Azospirillum IV

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INTERACTION BETWEEN AZOSPIRILLUM BRASILENSE CD AND WHEAT ROOT CELLS DURING EARLY STAGES OF ROOT COLONIZATION

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Summary: Following inoculation of wheat seedlings by A. brasilense Cd, the bacteria were strongly adsorbed to soil particles, but could migrate in the soil towards the plants. Upon reaching the wheat roots, the bacteria formed relatively small aggregates on the root surface, and a larger internal population within the cortex; no bacteria were found in the vascular system. Several bacteria penetrated into young live root cells, but most were free in the intercellular spaces or bound to cortical and epidermal cell walls by an electron dense material. The bacteria induced an increase in the rate of proton extrusion of the roots as well as an increase in cell division of the root tip.

Introduction

Inoculation of plants with bacteria of the genus Azospirillum has been tested world-wide in view of their potential contribution to plant productivity (1,2). This practice suffers from several disadvantages, of which the most notable ones are the inconsistent and unpredictable yield results, i.e., significant increases in yield besides negligible or even reduction in yield (3), and a high percentage of roots which fail to be colonized by the applied bacteria (4). Several factors, including the limited migration of the bacteria in the soil, adsorption of bacteria to soil particles and competition with the natural rhizosphere bacteria for nutrients that leak from the roots decrease the size of Azospirillum population and in turn their potential contribution to plant growth (4,5). The obtained erratic responses to inoculation led Schank and Smith (3) to assume that the success of Azospirillum inoculation was short-lived and this technology should not yet be transferred to farmers. However, more positive data was accumulated since (4), and a considerable interest in Azospirillum still persists world-wide, despite its controversial nature.

At least three types of factors, different in nature, participate in the plant-bacteria association, (i) soil factors, including physical, chemical and biological variables, (ii) bacterial factors, including metabolism and survival of the applied bacteria as well as bacterial factors that affect plant growth, and (iii) plant factors including the capability to respond to the metabolic activities of the bacteria. All these factors operate simultaneously from time of inoculation - usually performed at sowing or at the seedling stage (6). Therefore, inoculating

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plants with Azospirillum on a large experimental scale should not be recommended before these factors are elucidated.

Since we assumed that the initial stages in the association between the two organisms are crucial for the success of inoculation, we focused on these stages and attempted to identify factors that hinder this association. Our model for this study consisted of the wheat plant as a host and A. brasilense Cd as the inoculum. This bacterial strain was selected because it has been relatively successful in Israel, U.S.A. as well as in other countries (7,8) and because it is one of the most common reference strains in the world.

Adsorption to soil particles

Adsorption of A. brasilense Cd to soil was estimated in γ-irradiated soil columns filled with various types of soil, after washings with sterile tap water. Enumeration of bacteria in the soil and in the eluate was done as previously described (9,10). Live and dead cells of A. brasilense Cd strongly adsorbed to light and heavy soil and only slightly to quartz sand. An increase in bacterial adsorption was obtained by increasing soil content with clays and organic matter, by a decrease in soil pH or by flooding the soil with water, whereas presence of bacterial attractants, an increase in soil pH and drying the soil decreased bacterial adsorption. Intensive washings of the soil with water did not detach the bacteria from soil particles but only from sand particles, which acted as a bacterial filter. Overwashing the soil recovered relatively few bacteria, whereas most of the bacterial population in sand was removed. Bacteria were adsorbed to the upper layer of the soil profile but infiltrated deeper if the soil was very dry.

We propose that most of the population of A. brasilense Cd is adsorbed to soil particles immediately after inoculation and does not penetrate to deeper soil layers even after intensive water application. Bacterial adsorption counteracted bacterial motility. Factors affecting motility are described below.

Self motility and transport in the soil

A. brasilense Cd is known to be motile towards several synthetic attractants (11,12). We measured bacterial self motility in the soil and bacterial transfer in the field by methods described elsewhere (13,14). The main factor affecting motility towards germinating seedlings was soil moisture. The highest migration rate was obtained in brown-red degrading sandy soil at near field capacity (16% v/w water) and above. At 20% moisture, this soil tended to become flooded, facilitating motility of bacteria in the free water films even in the absence of seedlings (Fig. 1a). Of secondary importance were the soil type and the duration of plant growth prior to bacterial application: migration decreased with increasing soil density and increased when plants were grown in the soil several days (1-3 d) before inoculation. Migration was initiated following a lag period of 24-36h, and was characterized by a bacterial band migrating through the soil towards the target roots.

