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Responses of Soybean and Cowpea Root Membranes to Inoculation with *Azospirillum brasilense*

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Abstract

Inoculation with *Azospirillum brasilense* Cd of two leguminous species, soybean (*Glycine max*) and cowpea (*Vigna unguiculata*), demonstrated that: (1) *A. brasilense* significantly increased proton efflux from their roots; (2) inoculation reduced plant membrane potential (in soybean), (3) inoculation changed the phospholipid quantity in plant membranes of calli (in cowpea), and (4) a low molecular weight "bacterial signal" may be responsible for these effects. We suggest that plant membranes can serve as a reliable sensor for the effect of *Azospirillum* on plants and are probably a primary target for *Azospirillum* on plant roots.

Keywords: *Azospirillum*, beneficial bacteria, cowpea, membrane potential, plant-growth-promoting-rhizobacteria, proton efflux, rhizosphere bacteria, soybean

1. Introduction

The mode of action by which *Azospirillum* affects plant growth and productivity is vague and speculative. Common explanations include: a change

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in the plant's hormonal balance, an increase in mineral and water uptake, nitrate reductase activity, enhancement of root development in general, and an additive theory suggesting that several small-magnitude mechanisms operate simultaneously or in succession (Bashan and Levanony, 1990). Whatever the exact mechanism is, the fact that *Azospirillum* affects plant cell metabolism from outside the plant (Bashan et al., 1991; Levanony et al., 1989) suggests that the bacteria are capable of excreting and transmitting a signal(s) which crosses the plant cell wall and is recognized by the plant membranes. This interaction initiates a chain of events which results in the observed altered metabolism of the inoculated plants. Since plant membranes are extremely sensitive to any change, they may serve as a precise indicator for *Azospirillum* activity at the cellular level.

Proton efflux from roots is a very well known phenomenon associated with many metabolic root functions (Spanswick, 1981) and is essential for the well being of the plant. Inoculation with A. brasilense has been shown to alter and increase proton efflux in wheat plants (Bashan et al., 1989). This activity is dependent on plant metabolism which reflects environmental stimuli (Bashan and Levanony, 1989) and on the age, concentration and strain of the bacteria (Bashan and Levanony, 1991; Bashan et al., 1989). A very short exposure to bacteria is sufficient to increase proton efflux (Bashan, 1990) and reduce the membrane potential of the inoculated root cells by both *Azospirillum* (Bashan, 1991; Bashan and Levanony, 1991) and Rhizobium (Ersek et al., 1986).

The aims of this paper are: (1) to present evidence that *A. brasilense* Cd can alter membrane activities such as proton efflux and membrane potential (Novacky et al., 1976) in dicotyledonous legume roots, and (2) to emphasize that this bacterial activity is non-specific by providing evidence from dicotyledonous plants to supplement the known activity in monocotyledonous plants such as wheat (Bashan, 1990; Bashan and Levanony, 1991; Bashan et al., 1989).

2. Materials and Methods

Organisms, growth conditions and measurements of proton efflux

Azospirillum brasilense Cd (ATCC 29710) was used as a model in all experiments. Other *A. brasilense* strains were also demonstrated to have the ability to affect proton efflux and membrane potential in wheat (Bashan, 1991; Bashan and Levanony, 1991). Halotolerant cowpea plants (*Vigna unguiculata*, obtained from the Universidad Autonoma de Baja California Sur) and soybean plants (*Glycine max* cv. Pella) served as host plants. Bacteria were grown in OAB N-free medium (Bashan and Levanony, 1985). Bacterial growth

