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Immunocytochemical characterisation of endophytic bacteria *Azospirillum brasilense, Herbaspirillum seropedicae, Burkholderia ambifaria* and *Gluconacetobacter diazotrophicus*

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Abstract - In the present work an immunocytochemical characterisation of four endophytic bacterial species has been made by using polyclonal antiserum produced against each of the four bacterial strains previously heated at 60 °C. The aim of this research is to identify common elements among bacteria associated with their endophytic behaviour. Analysis of extracts of each strain by immunoblotting and ELISA confirmed the presence of proteins from different bacterial strains made up of common epitopes. However, antisera produced against *Herbaspirillum seropedicae* and *Burkholderia ambifaria* show a high number of bands recognised on each extracts, while antisera against *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus* show a low number of bands recognised on each extract. Immunogold labelling showed that epitopes are located both on the cell wall and in the cytoplasm; most likely they could be precursor cell wall proteins synthesized inside the cytoplasm and subsequently transported onto cell wall. Finally, the common bands among bacterial strains revealed by immunoblotting could play a role as active hydrolases involved in host tissue penetration.

Key words: endophytic bacteria, immunogold labelling, Western Blotting, ELISA.

INTRODUCTION

Soil microorganisms have an important influence on soil fertility and plan health (Gianinazzi and Schüepp, 1994). During its growth through the soil, the root releases organic material which provides the driving force for the development of active microbial populations around the root, known as the rhizosphere effect (Whipps, 1990; Morgan and Whipps, 2001). Root components and microbial populations can establish symbiotic interactions such as mutualism, parasitism, or commensalisms. Bacterial and fungal populations are commonly present in the rhizosphere and may have all the types of interactions with the root areas. However, since plant roots in natural ecosystems are commonly mycorrhizal, the concept of the 'rhizosphere' has been widened to include the fungal component of the symbiosis, resulting in the term 'mycorrhizosphere' (Rambelli, 1973; Johansson *et al.*, 2004). The loss of plant productivity due to plant pathogens, has led to formulating strategies that combat different pathogenic species, allowing a qualitative and quantitative improvement in crops. Significantly, disease suppression can be achieved by manipulation of the physicochemical and microbiological environment through management practices such as the use of soil amendments, crop rotations, use of fumigants or soil solarization. However, at present, the main interest concerns the application of specific biocontrol agents to prevent diseases in seeds and roots (Whipps, 2001; Whipps and Lumsden, 2001). Whereas legumes form typical symbiosis inside root nodules with N_2-fixing rhizobia in a nutrient-rich, oxygen-controlled microenvironment, grasses do not form specialised symbiotic structures, such as root nodules. However, diazotrophic bacteria have been found within tissues of some grasses. Indeed, many studies have demonstrated that most of rhizospheric bacterial species are present inside root structure (Grilli Caiola *et al*., 2004), suggesting that many soil bacteria have plant-invasive properties (Reinhold-Hurek and Hurek, 1998). Several taxa of N_2-fixing bacteria can penetrate plants through natural openings, such as stoma or hydathodes, through artificial or natural wounds, through the root emergency points, as well as using vegetative propagation techniques. They can also penetrate intact tissues (Sprent and James, 1995).

In this work four diazotrophic species, *Azospirillum brasilense, Herbaspirillum seropedicae, Gluconacetobacter diazotrophicus* and *Burkholderia ambifaria*, have been studied. These bacteria represent the main endophytes of numerous Angiospermae, many of which have an agricultural relevance such as *Lycopersicon esculentum.* These endophytes are well-known for their role in promoting plant growth and as biocontrol agents against many plant pathogens. Their possible adoption could reduce the use of pesticides and chemical fertilisers (Bar and Okon, 1992; Caballero-Mellado *et al*., 1992; Urquiaga *et al*., 1992; Fuentes-Ramirez *et al*., 1993; Parke and Gurian-Sherman, 2001).

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The literature reports numerous works of ecological interest (Johansson *et al*., 2004) and others of biochemical characterisation (Grilli Caiola *et al*., 2004; Gamalero *et al*., 2005), however, for two or more of the above-mentioned species, we lack comparative immunocytochemical studies. The present research proposes the use of immunocytochemistry as method to identify and localise common proteins of endophytic behaviour. This application at the ultrastructural level enables a much more precise analysis of endobacterial protein expression than using conventional techniques (i.e. bioassay, fluorescence optical microscopy).

