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The control of root-knot nematodes (*Meloidogyne* spp.) by *Pseudomonas oryzihabitans* and its immunological detection on tomato roots

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Summary – *Pseudomonas oryzihabitans*, a bacterium associated with the entomopathogenic nematode *Steinernema abbasi*, was evaluated for its potential to colonise roots and thereby control a field population of root-knot nematodes. Immunological techniques were developed to detect root colonisation of *P. oryzihabitans* on tomato roots using a specific polyclonal antibody raised against vegetative bacterial cells. *In vitro*, bacterial cell filtrates were also shown significantly to inhibit juveniles hatching. In a glasshouse pot experiment, there were 22 and 82% fewer females in roots of plants treated with suspensions containing 10^3 and 10^6 cells ml^{-1} of *P. oryzihabitans*, respectively. In addition, there were significantly fewer egg masses produced; however, the numbers of eggs per egg mass did not differ significantly. The relationship between root colonisation and nematode control is discussed.

Keywords – biological control, ELISA, fluorescence microscopy, immuno-blotting, rhizosphere.

The entomopathogenic nematode *Steinernema abbasi* has been shown to carry the bacterium *Pseudomonas oryzihabitans* (Elawad *et al.*, 1999) but not in the same manner as members of the genus *Xenorhabdus* (Boemare, 2002). *Pseudomonas oryzihabitans* or its toxic secretion has been shown to cause insect mortality when injected into the haemocoel (Vagelas, 2002; Mahar, 2003). This bacterium has potential as a biological control agent not only for insects but also for other target organisms. In particular, *P. oryzihabitans* and its metabolites have been reported as promising biological agents towards various plant root pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* (Vagelas, 2002; Vagelas *et al.*, 2003) and the plant-parasitic nematodes *Meloidogyne* spp. and *Globodera rostochiensis* (Samaliev *et al.*, 2000; Andreoglou *et al.*, 2003).

Colonisation of the rhizosphere and rhizoplane by these *Pseudomonas* spp. can limit the incidence of infection and systemic invasion by root pathogens, producing an increase in plant yield of certain crops when applied to seeds (Dupler & Baker, 1984; Davies & Whitbread, 1989; Lugtenberg *et al.*, 1999). The aims of this study were to test the ability of the *P. oryzihabitans* filtrates and cells to control root-knot nematodes and to develop a rapid serological technique for visualising the presence of the bacterium on the roots.

Materials and methods

NEMATODE AND BACTERIA CULTURES

Populations of *Meloidogyne* spp. were isolated from glasshouse soil at Larissa, Greece. All nematode populations were routinely maintained in a glasshouse in natural light at 27 ± 4°C on tomato (cv. Tiny Tim).

The bacterium *P. oryzihabitans*, found associated with the entomopathogenic nematode *S. abbasi*, was isolated from the haemolymph of infected greater wax moth larvae (*Galleria mellonella*) using the method described by Akhurst (1983). A pure colony of *P. oryzihabitans* and 33 isolates of other bacteria were obtained from the rhizosphere of tomato plants (grown in John Innes No. 2

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loam-based compost; 12 isolates), John Innes No. 2 loam-based compost (13 isolates), sandy loam (six isolates) and tap water (two isolates). These were differentiated by the Gram stain, cell shape and specific colony appearances according to Schaad (1988). The isolates were multiplied in nutrient broth No. 2 (Oxoid; 30 g l^{-1}) on a rotary shaker at 28°C for 24 h. Each suspension was centrifuged and the pellets were diluted with sterile tap water. Bacteria concentrations were determined using a spectrophotometer adjusted to 600 nm wavelength (Vagelas, 2002).

Hatching bioassay

A 24 h culture of *P. oryzihabitans* was injected through a 0.2 μm filter to remove the bacterial cells. These cell-free culture filtrates were diluted with sterile distilled water to provide concentrations of 50, 25, 10, 5 and 1%. Three egg masses containing 300 ± 48 eggs per egg mass were exposed to 10 ml of the cell-free culture filtrates in 35 mm diam. Petri dishes and incubated at 28°C for 5 days. Equal numbers of Petri dishes with egg masses in sterile distilled water were kept as controls. The numbers of hatched second-stage juveniles (J2) were recorded after 3 and 5 days. The experiment had three replicates and was repeated once.

