then washed 5 times with sterile water.

Growth in the hydroponic system

Twenty five surface-sterilized seeds were placed on a sterile cheesecloth-covered steel screen over 350 ml of sterile aerated 10-fold diluted Jensen nutrient solution (Vincent, 1970) in a 400 ml plastic container. A sterile clear plastic cover was positioned over the container and roots developed into the nutrient solution (Maxwell et al., 1989). For germination, containers were incubated for 72 or 96 h (see Results) at 30°C in the dark for maize seeds, and for 120 h at 25°C in the dark for common bean seeds. They were then transferred to a growth chamber with an irradiance of 90 $\mu E/m^2$ s, 16/8 h light/dark, and 25/20°C. *Azospirillum* cells were inoculated at different concentrations and times (see Results). Elicited controls were obtained by applying autoclaved

yeast extract to the growth container at a final concentration of 3 mg/ml (Jorrin and Dixon, 1990). Untreated controls were growth containers supplied with an equal amount of nutrient solution.

Measurements of root respiration

Root segments (300 mg) were incubated in 30 ml-sealed tubes, containing 5 ml of a solution described by Saligo and Pradet (1980), for 20 min at room temperature. CO_2 concentration was determined by injecting 2 ml-gas samples in a Gow-Mac 550 gas chromatograph apparatus (Gow-Mac Instrument Co., Madison, NJ) with a 120 × 0.63 cm Poropack-column and Helium as the carrier gas.

Protein extraction and measurements of β-glucosidase activity

Proteins were extracted from 1 g of root by homogenizing them with 4 ml of cold PC buffer (12 mM citric acid, 50 mM NaH_2PO_4, pH 7.0). A clear supernatant was obtained by centrifugation of the homogenate at 12,000 g for 25 min. Samples were taken for protein determination according to Bradford (1976).

β-glucosidase specific activity was assayed according to Tilbeurgh et al. (1988) with several modifications. The reaction mixture contained different MUG (4-Methyl umbelliferyl-β-D-glucoside) (Sigma, Chemical Co., St. Louis, MO, USA) concentrations (0.025–0.25 mM) and 10 µl of the enzyme (10 ng/µl), in PC buffer at a final volume of 0.5 ml. Enzyme activity was estimated by measuring the fluorescence of the reaction product, MU (methyl-umbelliferone) for 2 min (excitation at 534 nm, emission at 345 nm) at room temperature, using a fluorimeter (TKO-100, Hoffer, CA, USA). The activity was linear for 5 min with time and protein concentration up to 30 ng/µl. Km and Vmax values were calculated according to Lineweaver-Burk (Lehninger et al., 1993).

Native polyacrylamide gel electrophoresis (PAGE) and identification of β-glucosidase activity on the gel

Separation of 100–200 µg root proteins by native PAGE was performed according to Laemmli (1970). After electrophoresis, the gel was rinsed twice with PC buffer containing 5 mM MUG and then photographed under UV light for the detection of β-glucosidase activity. To confirm equal loading of proteins, the gel was stained with coomassie blue G-250.

RNA extraction and northern-blot analysis

Total RNA was extracted from root segments according to the method of Van

Tunen et al. (1988). RNA (10 μg) was size-fractionated on a formaldehyde-denaturing agarose gel (Maniatis et al., 1982) and blotted onto a Hybond-N+ filter (Amersham, Buckinghamshire, UK). Following electrophoresis, the formaldehyde gel was briefly stained with ethidium bromide and photographed before blotting to ensure that equal amounts of RNA had been used for each sample. The blots were hybridized with a ^{32}P-labeled full-size maize cDNA-probe for *Zm-p60.1* (Brzobohaty et al., 1993). Following hybridization, the blots were washed twice with 2xSSC and 0.1% SDS at 60°C for 10 min and then autoradiographed.

3. Results

Effect of Azospirillum on respiration of maize roots in Petri dishes

Seeds were inoculated with different concentrations of *Azospirillum* (10^6 to 10^8 cfu/ml). Inoculation did not affect seedling fresh weight 2 days after treatment, but 5 days after inoculation there was a decrease tendency in root fresh weight as bacterial concentration increased. Table 1 shows the respiration rates of seedling roots from one representative experiment. Significant difference ($p=0.05$) was obtained only when comparing respiration rates on the basis of fresh weight between inoculation with 10^7 cfu/ml and non-inoculated controls after 5 days. Lack of statistical significance was due to a large variability between replicates, however an optimal curve was obtained in this and in other five similar experiments, with the maximal respiration rate after inoculation with 10^7 cfu/ml.