conditions and plant inoculation were as described elsewhere (Bashan et al., 1989). Plants were grown from disinfected seeds (1% NaOCl for 5 min, thoroughly washed with sterile-distilled water) in large (100 ml test tubes filled with sterilized, moistened, coarse vermiculite. The seedlings were incubated at 25 ± 1°C, 50 f 5% relative humidity, 100 μE m^{-2} s^{-1} for 6 days prior to inoculation. After inoculation with 10^6 cfu ml^{-1} (suspended in the corresponding plant nutrient solution), the plants were further incubated for 2-3 days. Then, the plants were transferred into 150 ml flasks containing 50 ml sterile plant nutrient solution sealed with a transparent lid in which a small hole was cut to allow the stem to emerge from the flask. The decrease in pH was continuously monitored by a chart recorder for 18 hr. In addition, 4-10 identical replicates were manually recorded at 2 hr intervals. The quantity of protons released by the roots was determined by titrating the nutrient solution with 0.01 N NaOH to the initial pH value. Excess water was removed from the roots by blotting with soft paper prior to fresh weight determination. The amount of protons released into the nutrient solution was expressed as μmol H^+. (g fresh weight)$^{-1}$·h^{-1} (Bashan, 1990, 1991; Bashan and Levanony, 1989; Bashan et al., 1989).

Plant nutrient solutions

Nutrient solutions were prepared according to Marschner et al. (1982) as follows (mM): (1) K_2SO_4, 0.75; $MgSO_4$, 0.65; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.5; $CaSO_4$, 0.5; H_3BO_3, 1 x 10^{-2}; $MnCl_2{\cdot}4H_2O$, 1 x 10^{-4}, $ZnSO_4{\cdot}7H_2O$, 0.5 x 10^{-5}; $CUSO_4.5H_2O$, 0.5x10^{-5}; $H_2MoO_4{\cdot}H_2O$, 0.5x10^{-6}. (2) Similar to solution (1) plus FeEDTA, 0.1. (3) The same as (1) replacing $(NH_4)_2SO_4$ with $Ca(NO_3)_2$, 2. (4) The same as (3) plus FeEDTA, 0.1. The pH of all nutrient solutions was adjusted to 5.8 with NaOH.

Perfusion solution

Perfusion solution used to equilibrate the cells prior to membrane potential measurements was the one suggested by Érsek et al. (1986) and was composed of (mM): KCl, 1; $Ca(NO_3)_2$, 1; $MgSO_4$, 0.25; NaH_2PO_4, 66, final pH 5.7.

Measurement of membrane potential (E_m)

Intact seedlings having a single root (63 hr-old) were sampled and mounted horizontally on a plexiglass holder and washed for 3 hr in an aerated perfusion solution.

Microcapillaries with glass micro-fibers (WP Instruments) were pulled to micro-electrodes using a vertical electrode puller. Micro-electrodes having a tip diameter of 0.6 ± 0.2 *m*m, tip potential of -2 to -16 mV and tip resistance of 5 to 14 MΩ were used. Micro-electrodes were filled with 3 M KCl to eliminate air bubbles trapped inside the micro-electrode. Each micro-electrode was microscopically tested for tip perfection. A reference salt-bridge, a 4 cm long piece of tube (2 mm inner diameter), was filled with a 3 M KCl in 2% agar. Both salt-bridges were connected through Ag/AgCl wire with an electrometer amplifier and a chart recorder. Micro-electrodes were inserted into the selected root site with a micromanipulator, and continuously observed with a horizontally mounted stereoscopic microscope illuminated with fiber optics. The plexiglass chamber containing the seedling and the holder (total volume of 7 ml) was perfused with perfusion solution at a flow rate of 8 ml/min (Bashan, 1991); Bashan and Levanony, 1991; Érsek et al., 1986).

Continuous perfusion of inoculated roots

Continuous perfusion of the nutrient solution with fresh solution (4 mm/min) was carried out using a small pump. the overflow from the beaker was filtered through a 0.45 *m*m filter. This resulted in the continuous washing of bacteria and roots, yet the bacteria were not removed from the root vicinity. This procedure allowed replacement of a solution volume equivalent to the testing solution, every 5 min. The replaced solution was collected, its volume was reduced by roto-evaporation at 38 ± 2°C to its original volume, and the amount of released protons was determined (Bashan, 1990).

