CELLULAR ASPECTS OF ROOT COLONIZATION BY ANTAGONISTIC BACTERIA AND PHYTOPATHOGENIC FUNGI

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Abstract. In this study, the capacity of root colonization and plant defense activation by some *Pseudomonas aeruginosa* and *Bacillus licheniformis* strains against *Pythium debaryanum* HESSE was evaluated. There were tested a series of experimental variants in which *Cucumis sativus* L (Wisconsin SMR58 cultivar) plantlets were pre-treated with bacterial suspensions of P7, P14, P18 (*P. aeruginosa*) and B40 (*B. licheniformis*) strains, and a series in which pre-bacterized plants were infected with *P. debaryanum* HESSE. The interactions at the site of infection, was observed by light and electron microscopy. The results showed that the bacterial strains colonized intercellular spaces of cucumber root and hypocotyl. The colonization was correlated with defense-associated reactions in cucumber, like formation of cell wall appositions, also known as papillae, and obturation of intercellular areas.

Keywords: antagonist-phytopathogen-plant interaction, root colonization, light and electron microscopy.

Rezumat. Aspecte celulare ale colonizării rădăcinii plantelor cu bacterii antagoniste şi fungi fitopatogeni. În acest studiu, a fost evaluată capacitatea de colonizare a rădăcinii de unele tulpini de *Pseudomonas aeruginosa* şi *Bacillus licheniformis* cu activitate inhibitorie asupra unei game largi de fitopatogeni. Au fost testate o serie de variante experimentale, în care plantule de *Cucumis sativus* L. (soiul Wisconsin SMR58) au fost pre-tratate cu suspensii bacteriene ale P7, P14, P18 (*P. aeruginosa*) şi B40 (*B. licheniformis*) tulpini, precum si o serie în care plantele tratate cu suspensii bacteriene au fost infectate cu *P. debaryanum* HESSE. Interacţiunile de la site-ul de infecţie au fost observate prin microscopie optică şi electronică. Rezultatele au arătat că tulpinile bacteriene au colonizat spa□iile intercelulare ale rădăcinilor şi hipocotilului de castravete. Colonizarea a fost corelată cu reacţii de apărare, cum ar fi formarea de apoziţii la nivelul peretelui celular şi obturarea spaţiilor intercelulare.

Cuvinte cheie: interacţiune pathogen-antagonist-plantă, colonizarea rădăcinii, microscopie optică şi electronică.

INTRODUCTION

Successful control of soil phytopathogens using microorganisms presumes several characteristics of the biocontrol agent, like rhizosphere competence, antagonistic activity, plant growth enhancement and activation of plant defense mechanisms. Root colonization represents the primary step in almost all types of interaction between plants and soil microorganisms. Previous studies have shown a nonuniform distribution of bacteria on the root, in case of *Pseudomonas* the junctions between epidermal root cells, indented parts of the epidermal surface, or sites of root appearance, being the most populated (LUGTENBERG et al., 2001). Among the traits involved in efficient root colonization, bacterial motility and attachment to the roots are definitory (ZHENG & SINCLAIR, 2000; LUGTENBERG & BLOEMBERG, 2004). Thus, *Pseudomonas* is considered one of the best root colonizers, being used as a model of root colonizer (LUGTENBERG et al., 2001).

There are various plant responses that appear during plant-microbe interactions, like rapid generation of reactive oxygen species, synthesis of antimicrobial compounds, pathogenesis-related proteins, and phytoalexins, but also structural changes, like reinforcement of the cell walls and formation of cytoplasmic aggregates (SCHMIDT & PANSTRUGA, 2008). Defense mechanisms induced by microorganisms can be observed locally, at the ingression site of microorganism, but also systemically, in untreated plant parts. Locally, defense reactions like strengthening the cell walls through cell wall appositions, occlusion of intercellular spaces or formation of multivesicular bodies were previously described (BENHAMOU et al., 2002; AN et al., 2006). In this study, we have focused on plant cell structural modifications in plant-microbe interaction using light and electron microscopy techniques. Among the bacterial strains used in experiments, pseudomonads are new Romanian isolates from oil - polluted areas (CORNEA et al., 2006).

