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Full Paper

Microbial community in the rhizosphere of young maize seedlings is susceptible to the impact of introduced pseudomonads as indicated by FAME analysis

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Two species of *Pseudomonas* (i.e. *P. chlororaphis* or *P. putida*) derived from a maize rhizosphere were studied for their impact on the structure of the microbial community in the rhizosphere of young maize seedlings after inoculation. The culturable bacteria and total microbial communities were analyzed based on profiles of whole-cell fatty acid methyl esters (MIDI-FAME). The introduction of *Pseudomonas* species resulted in the shift from the Gram-positive dominated culturable community in the rhizosphere of uninoculated maize to more Gram-negative populations in the rhizospheres of the inoculated plants. For the total rhizosphere communities, 43, 47 and 42 FAMEs were detected in the uninoculated maize and the samples inoculated with *P. chlororaphis* or *P. putida,* respectively. In contrast to the culturable communities, low concentrations of marker FAMEs for Gram-positives (i15:0, a15:0, i16:0) were found in the profiles of the total rhizosphere communities. The maize inoculations resulted in an enrichment of some Gram-negative isolates; however, Gram-positive bacteria, *Cytophaga*/*Flavobacterium* and saprophytic fungi were found in the uninoculated rhizosphere.

Key Words——fatty acid profiling; introduced *Pseudomonas*; maize rhizosphere; microbial community

Introduction

In the rhizosphere, the microbial community may change in the composition under the influence of different factors among which exogenous bacterial inoculants have been of increased interest (Kokalis-Burelle et al., 2006; Kozdrój et al., 2004; Nacamulli et al., 1997). Considered as biofertilizers, different bacterial preparations have been prepared for direct promotion of plant growth and/or inhibition of plant pathogen development (El-Tarabily and Sivasithamparam, 2006; Lucy et al., 2004; Mehnaz and Lazarovits, 2006). These bacterial inocula introduced on seeds, seedling roots or released into soil are involved in different interactions with indigenous rhizosphere microorganisms (Canbolat et al., 2006; Kozdrój et al., 2004; Naseby and Lynch, 1998).

New methods such as phospholipid fatty acid (PLFA) or whole-cell fatty acid methyl ester (FAME) analysis have been used to study changes in microbial community composition without having to actually determine each species present in the soil or rhizosphere (Buyer et al., 2002; Drenovsky et al., 2004). Since different microorganisms characterize specific fatty acid profiles and some PLFA or FAME can be regarded as

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markers of particular microbial groups and genera, even small changes in the community structure are indicated (Ibekwe and Kennedy, 1999). Thus to take one example, FAME analyses of microbial communities in the rhizospheres of wheat inoculated with *Pseudomonas chlororaphis* or *Pseudomonas fluorescens* indicated differences in the community compositions considering the seedlings inoculated versus uninoculated (Gagliardi et al., 2001).

Pseudomonads are efficient colonizers of plant rhizospheres especially those rich in different nutrients, e.g. maize that produces exudates containing different sugars, amino acids and organic acids stimulating growth of saprophytic bacteria (Baudoin et al., 2001; Kozdrój and van Elsas, 2000). Since these rhizobacteria may be beneficial for the maize host, stimulating its growth or protecting the plant against some pests, they could be applied for inoculation of seeds or seedlings as biofertilizers during cultivation practices. However, successful application of pseudomonads as inoculants also depends on their impact on the indigenous microbial community of the maize rhizosphere (Kozdrój et al., 2004).

The aim of this study was to find whether colonization of young maize rhizosphere by introduced *Pseudomonas chlororaphis* or *Pseudomonas putida* could induce changes in the microbial community structures. In addition, differences in the composition of dominant culturable bacterial isolates between the inoculated and uninoculated seedlings were also determined. Considering that both species were maize rhizobacteria, it was interesting to assess the scale of changes caused by them in the community structures when they were reintroduced as inoculants.