Bacterial counts

This was done by the improved selection technique (Bashan and Levanony, 1985) on OAB Medium (Bashan et al., 1992).

Collection of bacterial "signal molecules"

Plants were grown in hydroponic solution (Nutrient solution (1); Bashan and Levanony, 1989) and inoculated with *A. brasilense* Cd. Two days after inoculation, the plants were removed and the solution was filtered through a filter paper and a 0.45*m* Millipore filter. The pH was re-adjusted to 5.8 with 0.1 N NaOH. Non-inoculated plants were introduced into this solution and the membrane potential and proton efflux were determined. Solutions which served as controls were obtained from non-inoculated plants, plant nutrient solutions inoculated with bacteria only, and a supernatant from a bacterial culture.

Dialysis of the solution containing "bacterial signal molecules" was done in cellulose dialysis tubes allowing molecules up to 12000 MW to leave the tube against 0.01 M potassium phosphate buffer pH 6.0, the same buffer supplemented with 15.4 mg 1-1 dithioerythritol and 300 mg 1-1 MgCl2, or 0.02 M Tris-HCl buffer pH 8.0.

Callus induction and reproduction

Cowpea seeds were surface disinfected by soaking in 0.5% NaOCl for 15 min. Then, they were thoroughly rinsed with distilled water. The seeds were germinated in glass beakers (150 ml), capped with translucent autoclavable lids; beakers contained 20 ml of Murashige and Skoog (1962) medium (MS) lacking plant growth regulators. The beakers were incubated at 26 ± 2°C at 100 μE m^{-2} s^{-1} for 10 days. Then, 0.5 cm^2 leaf pieces were cut and transferred into MS medium supplemented with 10 μM of 2, 4-dichlorophenoxyacetic acid. The calli were induced at 26 ± 2°C under continuous fluorescent light (100 μE m^{-2} s^{-1}) (Vazquez-Duhalt et al., 1991).

Dry weight determination

Plant material was weighted after drying in an oven for 24 hr at 60°C.

Lipid analysis

Calli were suspended in methanol and heated for 5 min at 100°C in a glass-stoppered tube to inactivate degradative enzymes. Then, the suspensions were cooled and chloroform was added to obtain 5 ml of methanol-chloroform (2:1, v/v) mixture. From this mixture, the biomass of calli was extracted for 24 hr at 4°C. The extract was filtered and the residue was washed two times with 5 ml of methanol-chloroform (2:1, v/v). Ten ml of distilled water were added to the combined filtrates in a glass-centrifuge tube, and the two phases of liquids were separated by centrifugation at 700 g. The chloroform layer was withdrawn and the aqueous (methanol/water) phase was washed two times with 3 ml chloroform. The chloroform solution containing the lipids was evaporated under vacuum (Rotavapor R-110, Büchi, Switzerland) at 45°C. The dry residue was weighed and immediately dissolved in 1 ml of chloroform containing 0.02% of 2,6-di-t-butyl-4-methyl phenol (Vazquez-Duhalt et al., 1991).

Fractionation of lipids was done as follows: The neutral lipids were obtained by fractionation in alumina column chromatography (Vazquez-Duhalt and Greppin, 1987). Then the glycolipid and phospholipid were eluted successively with 10 volumes of acetone and 10 volumes of methanol, respectively. The different fractions were dried under N_2 and immediately weighed.

Lipid analysis of bacteria was done in a similar manner to the calli.

Experimental design and statistical analysis

All experiments were carried out 2 to 5 times each in 3 to 10 replicates in similar size plants having 2-3 true leaves. A replicate consisted of one determination of lipids from 10-18 g of calli or proton efflux from one plant. Continuous measurement charts were plotted together and the presented graphs represent the average of proton efflux activity in all experiments. Results were analyzed by Analysis of Variance (ANOVA) one-way and by Student's t-test. Significance is given by $P \leq 0.05$.

