PROFILE OF FLUORESCENT PSEUDOMONADS FOR BIOCONTROL USE ISOLATED FROM A BRAZILIAN AGROECOLOGICAL PRODUCTION SYSTEM

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ABSTRACT

Major part of fluorescent Pseudomonads isolated from a mixed plantation of lettuce and carrot grown in an organic experimental station (Integrated Agroecological Production System) were identified as P. putida. *Phenotypic characterization of these isolates indicated their specificity to their host plant. A cluster formed by strains with high percentage of similarity was predominant on carrot while in lettuce the isolates were grouped in discrete clusters. Genotypic characterization carried by BOX-PCR distinguished these isolates more precisely, in terms of species or genera, while ITS-RFLP profiles confirmed specificity of the isolates for plants, or even for their compartments. These results suggested that there was a level of specificity in the interaction of fluorescent* Pseudomonads *and the host plant. Specificity may be an important feature to identify efficient BCA (biological control agents)/ PGPR (plant growth promoting rhizobacteria).*

KEYWORDS

rhizobacteria, bacterial typing, plant association

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PERFIL DE PSEUDOMONAS FLUORESCENTE PARA USO EM BIOCONTROLE ISOLADAS DE SISTEMA DE PRODUÇÃO AGROECOLÓGICO BRASILEIRO

RESUMO

Grande parte dos isolados de Pseudomonads fluorescente de um consórcio de alface e cenoura cultivado em uma estação orgânica experimental (Sistema de Produção Agroecológica Integrado) foi identificada como P. putida. *A caracterização fenotípica destes isolados indicou especificidade destes pelas plantas hospedeiras. Um grupo formado por estirpes com alta porcentagem de similaridade foi predominante na cenoura, enquanto na alface, os isolados foram agrupados em grupos discretos. Na caracterização genotípica feita por BOX-PCR separou estes isolados mais precisamente, em termos de espécie e gênero, enquanto os perfis de ITS-RFLP confirmaram a especificidade dos isolados de plantas ou mesmo seus compartimentos. Estes resultados sugerem que houve um nível de especificidade na interação de* Pseudomonas *fluorescente e a planta de onde foram isolados. A especificidade pode ser um aspecto importante para identificar ACB (Agentes do Controle Biológico)/ RPCV (Rizobactérias Promotoras de Crescimento Vegetal) eficientes.*

PALAVRAS-CHAVE

rizobactérias, tipificação bacteriana, associação com plantas

Introduction

The rhizosphere effect on the microbial community influences different taxonomic, physiological and morphological groups, which form a specific community where gram-negative bacteria represent significant proportion (Alexander, 1977). Among many bacterial genera present in this habitat, the fluorescent Pseudomonads have been intensively studied due to their capacity to survive in different environments, promote plant gro-

wth (Kloepper & Schipper, 1992; Botelho & Hagler, 2006) and suppress many plant diseases (Thomashow & Weller, 1995). Plant growth promoting rhizobacteria (PGPR) can stimulate plant growth directly or indirectly (Compant et al., 2005). Plant growth can be stimulated especially by their capacity to inhibit fungi that cause plant diseases. This potential of fluorescent Pseudomonads is related to the metabolites produced by these microorganisms such as siderophores (Lemanceau *e*t al., 1995), compounds that induce resistance by plants to antagonist microorganisms (Mariano & Kloepper, 2002), biosurfactant compounds (Stanghellini & Miller, 1997) and antibiotics (Thomashow & Weller, 1988; Möenne-Loccoz et al., 2001; Raaijmakers et al., 2002).

The SIPA[4] (Sistema Integrado de Produção Agroecológica – Agroecological Production Integrated System - Seropédica/RJ) has as the main goal the development and divulgation alternative agricultural practices based on the organic agriculture and agrobiodiversity. Over the last few years in this experimental area, a reduction in the incidence of some plant diseases has been observed. Until now, the cause of this phenomenon is poorly understood. The improvement of microbial community abundance and diversity, as well as cultural practices adopted may stimulate some of the bacterial community, such as fluorescent *Pseudomonas* spp. Fonseca (2003) isolated fluorescent *Pseudomonas* spp. from a mixed crop of lettuce and carrot planted in the SIPA. Some of the isolates suppressed the development of *Sclerotinia sclerotiorum in vitro* and *in vivo* and promoted lettuce growth. The fluorescent Pseudomonad community seems to play an important role in the SIPA soil microbiota. The utilization of microorganisms as BCA/PGPR requires phenotypic and genotypic characterization for their correct identification, as well studies of their impact on the soil community (Versalovic et al., 1991; Araújo et al., 1995; Botelho et al., 1998; Seurinck et al., 2003).

4 "Fazendinha Agroecológica Km 47" ("Agroecological Farm Km.47" - Accord Embrapa, UFRuralRJ, Pesagro - Rio)

Material and methods

Fluorescent *Pseudomonas* spp. isolates

Fifty-four isolates previously obtained from the rhizosphere (RF), rhizoplane (RP) and inner root tissues (ED - endorhizosphere) of lettuce and carrot cultivated in a mixed cropping system located in the SIPA were analyzed (Fonseca, 2003).

Phenotypic characterization

Isolates were identified and characterized by their metabolic profile by the kit API 20NE (Biomérieux®, France). The isolates were previously inoculated on plates containing KMB (King et al., 1954) for 24h at 28°C. After this period, one to four colonies were transferred to tubes containing 2mL of NaCl 0.9% (w/v). The optical density strip inoculations and their analyses were performed according to the manufacturer´s recommendation. Based on the metabolic pattern, similarity among isolates was calculated according to UPGMA algorithm and Jaccard coefficient by software BioDiversity Professional v.2.

Genotypic fingerprinting

DNA extraction

DNA extraction of the isolates was carried out by phenol: chloroform: isoamilic acid method according to Xavier et al. (2004). The suspensions were then quantified by optical density - 260 ηm (Ausubel et al., 1992) using UV 1201 spectrophotometer (Shimazu Corp., Japan). The suspensions were diluted to final concentration of 100μg/μL. Different molecular techniques were used.

