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Burkholderia community structure in soils under different agricultural management

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To “ vovó Lêda” and “ vovó Gaé”,
for their love and devotion

Contents

Chapter 1

General Introduction

Soils possess a vast and diverse microbial population, comprising both bacterial and fungal communities. The balance between these two communities may vary according to a number of factors, including soil management, and the dominance of one over the other seems to be correlated with the complexity of organic compounds present in the soil. As a result, in agricultural soils, which frequently contain simple organic compounds and great availability of nutrients, bacteria often are the dominant group (50). Bacterial populations in soil can range from 4.8 billion cells per cubic centimetre of soil collected from a forest to 18 and 21 billions in pasture and arable soils, respectively (97). Furthermore, DNA reassociation analysis has shown that pristine forest soil and agricultural soil may contain from 8000 to 10000 different bacterial genomes per cubic centimetre of soil, demonstrating the great diversity of the soil bacterial community. This diversity is remarkable when compared to that in aquatic environments, which typically exhibit from seven to 160 different bacterial genomes or species (27, 97). Although vast, only a small fraction of this total bacterial community, varying from 0.1 to 1% in pristine forest soil to 10% in arable soil, can generally be retrieved by cultivation techniques (96). In addition, the diversity of the culturable bacterial community was shown to be at least 200 times lower than the diversity of the total bacterial community (96).

As a result of its large bacterial diversity, soil has become a source of bacterial isolates or bacterial-related products. Soils are important sources of medically relevant compounds, such as antibiotics, and microorganisms with biological control capabilities are increasingly being used as replacements of pesticides in agriculture (7, 8). Furthermore, bacteria that are able to stimulate plant growth, either by fixing atmospheric nitrogen, by producing plant hormones, or via both processes, can improve crop production when introduced into soil (81, 84, 99). The use of bacterial inoculants has not been limited to agriculture and several bacterial species have been used for cleaning contaminated soil and groundwater, in a process called bioremediation (62). Among the bacteria occurring in soil, the genus *Burkholderia* is of particular importance as it is involved in most of the processes mentioned above. In the light of the significance of this genus, the aim of this thesis was to evaluate the diversity of both culturable and non-culturable *Burkholderia* communities in agricultural field plots where different management regimes had been applied.

The genus *Burkholderia*

Description

The genus *Burkholderia* was created by Yabuuchi *et al.* in 1992, who proposed that seven former *Pseudomonas* species belonging to the RNA homology group II should constitute a new genus (111). Among these species, *B. cepacia* was chosen as the type species. Since 1992, the list of species encompassing the genus *Burkholderia* has changed several times. Some species were removed from the genus (112), some were later added to it (46, 107), along with some newly described species (1, 10, 11, 21-23, 49, 100, 104, 105, 107, 110, 114). In addition, in 1997 a polyphasic taxonomic study revealed that *B. cepacia* was not one species, but constituted a species "complex" composed of several phenotypically similar species (denoted genomovars) (105). Currently, the *B. cepacia* complex comprises nine species, namely *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia* (genomovars I to IX, respectively) (24). In addition to the species from the *B. cepacia* complex, 24 other species constitute the whole genus: *B. glathei*, *B. sacchari*, *B. tuberum*, *B. kururiensis*, *B. phenazinium*, *B. terricola*, *B. sordidicola*, *B. fungorum*, *B. caledonica*, *B. graminis*, *B. caryophylli*, *B. phymatum*, *B. hospita*, *B. caribensis*, *B. andropogonis*, *B. gladioli*, *B. plantarii*, *B. glumae*, *B. thailandensis*, *B. pseudomallei*, *B. mallei*, *B. unamae*, *B. tropica* and *B. ubonensis* (1, 10, 11, 15, 21-23, 49, 66, 82, 100, 104, 107, 110, 114). The species *B. kirkii*, *B. brasilensis* and *B. phytofirmans* have not yet been formally described or are in the process of being published (103, 109). A phylogenetic tree showing the relationship among all these *Burkholderia* species can be found in Coenye and Vandamme (25).

Distribution and diversity

Burkholderia species have a broad distribution, occurring commonly in soil (1), water (106) and in association with plants (5, 8, 75), fungi (66), animals and humans (25). In general, *Burkholderia* species are known soil saprotrophic bacteria, but the exceptions are the species *B. mallei* and *B. pseudomallei*, which cause diseases such as "glanders" and "melioidosis" respectively, in animals and humans (30, 53). The species belonging to the *B. cepacia* complex have also been isolated from various clinical samples, mainly from cystic fibrosis (CF) patients, where they are considered to be opportunistic pathogens (25).

Although the type species of the genus *Burkholderia*, *B. cepacia*, was initially described as the causative agent of onion soft rot (13), the majority of *Burkholderia* species apparently lacks phytopathogenic traits. A few species however, namely *B. plantarii*, *B. glumae* and *B. gladioli* can be pathogenic on rice. The latter has also been isolated from diseased onions, *Gladiolus* sp. and *Iris* sp. (25). In addition, *B. andropogonis* was described as a pathogen of sorghum, velvet bean and carnation, while *B. caryophylli* is also considered a pathogen of carnation and onion (25).