Table 1. Effects of *Azospirillum brasilense* at different concentrations on respiration rates of roots of maize seedlings grown in Petri dishes, 2 and 5 days after inoculation. Values indicate mean of five replicates per treatment ± standard error.

Inoculum concentration (cfu/ml)	Respiration rate on the basis of fresh weight (μl CO_2/g.h)		Respiration rate on the basis of dry weight (μl CO_2/g.h)	
	2 days	5 days	2 days	5 days
0	300±72	305±43	3,420±752	3,591±503
10^6	318±75	479±183	4,001±718	4,345±1,257
10^7	420±156	726±183	5,917±2,394	4,489±503
10^8	357±72	433±110	4,720±787	4,094±503

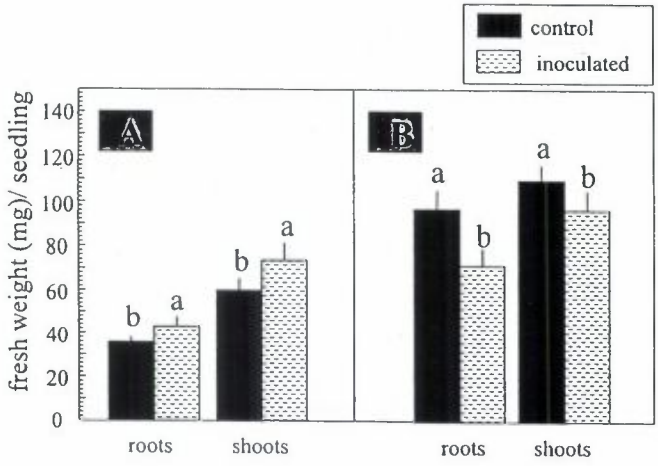

Figure 1. Effect of inoculation with *Azospirillum brasilense* (10^6 cfu/ml) on fresh weight of maize seedling roots 3 (A) and 5 days (B) after inoculation in hydroponic growth system. Each result represent the average of 5 replicates ± SE.

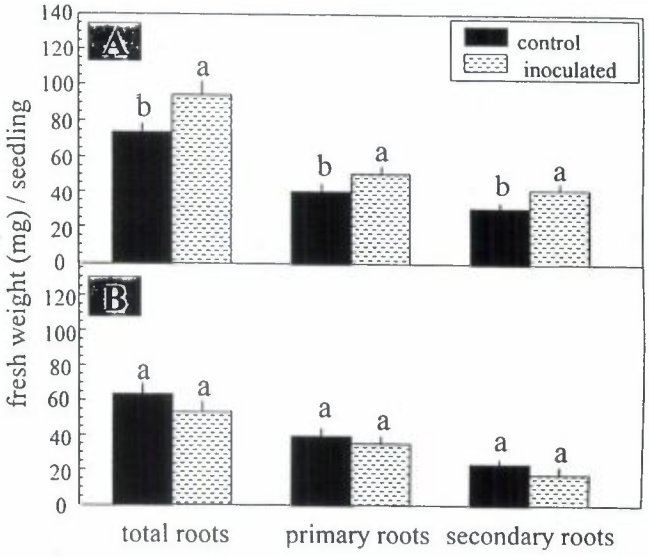

Figure 2. Effect of different *Azospirillum* concentrations, 10^6 (A) and 10^7 cfu/ml (B), on fresh weight of common bean seedling roots, 2 days after inoculation in hydroponic growth system. Results represent the average of 8 experiments (5 replicates each) ± SE. Different letters represent significant differences between inoculated and control treatments at p=0.05.

Effect of Azospirillum on the development of maize seedlings in hydroponic growth system

Azospirillum at 10^6 cfu/ml caused an increase in fresh weight of maize seedlings roots and shoots by 15–20%, when measured 3 days after inoculation (Fig. 1a). After 5 days, fresh weight of both roots and shoots decreased by 10 to 25% (Fig. 1b). The decrease was even more marked (30–40% from controls) in seedlings inoculated with 10^7 cfu/ml (data not shown).

