

CHARACTERIZATION OF ANTAGONISTIC ROOT-ASSOCIATED FLUORESCENT PSEUDOMONADS OF TRANSGENIC AND NON-TRANSGENIC CITRANGE TROYER PLANTS

G. Cirvilleri¹, S. Spina², G. Scuderi², A. Gentile³ and A. Catara^{1,2}

¹Dipartimento di Scienze e Tecnologie Fitosanitarie, Università di Catania, Via S. Sofia 100, Catania, Italy

²Parco Scientifico e Tecnologico della Sicilia S.c.p.a, Via A. Borrelli 10, Palermo, Italy

³Dipartimento di Ortoflorocarboricoltura e Tecnologie Agroalimentari, Università di Catania, Via Valdisavoia 5, Catania, Italy

SUMMARY

To study the influence of *rolABC* Troyer citrange plants on potentially beneficial root-associated bacteria, transgenic and non-transgenic plant lines were monitored over the course of one year. Culturable bacterial populations were not significantly different between *rolABC* and wild type lines, whereas the population of fluorescent pseudomonads varied according to the plant lines and the date of sampling. A total of 321 representative fluorescent pseudomonads were investigated to determine antagonistic activity against *Fusarium solani* and *Phoma tracheiphila*, and two different antagonistic groups were identified. IAA-producing *Pseudomonas fluorescens* strains were well distributed among transgenic and non-transgenic plant lines. A subset of 42 representative antagonistic *P. fluorescens* strains, characterized by ARDRA and ERIC-PCR, showed a high degree of DNA heterogeneity. Four main groups were identified. Three groups were heterogeneous and contained isolates from transgenic and non-transgenic plant lines whereas one group was homogeneous and included only strains from transgenic lines belonging to the same antagonistic group. On the whole, the fluorescent populations isolated from the *rolABC* lines showed some differences from those isolated from wild types, and a partial correlation was observed between grouping of strains based on antagonistic activity and on ERIC-PCR fingerprints and the transgenic character of the plants.

Key words: antagonistic bacteria, rhizosphere, *rolABC* genes, ERIC-PCR fingerprinting.

INTRODUCTION

The development of genetically modified plants (GMPs) has been a topic of considerable public debate in recent years. Environmental risk assessment regarding the cultivation of GMPs have focused on potential

aboveground effects, whereas little is known of the consequences of genetically engineered plants on soil-borne microorganisms (Bruinsma *et al.*, 2003) despite their recognized importance in terms of biomass and activity in soil.

Microorganisms are the dominant soil organisms both in terms of biomass and activity, and largely determine the functioning of terrestrial ecosystems being responsible, for example, for functions such as nutrient cycling and decomposition. In addition root-associated microorganisms interact directly and indirectly with plants and vegetation dynamics, may exert beneficial or deleterious on plant growth and health, and their composition and population size is influenced by a variety of factors such as plant species, cultivar and exudation of nutrients (Bachmann and Kinzel, 1992; Grayston *et al.*, 1998; Siciliano *et al.*, 1998).

Root-associated microorganisms have recently been investigated as sensitive indicators of GMP-induced variation. Differences in rhizobacterial communities between wild type and transgenic alfalfa (*Medicago sativa* L.) cultivars transformed for alpha-amylase and lignin peroxidase have been reported, although the consequences of these changes were unknown (Di Giovanni *et al.*, 1999). In a field study comparing conventional canola (*Brassica napus* L.) varieties with others transformed for glyphosate tolerance, the differences in the rhizobacterial communities over the course of two years appeared to be most influenced by soil type (Siciliano and Germida, 1999; Dunfield and Germida, 2001). In studies on potato (*Solanum tuberosum* L.) plants transformed to produce T4-lysozyme (Lottmann *et al.*, 1999; Heuer *et al.*, 2002), the differences in number and diversity of bacteria appeared as a natural shift within the soil microbial community. Other studies examined the effects of *Lotus corniculatus* L. plants, modified to produce mannityl opines and nopaline (Oger *et al.*, 2000). Only opine-utilizer populations were significantly greater in the root system of transgenic plants, whereas the remaining bacterial groups examined did not differ significantly between transgenic and wild type plants, clearly indicating the impact of plant modification on microbial populations and the possibility to modify the rhizosphere community through alterations in the quali-

ty and quantity of root exudates.

Troyer citrange [*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck], an important citrus rootstock, has been transformed with *rolABC* genes from *Agrobacterium rhizogenes* to modify the plant's growth habit (Gentile *et al.*, 2004) and possibly its behaviour against pathogens (La Malfa *et al.*, 2004). *rolABC* genes are involved in phytohormone balance, altering plant morphology, inducing hairy root syndrome (Spena *et al.*, 1987), and increasing the sensitivity of transgenic tissues to both cytokinins and auxins (Estruch *et al.*, 1991a,b).