MATERIAL AND METHODS

Biological material used

The bacterial strains used in this study were *Pseudomonas aeruginosa* (strains P7, P14, P18) and *Bacillus licheniformis* (B40) and were provided by the Faculty of Biotechnology, USAVM, (Bucharest, Romania). As fungal pathogen, we used *Pythium debaryanum*, obtained from the Institute of Plant Protection (Bucharest, Romania). Cucumber plants were obtained by germination of cucumber seeds (Wisconsin SMR58 cultivar).

Plant treatment with antagonistic bacteria and fungal pathogen

Cucumber seeds were sterilized for one hour in 1% sodium hypochlorite solution and then washed five times with sterile distilled water. The bacterial strains were grown for 4 days on CPM medium (1% mannitol, 0,1% casamino acids, 1% peptone, 0,5% calcium chloride, pH 7) at 28°C. Cucumber seeds were immersed for 12 hours in bacterial cultures and then germinated in sterile conditions. Subsequently, the seeds were grown in sterile perlite supplemented with Knop nutritive

solution, for two weeks. The fungal pathogen *P. debaryanum* was grown for seven days on PDA medium at 28°C. For the infection of plantlets, slices of 5 mm PDA medium with grown mycelium were placed at the basis of the plant, as near as possible to the plant root. After five days, the roots of treated plants were analysed by light and electron microscopy.

Squash analysis

Plant rootlets were used for squash preparations, which were visualized by phase contrast microscopy or contrasted with 4 ‰ (w/v) methylene-blue solution prior to examination using an MC 1 light microscope.

Electron microscopy

Five days after fungal contamination plant roots were processed using **the method of Mascorro and Bozzola (2007)**. Samples were subjected to a prefixation in a solution of 3% (v/v) glutaraldehide in 0.2 M sodium cacodylate buffer, pH 7.2 at 4°C, overnight, and rinsed with the same buffer. The biological material was washed several times with 0.05 M sodium cacodylate buffer, for 2-3 hours and then fixed in 1% (w/v) osmium tetraoxide solution in the same buffer, at 4°C, overnight. After washing for 2 h with distilled water, the samples were dehydrated in a graded series of 10-100% (v/v) ethanol. The samples were washed twice with propylene oxide and finally embedded in Epon 812 resin.

The samples were ultrasectioned at ultramicrotom (LKB, Sweden) with diamond knife and ultrathin sections were stained according to Reynold's double coloration (REYNOLDS, 1963), before examination with an EM-125 (Selemi, Ukraine) transmission electron microscope at 50 kV. For light microscopy semithin sections 1–2 µm thick were stained with a solution of 1% toluidine blue in 1% borax (PICKETT-HEAPS, 1966).

RESULTS AND DISCUSSIONS

Squash analyses showed the presence of fungal mycelium on plant roots in variants subjected to pathogen infection (Fig. 1a). On plant roots treated with both antagonistic bacteria and phytopathogenic fungi hyphae were not observed (Figs. 1b,c).

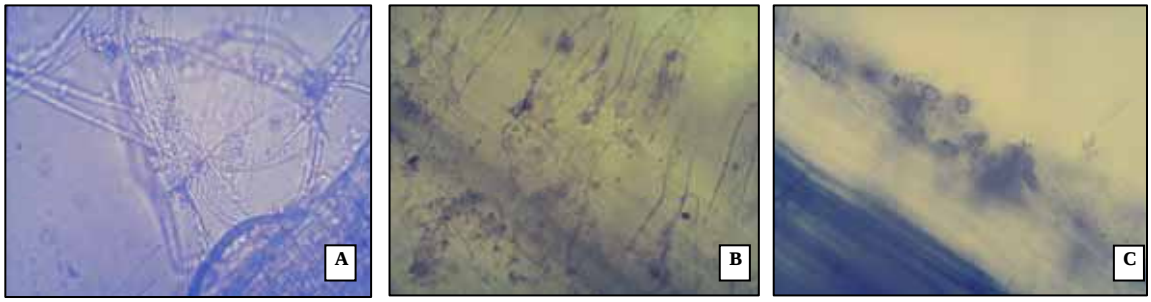

Figure 1. Microscopic aspects of squash preparates of plants treated with fungal pathogen (A) and plants treated with both antagonistic bacteria and fungal pathogen (B- P14-Py; C – B40-Py). Direct magnification 100X (A), 400X (B) and 200X (C).