ITS1 restriction analysis

Thirty mL of PCR mixture constituted of 1x buffer enzyme (Invitrogen Corp) 3mM de $MgCl_2$ (Invitrogen Corp.), 200μM dNTP, 2.5% formamide 250 μM of each primer, 5 U of Taq polymerase (Invitrogen Corp.) and 3ηg/μL of DNA. The primers fPs16S and rPs23S and PCR program were described by Locatelli

et al. (2002). After the PCR, 3μL of the reactions were deposited in 1% agarose gels at 90V for 30 min and checked at UV light in IMAGO Compact Imaging System, B&L system.

The restriction of amplified fragment was performed using TaqI (Promega Corp.). Fifteen mL of the final mixture constituted of 2x enzyme buffer, 2U/μL of TaqI and 6μL of PCR reaction adjusted previously by band intensity in the agarose gel. After incubating the mixture for 3h at 37°C, all product was placed on 5% polyacrilamide gel at 60V for 16h. Gel was stained with 1% SyberGold™ and checked with UV light in IMAGO Compact Imaging System, B&L system. Band profiles were analyzed by software Gelcompare II version 3.5 (Applied Maths, Inc.) that generated similarity dendrograms according to UPGMA algorithm and Jaccard coefficient.

BOX-PCR analysis

Twenty five mL of PCR mixture of 1x Gitschier buffer (Rademaker et al., 1998), 170μg/mL of BSA (Bovine Serum Albumin), 5% DMSO (Dymethil sulfoxide), 125μM dNTP, 400ρM of BOX A1R primer (Versalovic *et al*., 1994), 1.5 U of Taq polymerase (Invitrogen, Belgium) and 1ηg/μL of DNA. The PCR program was carried out as described by Versalovic *et al*. (1994). After the PCR, 3μL of the reactions were placed on 1% agarose gels at 90V for 30 min and checked with UV light in the IMAGO Compact Imaging System, B&L system. Eight mL of the PCR product were placed on 5% polyacrilamide gel at 60V for 16h. Gels were stained with 1% SyberGold™ and visualized with UV light on IMAGO Compact Imaging System, B&L system. Band profiles were analyzed by software Gelcompare II version 3.5 (Applied Maths, Inc.) that generated similarity dendrograms according to the UPGMA algorithm and Jaccard coefficient.

Results and discussion

Phenotypic profile

Most of the isolates were identified as *P. putida* by API 20NE (Biomeriéux®) (table 1). Eighty one percent of the isolates

were identified as *P. putida*, 7% as *P. fluorescens* and 11% as other Pseudomonads species or genera. Many of these other genera were identified as *Burkholderia* which is a genus derived from *Pseudomonas* (Yabuuchi *et al*., 1992). Despite of the low number of *P. fluorescens* isolates, they were obtained from both lettuce and carrot compartments (non-rhizospheric soil) but two of the isolates which displayed the highest degree of identification with *P. fluorescens*, (above 95%), were isolated from the rhizoplane.

Based on the metabolic fingerprinting of the isolates, the dendrogram of similarity clustered the isolates in three different groups (figure1). Group A1 comprised sub-clusters A1a e A1b that assembled almost exclusively P. *putida*. Sub-cluster A1a clustered isolates with a higher identity percentage (83-96%). On the other hand, A1b assembled isolates with lower identity percentage (>65%). *P. fluorescens*, other species and genera were spread in the groups A2 and A3. Two isolates, identified as *P. alcaligenes*, did not match the groups.

The A1a and A1b sub-clusters contained higher numbers of rhizoplane isolates from the two plants, followed by the population of non-rhizospheric soil and finally, from the rhizosphere (Figures 2a and 2b). The rhizosphere and rhizoplane populations were very similar for the two plant species. Nevertheless, it was observed that in the rhizoplane of lettuce, the size of the population from A1a sub-cluster was similar to the A1b, contrasting with the higher population of A1b in the rhizosphere and non-rhizospheric soil. In the carrot rhizoplane, isolates from A1b were predominant, as was the case in the rhizosphere and non-rhizospheric soil. The population of A1a in carrot rhizosphere was significantly lower than in the other compartments. It is important to notice that in the rhizoplane of the two plants, only *P. putida* and *P. fluorescens* were identified. The bacterial community isolated from the inner tissues of the plants was low but *P. putida* was present in both plants.

Our results confirmed the previous observations reported by Fonseca (2003). The author´s analysis clustered the isolates in 12 different morphological groups and analyzed 9 different

features: among them elasticity that was considered more appropriate to distinguish between *P. putida* and *P. fluorescens* (Zago, 2003). The previous observations were confirmed by our results, after identifying part of the isolates using the metabolic profile. Different groups generated by the biochemical features indicated that there is significant metabolic versatility among isolates that is a characteristic of genus *Pseudomonas* (Stanier *et* al., 1966). These data showed that the majority of the isolates originated from the non-rhizospheric soil, rhizosphere and rhizoplane were *P. putida*. The majority of isolates were obtained from the rhizoplane of the plants, as a result of the rhizosphere effect. Just one isolate from carrot rhizoplane was identified as *P. fluorescens*. In lettuce, there were two isolates identified as *P. fluorescens* and *Alcaligenis* spp. Despite of the low number of isolates from the endorhizosphere, *P. putida* was predominant as in other plant compartments. The diversity of species and genera identified could suggest that there was no specificity for its colonization. Nevertheless, refined studies should be performed to better understand the endorhizosphere colonization.