3. Results

Proton efflux of inoculated cowpea plants

In general, the increase in proton efflux from the inoculation of cowpea seedlings coincided with the type of nutrient solution used (Fig. 1). The most marked effect was observed 9-10 hr after transferring the plants to a new nutrient solution containing ammonium (Fig. 1A,B). About 18 hr after the transfer, the pH reached its lowest level and was in the range of 4.13±0.19 units (Fig. 1A,B). Nutrient solution containing nitrate generated a smaller proton efflux in inoculated plants and blocked this activity in non-inoculated plants (Fig. 1C,D). No effect from seedling age was observed when seedlings of 5 to 15 days old were used. The addition of ferrous ions slightly accelerated proton efflux in the nutrient solution containing ammonium during the first 5 hr. It had no effect later (Fig. 1A,B) or in the nutrient solution containing nitrate (Fig. 1C,D). A decrease in nitrogen content was detected before and after inserting the plants for proton efflux measurement. The initial N content of the solution was 0.0266 *m*g ml^{-1} and the final N content was 0.0199 *m*g ml^{-1}.

Changes in proton efflux and membrane potential after application of "bacterial signal(s)" molecules

When non-inoculated plants were placed in a solution which had been previously used by inoculated plants, they showed trends of proton efflux and decreased membrane potential similar to the inoculated plants, though to a lesser extent (Fig. 2A-B). The addition of a solution of "signal molecules" to the inoculated plants did not further enhance the proton efflux of these roots (data not shown). Supernatants from bacterial cultures or solutions obtained from non-inoculated plants did not affect membrane potential (Fig. 2A) or proton efflux activity (data not shown). Dialysis of the solution in three separate buffers diminished its activity on plants.