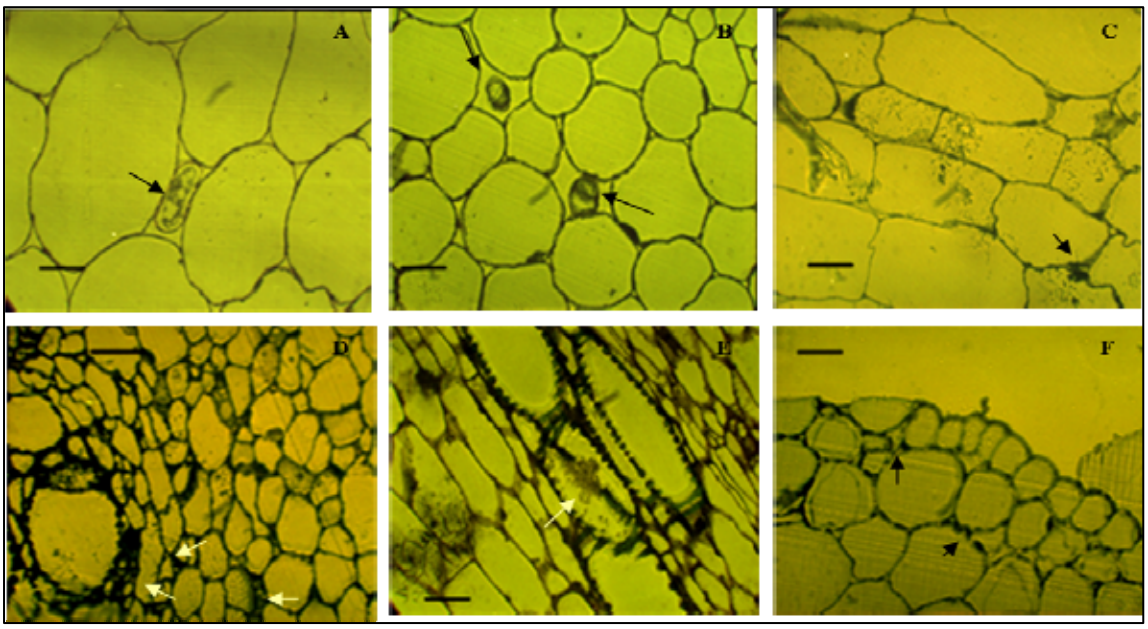

Figure 2. Aspects of cucumber plant infection with *P. debaryanum* (A, B), P14 strain (C, D), B40 strain and *P. debaryanum* (E) and P18 strain with *P. debaryanum* (F) visualized by optic microscopy on semifine sections stained with toluidine blue: intracellular proliferation of hyphae (A, B), deposits of fibrilar material (C), intercellular space occlusion (C, F), deposition of amorphous material (D), disorganized fungal cell in young xylematic vessels (E). Scale bars = 10µm.

Semithin sections through the hypocotyl of variants treated only with fungal pathogen showed the presence of fungal hyphae in intercellular spaces (Figs. 2a,b). In samples treated with bacterial suspensions, a thickening of the cell wall was observed (Fig. 2d). However, bacteria succeeded to penetrate the root tissues and invaded the intercellular spaces. In some cases, occlusion of intercellular spaces by cell wall appositions was observed (Figs. 2c,d). Sections of variants treated with antagonistic bacteria and phytopathogenic fungi showed the presence of amorphous material aggregations densely stained material toluidine blue in cortical tissue (Fig. 2f). Also, disorganized fungal cells were found in young xylematic vessels (Fig. 2e).

Electron microscopy

Transmission electron-micrographs of *P. debaryanum*-treated variant highlighted the penetration of plant cells by phytopathogen hyphae. Most of the sections showed the hyphae inside plant cells (Figs. 3a,b,c) and the pathogen abundantly invaded the intracellular area.

