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The Effect of *Azospirillum brasilense* Inoculation on Metabolic Enzyme Activity in Maize Root Seedlings

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Abstract

Application of *Azospirillum brasilense* to maize plants at an inoculum concentration of 10^7 colony forming units (CFU)/plant increased the specific activity in maize root extracts of the enzymes alcohol dehydrogenase, acid phosphatase, glutamine synthetase, isocitrate dehydrogenase, malate dehydrogenase, pyruvate kinase and shikimate dehydrogenase. Significantly higher specific activities were observed between the 2nd and 3rd week after sowing, in inoculated roots as compared to non-inoculated controls.

Azospirillum brasilense inoculation did not affect the activity of phenylalanine-ammonia-lyase and glucose-6-phosphate dehydrogenase, enzymes that are generally elevated in plants as a result of bacterial, fungal or viral infection. This suggests that *Azospirillum* does not behave as a pathogenic rhizosphere bacterium.

SDS-gel electrophoresis pattern of cell free extracts of roots, revealed no differences between inoculated and non-inoculated plants.

Keywords: alcohol dehydrogenase, acid phosphatase, glucose-6-phosphate dehydrogenase, glutamine synthetase, isocitrate dehydrogenase, malate dehydrogenase, phenylalanine-ammonia-lyase, pyruvate kinase, shikimate dehydrogenase, *Azospirillum brasilense*, maize seedlings

Abbreviations: ADH — alcohol dehydrogenase, AP — acid phosphatase, G6PDH - glucose-6-phosphate dehydrogenase, GS — glutamine synthetase, ICDH — isocitrate dehydrogenase, MDH - malate dehydrogenase, NPK — nitrogen, phosphorous and potassium, PAL — phenylalanine-ammonia-lyase, PK — pyruvate kinase, SHDH — shikimate dehydrogenase, SDS-PAGE — sodium dodecyle sulphate-poly acrylamide gel electrophoresis

1. Introduction

Inoculation of plants with bacteria of the genus *Azospirillum* affect plant development by several mechanisms, including biological N_2 fixation and enhancement of mineral and water uptake due, probably, to improved root systems of inoculated plants (Kapulnik et al., 1985; Lin et al., 1983; Okon, 1985). Between the 2nd and 3rd week after sowing, the surface area of inoculated maize and wheat roots is significantly increased by an optimal *Azospirillum* inoculation concentration of 10^7 colony forming units (CFU)/mL (Fallik et al., 1988). Root surface area in maize seedlings is a reliable criterion for the evaluation and measurement of maize inoculated with *Azospirillum* (Fallik et al., 1988).

The physiology and biochemistry of maize roots colonized by *Azospirillum* has not been extensively studied.

Results of preliminary experiments indicated that specific activity of the enzymes polyphenol oxidase, peroxidase and IAA-oxidase were lower in extracts of inoculated wheat roots compared to extracts from non-inoculated plants (Okon and Kapulnik, 1986).

The observed increases in root surface area, dry weight and NPK content are indicatives of changes in metabolic activity of the inoculated plants. To test this notion 9 enzymes representative of a number of different metabolic pathways were studied in maize seedlings.

The results of this study provide evidence to support the above notion.

2. Materials and Methods

Biological material

Azospirillum brasilense (ATCC 29729)-Cd was used in all experiments. The growth conditions of the bacteria, seed variety, inoculation and pot experiment were as described by Fallik et al. (1988).

Preparation of root extracts

Ten grams weight of roots from one plant were macerated at 4°C with a chilled Waring blender in 34 mM phosphate buffer, pH 6.8. The ratio of fresh weight to extraction medium was 3:3 (w/v). Extracts were centrifuged twice at 15,000 rpm for 10 min at 4°C. The clear supernatant was placed on ice in capped vials and aliquots were removed for the determination of enzyme activities and protein determination according to Bradford (1976) using bovine serum albumin (BSA) as standard. The enzyme activities of root extracts were determined once a week over a period of 4 weeks. Extracts were prepared from non-inoculated roots and roots inoculated with 10^7 CFU *Azospirillum*/plant. The experiment was repeated 3 times and each treatment consisted of 8 plants. For SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), roots were extracted in 34 mM phosphate buffer pH 6.8 containing 0.5 mM β-mercaptoethanol.

Statistical analysis

Data were analyzed for the mean and S.E. by the Duncan's multiple range test at the 0.05 probability level (Snedocor and Cochran, 1967).

