

Alterations in membrane potential and in proton efflux in plant roots induced by *Azospirillum brasilense*

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Abstract

Inoculation of soybean seedlings with *Azospirillum brasilense* Cd significantly reduced the membrane potential in every root part and was being maximal in the root elongation zone. Monitoring the proton efflux pattern of inoculated wheat roots by several *A. brasilense* strains and by *Pseudomonas* sp. for prolonged periods (up to 200 h) revealed a change from the bimodal pattern of proton efflux of non inoculated roots. This change was not related to root colonization ability but to bacterial capacity to induce changes in root surface area. Continuous perfusion of the plant nutrient solution with a fresh solution (from inoculation time), eliminated the enhancing effect of inoculation on proton efflux. We propose that *A. brasilense* inoculation influences membrane activity and subsequently proton efflux in roots, probably through the release of an as yet unidentified bacterial signal.

Introduction

Changes in plant rhizosphere pH which result from an imbalance of ion uptake can be detected in the rhizosphere of several dicotyledonous plants. Proton extrusion through membranes of root cells, which result in acidification of the rhizosphere, is suggested as a major mechanism in mineral immobilization in plants (Spanswick, 1981).

The mode of action of the beneficial rhizosphere bacteria of the genus *Azospirillum* has been under a continuous debate for more than a decade (Bashan and Levanony, 1990). Among the several mechanisms proposed so far, increased mineral uptake by the plant as a result of inoculation has been suggested to play an essential role (Bashan et al., 1990; Murty and Ladha, 1988). However, no attempt has been made to relate mineral uptake to root membrane activity.

The aim of the present report is to demonstrate that inoculation with *A. brasilense* can affect membrane potential of root cells and release protons from these roots.

Material and methods

Bacterial strains and wheat cultivar, plants and bacteria growth conditions, hydroponic systems, nutrient solution for plants, bacterial inoculation, bacterial counts on roots, proton efflux and root surface area measurements, experimental design and statistical analysis

These were done as previously described (Bashan, 1990; Bashan and Levanony, 1989; Bashan et al., 1989). Soybean seedlings (*Glycine max*, cv. Pella) were treated similarly to wheat seedlings.

Electrophysiological measurements

Intact soybean seedlings having a single root (63 h-old) were sampled and mounted horizontally on a plexiglass holder and washed for 3 h in an aerated solution composed of 1 mM KCl, 1 mM $\text{Ca}(\text{NO}_3)_2$, 0.25 mM MgSO_4 , and 66 mM NaH_2PO_4 , final pH 5.7 (1X) (Érsek et al., 1986). This rinsing was essential to equilibrate the cells in the perfusion medium.

Microcapillaries with glass micro-fibers (WP Instruments) were pulled to micro-electrodes using a vertical electrode puller. Micro-electrodes having a tip diameter of $0.6 \pm 2 \mu\text{m}$, tip potential of -2 to -16 mV and tip resistance of 5 to 14 M Ω were used. Micro-electrodes were filled with 3 M KCl, eliminating 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 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 with a micromanipulator into the selected root site, 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 1 X medium at a flow rate of 8 mL min^{-1} (Érsek et al., 1986; Findlay and Hope, 1976).

Continuous perfusion of plant nutrient solution

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 μm filter. This resulted in the bacteria and roots being continuously washed, but the bacteria were not removed from the root vicinity. This procedure allows replacement of a solution volume equivalent to the testing solution every 5 min. The replaced solution was collected, its volume was reduced by a roto-evaporation at $38 \pm 2^\circ\text{C}$ to its original volume, and the amount of released protons was determined.

Results*The effect of inoculation by *A. brasilense* on the membrane potential of soybean cells in different root parts*

Non-inoculated soybean root parts exhibited similar E_m values regardless of the root parts and within the range of $-146 \pm 6 \text{ mV}$. Inoculation of roots with *A. brasilense* Cd significantly reduced the E_m in every root part. The differences in E_m reduction significantly varied between root parts, being maximal in the root elongation zone (Fig. 1).

Changes in nutrient solution pH of wheat plants during prolonged periods by several rhizosphere bacteria

Changes in pH of the nutrient solution were monitored continuously for 200 h. During this period a bimodal proton efflux activity was observed in non inoculated plants: two periods of proton efflux and two periods when an alkaline compounds) was released. Inoculation of seedlings with *A. brasilense* Cd eliminated the fluctuation effect. Once the solution reached a pH of

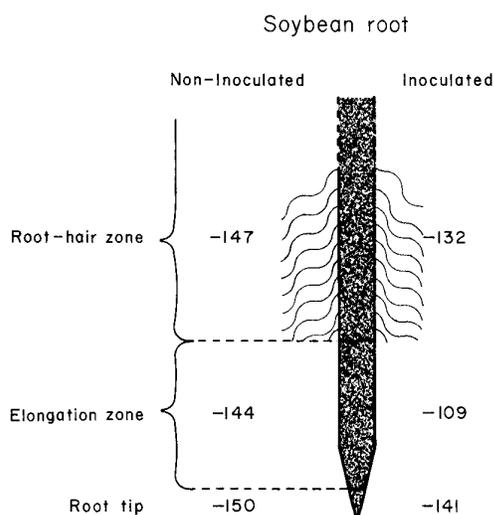


Fig. 1 Membrane potential values (mV) for soybean cells at different sites of the root 16 h after inoculation with *A. brasilense* Cd.