A large number of *Burkholderia* species has been isolated from soil, exhibiting different degrees of non-pathogenic interaction with plants (25). *B. graminis* has been described as a plant-associated species found in maize and wheat rhizoplanes, in Australia and France (107),

while *B. caledonica* was isolated from the rhizosphere of different plants in Scotland (23). Species belonging to the *B. cepacia* complex are often found in association with grass (77) and maize roots (32); in the latter they can represent up to 3.6% of the total culturable population (32). The newly described species *B. phytofirmans*, which was originally isolated from surface-sterilised onion roots, is able to colonise a range of plants, such as potato, tomato and grape, both in the rhizosphere and inside plant tissues (88). By using a gfp-tagged derivative of *B. phytofirmans* strain PsJN, Sessitsch *et al.* (88) observed that six days after inoculation on chick pea plants, this strain was able to colonize root epidermal cells, parenchyma cells, xylem vessels, stems and leaves endophytically. The presence of *Burkholderia* species as endophytes in different plants was observed in a survey conducted in South Australia and in France (5), where many isolates identified as *B. cenocepacia* were obtained not only from the rhizosphere, but also from the inner tissues of wheat, lupine and maize. Recently, *Burkholderia* strains were also isolated from inner tissues of maize plants (37) and analysis of the 16S rDNA indicated that these strains represent two new *Burkholderia* species, named *B. tropica* and *B. unamae* (15, 82)

Burkholderia species are also known to be present in bulk soil. Examples of these bacteria are *B. glathei, B. phenazinium, B. hospita, B. terricola* and *B. sacchari*, which were originally isolated from soil samples collected from agricultural areas (10, 49, 107). Regarding the organisms belonging to the *B. cepacia* complex, their presence in soil is often unequally distributed, most of the *B. cepacia* complex population generally being represented only by *B. cepacia, B. cenocepacia, B. ambifaria* and *B. pyrrocinia* (25).

Although many *Burkholderia* species have been isolated from soil or from plants (roots), they are also prevalent in water. By using enrichment broth, Vermis *et al.* (106) showed that five different genomovars of the *B. cepacia* complex (*B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. vietnamiensis* and *B. anthina*) could be isolated from two European rivers. However, the genomovar pattern from a given sample varied with the enrichment broth used (106).

Interestingly, some *Burkholderia* species are also found in close association with fungi. *B. fungorum* was isolated from the white-rot fungus *Phanerochaete chrysosporium*, and it has been suggested that there is a symbiotic relationship between these bacteria and the fungus (23). The species *B. sordidicola* was isolated from another white-rot fungus, *P. sordida.* Similarly to the situation with *B. fungorum*, attempts to obtain a pure fungal culture failed, although the bacterial culture could be easily isolated (66, 87). In another example of a potential symbiosis, a *Burkholderia* species was found at the junction of the midgut and intestine of *Tetraponera* ants, where it might be involved in the oxidative recycling of nitrogen-rich metabolic waste (101). In contrast to the white-rot fungus symbiont, the ant symbiont could not be cultured. Although this species is closely related to *B. fungorum* and *B. caledonica*, its exact taxonomic position remains unknown (25).

The close association between *B. cepacia* complex organisms and maize plants has strongly influenced *Burkholderia* ecological research, as it has mainly been focused on the interaction maize – *B. cepacia* complex organisms (8, 18, 28, 41). For instance, Chiarini *et al.* (18) showed that the diversity of *B. cepacia* complex isolates increased during plant development. In addition, the highest diversity values were obtained in the middle and terminal

portions of roots from mature plants (18). In another study, Dalmastri *et al.* observed that the majority of the isolates recovered from maize plants was identified as *B. ambifaria* while the remainder was distributed among *B. cenocepacia*, *B. pyrrocinia* and *B. cepacia*, a distribution which differed from those observed in clinical samples (29). Additionally, in a survey performed in maize fields with different management history, the species *B. cepacia*, *B. cenocepacia*, *B. vietnamiensis* and *B. ambifaria* were most frequently isolated (41). The highest degree of genomovar diversity was obtained in the field characterised by a low-input system, to which only manure had been added (41). In a survey performed in the United Kingdom, Richardson *et al.* evaluated the diversity of 75 *Burkholderia* isolates obtained mainly from rhizospheric environmental sources (83). They concluded that most of the isolates obtained from woodland rhizospheres were related to each other and had close affinity to *Burkholderia* species with known biocontrol or bioremediation abilities (83).

In conclusion, *Burkholderia* species represent a diverse group of microorganisms, present in many environments and involved in a range of different functions, some essential for the survival of the symbiotic partner.

Applications

Biological control of plant disease: Species belonging to the genus *Burkholderia* have been identified as biocontrol agents of many plant-pathogenic fungi, such as *Pythium aphanidermatum*, *Pythium ultimum*, *Fusarium* sp., *Phytophthora capsici* and *Rhizoctonia solani* (16, 52, 55, 65). Moreover, a strain identified as a *Burkholderia* sp. was able to inhibit the growth of bacteria, pathogenic yeasts and protozoa (16). The ability of *Burkholderia* strains to suppress plant disease was observed in many different crops, such as corn, sweet corn, cotton, pea, tomato and pepper, where increased crop yield was observed even in the absence of the pathogen (52, 55, 65). In most of the studies mentioned above, the mechanisms involved in disease suppression were unknown. Antibiotic compounds such as phenazine and pyrrolnitrin can be produced by *B. phenazinium*, *B. pyrrocinia*, *B. ambifaria* AMMD and *B. cepacia* NB-1 and a novel antifungal lipopeptide has been identified in *B. cepacia* strain BC11 (36, 59). However, although both phenazine and pyrrolnitrin can play important roles in disease suppression by *Pseudomonas* species, the confirmation that antibiosis was the primary mechanism involved in biocontrol by *Burkholderia* species was obtained only for strains *B. ambifaria* AMMDR1 and BC-11, by using antibiotic production-deficient mutants (54, 59).

Despite the great potential for biological control, the impact of the *B. cepacia* complex strains on the survival of CF patients has led to more strict safety issues. As a result, some biocontrol strains, which had already been approved by the U. S. Environmental Protection Agency, had their risk assessment modified and their use restricted (79).

