Rhizosphere bacterial communities of potato cultivars evaluated through PCR-DGGE profiles

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Abstract – The objective of this work was to determine the shifts on the PCR-DGGE profiles of bacterial communities associated to the rhizosphere of potato cultivars, in order to generate baseline information for further studies of environmental risk assessment of genetically modified potato plants. A greenhouse experiment was carried out with five potato cultivars (Achat, Bintje, Agata, Monalisa and Asterix), cultivated in pots containing soil from an integrated system for agroecological production. The experiment was conducted in a split plot randomized block design with five cultivars, three sampling periods and five replicates. Rhizosphere samples were collected in three sampling dates during plant development. DNA of rhizosphere microorganisms was extracted, amplified by PCR using bacterial universal primers, and analyzed through DGGE. Shifts on the rhizosphere bacterial communities associated to rhizosphere of different cultivars were related to both cultivar and plant age. Differences among rhizosphere bacterial communities were clearest at the earliest plant age, tending to decrease in later stages. This variation was detected among bacterial communities of the five tested cultivars. The characterization of soil microbial communities can be part of plant breeding programs to be used on studies of environmental risk assessment of genetically modified potatoes.

Index terms: *Solanum tuberosum*, associative bacteria, molecular marker, PCA.

Comunidades bacterianas associadas à rizosfera de cultivares de batata avaliadas por perfis de PCR-DGGE

Resumo – O objetivo deste trabalho foi determinar as alterações nos perfis de PCR-DGGE das comunidades bacterianas associadas à rizosfera de cultivares de batata, para obter informações para futuros estudos de avaliação de risco ambiental de plantas de batatas geneticamente modificadas. Foi conduzido experimento em casa de vegetação com cinco cultivares de batata (Achat, Bintje, Ágata, Monalisa e Asterix), cultivadas em vasos com solo de um sistema integrado de produção agroecológica. O delineamento experimental foi o de blocos ao acaso, em parcelas subdivididas, com cinco cultivares, três períodos amostrais e cinco repetições. As amostras de rizosfera foram coletadas em três diferentes épocas durante o desenvolvimento das plantas. O DNA dos microrganismos associados à rizosfera foi extraído, amplificado por PCR com uso de iniciadores universais para bactérias e analisados por DGGE. Foram observadas alterações, relacionadas à cultivar e à idade da planta, nos perfis das comunidades bacterianas associadas à rizosfera das diferentes cultivares. As diferenças entre as comunidades bacterianas foram maiores na fase inicial do crescimento das plantas, com tendência a diminuir no estágio final de desenvolvimento. Essa variação foi detectada na comunidade bacteriana das cinco cultivares estudadas. A caracterização da microbiota do solo pode ser parte de programas de melhoramento de plantas a ser utilizada em estudos de avaliação de risco ambiental de batatas geneticamente modificadas.

Termos para indexação: *Solanum tuberosum*, bactérias associativas, marcador molecular, PCA.

Introduction

Many countries worldwide have commercial production of genetically modified (GM) crops. The adoption of GM plants in the agricultural scenario aimed to improve yield production, through the incorporation of interesting agronomical traits and by the introduction of genes resistant to pest and diseases. However, most GM crops released under field conditions were not preceded by environment-specific biosafety studies, which today are considered of great importance for the reduction of environmental risks (Hilbeck & Andow, 2004).

Despite the role of potato crop in the world economic scenario, studies about its interactions with microbial communities are scarce. Since 1996, Embrapa developed a transgenic potato clone resistant to *Potato virus Y* (PVY), and the environmental risk assessment studies of this clone are underway. The relationship between plants and soil microbial community is very complex and occurs in many ways, resulting in beneficial effects such as stimulation of plant hormones synthesis and enhancement of biological nitrogen fixation and phosphate solubilization, or in negative effects like occurrence of diseases.