Materials and Methods

Soil and bacterial species used. A sandy loam soil used for plant cultivation (pH 6.2, 12.6% organic matter, 0.36% total N) was air-dried to about 15% (w/w) moisture content, sieved (2 mm-mesh), placed in plastic containers (400 g) and wetted with distilled water to about 35% (w/w) moisture content corresponding to about 50% (w/w) of water holding capacity. Soil nitrogen was determined using the Kjeldahl method, while organic matter was assessed after dry combustion at 550°C for 6 h. The soil portions were equilibrated for 2 weeks in a plant growth cabinet under a light/dark regime (26°C 16 h/21°C 8 h) at relative air humidity of 70%.

In a preliminary experiment, two rhizobacterial species identified as *Pseudomonas chlororaphis* (similarity index 0.853) and *P. putida* (similarity index 0.898) were isolated from the rhizosphere of maize seedlings growing in the sandy loam soil. The identification was conducted by the MIS microbial fatty acid identification system (MIDI Inc., Newark, USA) using the TSBA40 method and TSBA40 library (Microbial ID Inc., 1999). Both strains were stored at −20°C in 20% (v/v) glycerol and, when required for experimental use, the strains were transferred to Luria-Bertani broth (LB: Difco tryptone 10 g, yeast extract 5 g, glucose 1 g, NaCl 5 g per liter H_2O, pH 7.2). Cultures were incubated overnight with shaking at 28°C until late log phase, after which cells were harvested by centrifugation and washed three times in sterile 0.85% NaCl. Then, bacterial cells were suspended in 1% water solution of methyl cellulose (1:1, 10^9 cfu ml^{-1}) that was used to introduce the strains into the soil surface over sown maize.

Experimental design. Seeds of maize (*Zea mays* L.) were placed in soil, followed by introduction of either bacterial suspension or methyl cellulose solution to the soil surface over the sown seeds, establishing inoculant densities of log 7.60 (*P. chlororaphis*) or log 7.75 (*P. putida*) cfu g^{-1} dry soil. Thus, the planted pots were either untreated control or treated separately with *P. chlororaphis* or *P. putida*, giving a total of 15 pots in the experiment (i.e. 3 treatments with 5 replicates). All pots (two plants per pot) were wetted with distilled water to establish a moisture content of about 35% (w/w) during the cultivation time. The pots were incubated for 35 days under a light/dark regime (26°C 16 h/21°C 8 h) at relative air humidity of 70%. Five pots from each treatment were destructively sampled after 35 days of cultivation. Maize seedlings were carefully removed from soil and the roots with adhering rhizosphere soil were placed in sterile 0.1% sodium pyrophosphate (pH 7.0) for shaking (180 rev min^{-1}, 30 min, 20°C) and preparing serial tenfold dilutions to obtain colonies of culturable bacteria. Replicate aliquots from rhizosphere dilutions were spread-plated into one-tenth strength Trypticase Soy Broth Agar (0.1 × TSBA: Difco TSB 3 g, agar 15 g L^{-1} of water, pH 7.0).

FAME analyses of culturable bacteria and total community. To perform FAME analyses of culturable bacteria community, all colonies growing on an agar plate (i.e. typically 25 to 250 colonies; 10^{-5} rhizosphere dilu-

tion) were scraped together to a reaction tube and then the biomass was saponified and methylated following the procedure given for the Microbial Identification System (Microbial ID Inc., 1999).

Direct analysis of total microflora FAME profiles was conducted according to Kozdrój et al. (2004). The roots with adhering rhizosphere soil were air-dried overnight at 22°C and the soil (3 g) was removed with care to 20 ml glass test tubes and analyzed following the procedure modified from that used for the taxonomic identification of pure microbial isolates (Microbial ID Inc., 1999). Fatty acid extracts were analyzed and identified by gas-liquid chromatography (Hewlett-Packard 6890, USA) using the MIDI Microbial Identification System software (MIDI Inc.).