Biological nitrogen fixation and plant growth promotion: Biological nitrogen (N_2) fixation (BNF) by associative diazotrophic bacteria is a common process among *Burkholderia*. The ability to fix atmospheric nitrogen, which was initially observed only for *B. vietnamiensis* (98), has been extended to other species, including *B. kururiensis* (38), *B. unamae* (15), *B. tropica* (82, 109) and *B. brasilensis* (82, 109). In addition to the associative nitrogen fixation, the species *B. tuberum*, *B. phymatum* and *B. caribensis* were able to nodulate tropical legume plants (25, 104). The beneficial effects of inoculation of diazotrophic bacteria have been

observed on different crops. For instance, field trials performed in Vietnam showed that inoculation of rice with *B. vietnamiensis* resulted in up to 22% increases in grain yield (99). An endophytic *Burkholderia* species isolated from rice plants in Brazil was shown to be able to fix 31% of the total nitrogen captured by the plant (6) and the inoculation of rice with this endophytic *Burkholderia* species led to a 69% increase in the rice biomass (6). Moreover, increases in crop yield due to the introduction of diazotrophic *Burkholderia* strains have also been observed in sugarcane and maize (84).

It is known that the positive effects exerted by diazotrophic bacteria are not only confined to the fixation of atmospheric N_2, but to a combination of mechanisms including the synthesis of phytohormones and vitamins (33), including them in the group of the so-called plant growth promoting rhizobacteria. Rice plants inoculated with *B. vietnamiensis* strain TVV75, which is able to produce indole acetic acid (IAA) (92), showed a significant increase in plant growth already at the nursery stage (99). Moreover, inoculated plants flowered earlier than non-inoculated ones and inoculation affected grain filling positively (99). Plant growth promotion has also been observed for *B. ambifaria*, which led to a significant increase in the growth of maize when inoculated as seed treatment (19). A recently described *Burkholderia* species, *B. phytofirmans*, showed high activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, commonly found in plant growth promoting rhizobacteria and which is able to cleave plant ethylene ACC, therefore lowering the ethylene level in developing or stressed plant (88). In addition, plants inoculated with *B. phytofirmans* strain PsJN showed a more developed root system (78)

Bioremediation: *Burkholderia* species are also used for bioremediation, a process in which microorganisms are employed to reduce the concentration and toxicity of chemical pollutants from the environment (35). Many different strains, all identified as *B. cepacia*, have shown the ability to degrade xenobiotic compounds, such as cyanide and polychlorinated biphenyls (PCBs) (2, 69). One of the most studied examples is the PCB- degrading *Burkholderia* sp. LB400 and recently, a new species was proposed, *B. xenovorans*, to accommodate this strain (48). The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is another important soil pollutant and after the application of the herbicide in soils, the population of 2,4-D degraders increased (95). Sequence analysis of the 16S rDNA of the dominant bacterial community revealed that members of the genus *Burkholderia* were prevalent (95). Although this does not constitute causal evidence, it is likely that these organisms were involved in the degradation of 2,4-D.

B. cepacia *complex and cystic fibrosis*

Cystic fibrosis (CF) is a hereditary disease common in Caucasian populations and involves a mutation in the cystic fibrosis transmembrane conductance regulator gene, which finally leads to chronic microbial colonisation of the respiratory tract, and consequently, pulmonary infections (25, 26). Several opportunistic pathogens are involved in pulmonary infections, which are the main cause of morbidity and mortality among CF patients. However, it was only in the early 1980's that *B. cepacia* emerged as a CF pathogen (67). The fate of CF patients infected with species belonging to *B. cepacia* complex is unpredictable, varying from chronic infection to death (90), but the high virulence observed for certain species has led to strict measures for infection control (90). Although all species of the *B. cepacia* complex have

been identified in CF sputum cultures, surveys performed in many countries showed that *B. cenocepacia* and *B. multivorans* most often prevailed (25). This finding, together with the fact that *B. cenocepacia* is commonly found in association with plants (5), raised the question whether the plant/soil environment could represent a potential hazard for CF patients (14). The answer to this question remains unknown, since the lack of genetic markers to assess transmissibility or virulence makes any distinction between environmental and clinical strains, so far, impossible (79).

Microbial communities and their responses to agricultural management

Assessment of microbial diversity in soils

Due to the importance of soil microorganisms, many studies have been performed to determine the factors that regulate the structure of the microbial community across temporal and spatial scales and the impact of different plant species on microbial diversity (17, 50, 57, 60, 89). This has led to a strong increase in the numbers of publications from 1974 to 1999 (74), which assessed mainly the effects of different soil properties on microbial communities (74). However, over the last five years, the impact of human activities on the diversity of soil microorganisms and the impact of biodiversity on soil processes has become a more important issue (12, 20, 50, 58, 91). As a result, the number of studies addressing the impact of agricultural and other land management practices on microbial diversity has also increased.

The analysis of soil microbial communities has traditionally depended on cultivation techniques. However, the fact that only a small percentage of soil microorganisms can be cultured, has spurred the development of culture-independent techniques, resulting in a dramatic change in the generic approach used to analyse soil microbial communities (eg. 3). Among the culture-independent methods, DNA-based community characterisation techniques, particularly those targeting the 16S or 18S rRNA genes, have had the most dominant role in biodiversity studies over the past 15 years, compared to all other techniques used for characterising microbial diversity (74). For a complete description of the methods for assessing the composition and diversity of soil microbial communities, the reader is referred to Hill *et al.* (56), Akkermans *et al.* (3) and Kowalchuk *et al.* (61).