According to Sung et al. (2006), plants are capable to increase soil microbial population through root exudates, which are used by microorganisms as nutrient source for their growth. The chemical composition of root exudates vary not only among different plant species, but also among cultivars of the same plant species (Grayer et al., 2004; Kato-Noguchi & Ino, 2005; Kerdchoechuen, 2005). Besides, the chemical composition of root exudates is a result of different factor interactions, such as nutritional status, age, stress, diseases, and environmental factors that affect the microbial community associated to the rhizosphere (Mahaffee & Kloepper, 1997; Griffiths et al., 1999).

Different cultivars of the same species might present some physiological differences. Therefore, when cultivated under the same cropping conditions, they can potentially promote the selection of distinct microbial community associated to the plant tissues (Gomes et al., 2003), since microbial communities depend on plant cultivars (Silva et al., 2003). This suggests that part of these shifts on the microbial communities occurs due to direct interaction with the plants, or due to the stress occurred in the plants (MacDonald et al., 2004).

This objective this work was to determine the shifts on the PCR-DGGE profiles of bacterial communities associated to rhizosphere of different commercial potato cultivars, in order to generate baseline information for further studies of environmental risk assessment of GM potato plants.

Materials and Methods

The soil used in this experiment was collected from an agroecological integrated production system area, in the county of Seropédica, RJ, Brazil. The experiment was carried out under greenhouse conditions, with five replicates, in a split plot randomized block design with five potato cultivars (Table 1), and three sampling periods: 15 days after germination (DAG), corresponding to the final stage of germination; 30 DAG, corresponding to the vegetative development stage; and 60 DAG, related to the reproductive stage. The tubers were planted in pots containing 1.5 kg of soil, three plants per parcel, once the root sampling procedure was a destructive method. The tuber-seeds were produced at Embrapa

Table 1. Morpho-physiological characteristics of potato cultivars[1].

Characteristic	Achat	Bintje	Agata	Monalisa	Asterix
Stem					
Vigor	Little	Much	Little	Medium	Medium
Number	4 to 5	3 to 4	8 to 10	5 to 6	4 to 5
Leaf size	Small	Larg	Medium	Larg	Medium
Leaf color	Dark green	Dark green	Light green	Light green	Light green
Plant height	Medium	Medium-tall	Short	Tall	Tall
Cycle	Semi-early	Late	Early	Early	Semi-late
Flowering	No	Yes	Yes	Yes	Yes
Flower color	-	White	White	White	Redish purple
Tuber					
Shape	Elongated flat	Elongated oval	Oval	Elongated	Elongated oval
Skin color	Light yellow	Light yellow	Purple yellow	Yellow	Red
Texture	Smooth fosca	Smooth bright	Smooth bright	Smooth bright	Rough
Eyes	Shallow	Shallow	Shallow	Shallow	Deep
Pulp color	Pale yellow	Pale yellow	Pale yellow	Yellow	Yellow
Sprout					
Color	Redish purple	Blueish purple	Light redish purple	Redish purple	Redish purple
Diseases					
Early-blight[2]	Resistant	Susceptible	Susceptible	Moderate resistance	Moderate resistance
PLRV	Susceptible	Susceptible	Moderate resistance	Moderate resistance	Moderate resistance
PVY	Resistant	Susceptible	Moderate susceptibility	Moderate susceptibility	Resistant

[1]Adapted of: Jabuomski & Furumoto (1997); Ávila et al. (1999); Nivaa (2003); Abba (2005). [2]*Phytophthora infestans*.

Hortaliças (Embrapa Vegetables) and were indexed as free of virus and other pests.

The plant roots were taken from the pots and gently separated from the bulk soil. The roots were carefully washed with autoclaved saline solution (NaCl 0.85%), in order to eliminate the excess of rhizospheric soil, and were placed on autoclaved absorbent paper to remove the excess of water. Then, 1 g of washed roots was placed in a 50 mL falcon tube containing 20 mL of sterile saline solution. Samples were shaken at 150 rpm for 30 min, and an aliquot of 8 mL was transferred to a 15 mL falcon tube, followed by centrifugation at 9,000 g for 30 min at 4 ºC. The supernatant was discarded, and the pellet was raised in 1 mL of sterile water and vortexed. The bacterial cells suspension was transferred to a 1.5 mL microtube and centrifuged as described above for 15 min. The supernatant was discarded and the pellet stored at -20°C.