Measurements of *Azospirillum* concentration in the nutrient medium of the hydroponic system 5 days after inoculation showed an increase to a level of 10^8–10^{10} cfu/ml. These levels were inhibitory to maize growth when tested in other experiments (data not shown).

Effect of Azospirillum on root development and respiration of common bean seedlings grown in the hydroponic growth system

Significant increases in fresh weight of primary and secondary roots (25 to 35% above controls) were observed (Fig. 2a) two days after inoculation with *Azospirillum* (10^6 cfu/ml). A similar trend was obtained in secondary root dry weight (data not shown). Inoculation with 10^7 cfu/ml resulted in a decrease in root fresh weight when measured after 2 days, however these differences were not statistically significant (Fig. 2b). Shoot fresh weight was increased following inoculation at 10^6 cfu/ml however an inhibitory effect for this parameter was observed at 10^7 cfu/ml (data not shown).

Similarly to maize roots, an optimal curve in the respiration rate of common bean seedlings roots was obtained (not shown). Although not significantly ($p=0.05$), respiration rates were maximally enhanced at an *Azospirillum* concentration of 10^6 cfu/ml (approximately 27% above controls) under these conditions.

Effect of Azospirillum on β-glucosidase activity

Maize seedlings were inoculated with *Azospirillum* at 10^6 cfu/ml in the hydroponic growth system, the roots were collected after 3 and 5 days, and proteins were extracted from root segments (1 cm from the root tip). Aliquots were taken for β-glucosidase analyses and the kinetic values (Km and Vmax) were determined.

A clear decrease of Km and Vmax was found after measuring β-glucosidase activity from root tips of inoculated maize seedlings in comparison to non-inoculated controls, both after 3 and 5 days (Fig. 3). Km and Vmax were calculated from highly significant ($r>0.95$) regression curves and the same picture was observed in other two separate experiments. Similar results were

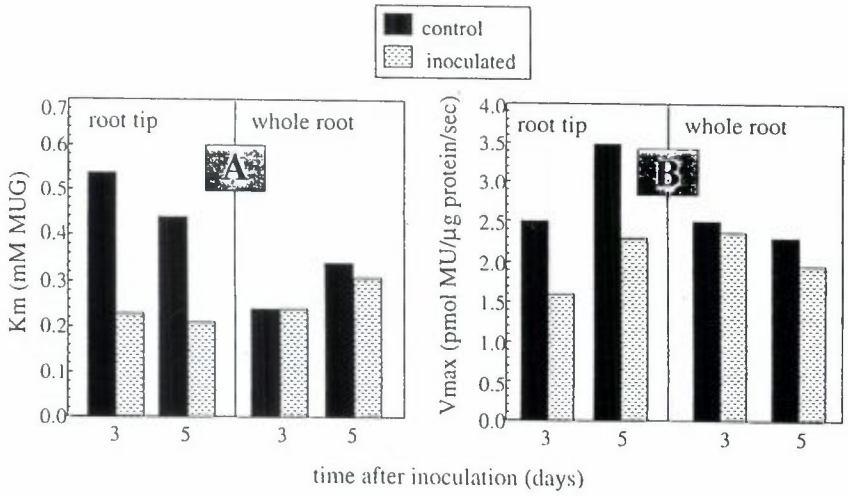

Figure 3. Effect of inoculation with *Azospirillum* at 10^6 cfu/ml, on β-glucosidase Km (A) and Vmax (B) values, in root tips of maize and in whole roots, 3 and 5 days after inoculation. Values were calculated from specific activities measured in 4 different substrate concentrations.