Effects of anthropogenic disturbance on soil microbial diversity

Microbial communities play important roles in many soil processes. For instance, they regulate nutrient cycling, organic matter decomposition, control plant growth and structure the soil environment. They are therefore considered to be the most important element in determining soil quality. Accordingly, the quality of the soil, together with the climate, directly affect the productivity and structure of the vegetation, which in turn, influence soil microbial communities through the abundance, quality and distribution of organic resources (45). Although this aboveground/belowground system seems to be well balanced, it is often disturbed by human activities. Human-induced soil degradation has now even reached about 40% of the world's agricultural land through soil erosion, environmental pollution and intensive soil cultivation (34). Along with this intensive soil cultivation, there is often an increase in the use

of mechanical power and agrochemicals in substitution of manual labour, organic fertilisers and environmentally-friendly (natural) pest management. As a consequence, the regulation of soil functioning through the soil microbiota is progressively being replaced by regulation through chemical and mechanical input (45).

Although man might improve soil fertility, for instance by adding fertilisers or plant or animal residues, or by improving soil drainage or altering soil pH, these management practices modify the soil in such a way that the soil's functional diversity is altered (108). Moreover, practices such as (excess) tillage, burning, removal of crop residues and irrigation with saline water promote a decline of soil quality, which is generally accompanied by a loss of biological diversity (108). Soil microorganisms are key components of soil biodiversity since they are involved in many biogeochemical cycles (85). Therefore, perturbations caused by soil contamination or agricultural practices may provide a selective advantage to some groups, mainly bacterial species, which are able to respond faster to the change imposed in the environment than others groups (96). While bacterial biomass and respiration might not be reduced in moderately perturbed environments, denaturing gradient gel electrophoresis (DGGE) and reassociation analysis have revealed that bacterial diversity can be reduced, and that certain bacterial groups can become dominant (96).

Effect of agricultural practices and management regimes on microbial communities associated with:

Grassland: Soil microbial communities associated with grassland are by far the most intensively studied systems in the context of soil management. Different techniques, such as direct ribosome isolation, 16S rDNA libraries and temperature/denaturing gradient gel electrophoresis (T/DGGE) analysis, phospholipid fatty acid profiles (PLFA) and community level physiological profiling (CLPP) have been used to assess these communities (12, 20, 50, 72, 91).

Different levels of soil fertility have been shown to correlate with microbial community structure, as shown by McCaig *et al.* who assessed the bacterial diversity in so-called "unimproved" (non-fertilised) and "improved" (fertilised) grassland (72). DGGE analyses of the PCR products revealed that the microbial communities in the improved grassland were less diverse than those in the unimproved grassland, due to a decrease in evenness, what could be explained by the selection of particular bacterial types in the former (72). Interestingly, the so-called "semi-improved" grassland, which did not receive any fertiliser but was grazed upon, was more similar to the corresponding improved (fertilised) grassland than to the unimproved (non-fertilised) grassland (72). Since these grasslands are characterised by different levels of sheep livestock grazing pressure, being highest in the improved grassland (10-20 sheep ha^{-1} yr^{-1}), and lowest in the unimproved one (1-4 sheep ha^{-1} yr^{-1}), it is possible that the differences in grazing pressure overcame the effect of the fertiliser. Grayston *et al.* (50) further observed that soil microbial biomass increased as soil fertility decreased (unimproved), and this increase was accompanied by a shift in microbial community structure. As demonstrated by PLFA and culturing techniques, there was an increase in the proportion of fungi relative to bacteria in the unimproved grassland. Bacteria dominated the improved grassland and there was a strong correlation between lipids typical of Gram-negative bacteria and the presence of the plant species *Lolium perenne* and *Trifolium repens* (50).

The fact that grassland management practice had an impact on the community structure of specific bacterial groups was confirmed by Clegg *et al*., who analysed PCR-DGGE banding patterns and PLFA profiles (20). Inorganic nitrogen fertilisation resulted in significant changes in microbial community structure, as determined by PLFA community profiles and PCR-DGGE analysis. The latter method indicated a significant impact of fertilisation on bacterial and actinomycete communities, but not on those of pseudomonads. Since the soil was poorly drained, permanent drainpipes were placed on a few plots to evaluate the effect of draining the soil on microbial community structure. Hence, it was observed that soil drainage had a significant impact on the diversity of actinomycetes, as determined by both PLFA and PCR-DGGE analysis, while the latter method showed that soil drainage also affected the community structures of pseudomonads (20).

Different types of grassland harbour different microbial communities, as shown by Steenwerth *et al.*, who observed that perennial grasslands had high microbial diversities, and were distinct from "old" grassland fields that had been formerly cultivated and at that moment supported perennial bunchgrasses. Unlike perennial grasslands, all sites that contained annual grassland had similar microbial communities, as shown by PLFA profiles, regardless of the time elapsed since the last tillage (from 8 to 50 years). The resemblance in PLFA profiles could be explained by the fact that the plant community in the annual grassland was composed of less than a dozen dominant plant species of similar phenology. Although these species might change in relative abundance every year, the plant community composition remained similar and no succession occurred (91). Interestingly, when annual grasslands were tilled for two consecutive years and were then kept fallow, their PLFA profiles diverged from those in the annual grassland.

The response of the soil bacterial community to grassland natural succession was also evaluated by studying five meadows taken out of agricultural production at different time points and one fertilised meadow plot (39). The results showed that the ribosome levels per g of soil approximately doubled a few years after agricultural production and fertilisation had stopped, which seemed to be correlated to changes in vegetation, mainly the increase in plant diversity generated by the collapse of the dominant species *L. perenne* (39). However, despite the changes in vegetation, no differences in the composition of the bacterial communities could be observed by PCR-TGGE analysis on the basis of (amplified) 16S rRNA fragments.