Effect of *Pseudomonas oryzihabitans* on development and reproduction of *Meloidogyne* spp.

Tomato plants cv. Tiny Tim were grown in 9 cm diam. plastic pots in soil 3:1 loam/sand mixture in a glasshouse in natural light at 27 ± 4°C. Plants at the two-leaf stage were inoculated with 800 ± 80 fresh J2 per pot. At the same time, 20 ml of bacterial cell suspensions, prepared in sterile tap water at concentrations of 10^3, 10^4, 10^6 and 0 cells ml^{-1}, were applied to the soil surface of each pot. There were 12 replicates for each treatment and the experiment was repeated once. Nematode inoculated root systems were washed after 32 days, placed in a cold solution of phloxine B stain and egg masses were counted (Southey, 1986). The roots were then chopped, boiled for 2 min in lactoglycerol plus 0.1% acid fuchsin to enable visualisation and counting of female and developing female nematodes in the tissue (Southey, 1986). Eggs from five egg masses taken from each root system were separated by adding a drop of domestic bleach (sodium hypochlorite) and counted from an aqueous suspension at 50× magnification.

Production of polyclonal antibody

Pseudomonas oryzihabitans was cultured as described above, cells were removed from their culture filtrate by centrifugation and re-suspended in sterile distilled water at 10^6 cells ml^{-1}. The bacteria were heat killed by placing in a water bath for 5 min at 60°C and then stored at −20°C until required. A Dutch black and white rabbit was then immunised intermuscularly at three sites with Freund's complete adjuvant and then boosted 3 weeks later similarly but using Freund's incomplete adjuvant according to manufacturer's instructions. Bleeds were taken every 3 weeks for a period of 18 weeks. All operations were conducted according to The Home Office's Project Health Licence No. 70/4356. After collection, the blood was allowed to clot at 37°C for 1 h and then placed in a refrigerator at 4°C overnight. The following day the clot was removed and further insoluble material removed by centrifugation at 10 000 *g* for 10 min. The serum, (designated 452#2) was then stored at −20°C until required.

Specificity of the antisera tested by ELISA

The polyclonal antibody sensitivity (PC 451#2) was tested by enzyme-linked immunosorbent assay (ELISA) against *P. oryzihabitans* and 33 bacteria strains which included species of *Agrobacterium*, *Xanthomonas*, *Bacillus* and *Pseudomonas* that were isolated and sub-cultured from soil and tomato roots as described above. The suspensions were adjusted to 10^4 cells ml^{-1}. Two replicates of each bacteria at three concentrations, 10^4 cells ml^{-1}(A), 5×10^3 (A/2) and 2.5×10^3 (A/4) were tested by ELISA (Davies & Lander, 1992; Davies & Carter, 1995) using primary antibody (PC 451#2) diluted 1 : 2000 and secondary antibody (goat anti-rabbit IgG; Sigma, Poole, UK) diluted 1 : 1000 both in PBSTM.

Detection of *Pseudomonas oryzihabitans* on roots by immunoblotting assay

A sensitive serological method based on 'Dot-plots' (Lazarovits, 1990a, b) dot immunobinding assay (DIA) in which antigen is applied to the filter paper were used to detect *P. oryzihabitans* on tomato roots. Tomato plants cv. Tiny Tim (3-week-old) grown in sterilised sand, were carefully removed, washed and dipped for 3 min in a *P. oryzihabitans* suspension containing 10^6 cells ml^{-1}. One set of plants was then processed as described below and two others were re-potted in the sand and returned to the glasshouse for 24 or 48 h (Table 3), when they