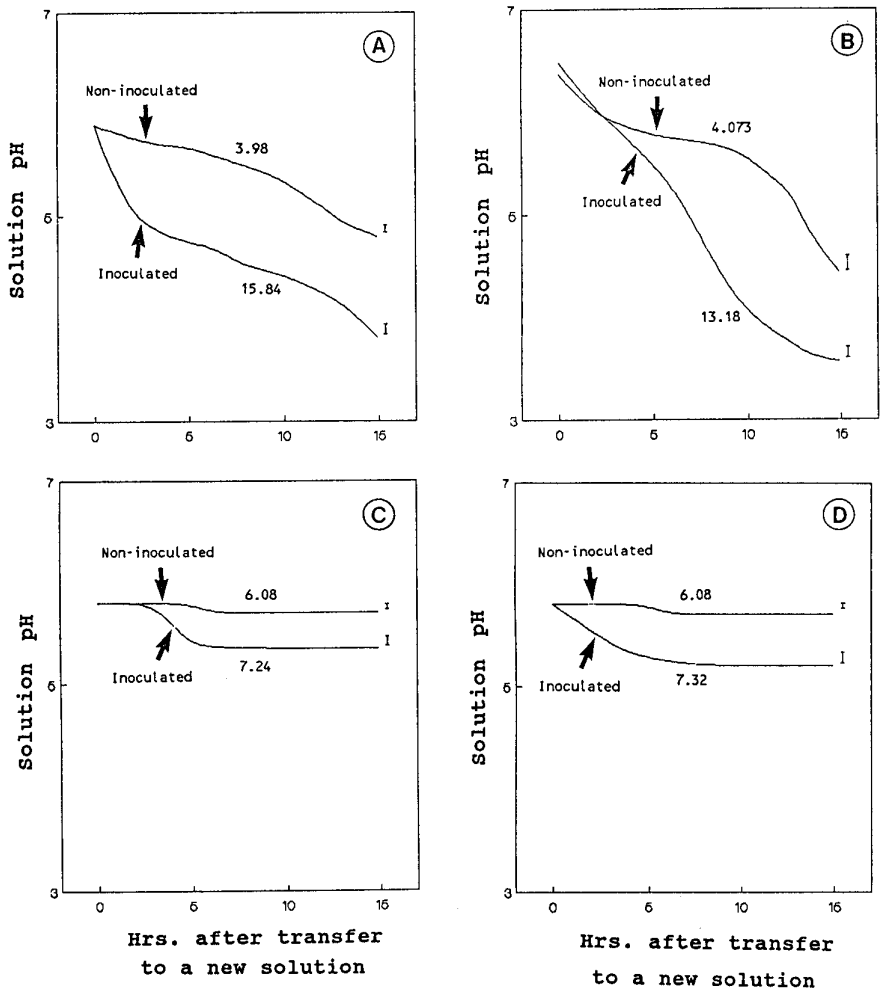

Figure 1. Effect of inoculation with *A. brasilense* Cd on proton efflux in young intact cowpea roots in different nutrient solutions.

(A) Ammonium with ferrous
(B) Ammonium without ferrous
(C) Nitrate with ferrous
(D) Nitrate without ferrous

All graphs were recorded automatically and drawn on a paper moving at a rate of 10 mm/hr. Each nutrient solution was tested 2 to 4 times. Results from all experiments were combined and redrawn by a computer. Lines represent the average lines of all the repetitions. Numbers represent the average of proton efflux (μmol H^+ $(g\ dw)^{-1}h^{-1}$) after 9 hr. Bars represent the SE of the lines.

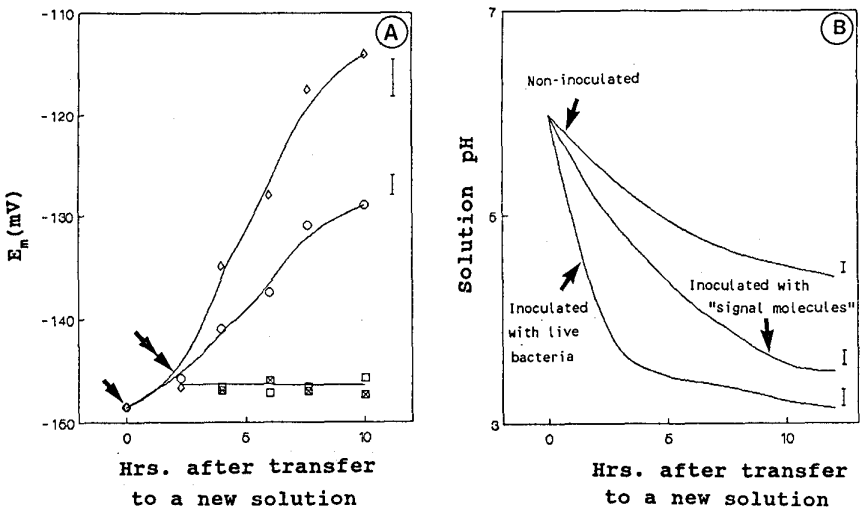

Figure 2. (A) Membrane potential (Em) differences between interior of soybean root cells and solution bathing the roots. ◇-E_m values for inoculated roots perfused 2 hr after inoculation. □ - E_m values for non inoculated roots. o – E_m values for non inoculated roots treated with solution containing "bacterial signal molecules". ⊠ - E_m values for non inoculated plants treated with bacterial supernatant. Arrow indicates the time of inoculation. Double arrow indicates beginning of perfusion. The values are means from 20 separate determinations, 4 determinations per seedling. Bars represent SE.

(B) Proton efflux of soybean roots inoculated with live *A. brasilense* Cd in nutrient solution 1 and with a solution containing "bacterial signal molecules". The experiment was repeated twice in a similar manner to Fig. 1. Lines presented are the average of two experiments redrawn by a computer.