Arable land: In order to evaluate how cereal/legume rotation affects the bacterial community associated with the rhizosphere, Alvey *et al.* performed microcosm experiments in which soils from legume rotations or continuous cereal plots were transferred to containers and sown with different crops (cereals and legumes) (4). Based on PCR-DGGE analysis, these authors showed that agricultural management had a stronger influence on bacterial community structure than plant species had. Hence, plants growing on continuous cereal soil had very similar bacterial communities in their rhizospheres, regardless of the plant species they were collected from, whereas bacterial communities associated with crop rotation showed greater variability and clustered according to plant species (4). Based on these results, Alvey *et al.* concluded that crop rotation can cause significant shifts in bacterial communities associated with the rhizosphere, an effect that could be observed already 14 days after sowing the species (4). Different rotations indeed exert a distinct effect on soil microbial communities, as FAME analysis of soils under different potato rotations showed that rotations including barley and

millet resulted in the highest fungi-to-bacteria ratio, while soybean and continuous potato induced the lowest ratio (63). In addition, microbial activity tended to be highest in potato following barley, canola and sweet corn and lowest in continuous potato (63).

The effect of crop rotation on soil microbial community structure was also evaluated by Lupwayi *et al.*, who measured the tillage effect by sampling soil from the wheat phase of different crop rotations established under zero or conventional tillage (68). The CLPP profiles showed that the (functional) microbial diversity was significantly higher in wheat under legume-based crop rotation than in wheat monoculture (68). In addition, tillage significantly reduced the microbial diversity, mainly in the bulk soil. Furthermore, bacterial communities under conventional tillage were more similar in structure than those under zero tillage (68). The effect of tillage on microbial community structure was also determined by Ibekwe *et al.*, who observed that no-till soils had a higher biomass (extractable PLFA) than soils under conventional tillage (57). Additionally, by using PCR-DGGE targeting the 16S rRNA genes, these authors showed that the soil under conventional tillage was the most dissimilar one, which differs from the results obtained from Lupwayi *et al.* (68). However, these contrasting results could be explained by the different methodological approaches used, since the results obtained by Lupwayi *et al.* were based on culturing techniques, while Ibekwe *et al.* used a culture-independent approach (57, 68). The influence of tillage on soil microbial communities was also evaluated by Feng *et al.* (40) in an area under long-term continuous cotton. Although no-till led to an increase in soil organic carbon, nitrogen and microbial biomass, PLFA analysis showed that changes in soil microbial communities during the growing season were primarily determined by root exudation and environmental conditions (moisture and temperature) rather than by the soil conditions modified by tillage (40).

The comparisons between crop rotation and continuous cropping indicated that crop rotation might increase microbial activity and diversity, and stimulate the population of fungi over bacteria in soil (4, 63, 68). But although monoculture might reduce soil microbial diversity (4, 68), the use of continuous cropping selects for certain bacterial species that are better adapted to that condition. In some cases, these species might have beneficial traits, allowing the farmer to profit from continuous crop systems. The best known example is the decline of take-all, an important disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* (51). Although this disease affects wheat, continuous wheat monocropping has led to the suppression of the pathogen. Take-all decline has been detected in different soils worldwide, and an increase in the population of antibiotic-producing *Pseudomonas* species seems to be a key mechanism involved in the process (31, 80). Similarly, the establishment of apple orchards in a field where wheat had previously been grown led to an decrease in suppressiveness of this soil towards *Rhizoctonia solani* with the increased age of the orchard. The decrease in suppressiveness was correlated with a decline of the *B. cepacia* and *P. putida* populations (71).

Although crop management affects microbial diversity, soil type and seasonal changes might also have an effect on microbial communities (43). According to the data of Bossio *et al.*, soil type and time were key factors that determined microbial community structures in soils from organic, low-input and conventional farming systems to a greater extent than management system (9). Similar results were obtained by Schutter *et al*. (86), who collected soil from two experimental fields in which winter cover cropping/reduced tillage versus winter

fallow were established. Although season and soil type were the major determinants of microbial community structure, alternative management practices like winter cropping also affected soil microbial community, as determined by FAME profiles and CLPP.

As a conclusion, different agricultural practices, such as fertilisation and tillage, lead to distinct microbial communities in both grassland and arable land. The effect of fertilisation on soil microbial community was extensively studied in grassland, where unimproved grassland (non-fertilised, low livestock grazing pressure) had higher soil microbial biomass and diversity than the improved grassland (fertilised, with high livestock grazing pressure) (50, 72). Tillage had a great impact on arable land, decreasing soil microbial diversity (68) and the comparison between conventional tillage and non-till practices indicated that the latter increased soil organic carbon, nitrogen and biomass (40, 57).

Effects of changes in agricultural management on soil microbial community structure

Agricultural practices and management regimes affect soil microbial community structure, but what happens to the microbial community when grassland is converted into arable land? How long will it take for an effect of such a change to become "visible" in the microbial communities? In order to answer these questions, an experiment was initiated in the Netherlands, in an area that consisted of permanent grassland, arable land under crop rotation, arable land under maize monoculture, grassland converted to arable land under crop rotation and to maize monoculture. After applying PCR-DGGE to assess both total bacterial and fungal communities, we (102) observed clear differences among grassland, arable land under crop rotation and arable land under maize monoculture, with the highest diversity being detected in the grassland. The use of genus-specific PCR-DGGE systems corroborated this result (42, 44, 102). In addition, the *Bacillus* and *Pseudomonas* communities associated with grassland converted into arable land (both crop rotation and maize monoculture) were more similar to the grassland communities than those of the arable land (42, 44). These results were not unexpected since the conversion from grassland into arable land had occurred only two years before the samples were taken. The effect of land use intensification on bacterial community structure was also observed by Gomez *et al.* (47), who compared an undisturbed field area with sites with increasing time elapsed since clearing of native vegetation and different further management. CLPP analysis showed that the highest functional diversity was present in bacterial communities from the native vegetation, whereas the lowest functional diversity was present in the soil that had been under agriculture for the longest period of time (47).