were removed from the pots, the sand gently shaken from the roots which were then pressed between two filter papers (Whatman No 1). The roots were carefully removed from the filter papers and the filter papers fixed with methanol/ethanol (1:1 M/E) for 30 min. After fixing the filter papers were then incubated with antibody 452#2 at 1:4000 in phosphate buffered saline containing Tween and dried milk powder (PBSTM, 10 mM sodium phosphate buffer, pH 7.2, 0.9% sodium chloride; T, 0.05% (v/v) Tween-20; M, 5% dried milk powder) for a further 30 min. Filter papers were washed twice (2×8 s) with PBST and then incubated with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma) at 1:2000 in PBSTM for 30 min. Following incubation the filter papers were washed again with PBST and then incubated with diaminobenzidine solution (SIGMA*FAST*) until colour developed following manufacturer's instructions. After colour development, filter papers were rinsed in tap water and dried in an incubator at 37°C for 15 min. The treatments were replicated four times.

Visualisation of *Pseudomonas oryzihabitans* cells

An immunofluorescence microscopy (IFC) procedure was used to identify and to visualise *P. oryzihabitans* cells using an epifluorescence microscope (Van Vuurde, 1990). Root tips and segments 2-3 cm long, that had been treated with *P. oryzihabitans* using the technique described above (DIA) were fixed with M/E for 3 min, washed twice with phosphate buffered saline (PBS) and incubated with 1:1000 primary antibody (PC451#2) diluted in PBSTM over night at 4°C. The root segments were then washed again with PBST (×3) and incubated with a goat anti-rabbit IgG antibody conjugated to FITC (1:100 for 2 h at 37°C in the dark). Finally the root tips and segments were washed again with PBST (×3) and placed on slides, mounted in a small drop of antifade mounting medium Citiflor™ (Agar Scientific, Stansted, UK) and observed under a microscope fitted with epifluorescence illumination with a 455 nm excitation filter and a 529 nm barrier filter.

Statistical analysis

Analyses were performed using the SPSS 10.1 statistical programme. Descriptive statistics was employed to identify central tendency and dispersion and characterised difference among treatments. Further, ANOVA and multiple range tests (Tukey's multiple comparisons) were applied to assess differences between treatments and identify statistical differences between means, respectively; level of significance, $P = 0.05$.

Results

Hatching bioassay

Hatching of J2 of *Meloidogyne* spp. was inhibited significantly by *P. oryzihabitans* cell-free culture filtrates at all concentrations (Table 1). Culture filtrates diluted down to 1% had a statistically significant effect on nematode hatching but dilution of culture filtrate to concentrations of 5% and above effectively halted hatching. The number of J2 hatching after 5 days was significantly greater than after 3 days; however, with those treated with culture filtrate at 5% or above there were no significant differences.

Effect of *Pseudomonas oryzihabitans* on development and reproduction of *Meloidogyne* spp.

Cells of *P. oryzihabitans* applied to tomato plants at the same time that J2 were inoculated resulted in significantly fewer females or egg masses in tomato roots 32 days later. The greatest effect was with the 10^6 cells ml^{-1} treatment (Table 2). However, in all *P. oryzihabitans* treatments, the egg masses that were produced contained the same number of eggs as from the non-treated plants.

Specificity of the antisera as tested by ELISA

The results of the specificity tests using an indirect ELISA show that only *P. oryzihabitans* was recognised by the antisera (OD > 1.4) and none of the other 33 different bacteria exhibited an OD greater than 0.3. A significant top curve was obtained for *P. oryzihabitans* for the three antiserum concentrations tested. All the other bacteria had significantly lower OD values compared with those of *P. oryzihabitans* and results are presented as combined means in Figure 1.