DNA extraction was performed according to the protocol described by Schwieger & Tebbe (1998) and modified by Xavier et al. (2004). The stored pellets were raised in 0.6 mL of TES buffer (0.05M NaCl; 0.01M EDTA; 0.05M Tris HCl pH 8; 1% SDS) and vortexed. Samples were submitted to five freezing and thawing cycles, consisting of freezing in liquid nitrogen (5 min) and heating under agitation (65ºC; 180 RPM; 5 min). Between each step, samples were mixed by vortexing. Then, 8.4 µL of proteinase K (20 mg mL^{-1}) were added to each sample, followed by incubation under agitation (65ºC; 180 RPM; 1 hour). After the incubation time, 0.6 mL of phenol, chloroform and isoamilic alcohol (25:24:1) was added to each sample and centrifuged (7,500 g; 6 min). The supernatant was transferred to a 1.5 mL microtube, and 0.6 mL of chloroform and isoamilic alcohol (24:1) was added, followed by centrifugation (7,500 g; 6 min). A 0.5 mL aliquot of the supernatant was transferred to a 1.5 mL microtube, and 0.5 mL of cold isopropanol was added. The samples were incubated at -20ºC for 60 min, and centrifuged (16,100 g; 20 min). The supernatant was removed, and the pellet was dried in a cold trap centrifuge and raised in 50 µL of TE buffer (10 mM Tris; 1 mM Na-EDTA; pH 8).

The PCR was performed with three different dilutions of the DNA samples: 1:20, 1:40 and 1:80 in ultrapure water. For each DNA dilution, PCR amplification was carried out on a final volume of 35 µL, containing 1 µL DNA dilution, PCR buffer (1X), MgCl$_2$ (1.5 mM), dNTP (0.2 µM of each), bovine serum albumin (0.17 µg µL^{-1}), *Taq* DNA polymerase (0.7 U), and the set of primers 1401-R and 968CG-F (0.2 µM of each). These primers and PCR program were described by Gelsomino et al. (1999). The amplified products of each replicate were mixed together in a single microtube.

To determine the best denaturing conditions for the samples, a preliminary assay was performed with 6% poliacrylamide gel, containing from zero to 100% of 7 M urea and 40% formamide. The best denaturing conditions were between 50 and 65% of the denaturing agents. For the assays, 50 to 65% denaturing gels were prepared by mixing two solutions, the first one containing 3.5 M urea and 20% formamide, and the second containing 4.55 M urea and 26% formamide. Depending on the efficiency of PCR amplification, 12 to 20 µL of the mixed amplified products were loaded in denaturing poliacrylamide gel 6% (N-acrylamide, N'-metilbisacrylamide, 37:1), in 0.5X TAE buffer final concentration: (Tris-acetate 20 mM and EDTA 0.5 mM). The electrophoresis was carried out in a *Dcode* Detection Mutation System, under constant voltage (120 V; 60ºC; 16 hours). Gels were stained using SYBR Gold (20X), and were visualized under UV light in an IMAGO photo-documentation system.

Binary matrixes based on the PCR-DGGE profiles for each replicate were created according to Kozdrój & Elsas (2001). The similarity matrixes of the five replicates were combined later to compose a single similarity matrix (Aboim et al., 2008) to be used on the similarity clustering and principal component analysis (PCA). The clustering analysis was performed using the Jaccard index and UPGMA method.

Results and Discussion

The bacterial community profiles associated to the rhizosphere of different cultivars at 15, 30 and 60 DAG are in Figure 1. Some bands (arrows) occur in all cultivars, in all evaluation dates. However, some bands (circles) are dependent on the cultivar or even development stage of the plant.