Although agricultural management, as well as other soil disturbances (such as pollution), can lead to changes in soil bacterial community structure, when the perturbation is removed, the bacterial community may increase in diversity again (96). But how long would it take to restore the initial diversity?

Buckley and Schmidt (12) compared fields that had been taken out of production for different periods of time and, therefore, were in different successional stages, to a historically-cultivated field, and a field that had never been cultivated. Based on rRNA abundance, assessed by probes targeting different bacterial groups, they concluded that the microbial community structure in the field that had never been cultivated differed significantly from the historically-cultivated ones. In addition, when comparing abandoned fields in different successional stages,

the field that had been abandoned for nine years was still quite similar to the historically cultivated areas (12). Only the field that had been abandoned for more than 45 years had a microbial community structure that was comparable to fields that had never been used for agriculture before (12). Similarly, Steenwerth *et al.* observed that grassland sites, which had been tilled from 3 to 33 years ago, were still different from native, never-tilled grassland (91). These observations indicate that recovery of soil microbial community structure from cultivation effects may require decades (12, 91).

In an experiment performed in China, soil was collected from areas in a field with different land use history but with the same soil type, and the microbial community structure was evaluated by PLFA profiling and CLPP (113). Both methods showed that the areas grouped according to land use history, confirming the effect of agricultural management on the microbial community structure (113). PLFA and CLPP analyses showed that two older orchard soils (8 and 12 years of citrus) were more similar to each other than to younger orchard soil (4 years of citrus). In fact, a progressive increase in fungal PLFAs was observed with increasing age of the orchard (113). Interestingly, the young orchard was very similar to an eroded soil, which is considered as a starting point of agricultural soils in the Chinese area studied. This observation suggests that the first four years of cultivating citrus were not enough to reach the state observed in orchards with 8 to 12 years of cultivation (113).

Multivariate analysis as a tool to investigate microbial diversity

Multivariate analysis have widely been used in macroecology (94), and in the past few years it has been also applied by microbial ecologists in order to describe microbial community structure. The methods used to analyse microbial community structure in soil vary according to the fraction of the microbial population they assess. However, irrespective of the method applied, multivariate analysis is a powerful tool to compare microbial community structures in soils under different agricultural management regimes (where multiple factors affect microbial community structure). The main advantage of this statistical technique is that a large number of species and environmental factors can be evaluated together, providing the means to assess broad-scale community structures, which are then correlated with multiple "controlling" factors, such as fertilisation, tillage and soil history (91). Although multivariate analysis can be applied to virtually any method that provides species distribution per collected sample, only recently it has been applied as a statistical tool to interpret DGGE fingerprinting (70, 73, 76). The traditional way to analyse DGGE profiles is by clustering analysis using UPGMA (unweighted pair group method with mathematical averages) as the algorithm. This method uses a hierarchical way to identify similar patterns based on the presence or absence of bands, but does not allow the quantification of bands nor the correlation between banding pattern and environmental variables, what can be achieved by using multivariate analysis. The usefulness of multivariate analyses of DGGE profiles was confirmed by Muylaert *et al.* (76) even after using an artificial data set where potential errors associated with PCR-DGGE analysis were introduced.

The goal of multivariate analysis as an ordination technique is to arrange sample points in space in such a way that the axes used represent the greatest variability in the community structure. The distribution of sample points and species is then visualised using an ordination diagram, which is interpreted following the basic assumption that graphical proximity means

close similarity (64). In general, ordination techniques are classified according to (1) “gradient analysis” and (2) “species response to the gradient”, and understanding this classification is crucial for choosing the most appropriate method. Classification according to “gradient analysis” refers to the presence or absence of explanatory variables, giving origin to either “unconstrained” or “constrained” ordination, respectively. In unconstrained ordination or “indirect gradient analysis”, variables that best explain the species composition are searched, since there are no measured environmental variables to explain the species distribution, and these variables are then represented by the ordination axes (64). Examples of unconstrained ordination techniques are principal component analysis (PCA), correspondence analysis (CA) and detrended correspondence analysis (DCA). In constrained ordination or “direct gradient analysis”, environmental variables are measured (different treatments) and used to explain species distribution (community structure) (64). In this case, the axes correspond to the directions of the greatest variability within the data set, which can be explained by the environmental variables (64). Redundancy analysis (RDA) and canonical correspondence analysis (CCA) are examples of constrained ordination techniques. The classification of ordination techniques according to “species response to gradient” refers to the fitted line or curve describing the species distribution (species response model). These techniques can be divided in linear (straight line; PCA, RDA) or unimodal (symmetric, bell-shaped curve; CA, CCA) (64). The decision whether or not the species distribution is linear or unimodal can be obtained by estimating the heterogeneity in the species data (64).

The results of multivariate analysis are shown as ordination plots, which might contain species distribution, samples and environmental variables. For unconstrained ordination plots, samples are represented by points (symbols) and species by arrows, which point in the direction where species abundance increases (64). In constrained ordination plots, both samples and species are represented by points (symbols) and in the case of species, the points estimate the species optima (weighted average of species distribution) (64). For both methods, quantitative environmental variables are represented by arrows or vectors, pointing in the direction where the value of the environmental variable increases. Both the length and the slope of the vector are significant parameters: longer vectors forming smaller angles with an ordination axis are more strongly correlated with that ordination axis (91). In addition, the angle between vectors provides an approximation of the correlation (93). Consequently, vectors pointing in the same direction are positively correlated, those pointing in opposite directions are negatively correlated and those forming a right angle are not correlated. For qualitative environmental variables, centroids are used for individual categories, and they represent the centroid of the samples where the category is present (64).