From the metabolic characterization, the population of *P. putida* from non-rhizospheric soil was approximately equally distributed between the two sub-clusters A1a and A1b. For lettuce, the rhizosphere population showed this same characteristic, while in the rhizoplane, there was a slight improvement of A1a population. On the other hand, for carrot, the A1b population was predominant in the rhizosphere and, especially, in the rhizoplane. These results suggested specificity of the groups of *P. putida* for plants. The carrot rhizosphere seemed to induce populations that had a larger range of identification percentage. It could indicate that: (a) specificity of strain colonization: particular metabolic pathways could cause high range of percentage of identification that could not be predicted by the kit for identification; or (b) the large number of strains capable of colonizing carrot rhizosphere, or even new species associated on it (Botelho & Hagler, 2006; Costa *et al*, 2006). This specificity is not so evident for lettuce, despite the slight increase in the number of

P. putida with higher percentage of identification. Some species colonize specifically a certain compartment of the root and/or even certain plants. This ability is also related to the root exsudate composition (Campbell & Greaves, 1990). Lemanceau et al. (1995) observed the root compartment affinity of species and biovars of fluorescent Pseudomonads in flax and tomato. By the phenotypic profile, they observed that three clusters included only flax isolates and two only tomato isolates. They described ten substrates that permitted them to discriminate strains belonging to these two clusters. Germida & Siciliano (2001) observed Pseudomonads were more abundant in the rhizosphere of older wheat cultivars, but were the most dominant endophytes in the newer cultivars. Kravchenko et al. (2003) observed that the utilization of root exometabolites by plant growth–promoting rhizobacteria might influence their growth and antifungal activity. They proposed that sugar and organic acid composition of the root exudates influenced the establishment of introduced rhizobacteria in the roots, as well as antifungal activity. Recently, Strigul & Kravchenko (2006) observed that an important factor for PGPR survival was the compatibility between composition of the host plant root exsudates, and ability of the PGPR to metabolize those compounds.

Genotypic fingerprinting

Primers used for ITS analysis amplified in 1 to 2 bands. These bands had 1100bp to 1300bp (figure 3a), indicating variation in the number of copies of ITS1 region. RFPL analysis of the amplified fragments demonstrated high polymorphism among the isolates (figure 3b). Most of the bands were between 350bp and 1300bp. This fingerprinting generated a dendrogram with two main groups (figure 4). The IT1 cluster consisted of bacteria from rhizoplane and rhizosphere for the two plants (Figures 5a and 5b), and non-rhizospheric isolates. In lettuce, the major part of the rhizoplane isolates were found in this group, while in carrot, they clustered mainly in the group IT2. The IT2 cluster was

formed by isolates from all sources, including endorhizosphere isolates. An isolate that did not fit on any group was obtained from lettuce endorhizosphere.

The dendrogram generated by BOX-PCR clustered the isolates in 4 different groups (Figure 6). Results indicated that this analysis better classified the isolates taxonomically than by plant/compartment affinity. Comparison between BOX – PCR and metabolic groups suggested this observation was valid (Figure 7). Groups B1 and B3 comprised mainly A1a and A1b (metabolic clustering) that contained all *P. putida*. B1 assembled mostly A1b that were classified as *P. putida* with larger percentage range of identification and this data suggested being significantly specific to this cluster. B3 gathered the two sub-groups but the presence of the A1a group(the group that aggregated mainly *P. putida* with higher percentage of identification) was higher. Despite their predominance, it was not so specific due to the presence of other clusters. B2 and B4 did not show any significant specificity to taxonomic groups. It is important to notice that some isolates did not react to BOX primers, so they were not included in this correlation.

To confirm the correlation of the two analysis results, the distribution of species in the BOX – PCR clusters were examined. Results were similar to those obtained by correlation. B1 and B4 clusters consisted of isolates identified as *P. fluorescens* and *P. putida* (Figure 8). B1 gathered mainly the *P. putida* classified in the larger percentage range of identification based on metabolic profiles. The B2 cluster contained both species, other species of *Pseudomonas* and genera, while B3 consisted mainly of *P. putida*, those with higher percentage of identification.

BOX-PCR and ITS-RFLP trials suggested the great diversity of isolates from the Agroecological Production System. These results are in agreement to those obtained by Costa et al. (2006) that observed large genotypic diversity and variability among *P. putida* in the Brazilian agrosystems. Analysis of BOX-PCR clustered isolates with regard to the species or genera better than specificity for compartment/plant. B1 and B4 BOX-PCR groups were formed only for fluorescent Pseudomonads (*P. putida* e *P. flu-*

orescens), while B2 and B3 group also included other species and genera. De Bruijn et al. (1997) described rep-PCR analysis (including BOX-PCR) as higher discriminatory power for classification and identification of bacteria. Our results are in agreement with this observation since BOX-PCR better clustered isolates in terms of species, despite of the significant diversity of fluorescent *Pseudomonas*. Group B1 and B4 that clustered exclusively fluorescent *Pseudomonas* showed similarity of 80% and 67% (Figure 7). This lower similarity in B4 would be related to the higher diversity of this group and/or lower number of bands that could make the analysis of the isolates difficult.

Many reports indicated rep-PCR (including BOX-PCR), as a good method to characterize strains/isolates of species. Seo and Tsuchiya (2005) clustered *Burkholderia cepacia* by rep-PCR (ERIC and BOX-PCR) and obtained five clusters. Three of them were more closely related to each other than the last two groups. The results were similar to those reached by PCR-RFLP analysis of *fliC* (flagellin gene) size. They did not detected significant differences among isolates from clinical and environmental sources. Vinuesa et al. (1998) found a finer taxonomic resolution to the genomic fingerprinting of *Bradyrhizobium* strains. Combining BOX, REP and ERIC PCR genomic analysis, it was possible to maximize strain discrimination and the phylogenetic coherency of the clusters. Cho & Tiedje (2000) observed high endemicity at the level of genome structure using BOX-PCR to analyze fluorescent *Pseudomonas* isolates from different regions.