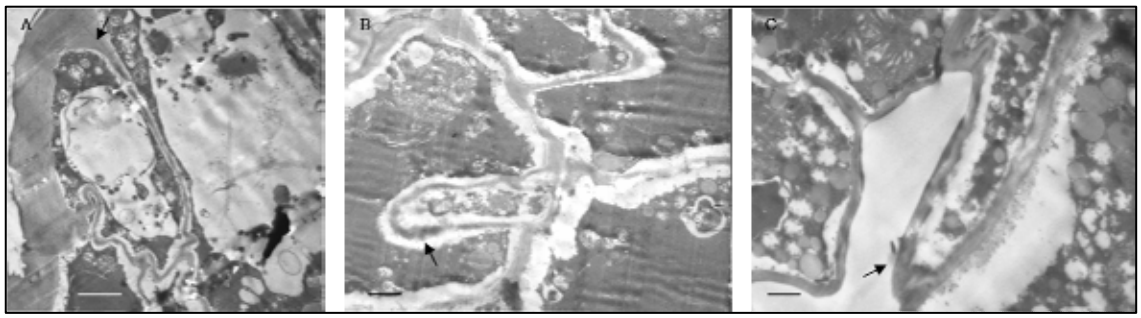

Figure 3. Ultrastructural aspects of invading cucumber plant cells by fungal pathogen (A, B, C). Scale bars = 1 μm.

Ultrathin sections of cucumber plants treated with antagonistic bacteria showed the presence of bacteria in the intercellular spaces, B40 strain colonizing abundantly these areas (Fig. 4b). The presence of bacteria correlates with the formation of irregular papillae or cell wall appositions (CWA) (Figs. 4b,d). Also, in the presence of B40 bacterial strain the excretion of vesicles and electron-opaque material from the invaded tissue cell walls was observed (Figs. 4a,c). Similar to semithin sections observations, ultrathin sections showed the obturation of intercellular spaces, probably to limit further spread of bacterial cells through the plant tissue (Fig. 4e). It was previously mentioned the fill of intercellular spaces with pectic substances in plant-arthropods interaction (POLITO et al., 2002) and with dense material likely enriched in phenolics in plant interaction with pathogenic and beneficial microorganisms (BENHAMOU et al., 2000; BENHAMOU et al., 2002; HIBAR et al., 2007). It is well known that intercellular spaces are preferred sites for pathogen ingress (BENHAMOU et al., 2002), this supporting our observation that generally these areas were filled with different materials that would restrict potential pathogen entrance in plant cells. Moreover, the fact that a fungitoxic role was attributed to these substances (BENHAMOU et al., 2002), strengthens the idea that intercellular space obturation plays an important role in plant defense against pathogens.