MATERIALS AND METHODS

Bacterial strains and media used. *Azospirillum brasilense* strain Cd, isolated from *Cynodon dactylon* (Eskew *et al*., 1977) was originally supplied by Yaacov Okon, Hebrew University Rehovot, Israel. It was grown on a modified Okon medium (OK) (Martinez-Dretz *et al*., 1984), solidified with 1.5% agar (Oxoid).

Gluconacetobacter diazotrophicus strain PAL 5 (Yamada *et al*., 1997) isolated from *Saccharum officinarum* was grown on a LGI-P solid medium (Cavalcante and Döbereiner, 1988).

Burkholderia ambifaria strain PHP7, isolated from *Zea mays* roots (Coenye *et al*., 2001), was grown on a PCAT solid medium (Burbage and Sasser, 1982).

Herbaspirillum seropedicae strain Z67 (Baldani *et al*., 1986) isolated from *Oryza sativa* was grown on a J-NFb solid medium (Olivares *et al*., 1996).

Preparation of bacterial extracts. Protein extraction from cells of each of the four bacterial strains was carried out by sonication with SONIPREP 150 (Bio Clinical Service). Bacteria in the stationary phase (one day-old) were withdrawn from plate culture on media above described and resuspended in 0.05% Phosphate-buffered saline (PBS)-Tween, then centrifuged at 3000 rpm for 5 min and resuspended twice in PBS. The pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, 2 mM EDTA, 100 mg/ml lysozyme, 1% Tween, then incubated at 30 °C for 15 min. Sonication was performed in an ice-bath according to a pattern of 6 sonication cycles of 30 s each, alternated to gaps of 60 s each. The sonicated solution was centrifuged at 12000 rpm for 15 min at room temperature. In the recovered supernatant, proteins were measured according to Bradford (1976).

Preparation of antisera. Each bacterial strain was cultured in its appropriate medium above-mentioned to the late stationary phase (one day-old). The cultures were centrifuged, the cells were resuspended in PBS and the resulting suspensions were heated at 60 °C for 60 min. Polyclonal antibodies against the resulting heated-inactivated bacterial cells were raised in rabbits as described by Leonardi *et al*., (1993). Four rabbits were injected intramuscular with 2 ml of a 1:1 mixture of heated bacteria and incomplete Freund adjuvant. Two booster injections of heated bacteria suspended in PBS were administered every 2 weeks thereafter. The final bleed for antisera was carried out 7 days after the last injections. The blood was allowed to clot and the sera, obtained by centrifugation at 6000 *x g* for 20 min at 4 °C, were used for the immunological assays in dilution 1:100. As a control for each antibody, preimmune rabbit sera were utilised.

ELISA. Bacterial proteins (50 µg) were adsorbed in 96-well microtiter plate: the plate was washed with PBS-Tween, preincubated with Bovine Serum Albumin (BSA) 5% in PBS-Tween for 1 h, then incubated with the primary antibody diluted 1:1000 and 1:3000 in BSA 1% in PBS-Tween 0,05% for 1 h. After washing, the plate was incubated with GAR-HRP 1:5000 in 1% BSA in PBS-Tween. The peroxidase activity of the secondary antibodies was visualised with o-fenylendiamine. Optical density was measured with ELISA reader at γ = 490nm.

SDS-PAGE and immunoblotting. Aliquots containing 20 µg of proteins were diluted 1:2 in the sample buffer. Electrophoretical separation was obtained with a 15% polyacrilamide gel (device Minigel BIORAD) under denaturing conditions, to 70 V in the stacking gel and 200 V in the separating gel for 1 h or until the dye reached the end of the gel (Laemmli, 1970). For each bacterial extract, two gels were simultaneously runned: one was stained with Comassie Blue Brilliant in order to verify the presence of proteins; the other one was transferred on nitrocellulose membrane at 260 mA for 2 h at 4 °C (device Bio-Rad Transblot) for immunoblotting procedures. After the transfer the strips of nitrocellulose were washed in PBS-Tween 0.05%, preincubated at room temperature with BSA 5% for 1 h, then incubated with primary antibody at 4 °C overnight. The next day the strips were incubated for 1 h at room temperature with secondary antibody Goat anti Rabbit conjugated with horseradish-peroxidase (GAR-HRP, Sigma-Aldrich, Italy) 1:4000. Peroxidases were detected using o-fenylendiamine. Controls were carried out omitting primary antibody and using preimmune rabbit serum.