Identification of culturable bacteria. After 72 h incubation at 27°C, a plate was selected from each replicate and approximately 20 different colonies were sampled from one plate for each plant treatment, giving a total of 300 isolates used for further identification. Isolates were streaked twice on 0.1× TSBA and purified strains stored on 1-ml stabs containing 0.1× TSBA, overlaid with sterile glycerol and maintained at 4°C. Bacterial isolates were identified based on FAME profiles using MIDI software (Sherlock TSBA40 method and TSBA40 library; MIDI Inc.). Strains with a similarity index (SI) between 0.3 and 0.5 were considered positively identified to the genus level (Buyer, 2002; Germida and Siciliano, 2001).

Results and Discussion

The results presented here confirm previous findings showing changes in the rhizosphere microbial community structure exposed to exogenous bacterial inoculants (Kozdrój et al., 2004; Nacamulli et al., 1997; Thirup et al., 2003). Furthermore, this study indicates that even two species of the same genus can differently disturb the indigenous microbial communities of young maize seedlings. In total, 45 and 63 FAMEs were detected in the culturable and rhizosphere samples, respectively. For culturable communities, a higher number of FAMEs (i.e. 33) was found in the uninoculated maize than in the plants inoculated with either *P. chlororaphis* (i.e. 20) or *P. putida* (i.e. 29). This observation would indicate that *P. chlororaphis* either decreased diversity of the community or shifted its species composition compared with *P. putida*. Only 16 FAMEs were common in the profiles of these communities, and i15:0, a15:0, i16:0, 16:0, cy17:0, 18:1 ω 7 c/ω 9 t/ω 12 t, and cy19:0 ω 8 c dominated in all profiles (Fig. 1). These fatty acids are characteristic of *Arthrobacter*, *Bacillus*, *Corynebacterium* and *Flavobacterium* that contain high concentrations of branched FAMEs, and different from Gram-negatives (i.e. *Pseudomonas*, *Alcaligenes*, *Enterobacter*) for the last three acids (Haack et al., 1994; Olsson and Persson, 1999; Zelles, 1999). Several isolates belonging to these genera were identified by the MIDI system after sampling selected colonies growing on the agar plates.

In the uninoculated sample, the highest amounts of FAMEs were detected for i15:0, a15:0, i16:0, 16:0 and 18:1 ω 7 c. In addition, such fatty acids as i14:0, i15:0, i16:0, 18:1 ω 9 c, and 18:0 showed higher amounts compared with those in the treated samples. Several FAMEs (i.e. i11:0, i11:0 3 OH, i16:1 H, 16:1 ω 5 c, 17:1 ω 8 c, 17:1 ω 6 c, 17:0 10 Me, i18:0, 18:2 ω 6,9 c, 18:1 ω 7 c, 18:0 10 Me TSBA, 19:1 ω 6 c/cy19:0 ω 10 c) were only isolated from the uninoculated maize (Fig. 1). These data reveal that several Gram-positive bacteria and representatives of the *Cytophaga*/*Flavobacterium* group may have dominated the rhizosphere of uninoculated maize. The MIDI identification of bacterial colonies isolated from the uninoculated maize indicated the presence of strains (SI 0.3 to 0.5) belonging to the genera of *Bacillus*, *Agrobacterium*, *Arthrobacter*, *Corynebacterium*, *Flavobacterium*, *Cytophaga*, *Actinomadura*, *Rhodococcus*, *Enterococcus*, *Pseudomonas* and *Xanthomonas*. It is widely supposed that culturable communities of bacteria in soil are dominated by Gram-positive species (Ibekwe and Kennedy, 1998; Kozdrój and van Elsas, 2001).