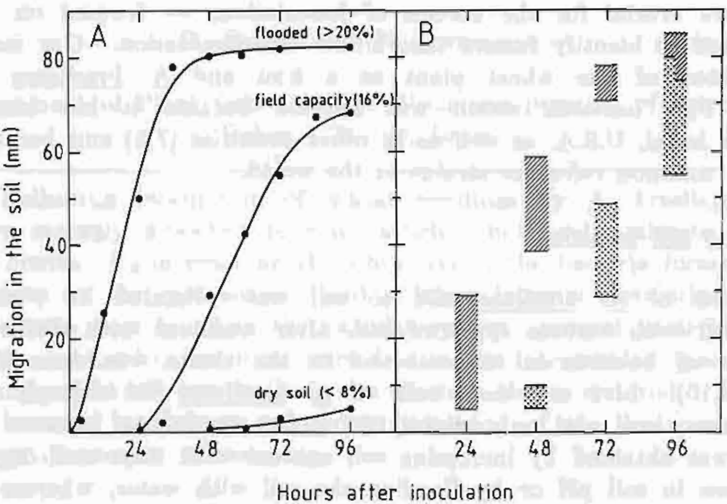

Fig. 1: Migration of A. brasilense Cd in the soil. A - in dry soil; soil in field capacity; and flooded soil. B - ▨ , Migration towards 11 wheat cultivars and , ▦ towards glycine and aspartic acid. Each column represents the maximal and minimal migration at the indicated hour after inoculation.

Eleven common and hard wheat cultivars were found to stimulate significantly bacterial migration. A similar effect was obtained by several bacterial attractants such as glycine and aspartic acid, known to comprise part of the grasses exudates, when encapsulated into solid alginate beads (15). These attractants, however, did not differ in the intensity of their effect (Fig. 1b). Therefore, we propose that A. brasilense Cd is stimulated to migrate non-specifically towards growing wheat plants.

Horizontal transport of A. brasilense Cd in the soil and its vertical transport along plant rhizosphere were measured. No transport was detected in the absence of living plants. Under controlled conditions the bacteria migrated horizontally at least 30 cm from the inoculation site towards the growing plant. Once the first root system was colonized, all the neighboring plants became colonized. Horizontal transport under field conditions was at least 160 cm, and depended on the presence of live plant roots. Weeds such as wild oat, wild barley, Phalaris paradoxa L., P. brachystachys Lk, Malva aegyptia L., Notobasis syriaca L. Cass.

and Silybum marianum L., that grew in the wheat field served as bacterial carriers. Vertical transport in soil columns under controlled environment, in the presence of a live plant was up to 40 cm. Under field conditions, bacteria were detected as deep as 50 cm, within the root systems of the plants. During the winter season A. brasilense Cd was found mostly in young roots at a depth of 20-50 cm and near soil surface. Profiles of depth distribution of A. brasilense Cd carried out at four different locations in Israel showed a non-homogeneous colonization pattern within the same root system or between adjacent plants.

We propose that A. brasilense Cd is transported in the soil horizontally and vertically and that these transports depend mainly on the presence of plants.

Adsorption on the root surface and on root hairs

Adsorption of Azospirillum cells to the root surfaces or to root hairs of several plant species were reported (16,17,18,19). When A. brasilense Cd was inoculated onto wheat roots or reached them by its own motility, the bacteria multiplied and formed an aggregate form of colonization. These bacterial cells were attached to each other and to the root surface by a fibrillar material (Fig. 2a, b). Slight rinsing of the roots (20) released most of this population. Disinfection of highly colonized roots eliminated most of the bacteria, whereas inoculating live or dead roots with γ-irradiated killed bacteria resulted in very small adsorption. However, killing roots before inoculation had no effect on bacterial adsorption to the roots. γ-irradiation following an intensive root colonization resulted in elimination of most of the dead bacterial cells from the root surfaces.

Microscopic inspection of external wheat roots surfaces revealed bacteria along the roots - mostly in the elongation zone. However, the root hairs were not heavily colonized. Small aggregates as well as single bacteria were found on root hairs. Few bacteria penetrated the root hairs, as observed by transmission electron microscopy. Usually, there were less bacteria on root hairs than on the root surface in the same region.