Preparation of bacterial cultures for Transmission Electron Microscopy (TEM). Cultures from each strain in the stationary phase were fixed by incubation overnight at 4 °C in 2.5% glutaraldehyde (v/v) in 0.2 M phosphate buffer (PB). Fixed bacteria were washed three times in 0.2 M PB and were centrifuged at 1600 rpm for 10 min at room temperature. Cells recovered were suspended in 1.5% agar previously warmed to a temperature of 45 °C. After solidification, small agar portions were postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon resin. Thin and ultrathin sections were cut and stained with lead citrate as described by Reynolds (1963).

Bacteria were also stained with phosphotungstic acid (PTA). Therefore, fixed bacteria were washed three times in 0.004% sucrose, collected by centrifugation and resuspended in 0.004% sucrose as described by Forni and Grilli Caiola (1992). A drop of this suspension was applied to a grid, dried and stained for 10 min with 2% PTA, pH 6.5. Grids were washed in double-distilled water, dried and observed in a Zeiss CEM902 electron microscope, operating at 80 kV.

Immunogold labelling. Ultrathin sections were immunogold labelled as described by Canini *et al*. (1992). Sections from each strain were applied to a grid, preincubated in 5% H_2O_2, washed with PBS-Tween, incubated with 5% BSA in PBS-Tween. The primary antibody, diluted 1:600, was applied to the grids, which were then incubated overnight at 4 °C. Grids were washed three times with PBS-Tween and incubated with the secondary antibody, GAR antibody conjugated with 10 or 15 nm gold particles diluted to 1:20. The den-

TABLE 1 – Optical densities after ELISA assays for the four antisera over each bacterium strain. Dilution 1:1000 and 1:3000 were assayed. S.D. values are less than 5%

Antisera	Dilution 1:1000	Dilution 1:3000
anti *Azospirillum* serum		
Gluconacetobacter	0.089	0.022
Herbaspirillum	0.200	0.200
Burkholderia	0.516	0.423
Azospirillum	1.411	0.644
anti *Gluconacetobacter* serum		
Gluconacetobacter	2.857	1.923
Herbaspirillum	0.485	0.314
Burkholderia	1.497	1.005
Azospirillum	0.125	0.137
anti *Burkholderia* serum		
Gluconacetobacter	0.577	0.400
Herbaspirillum	1.750	1.350
Burkholderia	4.533	4.258
Azospirillum	0.293	0.146
anti *Herbaspirillum* serum		
Gluconacetobacter	0.420	0
Herbaspirillum	3.244	3.022
Burkholderia	2.489	2.395
Azospirillum	0.057	0.093

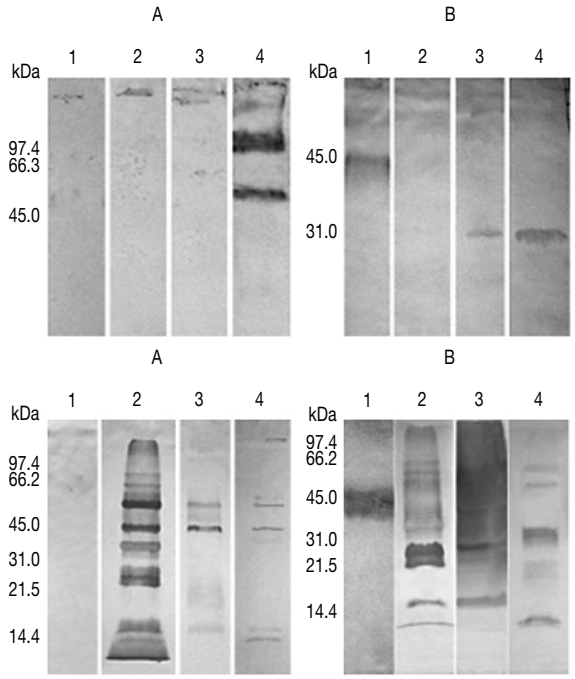

FIG. 1 – Immunoblot results of the four bacterial protein extracts recognised by their specific antisera. A: immunoblot with serum anti *Azospirillum brasilense*, B: immunoblot with serum anti *Gluconacetobacter diazotrophicus*, C: immunoblot with serum anti *Herbaspirillum seropedicae*, D: immunoblot with serum anti *Burkholderia ambifaria*. Lane 1: *Gluconacetobacter diazotrophicus* protein extract, lane 2: *Herbaspirillum seropedicae* protein extract, lane 3: *Burkholderia ambifaria* protein extract, lane 4: *Azospirilllum brasilense* protein extract.