An effect on root exudates and consequently on root-associated microorganisms should also be expected. Work on 23 transgenic *rolABC* Troyer citrange plants (Cirvilleri *et al.*, 2003) showed that *rolABC* genes modify some aspects of roots (higher number of hairy roots) but do not affect culturable bacterial populations. In addition, in transgenic Troyer Citrange clones, autotrophic ammonia oxidiser populations were modified, and phenolic compounds plus PAL and peroxidase activity increased in roots (La Malfa *et al.*, 2004).

The objective of this study was to evaluate the effects of *rolABC* Troyer citrange plants on potentially beneficial root-associated microorganisms. Bacteria were periodically monitored over the course of one year and characterized according to their antagonistic activity *in vitro* and to their ability to produce indole-3-acetic acid (IAA). A subset of 42 representative antagonistic fluorescent pseudomonads from *rolABC* and wild type lines were characterized by amplified 16S ribosomal DNA restriction analysis (ARDRA) and with respect to their enterobacterial repetitive intergenic consensus (ERIC-PCR) sequence polymorphism.

MATERIALS AND METHODS

Plant material. Three transgenic *rolABC* Troyer citrange lines (*rol* 1, 5, 7), regenerated from independent transformation events, and Troyer citrange wild types (WTs) were propagated *in vitro* and transplanted in pots with unpasteurized compost (1:1:1 sand:peat moss:soil). The plants were grown in a conditioned greenhouse at 24±2°C during the day and 18±2°C during the night; natural light was reduced by 25% covering the greenhouse with a black net. Morphological and physiological modifications previously detected (Gentile *et al.*, 2004) included dwarfing (up to 50% height reduction) and increase of root system density. Rhizosphere samples were collected three years after transplanting on three occasions: October 2002, April 2003, and January 2004.

Determination of microbial populations. Roots with adhering soil (10 g for each sample), taken from the rhizosphere of each plant line at each sampling time, were

immersed in 20 ml of washing buffer (0.1 M potassium phosphate buffer, pH 7). Tubes were sonicated in an ultrasonic cleaner (Brasonic 52, Branson Cleaning Equipment Co., Shelton, CT, USA) for 7 min and samples were spiral plated onto King's B medium (KB) (King *et al.*, 1954) using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Culturable bacteria and fluorescent colonies were counted after 48 h at 27°C to calculate the means of colonies (log₁₀ CFU) based on fresh weight. Four replicates from each plant line and three plants for each line were sampled.

For further testing, fluorescent bacteria were randomly selected at each sampling time and plant line and subcultured on KB, resulting in 321 fluorescent isolates (52 from *rol* line 1, 87 from *rol* line 5 and 7, respectively, and 95 from non-transgenic lines). All strains were stored at -80°C in nutrient broth (Oxoid, Basingstoke, UK) supplemented with 15% (vol/vol) glycerol. Analysis of variance (ANOVA) was performed by COSTAT software on log-transformed population sizes to determine any significant effects of plant lines and time of sampling influencing rhizosphere population sizes.

Screening for antagonistic fluorescent pseudomonads. All 321 fluorescent colonies were tested in dual culture assay on PDA and KB agar media against *Fusarium solani* 1A and *Phoma tracheiphila* LV, isolated from symptomatic citrus plants in Sicily. Aliquots (10 ml) of overnight bacterial suspensions (10⁸ CFU ml⁻¹) grown in NA were inoculated on surface-dry agar plates (4 spots per plate). After incubation at 27°C for 4 days, conidial suspensions (10⁶ CFU ml⁻¹) of fungi grown on PDA for 7 days were sprayed over the plates. Plates were incubated at 27°C and inhibition was scored 4 days after spraying by measuring the size of clear zones around the bacterial colonies. The assays were repeated twice with two replicates each. After transformation of the percentages to angular values, ANOVA was performed to determine any significant effects of plant lines and time of sampling.

Screening for IAA-producing fluorescent pseudomonads. The method of Gordon and Weber (1951) was modified and used to determine IAA production. All the fluorescent colonies were grown on KB broth supplemented with 0.5 mM L-tryptophan from a filter-sterilized 2 mg ml⁻¹ stock solution. After incubation for 48 h at 28°C and 180 rpm, cells were removed by centrifugation for 10 min at 5000 rpm and 0.5 ml of cell-free supernatant of each isolate was dispensed into 96-well microplates. One ml of Salkowski's reagent (3 ml 1.5 M FeCl₃; 100 ml of concentrated H₂SO₄; 63 ml sterile distilled water) was added and mixed vigorously. After 30 min in the dark at room temperature, the absorbance at 530 nm was measured by microplate reader (Spectra-max-250, Molecular Devices Corp., Sunnyvale, CA,

USA). The concentrations of IAA in each culture medium were determined by comparison with a standard curve (Gordon and Weber, 1951).

Identification of fluorescent pseudomonads. All the fluorescent pseudomonads were identified to species level according to the phenotypic characters and biochemical tests suggested for *Pseudomonas* spp. (Schaad *et al.*, 2001) and by the Analytical Profile Index procedure using the API 20NE system (BioMérieux, Marcy l'Etoile, France). A collection of representative fluorescent pseudomonads was identified to biotype, according to Schaad *et al.* (2001).