The bands indicated by arrows in the PCR-DGGE profiles (Figure 1) occur in all cultivars and sampling dates, suggesting that those bands are associated with unspecific bacterial groups associated with the rhizosphere of the plants. These profiles might be associated with bacterial groups such as growth promoters affected by the rhizosphere and being beneficial to plants (Bushby et al., 1993; Roesti et al., 2006).

The PCR-DGGE profile of bands of specific cultivars or their developmental stages, indicated by the circles at Figure 1, suggests that those bands were originated from

more specific bacterial groups, which are selected by the plants due to different root exudates. It has been observed that different cultivars of the same plant species exudate different amounts (Kato-Noguchi & Ino, 2005) and compounds (Grayer et al., 2004; Kerdchoechuen, 2005) that might be related to the observed shifts of microbial communities associated to the rhizoplane of these plants (Gu & Mazzola, 2003).

A study was conducted with *Trifolium repens* and *Lolium perenne*, in which the rhizosphere was divided into three different compartments (soil, rhizospheric soil and rhizoplane). The soil fraction was mostly colonized by gram-positive bacterial species, *Cytophaga/*

Flexibacter/Bacteroides (CFB group), *Proteobacteria* and *Holophaga/Acidobacterium*. At the rhizosplane and rhizospheric soil fractions, a selective effect favored the gama-*Proteobacteria* prior to the gram-positive and *Holophaga/Acidobacterium*, leading to the prevalence of the *Pseudomonas* bacterial group (Marilley & Aragno, 1999). These authors also observed that the phylogenetic diversity diminishes, as the samples are collected nearer to plant roots. Bacteria belonging to the *Pseudomonas* group are known as growth promoters (Gupta et al., 2005; Picard & Bosco, 2005), although some species or even some strains cause diseases or interfere negatively with plant development (Berggren et al., 2005).
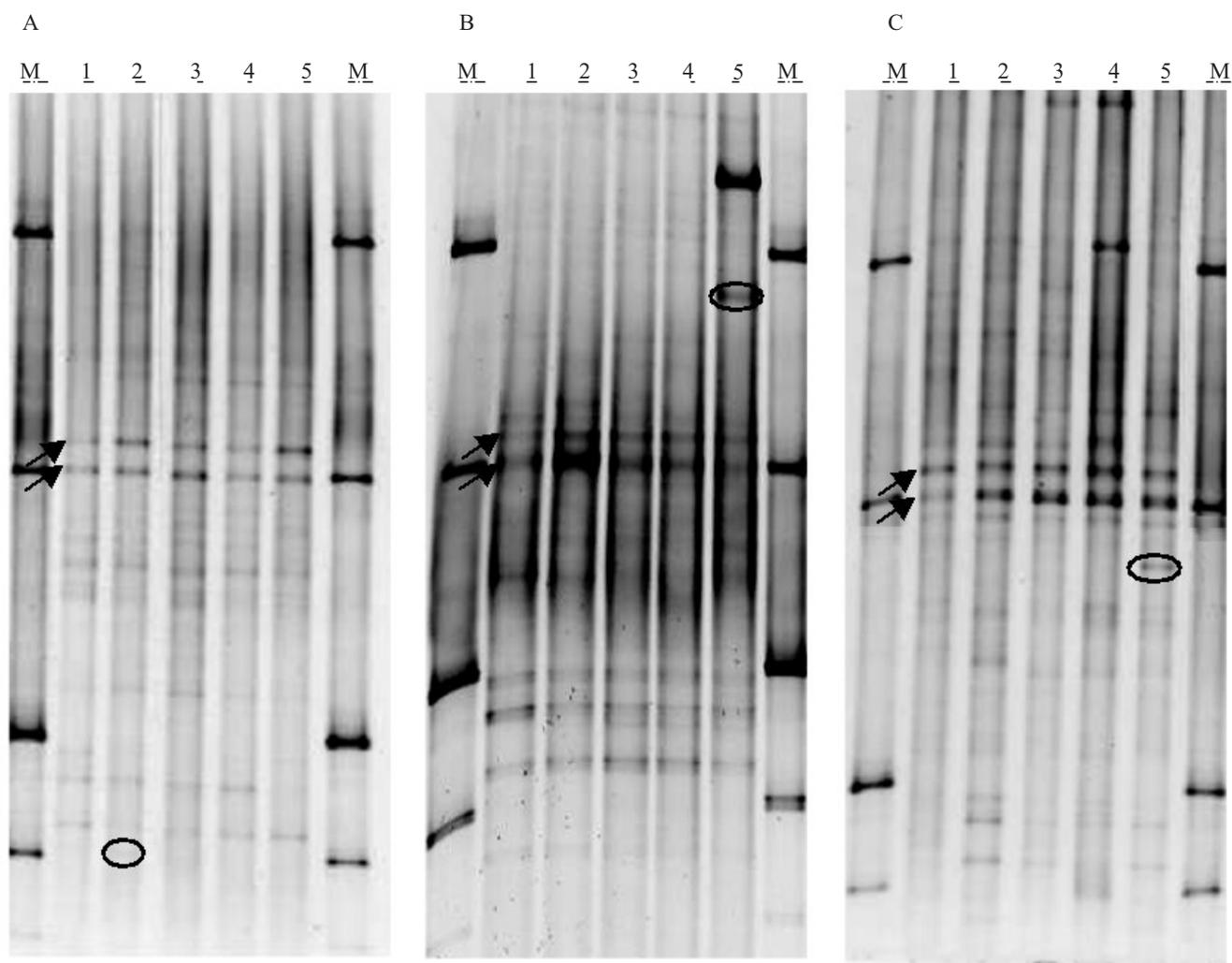
Figure 1. PCR-DGGE profiles of the bacterial communities associated to the rhizosphere of the different potato cultivars. A, first sampling date; B, second sampling date; C, trird sampling date. M, marker (from top to bottom: *Bradyrhizobium elkanii* BR 113, *Sinorhizobium fredii* BR 112, *Mesorhizobium plurifarium* BR 3804 and *Burkholderia* sp. BR 11340); 1, Achat; 2, Bintje; 3, Agata; 4, Monalisa; 5, Asterix.