sity of gold particles was estimated based on the analysis of 10-20 micrographs for each sample. The values were represented by the mean ± SD of data and analysed statistically with Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

ELISA

The results of the ELISA test are presented in Table 1. The ELISA assay showed that each bacterial strain is recognised distinctly by the antiserum produced against it self and also, even though in minor measures, by the antisera produced by the other three species as shown in Table 1. Each antiserum shows high reactivity towards its own protein extract. The strain most reactive to the respective antibody is *Azospirillum brasilense*, while *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria* and *Herbaspirillum seropedicae* were recognised not only by their respective antibody, but also by the other antisera.

SDS-PAGE and immunoblotting

In SDS-PAGE and immunoblotting assays, each polyclonal antiserum from the four bacterial strains reacted with proteic extract from bacterial strain originally utilised for antiserum production and with proteic extracts from other bacterial strains (Fig.1).

Western blotting with serum anti *Azospirillum* detected only two bands on its own extract (i.e., 97.4 and 53 kDa). No bands appeared using the other extracts (Fig. 1A).

When we used anti *Gluconacetobacter* antiserum, it recognised its own extract with a band at about 45 kDa and also the *Azospirillum* and *Burkholderia* extract with a band at 31 kDa. No reactivity was detected with *Herbaspirillum* extract (Fig. 1B).

Serum anti *Herbaspirillum* detected on its own extract numerous bands with molecular weight between 21.5 and 66.2 kDa (97, 66, 53, 45, 37, 29. 27, 18 and 14 kDa), four bands on *Azospirillum* extract (53, 45, 18 and 14 kDa), five bands on *Burkholderia* extract (53, 48, 45, 18 and 21.5 kDa). No bands are detected on *Gluconacetobacter* extract (Fig. 1C).

Serum anti *Burkholderia* revealed numerous bands on all extracts except on *Gluconacetobacter* extract one where a large band at 45 kDa was clearly observed (Fig. 1D). A lot of bands are detected on *Herbaspirillum* extract (66, 59, 53, 40, 39, 38, 37, 29, 26 and 18 kDa). A continuous scratch observed between 100 to 21.5 kDa was detected on *Burkholderia*. However, bands were observed at 39, 31 and 18 kDa. Four bands at 53, 37, 21.5 and 14.4 kDa appeared on *Azospirillum* extract.

Morphologic characterisation of bacteria at TEM

Bacteria extracted from their respective cultures, were stained with PTA, which highlighted cell morphology. In the TEM micrographs, *Azospirillum brasilense* showed distinctly gently curved rod form, a polar flagellum and numerous thinner peritrichous flagella (data not shown). *Burkholderia ambifaria* and *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus* bacterium showed a slightly curved rod form and no flagella.

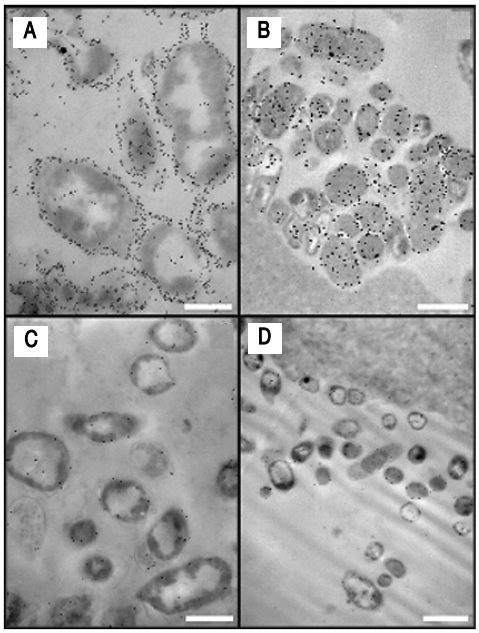

FIG. 2 – Immunogold labelling produced by serum anti *Azospirillum brasilense* on ultrathin sections from each bacterial strain. A: *Azospirillum brasilense*, B: *Burkholderia ambifaria*, C: *Herbaspirillum seropedicae*, D: *Gluconacetobacter diazotrophicus.* Bar= 0.6 mm (A, C), Bar = 1.1 mm (B, D).