We proposed that active metabolism of A. brasilense Cd is essential for root colonization. Since this species is able to colonize and adsorb to dead roots, the active metabolism of the root probably does not play an important role in this association. Thus, the adsorption of A. brasilense Cd to the surfaces of wheat roots can be defined as a weak, metabolism-dependent process.

Internal root colonization

There were indications that Azospirillum species could penetrate the roots and be established in the intercellular spaces of their host plant (20,21). However, there is no solid evidence that the observed bacteria were the inoculated ones.

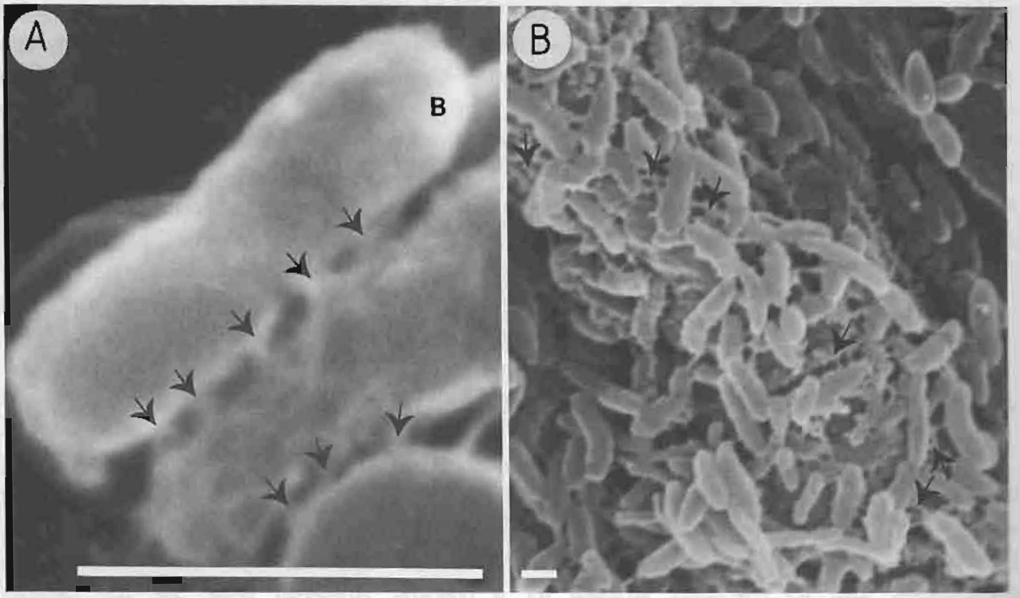

Fig. 2: Scanning electron microscopy of fibrillar material connections (A) between A. brasilense Cd cells, and (B) to the root surfaces. (Bars represent 1 μ)