ITS analysis distributed isolates in two main groups. One group consisted of only fluorescent *Pseudomonas* isolated mainly from rhizoplane of lettuce. The second group comprised other genera and species mainly isolated from the rhizoplane of carrot. This group included isolates from the endorhizosphere that were not present in the first group. ITS1 sequences are very similar for strains belonging to the same biovar or genomovar, while frequent insertion and deletion events are displayed in different sub-especies (Guasp et al. 2000; Locatelli et al., 2002). Restriction analysis of ITS1 can be used to differentiate Pseudomonas

strains at infra-specific level (Cho and Tiedje, 2000, Jeng et al., 2001). The restriction of the ITS region showed a high range of polymorphism amongst the isolates and clustering by plant/compartments. It could suggest that the *Pseudomonas* community is diverse and specific to each plant and/or even, to its root compartments. Concerning *Pseudomonas* diversity, it is important to note that the evidence indicated populations of *Pseudomonas* exchange DNA, when analyzing 16S rDNA (Stove *et al.*, 2000; Lomholt *et al.*, 2001), but despite this they can be defined by clear genetic and phenotypic clustering (Palleroni, 2003).

It was observed that each ITS group was sub-divided into three sub-clusters. One sub-cluster of group ITS1 (C) and two of ITS2 (A and C) comprised only isolates from plants. The sub-cluster ITS2 (A) mainly contained isolates from carrot, reinforcing that ITS-RFLP analysis is able to distinguish isolates by plant host affinity.

This study showed the specificity of fluorescent *Pseudomonas* isolates for plants and/or root compartments, especially with regard to the rhizoplane. This specificity was better evaluated by ITS/RFLP. Lettuce rhizoplane isolates had a polymorphic profile different from carrot rhizoplane isolates that permitted clustering them basically in two different groups.

The results reinforce the idea that phenotypic and genotypic characterization of microorganisms used in biological control and/or plant growth promotion are essential for choosing microorganisms well-adapted to the root, improving the success of applying them as inoculants. Besides it is possible to trace these introduced microorganisms to evaluate their fate in the environment, as well as their impact on soil biota.

Acknowledgments

We are grateful to FAPERJ – Foundation for Research Development of Rio de Janeiro (Brazil) and Embrapa (Brazilian Agricultural Research Corporation) for financial assistance. We acknowledge Dr. Phillippe Lemanceau (INRA-Dijon) for papers and suggestions, Dr. Leda C.S.M. Hagler (UFRJ/IMPPG) for re-

viewing and Dr. Jerri E. Zilli (Embrapa/CNPAfrr) for procedure suggestions.

References

ALEXANDER, M. Ecological interrelationships: Microbiology of rhizosphere. In:ALEXANDER, M., (Ed.) *Soil Microbiology*. Nova York: Ed. Wiley Eastern Limited, 1977. p. 423-437.

ARAÚJO, M. A.; MENDONÇA-HAGLER, L. C.; HAGLER, A. N.; VAN ELSAS, J. D. Competition between a genetically modified *Pseudomonas fluorescens* and its parent in sub-tropical soil microcosms. *Revista de Microbiologia*, v. 26, p.1-15, 1995.

AUSUBEL, F. M.; BRENT, R.; KINGSTON, R. E.; MOORE, D. D.; SEIDMAN, J. G.; SMITH, J. A.; STRUHL, K. *Short protocols in molecular biology*. New York: Ed. John Wiley & Sons, 1992. 740p.

BOTELHO, G. R.; HAGLER, L. C. M. Fluorescent Pseudomonads associated with the rhizosphere of crops - an overview. *Brazilian Journal of Microbiology*, v.37, n.4, p.401- 416, 2006.

BOTELHO, G. R.; GUIMARÃES, V.; DE BONIS, M.; FONSECA, M. E. F.; HAGLER, A. N.; HAGLER, L. C. M. Ecology of a plant growth-promoting strain of *Pseudomonas fluorescens* colonizing the maize endorhizosphere in tropical soil.

World Journal of Microbiology & Biotechnology, v.14, p.499- 504, 1998.

DE BRUIJN, F. J.; SCHNEIDER, M.; ROSSBACH, U.; LOUWS, F. J. Automated fluorescent and conventional Rep-PCR genomic fingerprinting and multiplex PCR to classify bacteria and track genes. In: MARTINS, M.T.; SATO, M.I.Z.; TIEDJE, J.M.; HAGLER, L.C.N.; DÖBEREINER, J.; SANCHEZ, P.S., (Ed.) *Progress in Microbial Ecology*. São Paulo: Ed. SBM, 1997, p.197-205.

COMPANT, S.; DUFFY, B.; NOWAK, J.; CLÉMENT, C.; BARKA, E. A. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied Environmental. Microbiology*, v.71, n.9, p.4951-4959, 2005.

COSTA, R.; GOMES, N. C. M.; PEIXOTO, R. S.; RUMJANEK N.; BERG, G.; HAGLER, L.C.S. M.; SMALLA, K. Diversity and antagonistic potential of Pseudomonas spp. associated to the rhizosphere of maize grown in a subtropical organic farm. *Soil Biology and Biochemistry*, **v.**38, n.8, p.2434-2447, 2006.

DELORME, S.; PHILIPPOT, L.; EDEL-HERMANN, V.; DEULVOT, C.; MOUGEL, C.; LEMANCEAU, P. Comparative genetic diversity of *narG*, *narZ*, and 16S rRNA genes in fluorescent Pseudomonads. *Applied and Environmental Microbiology*, v. 69, n.2, p.1004-1012, 2003.

FONSECA, M. C. C. Diversidade de *Pseudomonas* spp. fluorescente no Sistema Integrado de Produção Agroecológica (SIPA). Seropédica, 2003.136p. Thesis (Ph.D.) - Universidade Federal Rural do Rio de Janeiro.

GARCÍA – MARTINEZ, J.; ACINAS, S. G.; ANTÓN, A. I.; RODRÍGUEZ-VALERA, F. Use of 16-23S ribosomal genes spacer region in studies of prokaryotic diversity.

Journal of Microbiological Methods, v. 36, p.55-64, 1999.