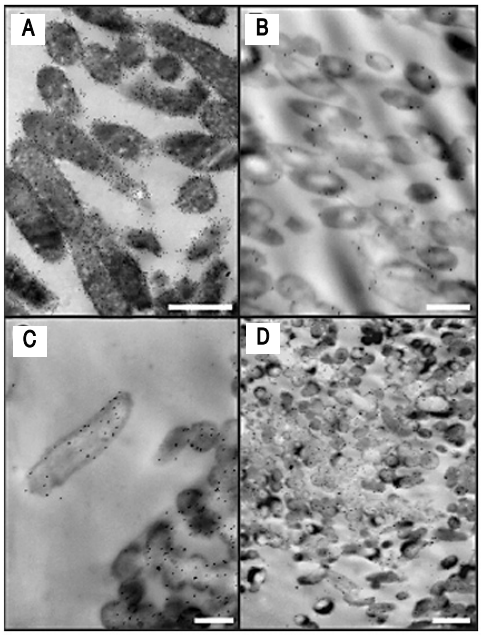

FIG. 3 – Immunogold labelling produced by serum anti *Herbaspirillum seropedicae*. A: *Herbaspirillum seropedicae*, B: *Azospirillum brasilense*, C: *Gluconacetobacter diazotrophicus*, D: *Burkholderia ambifaria*. Bar= 0.6 mm (A, B, C), Bar = 1.1 mm (D).

Immunogold labelling and immunocytochemical affinity valutation among bacterial strains

Immunogold labelling of four antisera was performed on every bacterium (Figs. 2 and 3). Serum anti *Azospirillum brasilense* produced a dense (i.e., 14.6 ± 1 gold particles/μm^2), homogeneously diffused labelling on cell walls from *Azospirillum* (Fig. 2A). Labelling appeared diffused on *Burkholderia* (Fig. 2B), whereas it is very slight (i.e., 2 ± 0.2 gold particles/μm^2) and localised on cell walls of *Herbaspirillum* (Fig. 2C) and *Gluconacetobacter* cells (Fig. 2D). Similarly, serum anti-*Gluconacetobacter* produced an intense immunolabelling (i.e. 20 ± 2 gold particles/μm^2) on its own cell walls, a low labelling (i.e. 1 ± 0.2 gold particles/μm^2) on the cytoplasm and cell walls of *Azospirillum*, *Burkholderi*a and *Herbaspirillum* (data not shown).

Serum anti *Herbaspirillum* produced an intense labelling on its own cell walls and cytoplasm (Fig. 3A); while it produced a low labelling on the cell walls of *Azospirillum* (Fig. 3B). A large number of epitopes from the cell walls and cytoplasm are localised on *Gluconacetobacter* (Fig. 3C) and *Burkholderia* (Fig. 3D). Serum anti *Burkholderia* produced a strong labelling on cell walls and a low cytoplasmic labelling on its own cells, while very low labelling was detected on each of the other three bacterial strains (data not shown).