Changes in lipid composition and quantity of inoculated cowpea calli

Inoculation of cowpea calli did not change the total lipid, neutral lipid, or glycolipid contents of the membrane. However, it significantly changed the phospholipid content of inoculated calli membranes (Table 1). The lipid content analysis of *A. brasilense* Cd cells was very low relative to the calli lipids and had no quantitative effect on the lipid content of inoculated calli (Table 1). The number of *A. brasilense* Cd colonizing calli was 1-5x10^6 cfu g callus^{-1}.

Table 1. Lipid content of cowpea calli inoculated with *A. brasilense* Cd and of *A. brasilense* Cd cells

	mg g (dry weight)$^{-1}$	
	Non-inoculated	Inoculated
Cowpea calli		
total lipids	16.7 a	18.7 a
neutral lipids	5.8 a	7.7 a
glycolipids	1.4 a	1.5 a
phospholipids	2.8 a	3.5 b
	*m*g/10^8 cfu	
Bacterial cells		
total lipids	19.7	
neutral lipids	6.47	
glycolipids	6.3	
phospholipids	6.9	

Numbers (in each lipid type separately) followed by a different letter differ significantly at $P \leq 0.05$ in Student's t-test.

4. Discussion

To clarify the role of *Azospirillum*, a novel approach is required since previous studies have inadequately explained *how* this bacteria affects plant growth (Bashan and Levanony, 1990). Such an approach should include a very sensitive plant part which might recognize the bacteria and be capable of transmitting bacterial signal(s) to the inner plant cell. Accumulated evidence suggests that plant membranes may act as a major component in *Azospirillum* interactions with plants. Our previous studies have shown that inoculation by *A. brasilense* increased the natural proton efflux of monocotyledonous wheat plants (Bashan, 1990; Bashan and Levanony, 1989, 1991; Bashan et al., 1989). The current study extends the range of influence of *Azospirillum* to dicotyledonous plants as well. Proton efflux from cowpea and soybean roots was significantly and positively enhanced by inoculation.

In addition to changes in the proton efflux of inoculated roots, the membrane potential of the roots was also affected. It has been recently shown that inoculation of soybean seedlings with several *Azospirillum* strains significantly reduced the membrane potential of roots cells, making it less negative. This effect prevailed particularly in cells of the elongation zone (Bashan, 1991), the preferred colonization site for *Azospirillum* (Bashan et al., 1986). This activity was attributed to an unidentified bacterial signal(s) (Bashan and Levanony, 1991). The present study adds evidence that (1) this bacterial signal is capable of enhancing proton efflux and changing the membrane potential of soybean

root cells, (2) this signal is a direct result of the *Azospirillum*-plant interaction; it was not produced in bacterial culture or by non-inoculated plants, and (3) the signal is of small molecular weight. The chemical nature of this molecule remains unknown, although involvement of siderophore should be considered (Loper and Buyer, 1991) since *Azospirillum* is known to increase iron uptake by plants (Barton et al., 1986; Bashan et al., 1990).

A previous study on proton efflux of intact wheat plants has shown that the environmental factors governing this activity are the nitrogen source, and to a lesser extent, light and ventilation (Bashan and Levanony, 1989). In dicotyledonous plants, it is well documented that ferrous ions deficiency played the major role (Römheld et al., 1984). Our results with cowpea and soybean plants showed that in the case of inoculation with *Azospirillum*, the major factor governing proton efflux is the nitrogen source and not the ferrous ions. It is still unknown whether inoculated plants efflux more protons because they grow faster.

In addition to its physiological activities on root membranes, this study provides preliminary evidence that the phospholipid content was changed in cowpea calli as a result of inoculation. The changes in the phospholipids have a direct relationship to ion transport in cowpea cells in vitro (Vazquez-Duhalt et al., 1991).

In conclusion, we propose that membranes play a role in the interaction of *Azospirillum* with plant roots and can serve as a sensitive indicator of physiological changes occurring during the plant-bacteria interaction.

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