Olsson & Persson (1999) reported the higher adherence of the soil to the roots of barley as a factor of increase of the CFB bacterial group, and of decrease of the *Pseudomonas* group, in rhizospheric soil.

Based on the PCR-DGGE profiles, dendrograms representing the bacterial communities at different sampling dates were drawn (Figure 2). Although tuber formation could be observed at 60 DAG, their developmental stage differed from one cultivar to another. Also, depending on the cultivar, the mother tubers were already decomposed, such as observed for 'Asterix' and 'Monalisa'.

'Asterix' and 'Monalisa' were grouped in the first and second sampling dates (Figure 2 A and B), with an average similarity of 65%. In the third sampling date, these cultivars split to different groups, with a similarity of 35%. 'Asterix' and 'Achat' were in the same group in all sampling dates, with 53, 65 and 40% for the first, second and third sampling dates, respectively. 'Agata' had the lowest similarity rates being placed apart from the other cultivars, in all sampling dates. Although the similarity indexes varied among the sampling dates (48, 60 and 36% in the first, second and third sampling dates, respectively; Figure 2 A, B and C), a general reduction trend was observed during the plants development, associated with the decomposition of the mother-tubers.

A reduction on the similarity index was observed in the third sampling date (Figure 2 C). The highest similarity was 43% between 'Asterix' and 'Achat', and the lowest similarity was 35% between the two clusters – first cluster: 'Achat', 'Asterix' and 'Bintje'; second cluster: 'Agata' and 'Monalisa' (Figure 2 C) –, resulting in a difference of 7% similarity. Using the same approach for the data from 15 DAG (Figure 2 A), the difference between the two clusters and the highest similarity between two cultivars is around 17%, indicating that at this stage bacterial communities are more easily distinguished than at later developmental stages of the plant.