DISCUSSION

Comparative analysis of ELISA assays revealed that each antiserum showed high reactivity against bacterial strains used for producing polyclonal antiserum. Instead reactivity resulted variable with proteic extracts of other species. Especially *Azospirillum brasilense* possessed an elevated selectivity in comparison to the other three bacterial strains, whose protein extracts are not only recognised each strains by the respective antiserum, also by the antiserum of the other three bacterial strains. An intermediary degree of selectivity was exhibited by *Gluconacetobacter diazotrophicus*, whose antiserum showed reactivity, even though of different degree, to each of the other three extracts. Immunogold labelling showed that each antiserum produced intense labelling against its own bacterial strain. This labelling was clearly located on cell wall and, secondarily, inside the cytoplasm. Use of antisera from different bacterial strains on ultrathin sections produced an antigenic localisation that changed according to the serum and the strain used. Analysis of immunogold labelling data showed that antiserum of *Azospirillum brasilense* primarily located epitopes on the cell wall, while antiserum of *Herbaspirillum* and *Gluconacetobacter* marked the cell wall or produced diffused labelling on sections. Finally, antiserum of *Burkholderia* produced a labelling diffused on cells of the other strains. Our hypothesis is that cytoplasmic or diffused labelling was due to epitopes recognised by antibodies, which are not only cell wall epitopes, but also cytoplasmic precursors of cell wall proteins (Lerouxel *et al*., 2006). After immunoblotting assays, antiserum of *Azospirillum* showed only one band specific for its extract; by contrast, *Burkholderia* antiserum displayed numerous bands on all extracts. *Gluconacetobacter* antiserum showed an intermediate selectivity since it reacted with three proteic extracts out of four, evidencing fewer bands than those evidenced by *Herbaspirillum* antiserum. Further information about the presence of common epitopes was obtained by the analysis of bands produced by Western blotting with the various sera. Such analysis identified: 1) many different bands on proteic extract recognised by different antisera; 2) many common bands recognised on different extracts by the same antiserum. Our results confirmed the presence of proteins from different bacterial strains made up of common epitopes. Moreover, by com-

parison of ELISA and immunoblotting tests, two opposite behaviours are revealed: anti *Burkholderia* and anti *Herbaspirillum* sera showed low selectivity; anti *Azospirillum* and anti *Gluconacetobacter* sera showed high selectivity. This behaviour led us to the formulation of a model whereby rabbit serum recognised a low number of cell wall epitopes from *Azospirillum* and *Gluconacetobacter* bacteria, while the number of epitopes recognised on *Herbaspirillum* and *Burkholderia* is considerably higher. In order to evaluate common epitopes having endophytic role, the following bands should be investigated: the band at 53 kDa detected in *Azospirillum* and *Herbaspirillum* extracts, recognised by anti *Herbaspirillum* and anti *Burkholderia*; the band at 18 kDa detected on the extracts of *Herbaspirillum*, *Burkholderia* and *Azospirillum,* recognised by the same antisera; and finally a band at 45 kDa, present on all extracts and evidenced by different antisera. Such bands deserve further investigation in order to establish their possible role in the penetration of host tissues.

In the literature there are no data about proteic characterisation of cell walls for the species studied in our work, with the exception of *Azospirillum brasilense* (Burdmann *et al*., 2001). Burdmann *et al*. identified a cell wall protein involved in radical adhesion. Cell wall protein characterisations have been made for different species of bacteria hosts of animal organisms (Kontusaari and Forsen, 1987; Patrick *et al*., 1990; Nakamura *et al*., 1993). In fact, Leclerc and Asselin (1989) identified a cell wall hydrolase of 45.7 kDa in cell extracts of *Streptococcus pyogenes.* Although the ecological behaviour of these species is clearly different from those of an endophytic bacterium, it is reasonable to assume that proteins with analogous functions are present in *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter* and *Burkholderia* and that they can have functions correlated to tissue host penetration. We hypothesized that the band at 45 kDa, present on all four extracts, could be investigated for this hydrolasic role. We can suppose that proteins involved in endophyte penetration are numerous and different in different species; it is also possible that antisera used in this work were not able to reveal their presence. An other hypothesis is that the rhizospheric atmosphere, where interactions between endophyte and host are multiple and complex, can induce and/or veiculate signals correlated with the expression of proteins corresponding to the bands detected. Such a process is partially clarified in *Azospirillum brasilense*, in which adhesin-like proteins are involved in adhesion to the host plant (Michiels *et al*., 1991; Croes *et al*., 1993). Recent studies (Burdmann *et al*., 2001) showed the existence of a membrane protein in *Azospirillum brasilense*, MOMP (Major Outer Membranes Protein, 38.5 kDa). This protein link with high affinity different radical extracts from cereals, legumes and tomato (Burdman *et al*., 2000). In our work, *Azospirillum* antiserum on *Azospirillum* extract did not reveal such a band, whereas immunoblotting the serum anti *Burkholderia* did reveal it. A band of analogous molecular weight in extract of *Herbaspirillum seropedicae*, evidenced by using anti *Herbaspirillum* and anti *Burkholderia* sera, could be tested for its adhesion role in *Herbaspirillum seropedicae* by a study of its aminoacidic sequence and comparing this with those in data banks.

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