Detection of *Pseudomonas oryzihabitans* on roots by immunoblotting assay

The results show that bacteria cells could be detected on tomato roots using the highly specific polyclonal antibody (PC 451#2) raised against *P. oryzihabitans*. The polyclonal antibody detected *P. oryzihabitans* on roots that had been pressed between filter papers and fixed

Table 1. *Effect of cell-free culture filtrate of* Pseudomonas oryzihabitans *on hatching of second-stage juveniles (J2) of* Meloidogyne *spp. after 3 or 5 days incubation.*

% Culture filtrate	Hatched J2			
	3 days		5 days	
	Experiment 1		Experiment 2	
	Mean ± SD*	Mean ± SD	Mean ± SD	Mean ± SD
0 (Control)	134^{a} ± 76.03	127^{b} ± 37.87	257^{a} ± 114.27	302^{a} ± 69.14
1	17^{b} ± 24.85	42^{a} ± 21.78	54^{ab} ± 99.79	29^{b} ± 13.58
5	1^{b} ± 0.58	0^{a} ± –	1^{b} ± 0.58	1^{b} ± 0.58
10	0^{b} ± –	0^{a} ± –	0^{b} ± 1.15	0^{b} ± 0.58
25	0^{b} ± –	0^{a} ± –	0^{b} ± –	0^{b} ± –
50	0^{b} ± –	0^{a} ± –	0^{b} ± –	0^{b} ± –
LSD 0.05	29.94	29.48	51.42	57.36

* SD = Standard Deviation.
Values within a column followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's multiple comparisons test.

Table 2. *Females and egg masses of* Meloidogyne *spp. on roots of tomato plants 32 days after inoculation with second-stage juveniles and treatment with different concentrations of* Pseudomonas oryzihabitans *cells.*

P. oryzihabitans cells ml^{-1}	Females	Egg masses	Eggs per egg mass
Control	179 c*	100 c	233
P. oryzihabitans 10^3	140 bc	66 ab	243
P. oryzihabitans 10^4	86 ab	88 bc	230
P. oryzihabitans 10^6	50 a	44 a	229
$LSD_{0.05}$	60.01	18.23	(NS)

* Values within a column followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's multiple comparisons test.

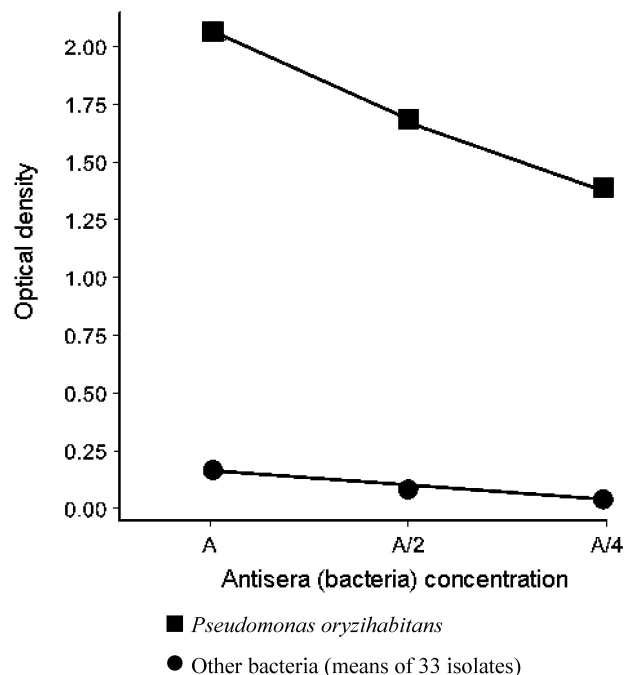

Fig. 1. *Optical density measurements of an indirect ELISA against* Pseudomonas oryzihabitans *and 33 other bacteria coated onto an ELISA plate at three concentrations (10^4 cells ml^{-1} A; 5×10^3 A/2; 2.5×10^3 A/4) using polyclonal antibody 452#2. The measurements for all 33 isolates were significantly different from that of* P. oryzihabitans.

and could be seen to produce a visible dark image of the tomato root system in all cases. No bacteria could be detected as visible spots on the untreated control roots (Table 3). After 3 min the *P. oryzihabitans* cells rapidly colonised the root surface (Table 3). Root tips were densely covered by the bacteria by 24 h and by 48 h, the bacterial population formed long strings of closely associated cells. A patchy distribution along the roots (Fig. 2) and root tips (Fig. 3) was also observed. *P. oryzihabitans* could not be found in the root segments and root tips of the untreated controls (Table 3; Figs 2, 3).