Enzyme activities

Enzyme activities were determined as follows:

Alcohol dehydrogenase (ADH) (E.C. 1.1.1.1) and malate dehydrogenase (MDH) (E.C.. 1.1.1.37) were measured by the rate of reduction of nicotinamide-adenine-dinucleotide (NAD) as described by Efron and Schwartz (1968) and Mollering (1974) respectively. One enzyme unit is defined as the amount of activity that reduces one micromole of NAD^+ per minute.

Iso-citrate dehydrogenase (ICDH) (E.C. 1.1.1.42), shikimate dehydrogenase (SHDH) (E.C. 1.1.1.25) and glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49) were estimated by the rate of reduction of nicotinamide-adenine-dinucleotide-phosphate (NADP) as described by Siebert (1965), Balinsky and Dennis (1970) and Decker (1977) respectively. One enzyme unit is defined as the amount of activity that reduces one micromole of $NADP^+$ per minute.

Acid phosphatase (AP) (E.C. 3.1.3.2) was measured by the rate of hydrolysis of O-carboxyphenyl phosphate as described by Decker (1977). One unit of the enzyme is defined as the amount of activity that hydrolyses one micromole of O-carboxyphenyl phosphate per minute.

Pyruvate kinase (PK) (E.C. 2.3.1.40) was determined in a lactate dehydrogenase coupled assay system by measuring the decrease in absorbance at 340 nm resulting from oxidation of reduced nicotinamide-adenine-dinucleotide (NADH), as described by Decker (1977). One unit of the enzyme is defined as the amount of activity catalyzing the oxidation of one micromole of NADH per minute.

Phenylalanine-ammonia-lyase (PAL) (E.C. 4.3.1.5) was measured by the formation of transcinnamate from phenylalanine as described by Havir and Hanson (1970). One unit is defined as the amount of enzyme catalysing the formation of one micromole of trans-cinnamate per minute.

Glutamine synthetase (GS) (E.C. 6.3.1.2) was determined by colorimetric assay measuring the formation of γ-glutamylhydroxamate as described by Rowe et al. (1970). Under these conditions, the extinction coefficient of one micromole of γ-glutamylhydroxamate at 540 nm is 0.532. A unit of the enzyme is defined as the amount which catalyses the synthesis of one micromole of γ-glutamylhydroxamate per minute.

The specific activity of each enzyme is expressed as units per milligram protein per minute.

Electrophoresis and staining methods

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% polyacrylamide according to Laemmli (1970). Proteins were detected on the gel by staining in Coommasie Blue-R.

3. Results

The effect of Azospirillum brasilense inoculation on enzyme activity in maize roots

The specific activity of ADH in root extracts prepared from plants inoculated with *A. brasilense* at 10^7 CFU/plant was significantly higher at the 2nd and 3rd week after sowing relative to controls. In the first week ADH was not significantly different from the control (Fig. 1A). At the 4th week, ADH specific activity was significantly lower in root extracts of inoculated plants compared to controls. The lower activity in the 4th week may be attributed to the faster ADH disappearance from seeds and roots due to accelerated growth (Fig. 1A).

One, two and three weeks after sowing, MDH specific activity in inoculated root extracts was significantly higher than in controls. At the 4th week, differences were not significant (Fig. 1B).

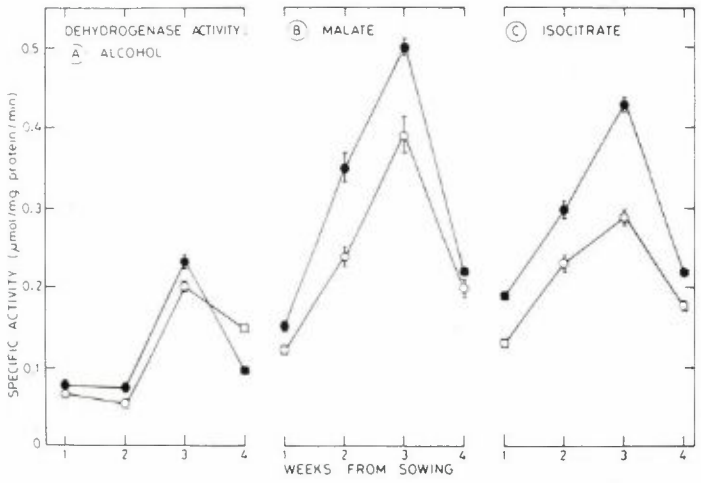

Figure 1. (A) ADH, (B) MDH and (C) ICDH enzymes specific activity in root extracts made from plants inoculated with *Azospirillum brasilense*. (10^7 CFU/plant •———• and non-inoculated control o———o. Enzyme specific activity expressed as μmole/mg protein/minute. Points represent the mean ± S.E. of 24 replicate samples.