To test the significance of the relationship of species distribution with environmental variables, the Monte Carlo permutation test is performed, with the null hypothesis that species composition is independent of environmental variables. By rejecting the null hypothesis, it is assumed that particular environmental variables affect the distribution of species composition significantly.

Aim and research questions

Aim

The general idea behind this study was to assess whether functional bacterial groups respond to agricultural management and if so, which groups would be selected by a particular management regime. By understanding the ecology of certain groups, for instance potential biocontrol species, it would be possible to manipulate soil microbial community structure by managing the system. Thus, biocontrol species might be favoured and the level of soil suppressiveness towards diseases enhanced. To achieve this goal, focus is on specific important bacterial groups and the genus *Burkholderia* is one of them. Therefore, the aim of this study was to evaluate the diversity and community structure of *Burkholderia* species in soils under different agricultural management and to determine how agricultural management regime (crop rotation, maize monoculture and grassland) would affect *Burkholderia* community structure.

Research questions

Four research questions were conceived.

1. Is it possible to develop a method that allows the direct molecular (cultivation-independent) analysis of *Burkholderia* species in environmental samples?

2. How is the diversity of the genus *Burkholderia* affected by land use history and crop species such as maize, oats, grass and barley? Which effect (land use history versus crop type) has a greater influence on *Burkholderia* community structure?

3. What effect does agricultural management regime exert on the diversity of the culturable *Burkholderia* community? Moreover, which species would be correlated with different management regimes? How do the species belonging to the *B. cepacia* complex respond to changes in the management?

4. What is the impact of different agricultural management regimes on the *Burkholderia* community structure? How do selected management regimes affect a subset of the *Burkholderia* community with potential for biological control of the soilborne pathogen *Rhizoctonia solani* AG-3?

The first research question concerns the possibility of assessing the community structure and diversity of the genus *Burkholderia* by direct molecular means. This question is addressed in chapter 2. The second research question is related to the response of the *Burkholderia* community to different crops and land use history and is tackled in chapter three. Research questions three and four are related to the effects of different agricultural management regimes on both the culturable and total populations of *Burkholderia* and are addressed in chapters four and five, respectively.

Outline of the thesis

A molecular method was developed to allow the direct assessment of *Burkholderia* community in environmental samples. Initially, a primer set specific for the genus *Burkholderia* was developed based on the 16S rRNA gene and tested *in silico* (databases) and *in vivo* (with DNA from pure cultures). DGGE analyses of the PCR products were then performed, indicating that there were sufficient differences in the migration of the fragments amplified by the primers to distinguish the majority of the *Burkholderia* species tested. The PCR-DGGE system was validated by amplifying DNA from rhizosphere and bulk soil collected from permanent grassland and by sequencing soil-derived clones. The *Burkholderia* community associated with permanent grassland was analysed (chapter 2).

The PCR-DGGE system developed was then used to evaluate the effect of different crop types and land use history on the *Burkholderia* community structure in soil. An experiment in microcosms was set up in the greenhouse, in which different crop plants (maize, barley, grass, and oat) were planted in pots containing soils with different land use histories (maize monoculture, crop rotation and grassland), for three consecutive growth cycles (chapter 3).

The effect of agricultural management regime on the diversity of *Burkholderia* species was then evaluated by using a culture-dependent approach. *Burkholderia* isolates were grouped in 47 clusters, according to a combination of their DGGE and BOX patterns. These clusters were used to evaluate the *Burkholderia* diversity (at culturable level) by applying species abundance models and the Shannon diversity index. The latter was used to evaluate the effect of agricultural management on the culturable fraction of the *Burkholderia* community (chapter 4).

By using a culture-independent approach, the influence of agricultural management regime on the dynamics of the *Burkholderia* community in the field was then evaluated. Additionally, a culture-dependent approach was used to correlate agricultural management with the occurrence of *Burkholderia* strains with potential for biological control of *Rhizoctonia solani* AG3 (chapter 5).

The research questions, which were posed in order to achieve the aim of this work, were answered based on the results obtained during this study (chapter 6).

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Chapter 2

A molecular method to assess the diversity of *Burkholderia* species in environmental samples

Abstract

In spite of the importance of many members of the genus *Burkholderia* in the soil microbial community, no direct method to assess the diversity of this genus has so far been developed. The aim of this work was the development of soil DNA based PCR-DGGE, a powerful tool to study the diversity of microbial communities, for the detection and analysis of the *Burkholderia* diversity in soil samples. Primers specific for the genus *Burkholderia* were developed based on the 16S rRNA gene sequence and evaluated in PCR reactions with genomic DNA from *Burkholderia* and non-*Burkholderia* species as templates. The selected primer system displayed good specificity and sensitivity for the majority of established species of the genus *Burkholderia*. DGGE analyses of the PCR products obtained showed that there was sufficient difference in migration behaviour to discriminate between the majority of the 14 *Burkholderia* species tested. Sequence analysis of amplicons generated with soil DNA exclusively revealed sequences affiliated with those from *Burkholderia* species, demonstrating that the PCR-DGGE method is suitable to study the diversity of this genus in natural settings. An analysis of the *Burkholderia* communities in two grassland plots via PCR-DGGE revealed differences in diversity mainly between bulk and rhizosphere soil samples.