PCR amplification of 16S rDNA (ARDRA). Forty-two antagonistic *P. fluorescens* strains representative of the 3 *rolABC* and the WT lines were used. DNA was extracted using the DNA Purification Kit (Puregene, Gentra, Minneapolis, MN, USA) following the manufacturers' instructions. Each isolate was amplified with *Pseudomonas*-specific primers PsF (5'-TAGCTCCAC-CTCGCGGC) and Ps-R (5'-GGTCTGAGAGGATGATCAGT) (Invitrogen-Life Technologies, Rockville, MD, USA) and PCR performed in an Applied Biosystems (Foster City, CA USA) thermocycler (GeneAmp® PCR System 9700) as described by Widmer *et al.* (1998). Amplification mixtures were digested with *Hae* III and *Alu* I (Invitrogen-Life Technologies, Carlsbad, CA, USA) and analysed in 3.5% agarose in 1x TAE buffer at 5 V cm⁻¹ over 6 h. The gels were stained with 0.2 mg ml⁻¹ ethidium bromide, visualized on a UV transilluminator, photographed and digitized by PowerShot G2 (Canon Inc., Lake Success, NY, USA). A 1 kb DNA Ladder (Invitrogen-Life Technologies, Carlsbad, CA, USA) was used as a size standard.

ERIC-PCR. This method was adapted from Louws *et al.* (1994). The primers ERIC 1R (5'-ATGTAAGCTC-

CTGGGGATTAC-3') and ERIC 2 (5'-AAGTAAGT-GACTGGGGTGAGCG-3') were synthesized by Invitrogen-Life Technologies (Carlsbad, CA, USA). The reaction mixture consisted of 1 µl of DNA template, 2.5 µl of 10x reaction buffer, 0.5 µl of dNTP mix (10 mM), 50 pmol of primer ERIC 1R, 50 pmol of primer ERIC 2, 1.25 µl of MgCl₂ (50 mM), and 1.5 U of *Taq* polymerase (Invitrogen-Life Technologies, Carlsbad, CA, USA) made up to 25 µl with sterile distilled water. The reaction mixture was denatured for 7 min at 95°C and then subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 8 min at 65°C, and a final extension for 16 min at 65°C. The products were separated by electrophoresis on 1.5% agarose (Bio-Rad Laboratories, Hercules, CA, USA) in TAE 1x buffer at 5 V cm⁻¹ over 4 h. A 1 kb DNA Ladder (Invitrogen-Life Technologies, Carlsbad, CA, USA) was used as a size standard. The gels were stained, visualized and handled as above. Banding patterns were analysed using GelCompar software version II (Applied Maths, Kortrijk, Belgium). Similarity coefficients were determined using Dice's coefficient (Dice, 1945). Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) using GelCompar software version II (Applied Maths, Kortrijk, Belgium).

RESULTS

Population trends. The number of culturable bacteria was not significantly different between transgenic and non-transgenic plant lines, and remained in the range of log 6.78 to log 7.17 per g fresh weight (fw) of root during all the sampling times (Table 1). There was no significant difference between the means of the four plant lines over all sampling times, and between the means of the different sampling times over all plant lines. Plant lines and date of sampling significantly af-

Table 1. Interaction of plant lines and date of sampling on population estimates of culturable bacteria and fluorescent *Pseudomonas*.

Sampling time ^b	Bacteria recovered (log CFU g ⁻¹ fw) ^a									
	Culturable bacteria					Fluorescent <i>Pseudomonas</i>				
	<i>Rol1</i>	<i>Rol5</i>	<i>Rol7</i>	WT	Mean ^c	<i>Rol1</i>	<i>Rol5</i>	<i>Rol7</i>	WT	Mean ^c
I (Oct. 2002)	6.88	6.86	7.02	6.72	6.87 a	4.50	4.76	6.00	6.14	5.35 b
II (Apr. 2003)	6.86	6.88	7.80	6.71	7.06 a	2.79	4.48	4.67	5.04	4.24 a
III (Jan. 2004)	6.81	6.65	6.68	6.91	6.67 a	5.45	5.25	5.45	5.16	5.33 b
Mean ^x	6.85 a	6.79 a	7.17 a	6.78 a		4.24 a	4.83 ab	5.37 b	5.45 b	

^a Values are means of three plant lines (four replications of the same plant line); ^b Sampling time (I: October 2002; II: April 2003; III: January 2004); ^c Means reported in a row or column followed by different letters differ according to the Student-Newman-Keuls test (P=0.05).

fects the fluorescent pseudomonad population size. Significantly more fluorescent pseudomonads were recovered from *rol* line 7 and WT (log 5.37 and 5.45, respectively) than from *rol* lines 1 and 5 (log 4.24 and 4.83, respectively) over all sampling times (Table 1). Overall, significantly more fluorescent pseudomonads were detected from the rhizosphere of the four plant lines at the first and third sampling times (log 5.35 and log 5.33, respectively) than in the second sampling time (log 4.24) (Table 1).