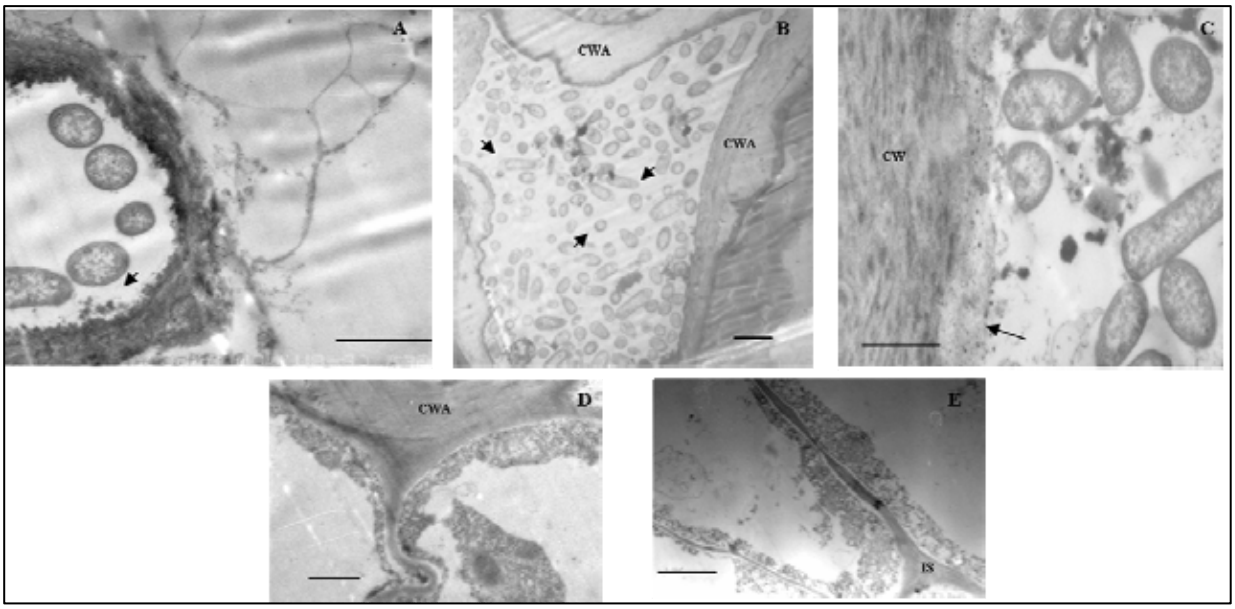

Figure 4. Some aspects regarding the interaction of the bacterial strains (A, B, C - B40 strain; D, E - P14 strain) with plant cells: the presence of bacteria near cell wall (A, C), colonization of plant intercellular territories by bacteria (B, C) cell wall appositions (CWA) in bacterial-colonized areas (B, D), fibrilar material deposition and thickness of plant cell walls (A, C), intercellular space (IS) occlusion (E). Scale bars = 1μm.

In contrast with the major part of the sections from the plant infected only with *P. debaryanum*, the variants treated both with pathogenic fungi and antagonistic bacteria, highlighted that fungi adhere to plant cell walls and are found in intercellular spaces, but very rare in intracellular spaces (Fig.5b,d,e,f). Most of the observed fungal hyphae presented disorganized cell content, were highly vacuolated, and had an abnormal shape. Moreover, fungi are trapped in an osmiophilic material, which prevents the penetration of host cell wall and further spreading through the plant (Figs. 5b,d,f).