Going on to the inoculated plants, specific fatty acids (i.e. 10:0 2 OH, 12:0, 12:0 2 OH, 12:0 3 OH, 14:0 3 OH/i16:1 I) were exclusively detected, indicating their possible origin from the introduced bacterial strains. Other FAMEs (i.e. 16:0, cy17:0, 18:1 ω 7 c/ω 9 t/ω 12 t and cy19:0 ω 8 c) often represented in profiles of pseudomonads were found in higher amounts for the inoculated samples. The introduction of exogenous bacterial species may have resulted in the shift from the Gram-positive dominated community in the rhizosphere of uninoculated maize to more Gram-negative populations in the rhizospheres of inoculated plants. This conclusion was supported by the MIDI identification of isolates (SI 0.3 to 0.5) that belonged to the genera of *Pseudomonas*, *Acetobacter*, *Xanthobacter*, *Burk-*

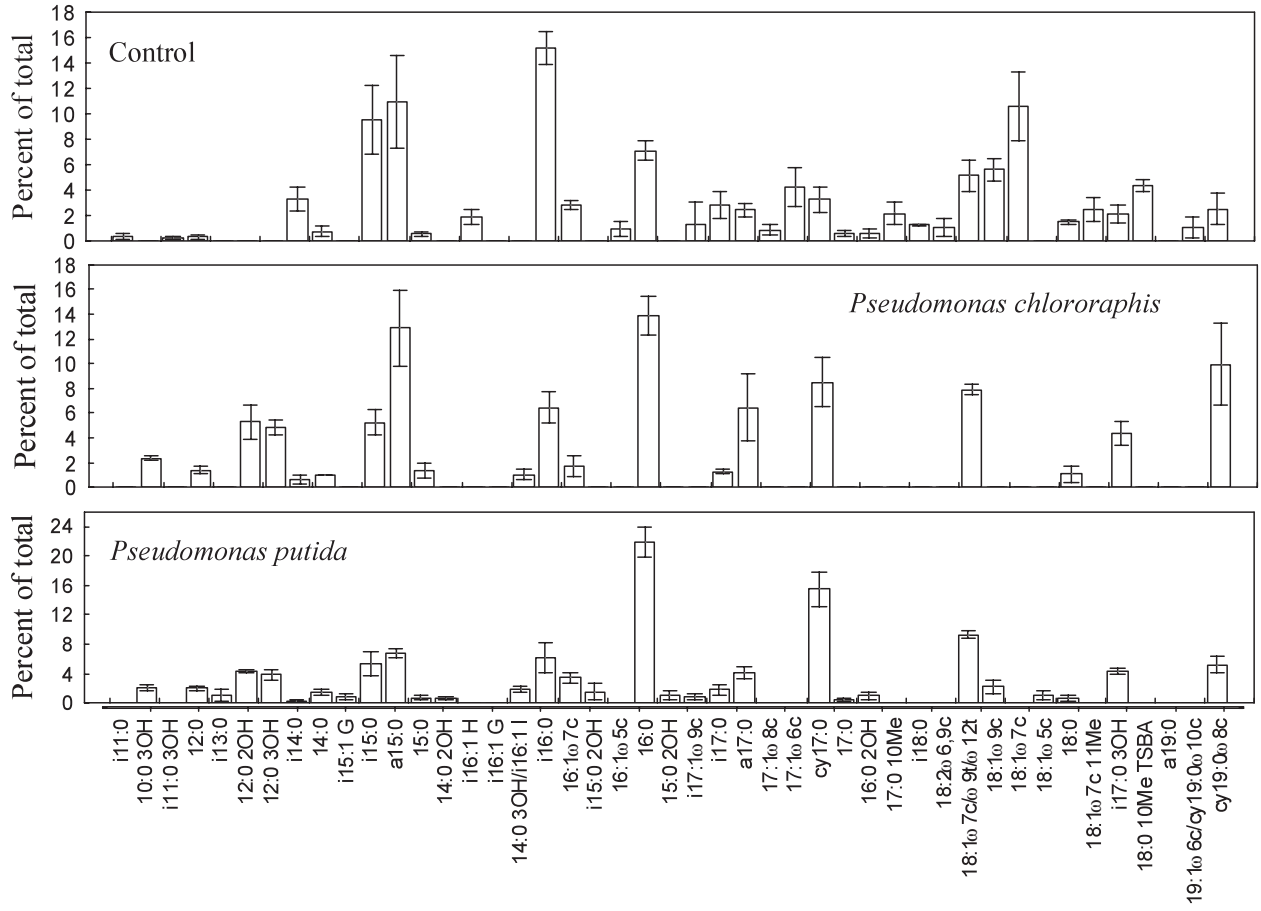

Fig. 1. FAME profiles of culturable microbial communities in the rhizospheres of young maize seedlings exposed to the impact of *Pseudomonas* species introduced.

The analyses were performed after 35 days of cultivation in a plant growth cabinet. Bars show means and 1 standard deviation (n = 5).

holderia, *Alcaligenes* and *Erwinia*. The community structure shift may have been associated with a progressive change from oligotrophic to more copiotrophic conditions that are suitable for Gram-positives and Gram-negatives, respectively, due to nutrients released from dead inoculant cells (Yao et al., 2000). Changes in the community structure of culturable bacteria in the maize rhizosphere inoculated with exogenous *P. chlororaphis* or *P. putida* were also found in a previous study (Kozdrój et al., 2004). From this we can assume that the same species regardless of their origin (i.e. indigenous/maize versus exogenous/tomato) are able to cause similar effects after colonization of the rhizosphere.

Going on to the total rhizosphere communities, 43, 47 and 42 FAMEs were detected in the uninoculated maize and the samples inoculated with *P. chlororaphis* or *P. putida*, respectively. The total profiles provided different information on the rhizosphere microflora compared with the patterns obtained from the culturable fraction. The fact of different composition of populations between culturable and total rhizosphere communities have also been reported in other studies (Cavigelli et al., 1995; Ibekwe and Kennedy, 1998; Kozdrój et al., 2004). Given all FAMEs extracted, 34 fatty acids were common in all samples. Several fatty acids (i.e. 18:2 ω 6,9 c, 16:0, cy19:0 ω 10 c/19:1 ω 6 c and 16:0 N alc) dominated in all profiles (Fig. 2). The data would seem to suggest that the soil and rhizosphere of young maize seedlings may have been suitable habitats for the development of saprophytic fungi, Gram-negative bacteria and specifically *Moraxella* species containing relatively large amounts of 16:0 N alc (Ritchie et al., 2000). In contrast to the culturable communities, low concentrations of marker FAMEs for Gram-positives (i15:0, a15:0, i16:0) such as *Bacillus* and *Clostridium* (Dunfield and Germida, 2001) were found in the profiles of the total rhizosphere communi-