Table 3. *Colonisation of tomato roots by* Pseudomonas oryzihabitans *observed by immunofluorescence.*

Treated period	Replicates	Immunofluorescence of bacteria cells on roots			
		Roots treated with *P. oryzihabitans*		Untreated roots	
		Roots	Root tips	Roots	Root tips
3 min	1	+	−	−	−
	2	+	−	−	−
	3	+	−	−	−
	4	+	−	−	−
24 h	1	++	+	−	−
	2	+	−	−	−
	3	++	+	−	−
	4	++	+	−	−
48 h	1	++	++	−	−
	2	++	++	−	−
	3	++	++	−	−
	4	++	++	−	−

++ Good colonisation; + Weak colonisation; − No colonisation.

VISUALISATION OF *PSEUDOMONAS ORYZIHABITANS* CELLS

Bacterial cells were observed on the root surface (Fig. 2) and the population increased and produced more patches with time (Table 3; Figs 3A, B). This observation confirmed the dot immunobinding assay (DIA) where bacteria-treated root samples after 24 and 48 h produced a stronger,more easily observed blot than on root samples treated for only 3 min. This provided evidence that the *P. oryzihabitans* cells are able to reproduce on tomato roots.

DISCUSSION

Serological methods were used to gain an understanding of the spatial and temporal distribution of *P. oryzihabitans* along the root. With the DIA method we confirmed the specificity of the antibody PC 451#2, and have produced a method capable of detecting and monitoring the distribution of *P. oryzihabitans*. When the antibody was combined with specific cell staining techniques (IFC) the simultaneous visualisation of specific bacteria cells was possible and the DIA results were confirmed. Similar data to ours were presented by other authors who used immunofluorescence and confocal scanning laser microscopy (CSLM) techniques to observe *Pseudomonas fluorescen*s on pine roots (Bent *et al.*, 2000), or CSLM to visualise *P. fluorescens* strain WCS365 on tomato roots (Chin-A-Woeng *et al.*, 1997), or a green fluorescent protein (GFP) as a marker to visualise and distinguish *P. fluorescens* WCS365 and *P. chlororaphis* strain PCL1391 in the tomato rhizosphere (Bloemberg *et al.*, 2000).

Pseudomonas oryzihabitans culture filtrates contain compounds that inhibit hatching of root-knot nematodes *in vitro*. Also, *P. oryzihabitans* cells decrease the numbers of female nematodes and egg masses when applied to soil at the time of nematode inoculation further demonstrating that *P. oryzihabitans* produces metabolites, which have nematostatic effects which probably affected the invasion by the infective J2. The failure to demonstrate differences in the numbers of eggs per egg mass suggests that nematodes that invaded the roots progressed to maturity normally and that the metabolites had no lasting systemic activity. *Pseudomonas oryzihabitans* has been reported acting as a biological agent against plant-parasitic nematodes (Samaliev *et al.*, 2000; Vagelas, 2002; Andreoglou *et al.*, 2003; Vagelas *et al.*, 2003), and other *in vitro* studies using the same strain of bacterium showed that it affects nematodes by causing paralysis and convulsive movements (Samaliev *et al.*, 2000).

We have developed a visualisation method for the colonisation of a root by a rhizosphere bacterium using a polyclonal antibody that is sufficiently sensitive to detect single bacteria. The results suggest that when applied to soil, the cells of *P. oryzihabitans* or the metabolites released, provided some protection to tomato roots from root-knot nematodes, preventing invasion by disabling or disorientating the infective J2. The immunological evidence enables a better understanding of the role of