Antagonism and IAA production. The percentage of antagonistic bacteria varied according to culture media and plant lines (Fig. 1). When tested on PDA, a medium high in iron and low in phosphate, in which fluorescent siderophores are repressed and antibiotics are produced (Gross, 1985), only strains isolated from *rolABC* lines showed antagonistic activity against *F. solani* (from 8.7 to 19.4% of isolates) and *P. tracheiphila* (from 2.2 to 14.8% of isolates) (Fig. 1), with inhibition zones ranging between 3 and 20 mm. By contrast, none of the 95 fluorescent pseudomonads isolated from wild type lines showed antagonistic activity on PDA. When tested on KB, which is low in available iron and favours siderophore production (Xu and Gross, 1986), fluorescent pseudomonads from transgenic and non-transgenic lines inhibited *F. solani* and *P. tracheiphila* (Fig. 1). On KB the percentage of anti-*Fusarium* isolates varied between 52 and 68% and the percentage of anti-*Phoma* isolates between 50 and 100% with inhibition zones ranging from 5 to 20 mm.

Two antagonistic groups were identified. Strains identified as antagonistic group I showed antagonistic activity only on KB agar (156 inhibitory to *F. solani* and

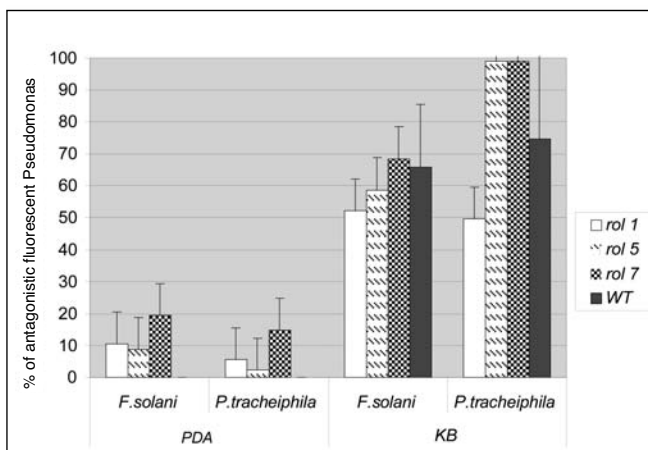


Fig. 1. Mean values of antagonistic fluorescent *Pseudomonas* on PDA and KB against *Fusarium solani* and *Phoma tracheiphila*. Percentage of antagonistic fluorescent *Pseudomonas* isolated from the rhizosphere of Troyer citrange plant lines *rolABC* 1, 5, 7 and wild type (WT) was determined. The test was performed on previously transformed data arc sen v%. Vertical lines represent standard errors.

Table 2. Influence of fluorescent *Pseudomonas* on the growth of *Fusarium solani* and *Phoma tracheiphila* on PDA and KB agar.

Source of strains	Strains tested (N.)	Number of fluorescent <i>Pseudomonas</i> ^a inhibitory to			
		<i>Fusarium solani</i> on		<i>Phoma tracheiphila</i> on	
		KB	PDA	KB	PDA
<i>Rol</i> 1	52	34	3	49	3
<i>Rol</i> 5	87	35	6	79	1
<i>Rol</i> 7	87	44	15	72	9
WT	95	43	0	84	0
Total	321	156	24	284	13

^a Fluorescent *Pseudomonas* were inoculated onto duplicate plates, incubated at 24°C for 4 days, and then oversprayed with a suspension (10^6 CFU ml⁻¹) of *Fusarium solani* or *Phoma tracheiphila*.

284 inhibitory to *P. tracheiphila*) (Table 2) and were isolated both from *rolABC* and WT lines.

Strains identified as antagonistic group II produced growth inhibition zones on PDA and were isolated only from *rolABC* lines (24 strains inhibitory to *F. solani* and 13 inhibitory to *P. tracheiphila*).

P. fluorescens and *P. putida* were the most common species among the fluorescent *Pseudomonas* analysed. The majority of the representative *P. fluorescens* strains identified to biotype was subdivided in biovar 3 and 5 (data not shown). The remainder could not be clearly differentiated as to biovar.

Fluorescent pseudomonads were analysed for their ability to produce auxin (IAA equivalents) in the presence of L-tryptophan as precursor. IAA-producing *Pseudomonas* from *rolABC* lines (38% of strains from *rol* 1, 18% from *rol* 5, 56% from *rol* 7) and those from wild type lines (up to 38%) were equally proficient in the production of IAA *in vitro*, and the level of IAA produced from most isolates varied between 0.4-15 mg ml⁻¹. Seven isolates from *rolABC* lines and four from WT produced high levels of IAA (between 16 and 28.5 mg ml⁻¹).