The bacterial community composition of 'Monalisa' and 'Asterix' rhizosphere, considering the phylogenetic dendrogram (Figure 2 A and B), was more similar at the first and second sampling dates. This suggests that their root exudates might be selecting more similar bacterial groups, when comparing with the other cultivars. Usually, a higher influence of the plant on the rhizosphere associated microbial communities is observed at the early stages of development (Gomes et al., 2003; Ibekwe & Grieve, 2004).

'Monalisa' and 'Agata' presented the mother-tuber at an advanced decomposition stage prior to the other cultivars. This might have stimulated specific saprophytic bacterial groups to develop. Also, the variation in the diversity of the group formation, at each sampling date,
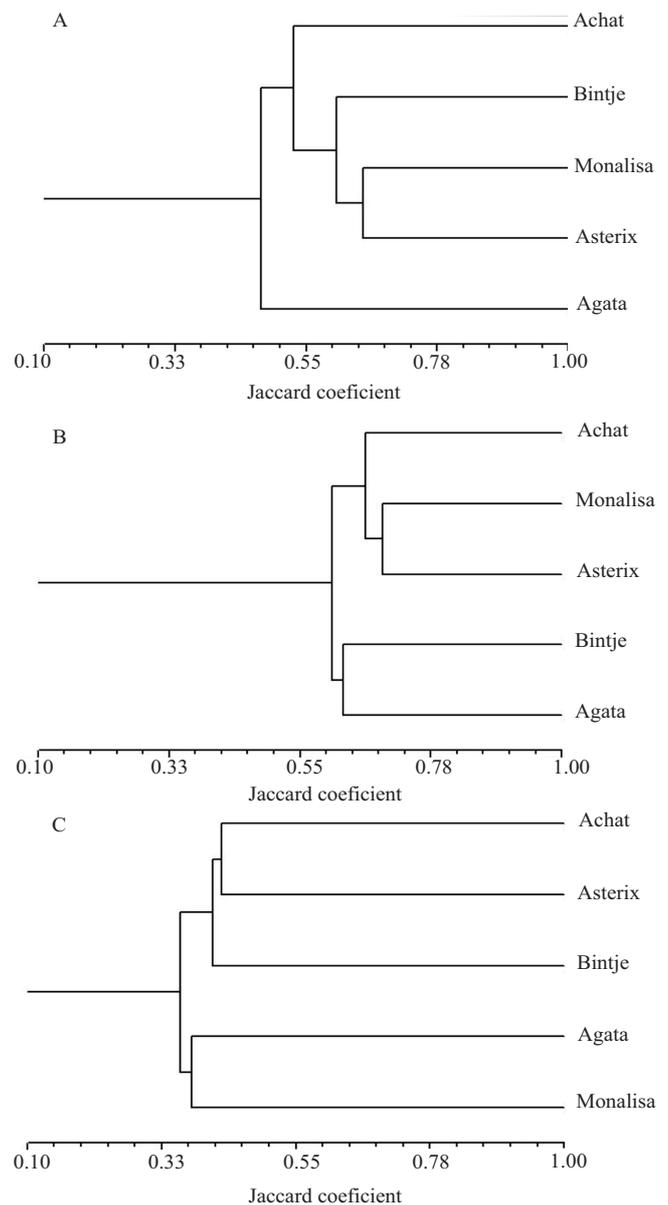
Figure 2. Similarity dendrograms representing the bacterial communities associated to the rhizosphere of different potato cultivars, constructed with data from the matrix generated by the Jaccard similarity coefficient, using UPGMA grouping method. A, plants collected at 15 days after germination (DAG) – first sampling date; B, 30 DAG – second sampling date; C, 60 DAG – third sampling date.

might be a function of the different maturation stages specific to each cultivar, which might have influenced the different types and amounts of root exudates. The root associated bacterial community is usually dynamic and can be highly affected by the plant developmental stage (Roesti et al., 2006).