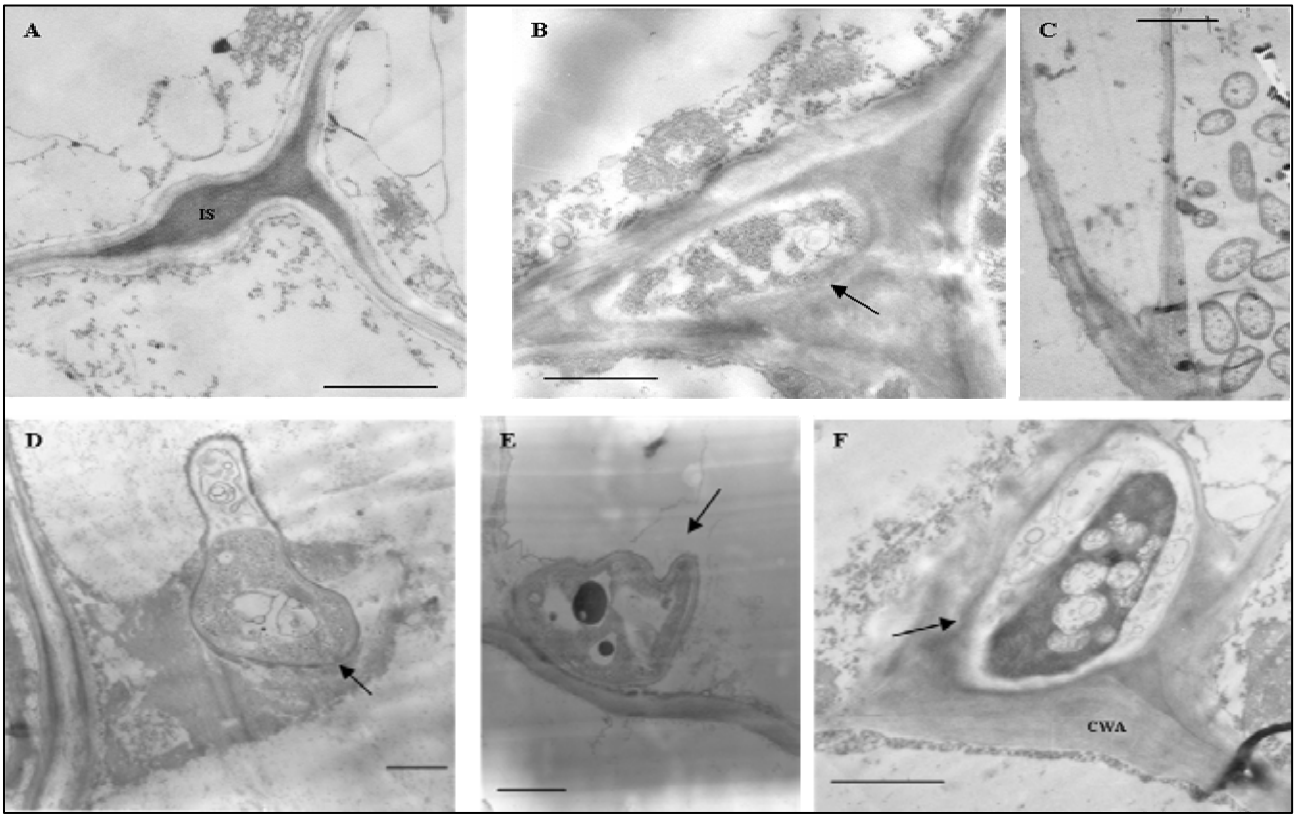

Figure 5. Ultrastructural aspects of cucumber plants treated with both bacteria and fungi (A, B – B40-Py; C – P18-Py; D, E – P7-Py; F – P14-Py): occlusion of intercellular space by osmiophilic material (A), the presence of fungi in intercellular space (IS) (B, D, E, F), fungal cell trapped in electronodense material (B, F), the presence of bacterial cells in areas other than those occupied by fungi (C), osmiophilic polymorphic material trapped the fungus near the primary host cell wall and hypha with severe alteration (D), distorted fungal cells near plant cell wall (E), the hyphae trapped by fibrillar material and vesicles accumulate in the paramural space of invading hypha and cell wall appositions (CWA) (F). Scale bars = 1µm.

Papilla formation and thickening of the plant cell walls (Fig. 5f) were correlated with fungal cell presence in plant intercellular spaces, this contributing to an increased resistance of plant to fungal pathogen. Examination of the variants that received mixed treatment allowed us to observe that fungal cells are not present in areas colonized by beneficial bacteria, this indicating the protective role of the strains used against pathogen infection.

Examination of both semithin and ultrathin sections showed papilla formation at plant cell wall level. Papillae are very complex structures (COLLINGE, 2009) and although the specific biochemical constituents of papillae vary between plant species, there are classes of compounds associated with papillae: callose, phenolics including lignin, phenolic conjugates such as phenolic–polyamines, reactive oxygen species (ROS), peroxidases, cell wall structural proteins such as arabinogalactan proteins and hydroxyproline-rich lycoproteins, cell wall polymers including pectin and xyloglucans (UNDERWOOD, 2012). The biochemical composition of papillae assigns them a double role in plant protection against pathogens. On the one hand, callose, lignin, cell wall polymers constitute a structural barrier which physically limits the pathogen entrance. On the other hand, phenolics, proteins and reactive oxygen species act as fungicide or fungistatic components, interacting with fungal cells and inducing cellular destruction in different degrees. Consequently, papillae are considered important elements and an integral part of the response of plants to microbial challenge (LEROUX et al., 2011).

CONCLUSIONS

The selected bacterial strains colonized root surface and also the intercellular areas. Plant treatments with bacteria induced structural modifications at the cellular level associated with plant resistance like the presence of cell wall appositions and thickened cell walls. Also, the occlusion of intercellular spaces was observed, and in areas colonized by bacteria no fungi presence was observed. The obtained results indicate that the selected bacterial strains had rhizosphere competence and induced structural defense responses in plants.

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