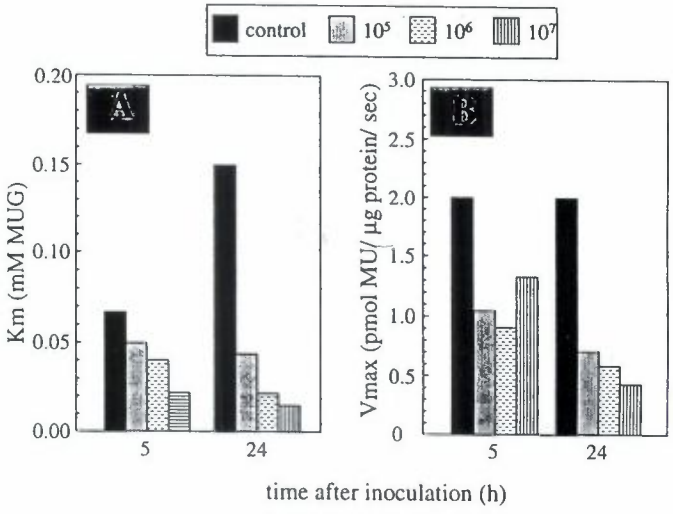

Figure 4. Effect of various concentrations of *Azospirillum* on β-glucosidases Km (A) and Vmax (B) of detached maize roots, 5 and 24 h after inoculation. Values were calculated from specific activities measured in reactions with 4 different substrate concentrations.

obtained in crude extracts taken from root tips inoculated with 10^7 cfu/ml (not shown). No differences were found when determining the kinetic values using whole roots (Fig. 3).

In order to confirm that the measured β-glucosidase activities were of plant origin and not from the colonizing bacteria, cells of *Azospirillum* were added to non-inoculated roots before protein extraction. Addition of bacterial cells to control roots did not change the specific activity, Km and Vmax values, indicating that the activity originated from plant tissues.

β-glucosidase activities were measured also in inoculated (10^6 and 10^7 cfu/ml) common beans roots grown in hydroponic growth system. No marked differences in Km and Vmax values between inoculated plants and non-inoculated controls could be observed 48 hours after inoculation and this was true for both primary and secondary roots (not shown). In some experiments, where root tips were assayed, a reduction of Km and Vmax values was obtained, but results were not consistent.

Effect of inoculation on β-glucosidase activity in detached maize roots

To obtain a more homogenous tissue for biochemical and molecular assays, roots (3 cm from the root tip) were detached from 3 day-old maize seedlings grown in hydroponics. The detached roots were first incubated in the hydroponic growth media, to minimize wounding effects. After 24 h the detached roots were inoculated with *Azospirillum* (10^5, 10^6 and 10^7 cfu/ml). The effect of *Azospirillum* on β-glucosidase activity was assayed 5 and 24 h after inoculation.

Inoculation with *Azospirillum* reduced both Km and Vmax values (Fig. 4). These effects were already observed 5 h after inoculation, and they become clearer after 24 h. The regression coefficients obtained from the different treatments were significantly high ($r>0.95$). From the picture observed in this and other two similar experiments, it seems that those effects are inoculum concentration-dependent (Fig. 4).

Effect of Azospirillum on the level or properties of specific β-glucosidase

To investigate the possibility that changes in the kinetic values were caused by changes in protein composition, maize protein extracts were separated by native gel electrophoresis. The gel was then stained with MUG to determine β-glucosidase activity on the gel.

Although 3 isoformes of β-glucosidases were previously reported in maize (Campos et al., 1993), only one band could be observed after specific β-glucosidase staining (Fig. 5). There were no differences in the intensity of the

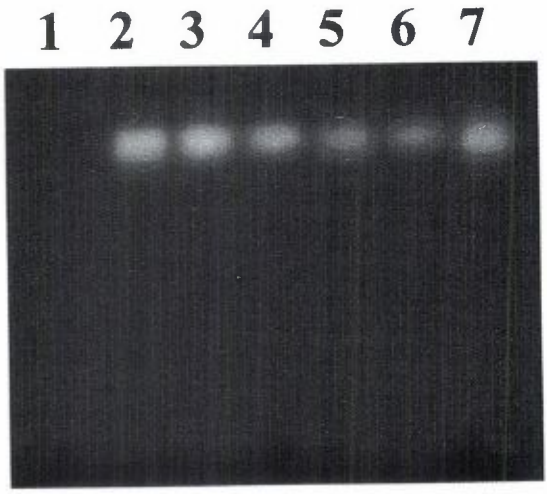

Figure 5. Native gel electrophoresis of extracts obtained from root tips of maize after inoculation with *Azospirillum* at 10^6 cfu/ml. The gel was stained with MUG (for β-glucosidase activity). Treatments are: markers (1); control (2) and inoculated roots (3) 3 days after inoculation; control (4) and inoculated roots (5) 5 days after inoculation; control roots with addition of *Azospirillum* just before extraction (6); yeast extract-inoculated roots 5 days after treatment (7).