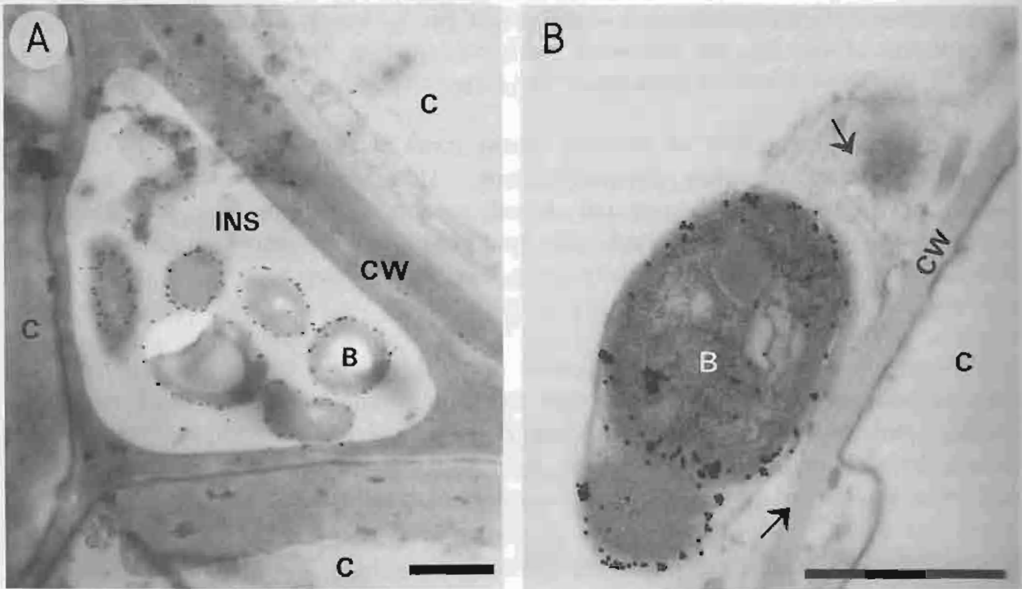

Fig. 3: (A) Transmission electron micrographs of specific identification of A. brasilense Cd cells within the intercellular spaces by coloidal-gold labeling.
(B) Binding of A. brasilense Cd cells to cortex intercellular spaces by an electron dense material (arrows). (Bars represent 1 μ). Abbreviations: B - bacteria; C - cortex cells; CW - plant cell wall; INS - inter cellular spaces.

When an external bacterial population was established on the root surfaces an internal bacterial population developed too. Washings and strong agitation did not remove the internal population. Moreover, counts made by the specific enzyme-linked immunosorbent assay (ELISA), especially developed for the detection and enumeration of this strain (10), revealed that the internal bacterial population comprised the majority of the bacteria that colonized the roots. Cd population present on the roots, the internal bacteria comprised the majority of that population.

The population of A. brasilense Cd within wheat roots was detected by light and transmission electron microscopy, using the coloidal-gold immunocytochemical technique, which is based on specific recognition by antibodies (10) and labeling the cells with gold particles as described by Roth (22). The external and the internal root population of A. brasilense Cd was as intensively gold-labeled as its liquid culture, with a very low background. Other bacterial species on root surfaces or in intercellular spaces were not labeled at all. Cells of A. brasilense Cd were distributed along root surfaces, while the internal population was restricted to the elongation and to the root-hair zones. A. brasilense Cd was located in the cortex intercellular spaces, as deep as the endodermis, but did not penetrate either the endodermis or the vascular system (Fig. 3a). These tissues in corn plants were reported to be colonized by this genus (20). The bacteria were bound to cell walls in the cortex intercellular spaces as well as to epidermal cells by an electron dense material (Fig. 3b). Several cells of A. brasilense Cd were found within young, live root cells without any apparent damage to the plant cells.

We suggest that A. brasilense Cd forms an unharmful inter- and intra-cellular association with wheat roots, in addition to its root surface colonization.

Other effects of A. brasilense Cd inoculation on wheat roots

Several mechanisms and phenomena have been proposed to characterize inoculation by Azospirillum i.e., hormonal activity, nitrogen fixation, enhancement and improvement in mineral and water uptake, promotion of root hair development and root branching, alterations of cell arrangement of the cortex, increase in dry matter and in mineral accumulation in leaves and improvement of yields of cereals and forage grasses (1,2,5,17).

We present here other effects of A. brasilense Cd on wheat roots: (i) increase in root membrane excitation causing an increase in proton extrusion from the root to the rhizosphere (ii) enhancement of cell division in root tips.

We identified a physiological mechanism of proton pumping in wheat roots. The effect was measured as a decrease in pH at the root surrounding, using a pH

Fig. 4: Acidification of agar medium by wheat roots inoculated with A. brasilense Cd. Dark color represents pH 6.8; white color represents pH 3.7-4.5.

electrode, and visualized by the agar-indicator technique (23). Inoculation with A. brasilense Cd caused a measurable increase in the proton leakage from wheat roots (Fig. 4). Therefore, we concluded tht inoculation affects cell membranes, even in the absence of attachment of bacteria to plant membranes. This effect might be related to the known changes in mineral uptake in the plant after inoculation (24).

We determined the mitotic index (no. of cells at mitosis/total number of cells) of inoculated root tips (25), 72h after germination and inoculation. Analyses were carried out on several thousands cells in different roots. Significant increase in the mitotic index was observed in roots inoculated at 10^6 colony-forming unit/ml, which is known to be an optimal level of inoculation (6).

Conclusions

The biophysical and ecological features of A. brasilense demonstrated here, combined with the considerable progress made world-wide during the past decade, are not sufficient to account for the inconsistency in yield response obtained by many research groups. Further studies on these aspects are essential before inoculation technology can be released for commercial use.

Acknowledgements

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