Genetic variability. A subset of 42 *P. fluorescens* strains representative of plant lines, time of sampling and antagonistic behaviour on KB and PDA (antagonistic groups I and II) were genotypically characterized. Ps-PCR primers selectively amplified an approximately 990-bp fragment and restriction analysis with *Alu* I revealed two fragmentation patterns: pattern I, comprising 3 strains from *rolABC* plant lines 1 and 5 (Fig. 2A, lanes 2, 3, 5), and pattern II comprising all other strains (95% of tested strains) from *rolABC* lines and from WT (Fig. 2A, lanes 6 to 19). A single *Hae* III pattern was obtained with all 42 fluorescent pseudomonas tested (Fig. 2B).

PCR amplification with ERIC primers yielded 6 to 12 distinct PCR products, ranging in size from approximately 150 bp to over 3,000 bp and allowed differentia-

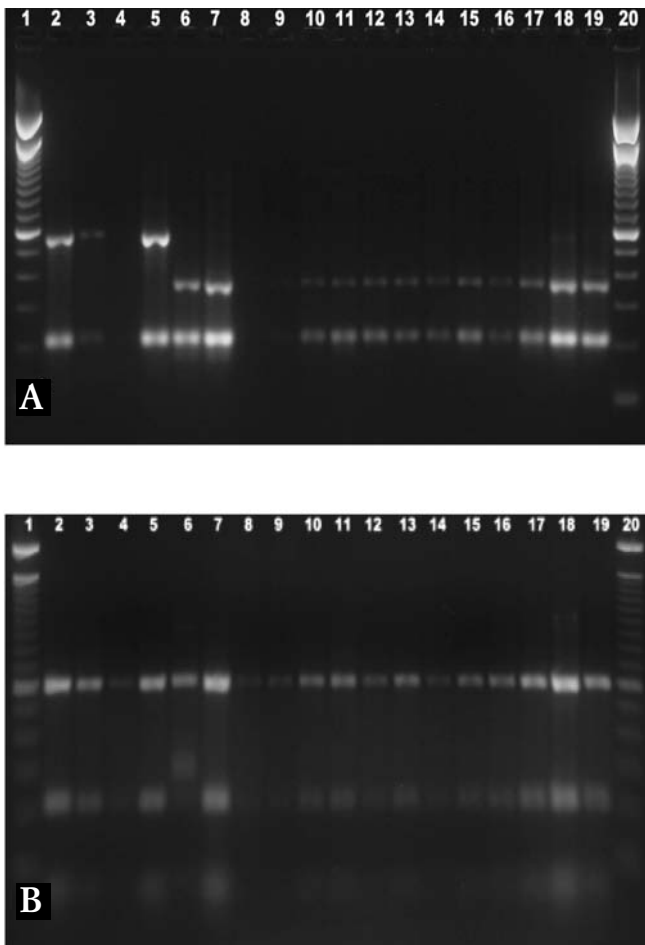


Fig. 2. ARDRA banding patterns obtained with *Alu* I (**A**) and *Hae* III (**B**) on *P. fluorescens* strains. The molecular weight marker was a 100 bp ladder. Lanes 2-4: strains isolated from *rol* 1; lanes 5-8: strains isolated from *rol* 5; lanes 9-13: strains isolated from *rol* 7; lanes 14-19: strains isolated from WT.

tion of strains (Fig. 3). The ERIC-PCR profiles were compared by numerical methods and the dendrogram revealed genetic diversity (Fig. 4). Cluster analysis resulted in four main cluster groups designated 1, 2, 3 and 4. Group 1 was heterogeneous and contained isolates from transgenic and wild type plant lines belonging to antagonistic groups I and II. Groups 2 and 4 included isolates from transgenic and non-transgenic plant lines belonging to the same antagonistic group (I). Group 3 was homogeneous and included only strains from transgenic lines belonging to the same antagonistic group (II). Most of the strains from *rolABC* lines were exclusively grouped under subclusters 2, 3, 4, 5, and cluster 3 (Fig. 4).

Cluster analysis also distinguished *rolABC* strains belonging to antagonistic group II, grouped exclusively under subcluster 3 and cluster 3, and the strains belonging to antagonistic group I, grouped under subcluster 1, 2, and clusters 2 and 4 (Fig. 4). On the whole, grouping of isolates based on ERIC-PCR profiles was partially correlated with strain origin, transgenic and non-transgenic plants, and antagonistic activity.