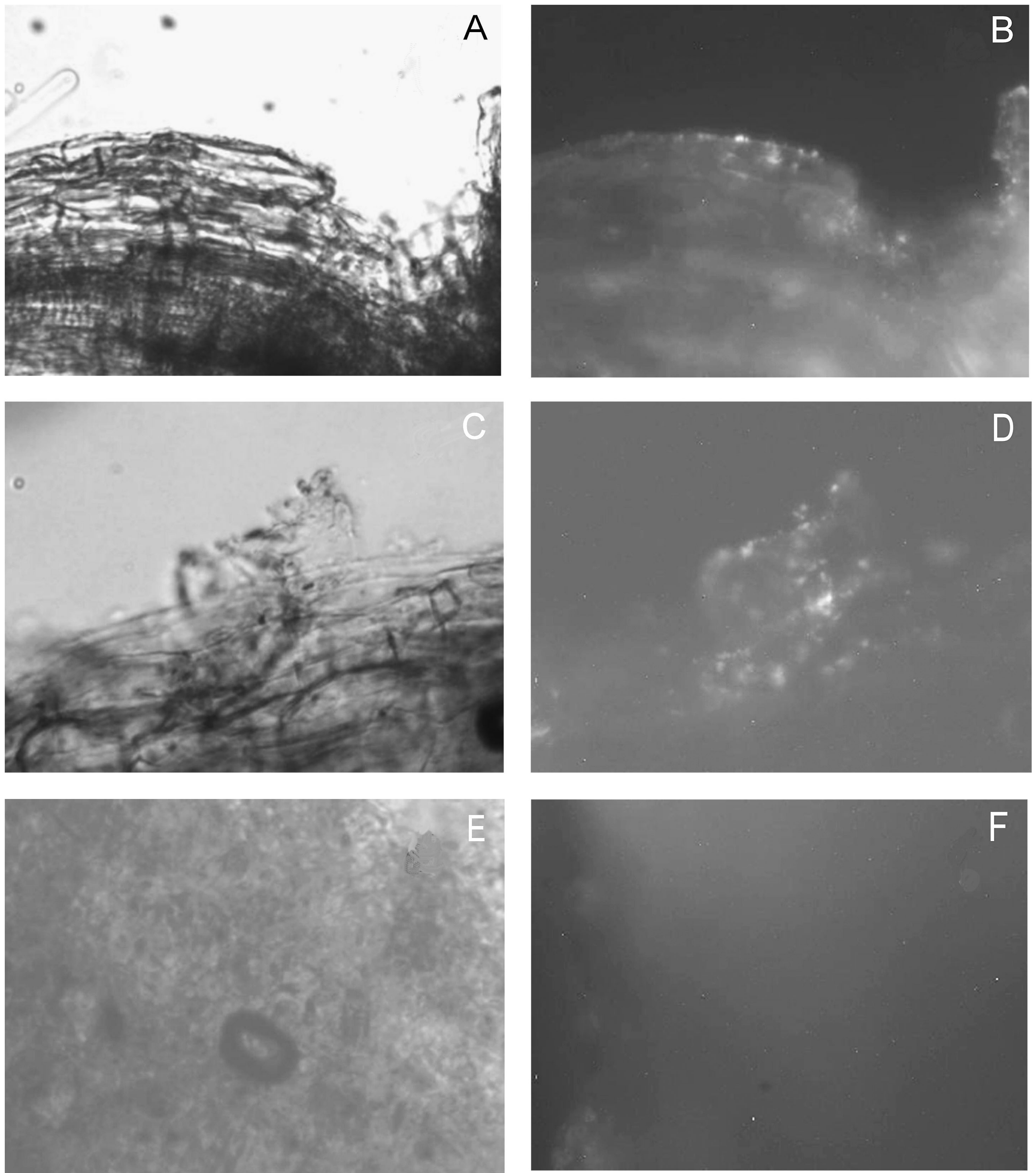

Fig. 2. *Surface colonisation of tomato roots by* Pseudomonas oryzihabitans *visualised using Immunofluorescence; B, D: Immunofluorescence of root segments showing* P. oryzihabitans *cells on the roots; A, C, E: Root segments under light microscopy; F: (Control) immunofluorescence of root segment incubated with conjugated antibody.*