GERMIDA, J. J.; SICILIANO, S. D. Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. *Biology and Fertility of Soils*, v.33, p.410–415, 2001.

GUASP, C.; MOORE, E. R.; LALUCAT, J.; BENNASAR, A. Utility of internally transcribed 16S-23S rDNA spacer region for the definition of *Pseudomonas sttzeri* genomovars and other *Pseudomonas* species. *International Journal of Systematic and Evolutionary Microbiology*, v.50, p.1629-1639, 2000.

GÜRTLER, V.; STANISICH, V. A. New approaches to typing and identification of bacteria using the 16S – 23S rDNA spacer. *Microbiology*, v.142, p.3-16, 1996.

HULTON, C. S. J.; HIGGINS, F.; SHARP, P. M. ERIC sequences: a novel family of repetitive elements in the genome of *Escherichia coli, Salmonella typhimurium* and others enteric bacteria. *Molecular Microbiology*, v.5, p.825-834, 1991.

JAE-CHANG CHO, J.C.; TIEDJE, J. M. Biogeography and Degree of Endemicity of Fluorescent *Pseudomonas* Strains in Soil. *Applied and Environmental Microbiology*, v.66, n.12, p.5448-5454. 2000.

JENG, R. S.; SVIRCEV, A. M.; MYERS, A. L.; BELIAEVA, L.; HUNTER, D. M.; HUBBES, M. The use of 16S-23S rDNA to easily detect and differenciate com gram-negative orchard epiphytes. *Journal of Microbiological Methods*, v.44, p.69-77, 2001.

JENSEN, M. A.; WEBSTER, J. A.; STRAUS, N. Rapid identification of bacterial on the basis of polymerase chain reaction amplified ribosomal DNA spacer polymorphisms. *Applied Environmental Microbiology*, v. 59, n.4, p. 945 – 952, 1993.

KING, E. O.; WARD, M. K.; RANEY, D. E. Two simple media for demonstration of pyocianin and fluorescein. *Journal of Laboratory and Clinical Medice*, v.44, p.301-307, 1954.

KLOEPPER, J. W.; SCHIPPERS, B.; BAKKER, P. A. H. M. Proposed elimination of the term endorhizosphere. *Phytopathology*, v.82, n.7, p.726-727, 1992.

KRAVCHENKO, L. V.; AZAROVA, T. S.; LEONOVA-ERKO, E. I.; SHAPOSHNIKOV, A. I.; MAKAROVA, N. M.; TIKHONOVICH, I. A. Root Exudates of Tomato Plants and Their Effect on the Growth and Antifungal Activity of *Pseudomonas* Strains. *Microbiology*, v.72, n.1, p.37–41, 2003.

LEMANCEAU, P.; CORBERAND, T.; GARDAN, L.; LATOUR, X.; LAGUERRE, G.; BOEUFGRAS, J. M.; ALABOUVETTE, C. Effect of two plant species, flax (*Linum Usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. *Applied Environmental Microbiology*, v.61, n.3, p.1004-1012, 1995.

LOCATELLI, L.; TARNAWSKI, S.; HAMELIN, J.; ROSSI, P.; ARAGNO, M.; FROMIN, N. Specific PCR amplification for the genus *Pseudomonas* targeting the 3' half of 16S rDNA and the whole 16S-23S rDNA spacer. *Systematic and Applied Microbiology*, v.25, p.220-227, 2002.

LUPSKI, J. R.; WEINSTOCK, G. M. Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bacteriology*, v. 174, p.4525-4529, 1992.

MARIANO, R. L. R.; KLOEPPER, J. W. Método alternativo de biocontrole: resistência sistêmica induzida por bactérias. *Revisão anual de patologia de plantas*, v. 8, p.121-137, 2000.

MARTIN, B.; HUMBERT, O.; CAMARA, M.; GUENZI, E.; WALKER, J.; MITCHELL, T.; ANDREW, P.; PRUDHOMME, M.; ALLOING, G.; HAKENBECK, R.; MORRISON, D. A.; BOULNOIS, G. J.; CLAVERS, J. P. A higly conserved repeated DNA element in the chromossome of *Streotococcus pneumoniae*. *Nucleic Acids Research*, v.20, p.3479-3483, 1992.

MOËNNE-LOCCOZ, Y.; TICHY, H. V.; O'DONNELL, A.; SIMON, R. O'GARA, F. Impact of 2,4-Diacetylphloroglucinol-Producing Biocontrol Strain *Pseudomonas fluorescens* F113 on Intraspecific diversity of resident culturable fluorescent Pseudomonads associated with the roots of field-grown sugar beet seedlings, v. 67, n.8, p.3418-3425, 2001.

NORMAND, P.; PONSONNET, C.; NESME, X.; NEYRA, M.; SIMONET, P. ITS analysis of prokariotes. In: AKKERMANS, D.L.; VAN ELSAS, J.D.; BRUIJN, F.J., (Ed.) *Molecular Microbial Ecology Manual*. Dordrecht: Kluwer Academic Publishers, 1996, Chapter 3.4.5, p. 1-12.

OLSEN, G. J.; WOESE, C. R.; OVERBEEK, R. The winds of (evolutionary) change:breathing new life into microbiology. *Journal of Bacteriology*, v.176, p.1-6, 1994.

RAAIJMAKERS, J. M.; VLAMI, M.; DE SOUZA, J. T. Antibiotic production by bacterial biocontrol agents. *Antonie Leeuwenkoek*, v.81, p.537-547, 2002.

RADEMAKER, J. L. W.; LOUWS, F. J.; DE BRUIJN, F. J. Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: AKKERMANS, A. D. L.; VAN ELSAS, J. D.; DE BRUIJN, F. J .(Eds.) *Molecular Microbial Ecology Manual*. Dordrecht: Kluwer Academic Publishers, 1998, supplement 3, chapter 3.4.3, p. 1-26.