DISCUSSION

rolABC Troyer citrange lines did not have any detectable effect on culturable bacterial populations, whereas fluorescent pseudomonads varied according to the plant lines and sampling date. The data also indicated that presence of *rolABC* genes did not influence fluorescent pseudomonads populations. In fact, mean population sizes of *rol* 7 and WT lines were similar, and higher than that of *rol* lines 1 and 5, suggesting that a more important factor influencing bacterial numbers could be the insertion point of the *rolABC* genes, since

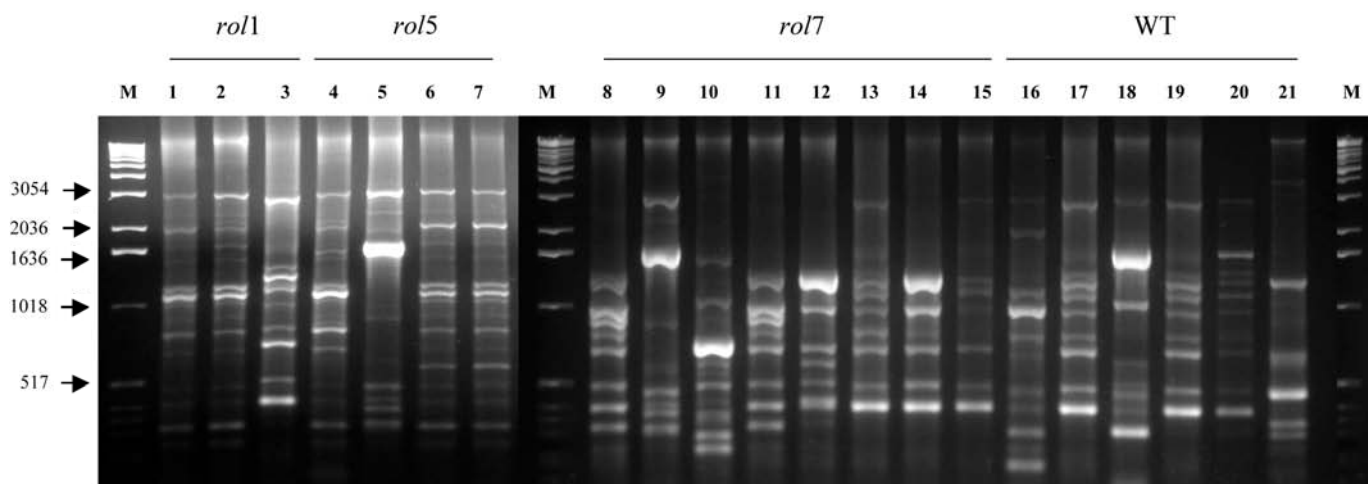


Fig. 3. ERIC-PCR DNA fingerprint patterns of fluorescent *Pseudomonas* representing isolates from *rol* 1 (lanes 1-3, strains 1AM, 1AO, 1AV), *rol* 5 (lanes 4-7, strains 5AS, 5AT, 5BA, 5CP), *rol* 7 (lanes 8-15, strains 7N, 7-14D, 7-8L, 7-17A, 7-17E, 7BD, 7-11C, 7-14C) and wild type (lanes 16-21, strains WT3I, WTB, WTR, WTAA, WTAD, WTCKE). Lane M is a standard 1 kb DNA ladder; the sizes are indicated in base pairs.

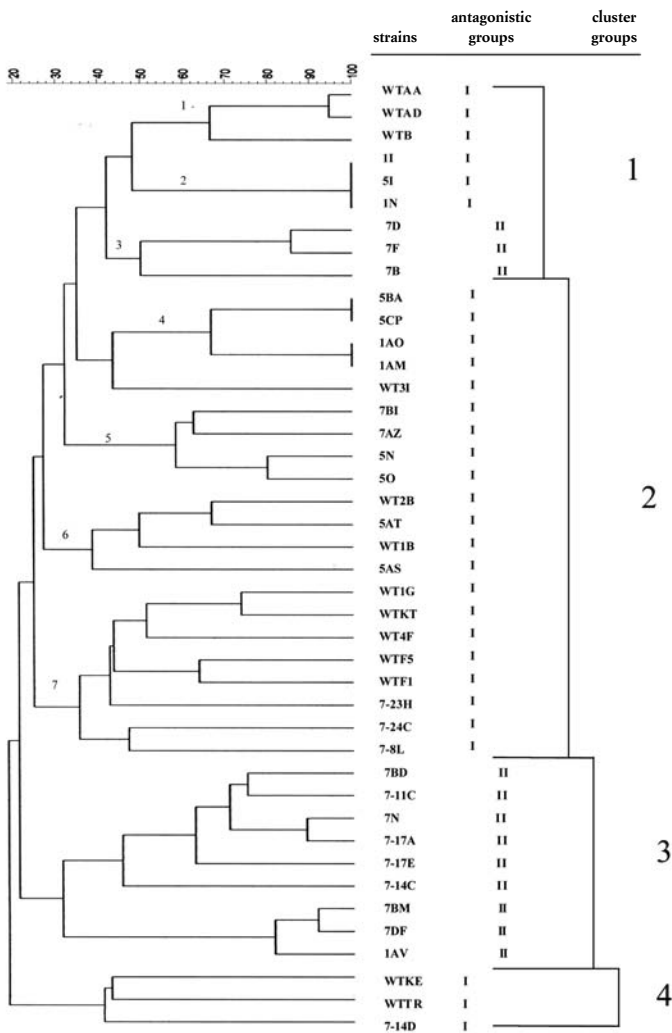


Fig. 4. Dendrogram showing relationships among 42 *P. fluorescens* strains from *rolABC* and WT Troyer citrange lines constructed using the ERIC-PCR results. Cluster analysis was performed by UPGMA with a matrix calculated with Dice's coefficient. Antagonistic groups: I, antagonistic activity on KB; II, antagonistic activity on PDA.

rol lines 1, 5 and 7 were generated by independent transformation events (Gentile *et al.*, 2004).