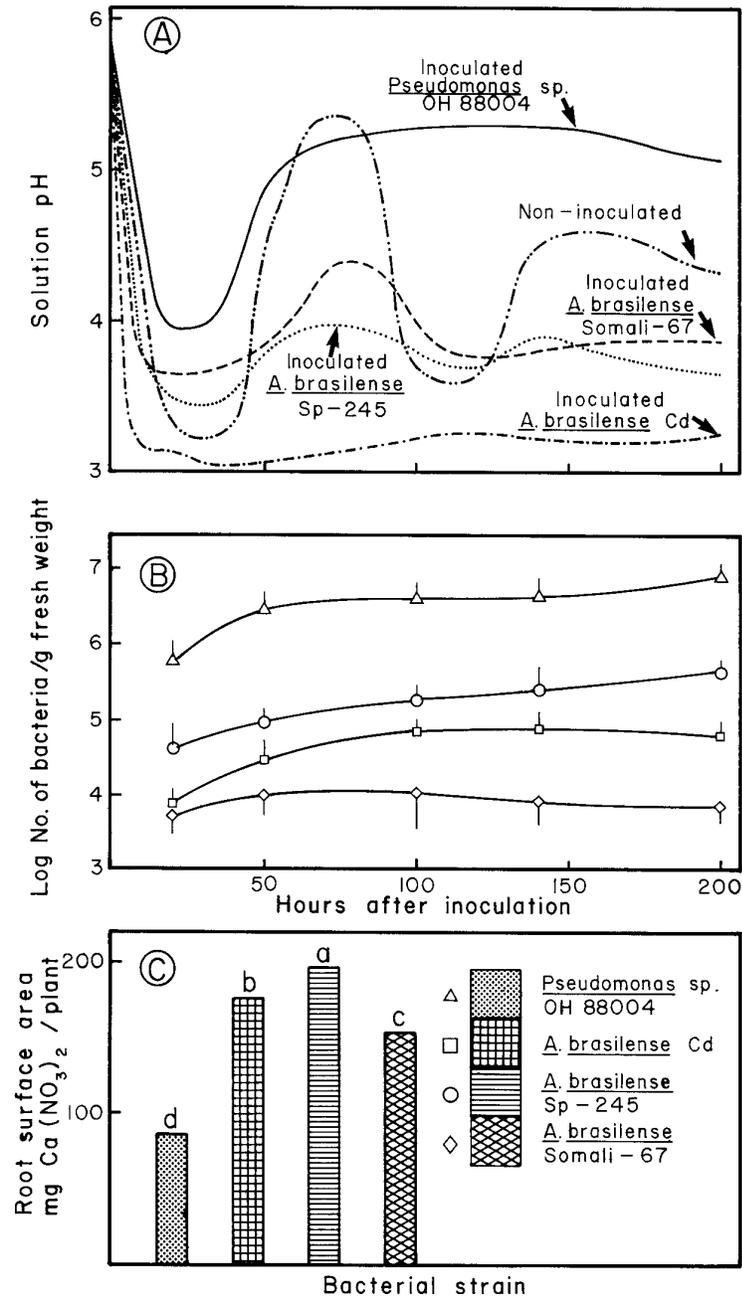


Fig. 2. Changes in nutrient solution pH (A) and in root colonization (B) caused by inoculation of wheat seedlings with several rhizosphere bacteria. All graphs were recorded automatically and drawn on a paper moving at a rate of 10 mm/h. (C) Changes in root surface area induced by inoculation with three rhizosphere bacteria.

3.1 ± 0.1 units, it remained at this low level throughout the entire period. Inoculation of wheat seedlings with *A. brasilense* strains Sp-245 and Somali-67 produced an intermediate result: the bimodal pattern of non inoculated plants was retained but at lower magnitudes. Inoculation with *Pseudomonas* sp. yielded a different pattern. After the initial pH decrease (up to 20 h from inoculation), the pH increased and remained at its highest level throughout the duration of the experiment (Fig. 2A).

Fluctuations in proton efflux were not related to colonization levels by the rhizosphere bacteria evaluated. All strains reached their maximal colonization level 50 h after inoculation. The best root colonizer (*Pseudomonas* sp.) produced the smallest effect. *A. brasilense* Cd which induced the most marked effects was a moderate colonizer (Fig. 2B).