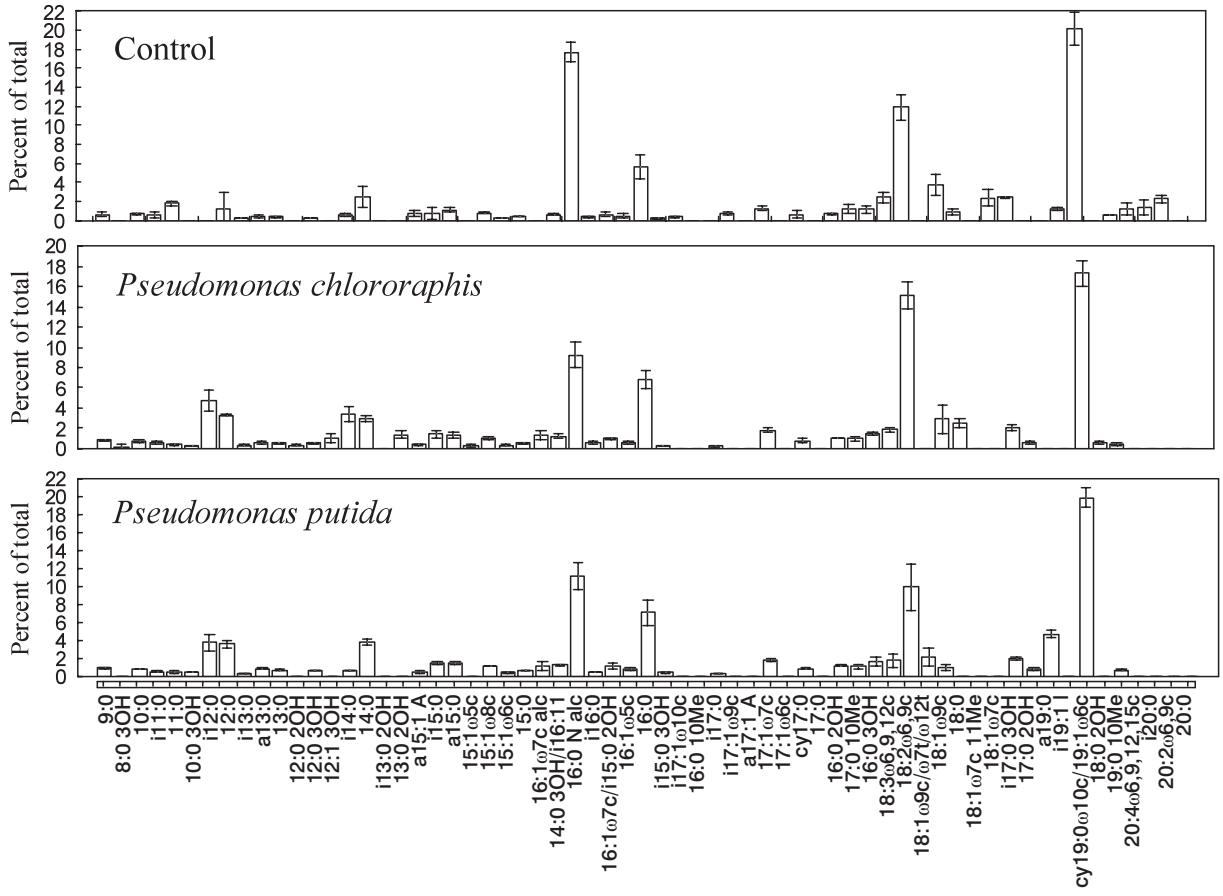

Fig. 2. FAME profiles of total microbial communities in the rhizospheres of young maize seedlings exposed to the impact of *Pseudomonas* species introduced.

The analyses were performed after 35 days of cultivation in a plant growth cabinet. Bars show means and 1 standard deviation ($n = 5$).

ties. Some hydroxylated FAMEs (i.e. 12:0 2 OH, 12:1 3 OH, 13:0 2 OH, 18:0 2 OH) and 15:1 w 5 c were only present in the profile of a rhizosphere inoculated with *P. chlororaphis*. For the maize inoculated with *P. putida*, 18:1 ω 9 c/ω 7 t/ω 12 t and a19:0 were exclusively identified. In comparison with the inoculated samples, some polyunsaturated FAMEs and i17:1 ω 10 c, i17:1 ω 9 c, 18:1 ω 7 c, and i19:1 I were only found in the uninoculated maize. From these observations it follows that the maize inoculations resulted in an enrichment of some Gram-negative bacteria, including surviving inoculants, in the rhizospheres. However, some strains of *Bacillus*, *Cytophaga*/*Flavobacterium*, *Arthrobacter, Agrobacterium* as well as saprophytic fungi may have exclusively been present in the uninoculated rhizosphere (Olsson and Persson, 1999; Zelles, 1997).

Altogether, this study indicates that the rhizosphere of young maize is inhabited with a microbial community that is unstable in structure and susceptible to a shift under the influence of *Pseudomonas* inoculants. In addition, the community shifts seem to be discerned based on the species of *Pseudomonas* introduced; however, the origin of these species (indigenous versus exogenous) is not significant for the community patterns found.

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