Fluorescent pseudomonads may be appropriate for analyzing microbial shifts in the rhizosphere since they are important and beneficial members of it, aggressive root colonizers, responsible for plant growth promotion and pathogen control (Weller, 1988; O'Sullivan and O'Gara, 1992; Lugtenberg and Dekkers, 1999).

Phenotypic analysis of *Pseudomonas* isolates based on antagonistic activity against *F. solani* and *P. tracheiphila* resulted in identification of two antagonistic groups including respectively strains isolated from transgenic and non-transgenic plant lines and strains only from transgenic lines with different biological features. Screening for antagonism on PDA and KB agar resulted in the selection of strains that produce a diverse array of inhibitory compounds and some differences be-

tween the *rolABC* and WT plant lines were observed. Antibiotic production, tested on PDA, occurred only in about 20% of fluorescent *Pseudomonas* isolated from *rolABC* lines, but not in fluorescent *Pseudomonas* isolated from wild type lines. In contrast, when tested on KB, strains were inhibitory without differences between lines. It has been amply demonstrated that the nature of test strains (e.g. strains of phytopathogen and strains of antagonist) as well as several abiotic factors like composition of culture media significantly influence antagonistic action against pathogens and affect the size of inhibition zones (Xu and Gross, 1986; Borowicz and Omer, 2000). Also in our studies the composition of culture media had an effect on the *in vitro* antagonism and allowed identification of differences between strains that were partially correlated with the transgenic character of plant lines. The reason for this difference is unclear. Future studies of the influence of *rolABC* genes on root exudate composition could clarify their influence on colonization of antagonistic pseudomonads with different modes of action.

No differences in IAA-producing strains were observed among the means of the transgenic and non-transgenic plant lines over all sampling times. The abundance of IAA-producing fluorescent pseudomonads was lower than that of isolates which were antagonistic, and the most active antagonistic strains were non IAA-producers (data not shown). Microbially derived IAA is implicated in the stimulation of plant growth (Patten and Glick, 1996) and formation and growth of lateral roots. Since *rolABC* plants are much more sensitive to cytokinins and auxins (Estruch *et al.*, 1991a,b) than non-transgenic ones, beneficial rhizobacteria able to produce IAA, considered an additional advantage for antagonistic strains, could amplify the susceptibility of transgenic plants. Future studies will be needed to assess the influence of IAA-producing rhizobacteria by comparing plant growth and root growth of *rolABC* and WT plants grown in presence of IAA-producing and non-producing strains.

The fluorescent pseudomonads isolated from *rolABC* and WT Troyer citrange rhizospheres were identified as either *P. fluorescens* or *P. putida*. These two *Pseudomonas* spp. were reported by Gardner *et al.* (1984) to be the most abundant fluorescent pseudomonads found on roots of rough lemon. These species are reported to have antagonistic activity and growth-promoting effects (Gardner *et al.*, 1984), and in our studies several *P. fluorescens* strains were able to inhibit the growth of two important pathogens of citrus, *F. solani* and *P. tracheiphila*. Furthermore, the genetically modified *rolABC* Troyer citrange plants did not appear to be of any influence in decreasing potentially beneficial populations of these bacteria, but rather the *rolABC* plants seemed to select (or promote) root colonization of antagonistic fluorescent pseudomonads producing an array of inhibitory

compounds not present in WT roots. All the data suggest that this specific transgenic plant genotype partially affected the distribution and functions of fluorescent pseudomonads in the rhizosphere in terms of antagonistic activity.

The main ARDRA restriction pattern group (group II) contained 95% of the isolates without differences between transgenic and non-transgenic lines. The ERIC-PCR fingerprints showed a high degree of DNA heterogeneity over all 42 randomly selected *P. fluorescens* strains and differences between transgenic and non-transgenic lines. Cluster analysis revealed one homogeneous group including only isolates from *rolABC* lines and belonging to the same antagonistic group, and three heterogeneous groups containing strains from transgenic and non-transgenic lines.

On the whole, the transgenic *rolABC* plants examined appeared to have little effect on antagonistic root-associated fluorescent pseudomonads. Only a partial correlation was found between clustering of strains based on their antagonistic activity, clustering of strains based on ERIC-PCR fingerprints, and transgenic and non-transgenic character of plants.

ACKNOWLEDGEMENTS

This work was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Progetto PON N°12839 "Innovazione tecnologica per il miglioramento delle produzioni e dei processi agroalimentari nelle PMI".