SEO, S-T.; TSUCHIYA, K. Genotypic characteriztion of *Burkholderia cenocepacia* strains by rep-PCR and PCR-RFLP of *fliC* gene. *FEMS Microbiology Letters*, v.24, p.19-24, 2005.

STANGHELLINI, M. E.; MILLER, R. M. Biosurfactants: their identity and potential efficacy in the biological control of zoosporic plant pathogens. *Plant disease*, v.81, p.4-12, 1997.

STERN, M. J.; AMES, G. F-L.; SMITH, N. H.; ROBINSON, E. C.; HIGGINS, C. F. Repetitive extragenic palindromic sequences: a major component of bacterial genome. **Cell** v.37, p.1015-1026, 1984.

STRIGUL, N. S.; KRAVCHENKO, L. V. Mathematical modeling of PGPR inoculation into the rhizosphere. *Environmental Modelling & Software*, v.21, n.8, p.1158 – 1171, 2006.

THOMASHOW, L. S.; WELLER D. M. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in Biological Control of *Gaeumannomyces graminis* var. *tritici*. *Journal of Bacteriology*, v.170, n.8, p.3499-3508, 1988.

VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J. R. Distribution of repetitive DNA-sequences in eubacteria and application to fingerprinting of bacterial genomes.

Nucleic Acids Research, v.19, p.823-6831, 1991.

VERSALOVIC, J.; SCHNEIDER, M.; DE BRUIJN, F. J.; LUPSKI, J. R. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods in Molecular and Cellular Biology*, v.5, p.25-40, 1994.

VINUESA P, RADEMAKER J L W, DE BRUIJN F J AND WERNER D. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes on the Canary Islands by PCR-Restriction Fragment Length Polymorphism Analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting and partial 16S rDNA sequencing. *Applied and Environmental Microbiology Journal*, v.64, n.6, p.2096-2104, 1998.

WHITAKER, R. J.; BANFIELD, J. F. Population genomics in natural microbial communities. *Trends in Ecology and Evolution*, v.21, n.9, p.508-516, 2006.

WOESE, C. R. Bacterial evolution. *Microbiological Reviews*, v.51, p.221-271, 1987.

YABUUCHI, E.; KOSAKO, Y.; OYAIZU, H.; YANO, I.; HOTTA, H.; HASHIMOTO, Y.; EZAKI, T.; ARAKAWA, M. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes, 1981) comb. nov. *Microbiology and Immunology*, v.36, p.1251-1275. 1992.

XAVIER, G. R.; SILVA, F.V.; ZILLI, J. É.; RUMJANEK, N.G. Série Documentos 171: Adaptação do método para extração de DNA de microrganismos associados a raízes de plantas. *Seropédica:* Embrapa Agrobiologia, 2004. 24 p.

Table 1 – Specie identification of SIPA isolates from carrot-lettuce consortium (ND –Not Determined)

Isolate	Specie	Plant	Plant compartment	% identity
EDA2.1	*P. putida*	Lettuce	Endorhizosphere	96
EDA3.1	*P. alcaligenes*	Lettuce	Endorhizosphere	ND
EDA4.1	*P. fluorescens*	Lettuce	Endorhizosphere	69
EDC1.1	*P. putida*	Carrot	Endorhizosphere	ND
EDC1.2	*P.alcaligenes*	Carrot	Endorhizosphere	ND
EDC4.3	*B. pseudomallei*	Carrot	Endorhizosphere	87
RF1.2	*P. fluorescens*	Soil	non-rhizospheric	88
RF1.5	*P. putida*	Soil	non-rhizospheric	ND
RF2.8	*P. putida*	Soil	non-rhizospheric	94
RF2.1	*P. putida*	Soil	non-rhizospheric	65
RF2.2	*P. putida*	Soil	non-rhizospheric	65
RF2.3	*Aeromonas*	Soil	non-rhizospheric	ND
RF2.4	*P. putida*	Soil	non-rhizospheric	93
RF2.5	*P. fluorescens*	Soil	non-rhizospheric	69
RF3.4	*P. putida*	Soil	non-rhizospheric	65
RF3.5	*P. putida*	Soil	non-rhizospheric	65
RF3.7	*P. putida*	Soil	non-rhizospheric	93
RF4.2	*P. putida*	Soil	non-rhizospheric	93
RFA1.1	*P. putida*	Lettuce	Rhizosphere	65
RFA2.2	*P. putida*	Lettuce	Rhizosphere	65
RFA2.5	*P. putida*	Lettuce	Rhizosphere	94
RFA3.3	*P. putida*	Lettuce	Rhizosphere	ND
RFA3.5	*P. putida*	Lettuce	Rhizosphere	86
RFA4.1	*P. aeruginosa*	Lettuce	Rhizosphere	95
RFC1.1	*P. putida*	Carrot	Rhizosphere	65
RFC1.3	*P. mendocina*	Carrot	Rhizosphere	44
RFC1.4	*P. putida*	Carrot	Rhizosphere	65
RFC1.5	*P. putida*	Carrot	Rhizosphere	65

Continua...