The PCA grouping of the rhizosphere associated bacterial community is presented at Figure 3. The sum of the two first components explain 70.5, 58.2 and 60.6% of the information for the first, second e third sampling dates, respectively. Once the PC explain the maximum variance of the studied variables (Jöreskog et al., 1976), the PCA allows a better demonstration of the observed differences between the potato cultivars.

The PCA compared with the similarity index grouping analysis provides more details of the interactions between the studied variables, once it mitigates data variation into several components, and allows a multidimensional view of the data. The PCA complements the information of the similarity dendrograms, helping the interpretation of data. The X axis is usually the most important to explain most of data variation.

Difference between the X and Y axes with our data was 10% maximum, indicating that both axes, in the three sampling dates, provided the same information. 'Achat', 'Monalisa' and 'Asterix' are usually at the same cluster, while 'Agata' is always apart (Figure 3 A). 'Bintje' only in the second sampling date, is at a different cluster from 'Achat', 'Monalisa' and 'Asterix' (Figure 3 B). At Y axis, 'Achat' tends to locate far from 'Monalisa' and 'Asterix' (Figure 3). From a total of 17 morpho-physiological characteristics, 'Achat' shares only three of them with 'Monalisa' and 'Asterix', while the later two cultivars share seven of the characteristics (Table 1). This higher number of shared characteristics between 'Monalisa' and 'Asterix' indicates that they might be physiologically similar, and this is probably the cause of their grouping at the PCA.

The different grouping observed at the PCA (Figure 3) was a function of the sampling dates. However, for 'Agata', a distinct group was detected, placing this cultivar apart from the others in the three sampling dates. 'Agata' presents the most distinct morpho-physiological characteristics, when compared with the other cultivars (sprout color, number of stems per plant, plant height, tuber shape and tuber skin color), which might be associated with the observed clustering. Similar results were presented by Thomas et al. (2006) for somaclonal variants of *Camellia* spp. The authors reported that these variants could be divided into two morphological groups, and that some of them with similar morphological
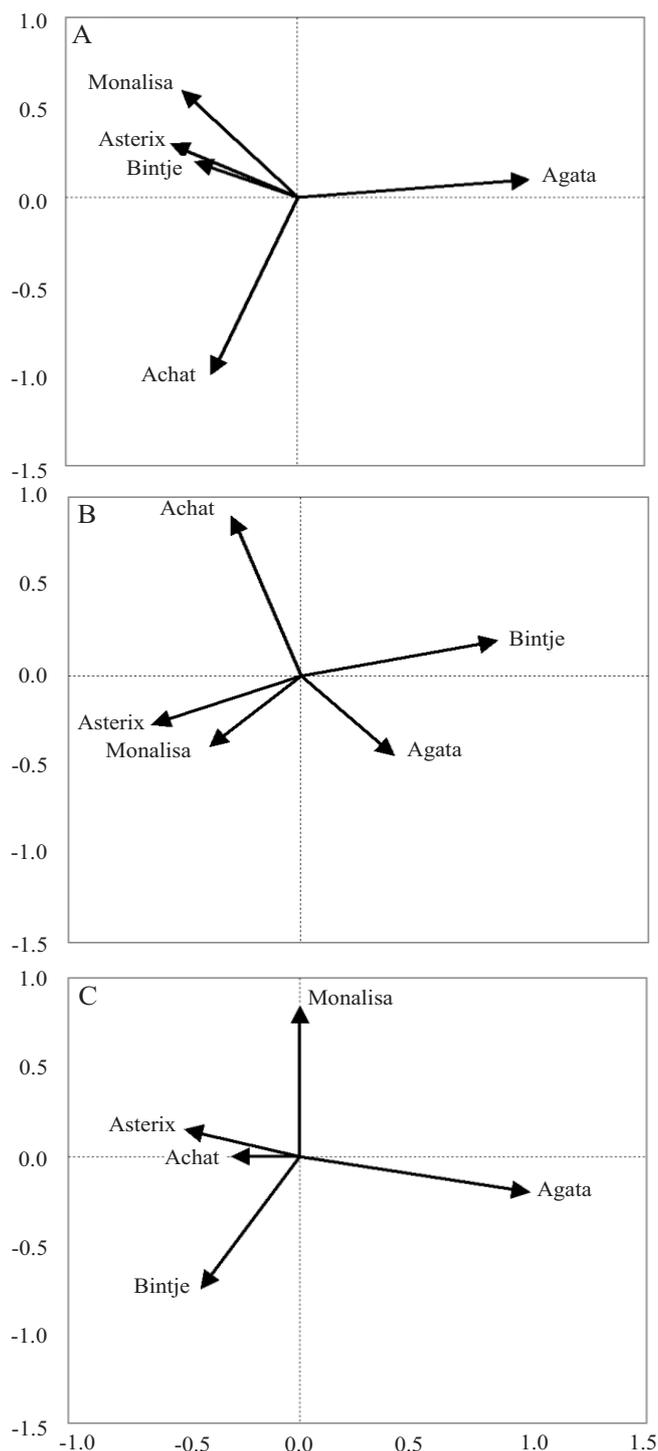
Figure 3. PCA grouping of the bacterial community associated to the rhizospheres of different potato cultivars: A, first sampling date; B, second sampling date; C, third sampling date. First sampling date: CP1, 40.1%, CP2, 30.4%; second sampling date: CP1, 31.3%, CP2, 26.9%; and third sampling date: CP1, 33.8%, CP2, 26.8%.