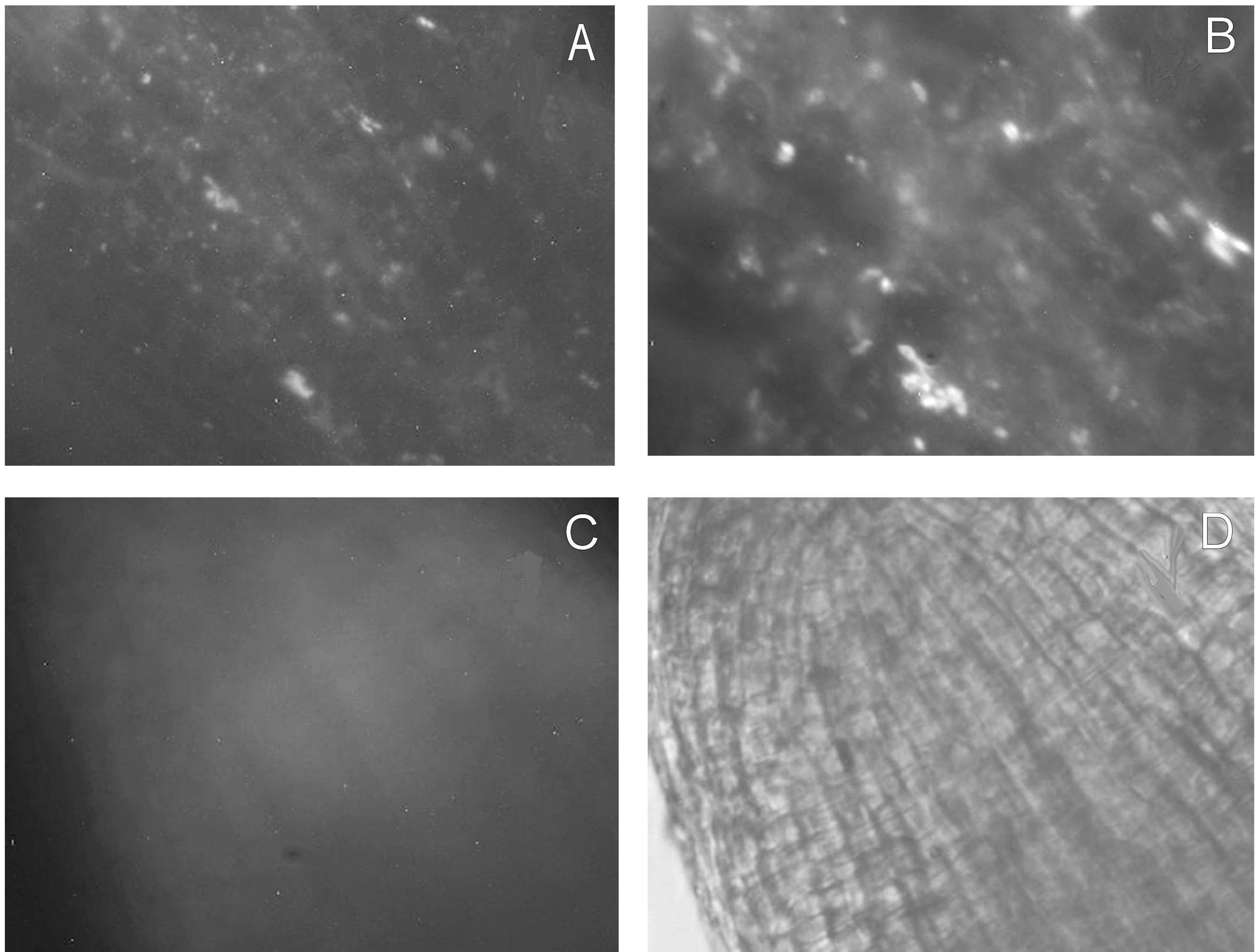

Fig. 3. *Surface colonisation of tomato root tips by* Pseudomonas oryzihabitans *visualised using Immunofluorescence; A, B: Immunofluorescence of roots showing* P. oryzihabitans *cells on the root tips; C: (Control) immunofluorescence of root incubated with conjugated antibody; D: Root-tip segment under light microscopy.*

P. oryzihabitans in the rhizosphere and shows that the bacteria cells will adhere to tomato roots and that *P. oryzihabitans* could be deployed for the protection of seedlings against attack by root-knot nematodes.

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