REFERENCES

- Bachmann G., Kinzel H., 1992. Physiological and ecological aspects of the interactions between plant roots and rhizosphere soil. *Soil Biology and Biochemistry* **24**: 546-552.
- Borowicz J.J., Omer Z.S., 2000. Influence of rhizobacterial culture media on plant growth and on inhibition of fungal pathogens. *BioControl* **45**: 355-371.
- Bruinsma M., Kowalchuk G.A., van Veen J.A., 2003. Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils* **37**: 329-337.
- Cirvilleri G., Gentile A., Gennari M., Deng Z.N., Rizzitano A., Spina S., Domina F., Abbate C., La Rosa R., 2003. Influence of transgenic *rolABC* citrus plants on root-associated bacteria. *Journal of Plant Pathology* **85**: 288.
- Di Giovanni G.D., Watrud L.S., Seidler R.J., Widmer F., 1999. Comparison of parental and transgenic alfalfa rhizosphere bacterial communities using Biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). *Microbial Ecology* **37**: 129-139.
- Dice L.R., 1945. Measurement of the amount of ecological association between species. *Ecology* **26**: 297-302.
- Dunfield K.E., Germida J.J., 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. *FEMS Microbiology Ecology* **82**: 1-9.
- Estruch J.J., Chriqui D., Grossman K., Schell J., Spena A., 1991a. The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. *Embo Journal* **10**: 2889-2895.
- Estruch J.J., Schell J., Spena A., 1991b. The protein encoded by *rolB* plant oncogene hydrolyses indole glucosides. *Embo Journal* **11**: 3125-3128.
- Gardner J.M., Chandler J.L., Faldman A.W., 1984. Growth promotion and inhibition by antibiotic-producing fluorescent pseudomonads on citrus roots. *Plant Soil* **77**: 103-113.
- Gentile A., Deng Z.N., La Malfa S., Domina F., Germanà C., Tribulato E., 2004. Morphological and physiological effects of *rolABC* genes into citrus genome. *Acta Horticulturae* **632**: 235-242.
- Gordon S.A., Weber R.P., 1951. Colorimetric estimation of indolacetic acid. *Plant Physiology* **26**: 192-195.
- Grayston S.J., Wang S., Campbell C.D., Edwards A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* **30**: 369-378.
- Gross D.C., 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. *Journal of Applied Bacteriology* **58**: 167-174.
- Heuer H., Kroppenstedt R.M., Lottmann J., Berg G., Smalla K., 2002. Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. *Applied Environmental Microbiology* **68**: 1325-1335.
- King E.D., Ward M.K., Raney D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**: 301-307.
- La Malfa S., Cirvilleri G., Rizzitano A., Spina S., Domina F., Abbate C., Deng Z., Gentile A., 2004. Evaluation of transgenic *rolABC* Citrange troyer for growth habit and root-associated bacteria. In: *X International Citrus Congress, Agadir*, 2004, 30.
- Lottmann J., Heuer H., Smalla K., Berg G., 1999. Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. *FEMS Microbiology Ecology*, **29**: 365-377.
- Louws F.J., Fullbright D.W., Stephens C.T., De Bruijn F.J., 1994. Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied Environmental Microbiology* **60**: 2285-2296.
- Lugtenberg B.J.J., Dekkers L.C., 1999. What makes *Pseudomonas* bacteria rhizosphere competent?. *Environmental Microbiology* **1**: 9-13.
- O'Sullivan D.J., O'Gara F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *FEMS Microbiology Review* **56**: 662-676.

- Oger P., Mansouri H., Dessaux Y., 2000. Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. *Molecular Ecology* **9**: 881-890.
- Patten C.L., Glick B.R., 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian Journal of Microbiology* **42**: 207-220.
- Schaad N.W., Jones J.B., Chun W., 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third Edition. APS Press, St. Paul, MN, USA.
- Siciliano S.D., Germida J.J., 1999. Taxonomic diversity of bacteria associated with roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. *FEMS Microbiology Ecology* **29**: 263-272.
- Siciliano S.D., Theoret C.M., de Freitas J.R., Hucl P.J., Germida J.J., 1998. Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Canadian Journal of Microbiology* **44**: 844-851.
- Spena A., Schmülling T., Koncz Z., Schell J., 1987. Independent and synergistic activity of *rol A*, *B* and *C* loci in stimulating abnormal growth in plants. *Embo Journal* **6**: 3891-3899.
- Weller D.H., 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26**: 379-407.
- Widmer F., Seidler R.J., Gillevet P.M., Watrud L.S., Di Giovanni G.D., 1998. A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (*sensu stricto*) in environmental samples. *Applied Environmental Microbiology* **64**: 2545-2553.
- Xu G.W., Gross D.C., 1986. Selection of fluorescent Pseudomonads antagonistic to *Erwinia carotovora* and suppressive of potato seed piece decay. *Phytopathology* **76**: 414-422.

Received 3 February 2005

Accepted 16 May 2005