RFC2.11	*P. putida*	Carrot	Rhizosphere	65
RFC3.2	*P. putida*	Carrot	Rhizosphere	65
RFC4.6	*P. putida*	Carrot	Rhizosphere	94
RFC4.7	*A. xylosoxodans*	Carrot	Rhizosphere	74
RPA1.5	*P. putida*	Lettuce	Rhizoplane	65
RPA1.9	*P. putida*	Lettuce	Rhizoplane	83
RPA2.1	*P. putida*	Lettuce	Rhizoplane	94
RPA2.5	*P. putida*	Lettuce	Rhizoplane	65
RPA3.10	*P. putida*	Lettuce	Rhizoplane	93
RPA3.11	*P. putida*	Lettuce	Rhizoplane	65
RPA3.12	*P. putida*	Lettuce	Rhizoplane	65
RPA3.3	*P. putida*	Lettuce	Rhizoplane	94
RPA3.9	*P. putida*	Lettuce	Rhizoplane	83
RPA4.4	*P. fluorescens*	Lettuce	Rhizoplane	95
RPA4.5	*P. putida*	Lettuce	Rhizoplane	98
RPC1.2	*P. putida*	Carrot	Rhizoplane	65
RPC1.4	*P. putida*	Carrot	Rhizoplane	94
RPC2.10	*P. putida*	Carrot	Rhizoplane	96
RPC2.3	*P. putida*	Carrot	Rhizoplane	65
RPC2.6	*P. putida*	Carrot	Rhizoplane	65
RPC3.2	*P. putida*	Carrot	Rhizoplane	93
RPC3.4	*P. putida*	Carrot	Rhizoplane	65
RPC3.9	*P. putida*	Carrot	Rhizoplane	65
RPC4.10	*P. putida*	Carrot	Rhizoplane	93
RPC4.12	*P. putida*	Carrot	Rhizoplane	65
RPC4.4	*P. fluorescens*	Carrot	Rhizoplane	99

Conclusão

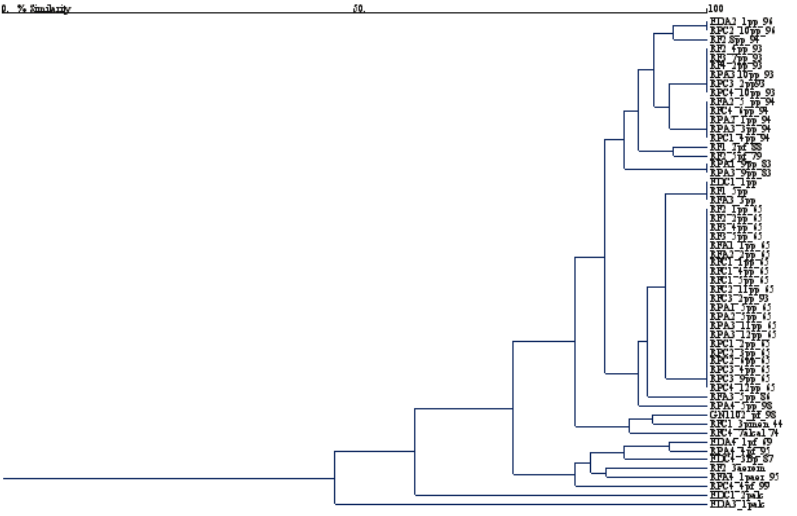


Figure 1 – Metabolic fingerprinting of fluorescent *Pseudomonas* spp. (Dendrogram of similarity using UPMGA and Jaccard coefficient).

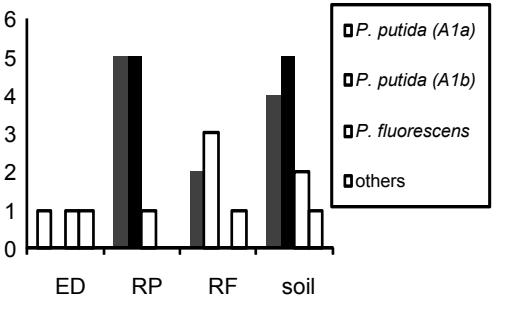
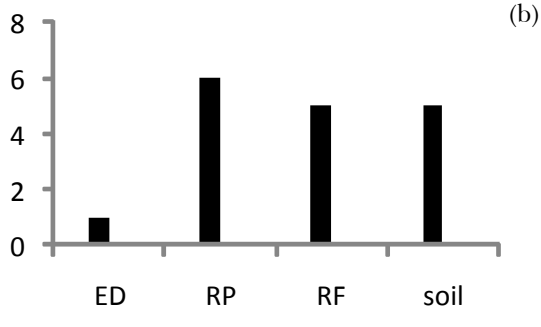


Figure 2 – Distribution of fluorescent Pseudomonads species on lettuce and carrot roots. (a) – Compartment distribution at lettuce root; (b) - Compartment distribution at carrot root. (RF) – rhizosphere; (RP) - rhizoplane ; (ED) – endorhizosphere; Soil - non-rhizospheric soil.



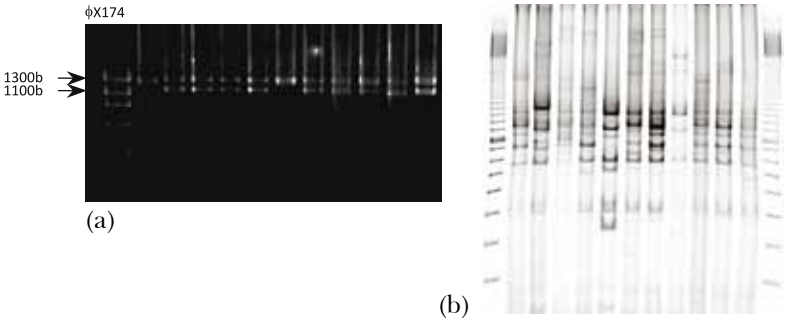


Figure 3 – ITS region amplification of fluorecent Pseudomonads from SIPA. (a) – ITS PCR; (b) – ITS/RFLP using Taq I. enzyme

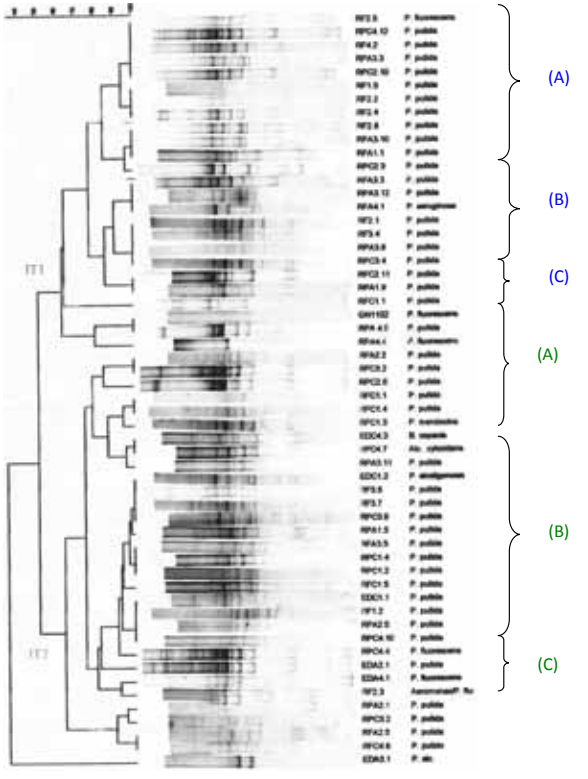


Figure 4 – Genotypic fingerprinting of fluorescent *Pseudomonas* spp. by ITS - RFLP (Dendrogram of similarity using UPMGA and Jaccard coefficient).