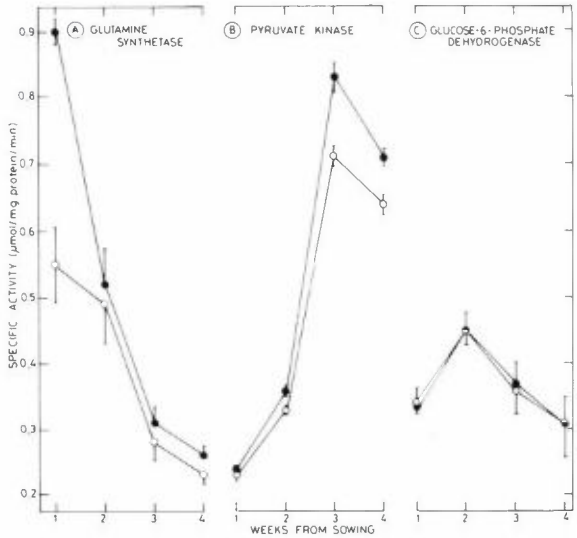

Figure 2. (A) GS, (B) PK, (C) G6PDH enzyme specific activity in root extracts made from plants inoculated with *Azospirillum brasilense*. (10^7 CFU/plant •———• and non-inoculated control o———o. Enzyme specific activity expressed as μmols/mg protein/minute. Points represent the mean ± S.E. of 24 replicate samples.

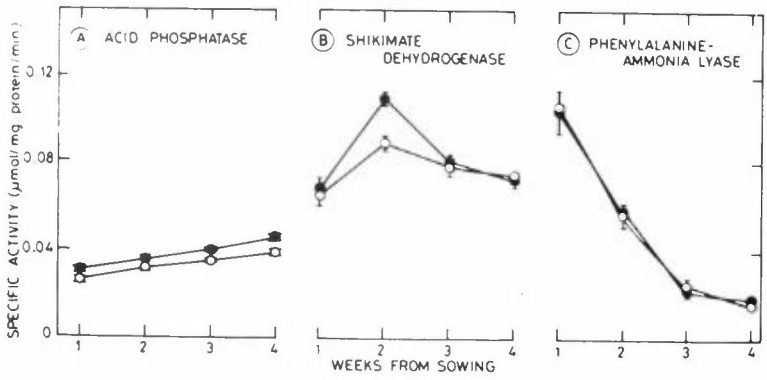

Figure 3. (A) AP, (B) SHDH and (C) PAL enzyme specific activity in root extracts made from plants inoculated with *Azospirillum*. (10^7 CFU/plant •———• and non-inoculated control o———o. Enzyme specific activity expressed as μmole/mg protein/minute. Points represent the mean ± S.E.. of 24 replicate samples.

The specific activity of ICDH was consistently and significantly higher in inoculated roots over controls during the 4 weeks of the experiment (Fig. 1C).

The specific activity of GS (Fig. 2A) in root extracts prepared from inoculated plants was significantly higher than controls only at the 1st week. No significant differences were observed between the treated group and controls from week two until the end of the experiment.

PK specific activity (Fig. 2B) in the treated group increased progressively during the first 3 weeks and then decreased. The results of the 3rd and 4th week after sowing were significantly higher from that of controls.

The specific activity of SHDH (Fig. 3B) in root extracts prepared from inoculated plants was significantly higher than controls only at the 2nd week from sowing.

The specific activity of AP (Fig. 3A) in inoculated root extracts was significantly higher at the 3rd and 4th week after sowing relative to controls.

No significant differences in the specific activities of the enzymes G6PDH and PAL were observed between the treated and control root extracts through the entire period of the experiment (Fig. 2C and 3C respectively).

Effect of Azospirillum inoculation on total isozymes and protein in maize roots

In order to test the possibility that *Azospirillum* triggers the expression of gene systems in inoculated plants in comparison to non-inoculated plants, isozymes and protein patterns were examined by starch gel or SDS-PAGE.

Since most pronounced differences in enzyme specific activities were observed at the 2nd week after sowing, it was reasonable to assume that at this stage one may detect differences between zymograms of extracts from inoculated and non-inoculated roots.