Effect of inoculation, expressed as a change induced in root surface area, was not related to colonization levels. It was related to changes in proton efflux only in *A. brasilense* strains. The

most marked effect on root surface area was induced by *A. brasilense* Sp-245, which affects proton efflux only moderately (Fig. 2C).

Effect of continuous perfusion of the nutrient solution surrounding roots of inoculated wheat plants on the root proton efflux

Inoculation with *A. brasilense* significantly increased proton extrusion over non-inoculated roots. Continuous perfusion of the plant nutrient solution with fresh solution did not affect proton efflux activity of non inoculated roots and the results resembled proton efflux in the still solution. When the nutrient solution of inoculated plants was continuously perfused (from inoculation time), the enhancing effect of inoculation on proton efflux was eliminated (Fig. 3A). However, the number of *A. brasilense* cells colonizing the wheat roots under these conditions was not different from the colonization numbers of roots in still nutrient solution (Fig. 3B, also compare to Fig. 2B).

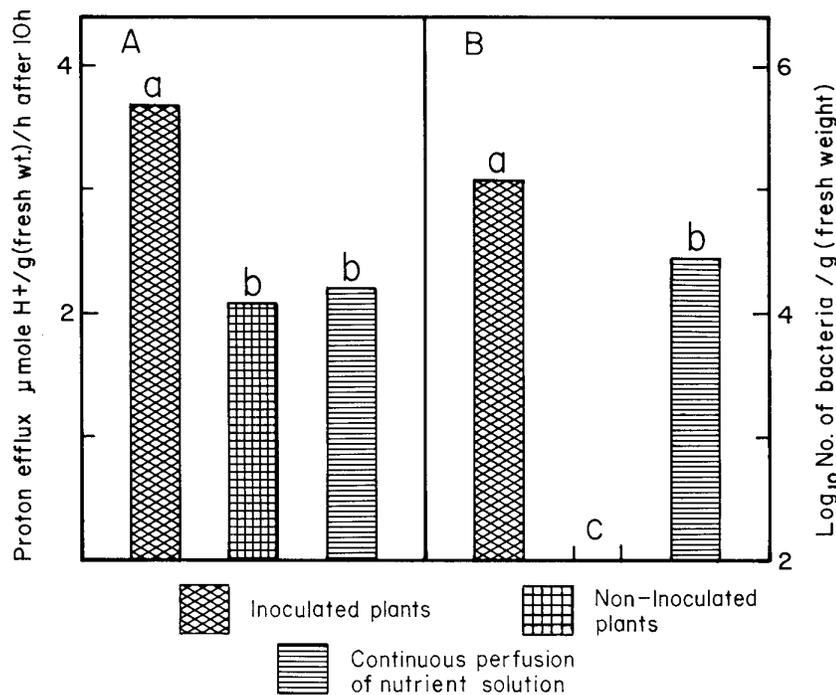


Fig. 3. Effect of continuous perfusion of the plant nutrient solution with fresh solution on: (A) proton efflux of wheat roots, and (B) the number of *A. brasilense* developed under these conditions.

Discussion

The *Azospirillum* mode of enhancing plant growth is an open question. One of the mechanisms suggest thus far (Murty and Ladha, 1988), but not fully confirmed (Bashan et al., 1990), is enhancing the mineral uptake of plants induced by the inoculated bacteria. Changes in mineral uptake may be directly linked to root membrane activity and to its direct consequence, proton efflux from the roots. Significant changes in proton efflux activity of inoculated roots were recently demonstrated (Bashan, 1990; Bashan et al., 1989). This report adds evidence that concomitantly with proton efflux changes induced in the root by *Azospirillum* inoculation, membrane potential is also changed.

A. brasilense is known to have a preference to colonize the elongation zone of several plant species despite its ability to colonize every part of the root system. This report provides evidence that this preference affects membrane activity as well. Although changes in membrane potential were recorded in every part of the inoculated root, the greatest change was detected in the elongation zone of soybean roots.

The presence of *A. brasilense* in roots increased proton efflux. However, the inoculation did not increase the total capacity of root proton efflux. On the other hand, it did alter proton efflux pattern of non-inoculated roots.

Ultrastructural studies of the interaction between *A. brasilense* and root cells revealed that plant cell-walls prevent direct physical contact between the bacterium cell and the plasma membrane responsible for proton efflux (Levanony et al., 1989; Whallon et al., 1985). Thus, to alter membrane activity, the bacteria should release a diffusible signal(s) that has the ability to cross the plant cell-wall and be recognized by the membrane. This report provides preliminary evidence that *A. brasilense* may release such a signal(s). When the plant nutrient solution was continuously replaced by a fresh solution, even the presence of optimal bacterial numbers on the root surface did not enhance proton efflux.

In conclusion, this report shows that *A. brasilense* has the ability to reduce the membrane potential of inoculated roots and in particular of cells in the root elongation zone. Several strains of *A. brasilense* are capable of chang-

ing the pattern of proton efflux of wheat roots. The bacteria probably affect cell membranes through a release of bacterial signal(s).

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