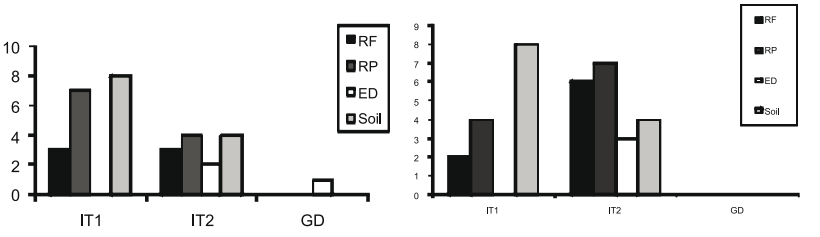


Figure 5 – Distribution of fluorescent Pseudomonads by ITS analysis. (a) – lettuce; (b) – carrot; Soil – non-rhizospheric soil; RP – rhizoplane; RF – rhizosphere; ED – Endorhizosphere.

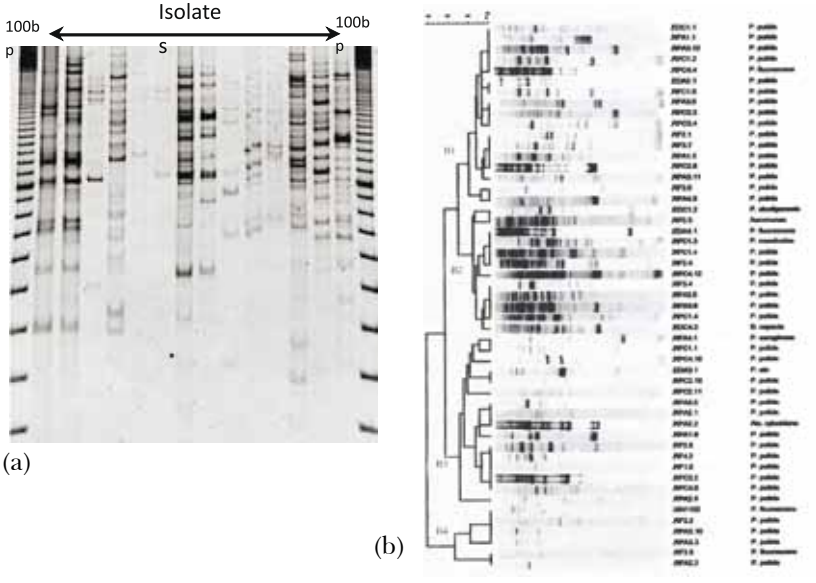


Figure 6 – (a) Genotypic fingerprinting of fluorescent *Pseudomonas* spp. by BOX – PCR. (b) - dendrogram of similarity using UPMGA and Jaccard coefficient).

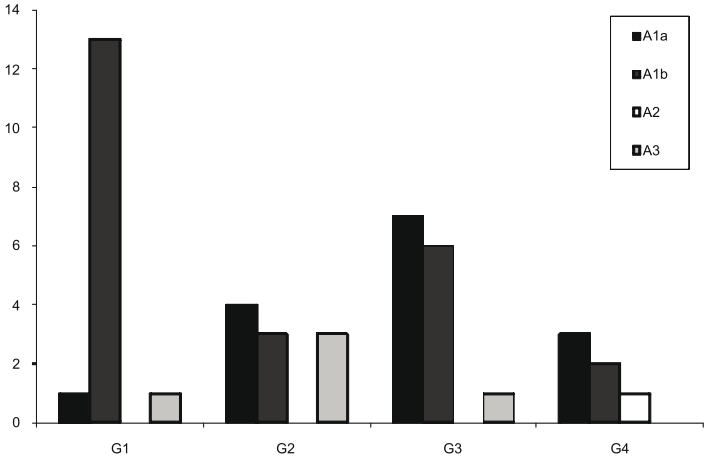


Figure7 – Correlation between BOX-PCR and metabolic profiles

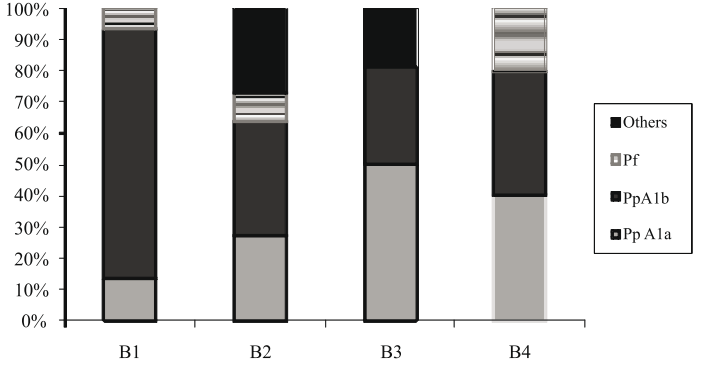


Figure 8 – Distribution of fluorescent Pseudomonads by BOX-PCR analysis. (Pp A1a) – *P. putida* - higher percentage of identification; (Pp A1b) – *P. putida* - larger identification range; (Pf) – *P. fluorescens*; Others – species and genera