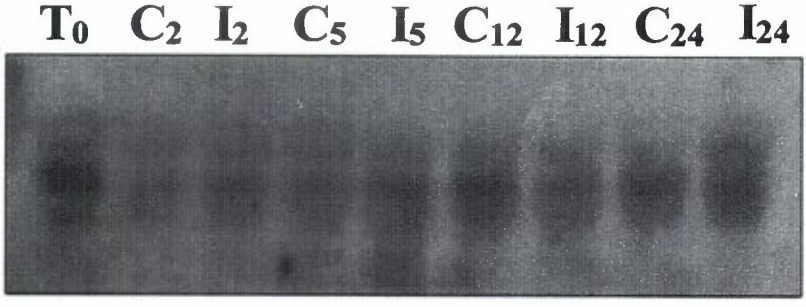

Figure 6. Northern-blot analysis of *Zm-p60.1* expression in maize roots, immediately after detachment of the seedling root (T_0), and 2, 5 and 24 h after inoculation with Azospirillum at 10^7 cfu/ml (I_n). C_n are non-inoculated controls. Quantitative analysis using a phosphorimager (not shown) indicated no marked differences in intensity between bands, with exception for T_0.

stained bands between inoculated roots and controls, even in roots treated with yeast extract, which is an universal elicitor of plant defense responses (Fig. 5).

Effect of Azospirillum on Zm-p60.1 mRNA accumulation

The possibility that *Azospirillum* affects the expression of a gene encoding for a specific β-glucosidase in maize was tested by northern-blot analysis, using the *Zm-p60.1* cDNA as a probe. *Zm-p60.1* encodes for a β-glucosidase involved in deglycosilation of plant growth promoting substances in maize (Brzobohaty et al., 1993). Detached roots were incubated for 24 h in the hydroponic growth media, to minimize wounding effects and then inoculated with *Azospirillum* at 10^7 cfu/ml. Total RNA was extracted from fresh roots, immediately after detachement, and from detached inoculated roots incubated for different periods of time.

Apart from a general decrease in *Zm-p60.1* expression, as a result of the detachment, it was not possible to detect marked differences in transcript level between the inoculated and non-inoculated roots (Fig. 6).

4. Discussion

Plant growth promotion following *Azospirillum* inoculation has been reported for many grass and legume species (Okon and Vanderleyden, 1997). Coinoculation of legumes with *Rhizobium* and *Azospirillum* may improve the nitrogen-fixing symbiosis and enhance plant growth and yield parameters (Burdman et al., 1997). In this work we investigated the effect of *Azospirillum* inoculation on maize and common bean seedlings. This study was made possible by growing plants in a gnotobiotic, hydroponic system with optimal aeration with sterile oxygen.

The effects of optimal inoculum concentrations of *Azospirillum* on root respiration rates and development, were observed in maize seedlings grown in Petri dishes and in hydroponic growth systems. After prolonged periods of incubation in hydroponics, an inhibition of plant growth was observed, but this was derived from a typical proliferation of *Azospirillum* to an inhibitory level of 10^8–10^{10} cfu/ml in the hydroponic medium during plant growth. Similar effects on growth promotion and respiration rates were observed in inoculated common bean seedlings in hydroponics.

The observed relative reduction of phytohormones and flavonoid conjugates in plants, following inoculation with *Azospirillum* (Fallik et al., 1989; 1996; Volpin et al., 1996), supports the hypothesis that there might be an increase in hydrolysis of conjugates, mediated by β-glucosidases. β-glucosidase activity could affect not only the release of aglycones with biological activity, but also the release of sugar radicals that might be involved in the higher respiration rates, as obtained in inoculated roots of maize and common bean. It was consistently observed in both root tips and detached roots of maize that

inoculation with *Azospirillum* significantly reduced the Km of β-glucosidase activity, and to a lesser extent, Vmax values. Although the same tendency was observed in root tips of common bean, enzyme activities were very low and treatments could not be properly compared.