Zymograms of ADH, MDH and AP of two weeks old root extracts, did not reveal differences in pattern between the inoculated and non-inoculated samples (data not shown). The lack of any change in zymograms strongly suggests that the observed increase in specific activity of ADH, MDH and AP is associated with increased gene activity or enzyme activation.

The supernatant of root extracts were fractionated by ammonium-sulfate at 0–25%, 25–75% and brought to identical protein concentration prior to electrophoresis (data now shown).

No new protein bands were observed on SDS-PAGE with inoculated, or non-inoculated root extracts in any of the fractions. We can not exclude the possibility that new proteins in undetectable amounts are present, or that they comigrated with other existing proteins. Further verification is needed by using 2-Dimensional SDS-PAGE and silver staining.

4. Discussion

The complexity of maize inoculation with *Azospirillum brasilense* has been studied extensively. In most of the reports, research emphasis was on biological nitrogen fixation, root colonization and grain yields increase under a variety of field conditions (Okon, 1985). Surprisingly, little attention has been devoted to inoculated plant metabolism.

Azospirillum, at an inoculum concentration of 10^7 CFU/plant causes significant increases in the specific activity of several enzymes which were extracted from inoculated roots.

The enzymes PK, MDH, ICDH are known to participate in energy deriving and respiratory pathways (Hackett, 1963; Hayden and Cook, 1972), while SHDH is involved in the synthesis of aromatic amino acids which are precursors for lignin and indole biosynthesis (Gilchrist and Kosuge, 1980). The specific activity of these enzymes increased significantly in root extracts

made from inoculated plants between the 2nd and/or 3rd week after sowing, relative to control.

GS is the main enzyme in assimilation of nitrogen during seed germination (Mayer and Poljakoff-Mayber, 1982; Miflin and Lea, 1980). Baskakova and Izmailov (1984) reported that the specific activity of GS decreases in maize and pea roots during plant development as a result of proteins breakdown or protein pools dilution in the seed. Except for the first sampling point, our results on GS specific activity, are in close agreement with that of Baskakova and Izmailov (1984). The higher specific activity in inoculated roots one week after sowing was unexpected. This exceptionally high specific activity may be associated with a short burst of GS synthesis or a slower rate in GS breakdown relative to other proteins.

Acid phosphatase is involved in the breakdown of organic phosphate compounds (Sutcliffe and Sexton, 1968). We found that the specific activity of AP increased at the 3rd and 4th weeks after sowing. O'Connell and Grove (1985) have noted that AP activity in soil deficient in phosphate content is elevated. Lin et al. (1983) reported that inoculation of *Azospirillum* increased phosphate uptake. The higher specific activity of AP especially at the 3rd and 4th weeks, could be related to phosphate exhaustion, which signals the plant to increase the phosphate utilization machinery.

The specific activity of ADH increased significantly above the control at the 2nd week after sowing. ADH has been shown to be activated by the auxin-like herbicide, 2,4-D (Freeling, 1973). By analogy, our results could be related to the higher levels of IAA in inoculated roots, as detected recently by Fallik et al. (unpublished).

These findings suggest that once roots emerge from the seed and *Azospirillum* has colonized the root surface, pathways such as glycolysis, TCA cycle, assimilation of nitrogen and the synthesis of aromatic amino acids, are stimulated. This enhanced metabolic activity is the principal driving force leading to faster plant growth.

The specific activities of G6PDH and PAL in root extracts of inoculated and control plants were very similar. It is well established that the levels of these 2 enzymes are elevated in plants infected with bacteria or viruses. For example, infection with *Agrobacterium tumefaciens* increased the specific activity of G6PDH two to three-fold in bean roots (Goodman et al., 1986). Similarly, potato virus infection increased the specific activity of G6PDH in infected plants by 89% relative to healthy leaves (Dwurazna and Weintraub, 1969). In the case of bean leaves infected with tobacco necrosis virus, PAL

activity was significantly elevated (Goodman et al., 1986).

The similar levels of G6PDH and PAL in extracts of inoculated and non-inoculated roots suggests that *A. brasilense* colonization of maize roots does not mimic symptoms associated with plant pathogenic organisms.

In view of the consistent differences in MDH, ICDH, PK and SHDH observed, these enzymes can be used as good parameters to evaluate the response of maize plant to *Azospirillum* inoculation at early stages of plant development.

It is worthwhile to point out that all the results and observations described in this report were obtained from plants grown in poor soil. It remains to be elucidated whether enzyme specific activities of plants grown and inoculated in other soils exhibit the same characteristics.

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