characteristics could be also grouped by their similar physiological characteristics.

In the first sampling date, a cluster with the cultivars Monalisa, Asterix and Bintje (Figure 3 A) was formed. In the second sampling date (Figure 3 B), 'Bintje' migrated to another group, apart from the other cultivars, shifting a little back in the third sampling date (Figure 3 C). This cultivar is distinct from the other ones, in relation to its morpho-physiological characteristics, although it shares some few similar characteristics with the 'Achat', 'Monalisa' and 'Asterix' (Table 1), probably leading to the observed grouping with the former cultivars at least at two developmental stages. 'Monalisa' also shifts, on the X axis, in direction of the cultivar Agata from the first to the third sampling date. Both are early cultivars and, probably, this characteristic is reflected by the similar profile of the soil bacterial community, at the end of the growth cycle of the plants.

'Achat' is different from 'Monalisa' and 'Asterix', mainly in leaf color, plant height and vigor. This might be reflected at the physiological level, resulting in the exudation of different compounds, which, in turn, affect the root bacterial community structure.

Considering the clustering at the Y axis, the early cycle characteristic of 'Monalisa' and 'Agata' contributed to their grouping, which was also observed for maize (Silva et al., 2003) and wheat (Roesti et al., 2006).

The data of this work indicate that the grouping of the bacterial communities associated to potato cultivars is related to some morpho-physiological characteristics. This is also supported by the fact that the chemical composition of root exudates is dependent on plant species, cultivar, nutritional status, age, biotic or abiotic stress, and other environmental factors, which will, at last, affect the microbial community present at the rhizosphere (Mahaffee & Kloepper, 1997; Griffiths et al., 1999).

Conclusions

1. Different potato cultivars show distinct PCR-DGGE profiles, as a result of a selection process of the rhizosphere bacterial community, controlled by potato cultivar; cultivars resembling their morpho-physiological characteristics show the closest PCR-DGGE profiles.

2. The similarity among bacterial communities associated to the rhizosphere of different potato cultivars decreases along the plant growth period.

3. The PCR-DGGE technique is suitable to detect the effect of potato cultivar on the bacterial community associated to potato rhizosphere.

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