In contrast to other plant-bacterial systems where a direct effect of bacterial β-glucosidases have been observed (Hartwig and Phillips, 1991; Spena, 1993), it seems that this does not occur in the *Azospirillum*-maize interaction. No differences in the kinetic values were obtained after adding *Azospirillum* culture to control roots, just before root protein extraction. The differences in the kinetic values were detected mainly in root tips but not in extracts of whole maize root system. This might be due to higher β-glucosidase activity in cells of the root elongation zone, where metabolic activity is higher (Salisbury and Ross, 1992). It has also been found that β-glucosidase Zm-p60.1 enzyme, isolated from maize roots, is present mainly in meristematic cells of the root tip, and participates in hydrolyzing glycoside conjugates of cytokinins, involved in cell division (Brzobahaty et al., 1993) This makes this enzyme an attractive candidate to test the hypothesis that β-glucosidase activity is increased upon inoculation with *Azospirillum*.

A decrease in the Km indicates a higher affinity of the enzyme(s) of this group to the substrate used (MUG). This increase in affinity might be similar for physiological substrates. Decrease in Vmax is generally related to a possible change in the maximum potential activity of the enzyme *in vitro*. This derives from either a change in the amount of the enzyme or from changes in the enzyme catalytic site (Lehninger, 1993). Thus, decrease in both Km and Vmax values expresses, on one hand, an increase in affinity to the substrate, but on the other hand, a decrease in the total potential activity. Since Km and Vmax values can derive from different sites of the enzyme, these results are not contradictory. The changes in Km values may also indicate changes in the relative amount of specific β-glucosidase. Campos et al. (1992) and Feldwisch et al. (1994) reported the presence of at least 3 different β-glucosidases in maize roots, all of them with a very close molecular weight (58–60 kDa). Native gel electrophoresis with specific β-glucosidase staining (Fig. 6), did not reveal changes in β-glucosidase profile or levels, and only one band of the enzyme was detected. We did not observe changes in the expression of the *Zm-p60.1* gene following *Azospirillum* inoculation. This gene codes for a β-glucosidase, apparently involved in the regulation of cytokinin levels in maize roots (Brzobohatny et al., 1993). In this experiment, we used detached root tips and followed *Zm-p60.1* expression for 24 hours after inoculation. It might be that experiments with whole inoculated roots, in different time scale would lead to different results. There is a possibility that changes in kinetic values, following *Azospirillum* inoculation, take place not only in the root tips but also

in whole roots. It might be that the activities are being diluted by the high heterogeneity of the extracted root tissue.

Azospirillum inoculation at a concentration of 10^6 cfu/ml enhanced growth of common bean seedlings (root and shoot fresh weight) and respiration rates, 2 days after inoculation. These results support previous conclusion on the positive direct effects of *Azospirillum* on growth of common bean, indirectly causing a benefit to the *Rhizobium*-common bean symbiosis (Burdman et al., 1996). Moreover, the main effect was observed on the development of secondary roots and this is in accordance with improvement in the development of adventitious roots in other plants (Sarig et al., 1992).

Azospirillum-derived growth promotion was accompanied by higher root respiration rates of common bean seedlings. This effect has previously been observed in roots of sorghum, maize and tomatoes along with an increase in the specific activities of enzymes involved in respiration processes (Fallik et al., 1994).

Inoculation of common bean seedlings at a concentration of 10^7 cfu/ml, decreased respiration rates and inhibited seedling growth. The importance of optimal inoculum level of *Azospirillum* for both grasses (Okon and Labandera-Gonzalez, 1994) and legumes (Burdman et al., 1997) is well documented. It has been proposed that *Azospirillum* causes changes in phytohormonal balances, contributing to positive effects on plant growth (Fallik et al., 1994). Supra-optimal phytohormonal concentrations can inhibit these processes (Salisbury and Ross, 1992) and therefore it is possible that under gnotobiotic growth conditions there might be supra-optimal levels of phytohormones either produced by the bacterium or elicited by its presence in the roots. It is unlikely that high concentrations of *Azospirillum* cause an inhibitory pathogenic type reaction. No increase in the expression or synthesis of plant defense enzymes, such as those involved in the phenyl-propanoid metabolism has been detected in grasses (Fallik et al., 1994) or legumes (Volpin et al., 1996) inoculated with supra-optimal levels of *Azospirillum*.

By testing β-glucosidase activity in extracts of inoculated roots, using natural substrates such as glycosides of phytohormones and flavonoids, it would be possible to clarify the role of this enzymes in the promotion of plant growth by *Azospirillum*.

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