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Introduction

The genus *Burkholderia* is an important component of the soil microbial community (19). For instance, *B. cepacia* has first been described as the causative agent of onion soft rot (10), but several strains of this species lack this phytopathogenic trait and play an important role in promoting plant health (5). Moreover, many species belonging to the genus *Burkholderia* have the ability to produce compounds with antimicrobial activity (13, 21, 29, 31) and thus, to serve as biocontrol agents of phytopathogens. In addition, other *Burkholderia* strains have been shown to be plant-growth-promoting rhizobacteria (PGPR) (44), and the introduction of *Burkholderia* species in crops such as maize and sorghum has, thus, resulted in increases of both root and shoot dry weights (4, 14). The mechanisms involved in the PGP property may range from the production of phytohormones to the fixation of atmospheric nitrogen, as shown for *B. vietnamiensis* (44). Estrada-De los Santos et al. (22) recently indeed showed that nitrogen fixation is a common property among the genus *Burkholderia*, after isolating new diazotrophic *Burkholderia* species from coffee and maize plants, which were phylogenetically unrelated to *B. vietnamiensis*. Furthermore, unculturable bacteria belonging to *Candidatus Glomeribacter gigasporarum*, closely related to the genus *Burkholderia*, have been found as endosymbionts of arbuscular mycorrhizal fungi (AMF) (6) and genes involved in nitrogen fixation were shown to be active at least during the germination of spores (34). The endosymbionts were detected mainly in the family *Gigasporaceae*, being present as homogeneous populations throughout the fungal life cycle (7). In addition to all these features, the high nutritional versatility of the genus *Burkholderia*, reflected in its ability to use a wide range of organic compounds as a carbon source (25), certainly contributes to its capacity to successfully compete for root exudates and so, to efficiently colonise habitats such as the plant root. This nutritional versatility has also led to the use of *Burkholderia* strains in the biodegradation of environmental pollutants (23).

Concomitant with the high applicability of members of the genus *Burkholderia*, there is an increasing concern about the risk of using this group of bacteria in processes such as biological control and bioremediation (11) since some species are important pathogens in cystic fibrosis patients (26, 49).

The list of species belonging to the genus *Burkholderia* has changed several times since 1992, when Yabuuchi *et al*. (51) proposed that 7 former *Pseudomonas* species belonging to the so-called rRNA group II should be grouped in this new genus, based on the results of a polyphasic taxonomic study. Nowadays, the genus *Burkholderia* comprises 36 species (1, 8, 18, 37, 49, 50, 52). Moreover, several strains previously identified as *B. cepacia* were grouped in the so called *B. cepacia* complex, which comprises at least nine genomic species or genomovars (18).

The microbial community in soil is inherently complex and assessments performed in such a complex population do not always reveal its specific components. Moreover, cultivation-based methods are limited in that they do not assess the non-culturable fraction of the soil microbiota (45). Hence, an analysis of distinct phylogenetic groups of bacteria on the basis of soil DNA is required, as it reduces the complexity and thereby facilitates the assessments of these subgroups of the total diversity (36). This can be achieved by denaturing gradient gel

electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments, a technique that has been widely used to assess the diversity of various phylogenetic groups (35).

Burkholderia spp. have been identified by techniques such as DNA-DNA hybridisation, SDS-PAGE, AFLP fingerprinting and PCR with primers with different degrees of specificity (3, 17, 49). In addition, the assessment of *Burkholderia* species in environmental samples has been mainly based on analyses of the *B. cepacia* complex, using restriction fragment length polymorphism (RLFP) analyses of the *recA* gene or 16S rDNA (4, 19, 24). However, none of these methods, including the PCR based approaches, is applicable to directly evaluate the diversity of the genus *Burkholderia* in natural settings.

The main objective of this work was the development of a method, based on PCR-DGGE, to allow the direct analysis of the diversity of *Burkholderia* species in environmental samples. To achieve this goal, primers specific for the genus *Burkholderia* were developed based on the 16S rRNA gene. The PCR system was first evaluated for specificity and sensitivity using DNA isolated from *Burkholderia* and non-*Burkholderia* species. After optimisation of the method, the PCR-DGGE system specific for *Burkholderia* was used to assess the diversity of this genus in soil samples.

Materials and methods

Bacterial strains

The strains used in this study and their growth characteristics are listed in Table 1. All species were stored at -80°C in 20% glycerol.

Soil samples

Samples from grassland bulk and rhizosphere soil were collected in a field (Wildekamp) located in Wageningen, the Netherlands. This site has been under permanent grassland for approximately 50 years. The soil used is a loamy sand soil (3% clay, 10% silt, and 87% sand), with about 2% of organic matter and pH 4.2. Samples were taken with a soil core sampler (∅ 3 cm), from the surface (0-10 cm) of two replicate plots (47 and 31) in the same area. One composite sample was prepared for each plot, by combining 100 of such samples. Bulk soil was obtained from each composite grassland sample by removing loosely adhering soil from the plant material and mixing it thoroughly. From the remaining root material with tightly-adhering soil, rhizosphere samples were prepared by removing the soil from the roots.

Table 1. Strains used in this study.

Group	Taxon	Strain(s)	Growth conditions[a]
-Proteobacteria	*Burkholderia andropogonis*	ATCC19311, LMG 6872, ATCC23061^T	TB-T, 27°C
	Burkholderia caribensis	LMG18531^T, WD3	TB-T, 27°C
	Burkholderia caryophylli	NCPPB353, ATCC25418^T	TB-T, 27°C
	Burkholderia cenocepacia	LMG16656	TB-T, 27°C
	Burkholderia cepacia	IPO1718, NCPPB945, NCPPB946, ATCC25416^T, P2[b]	TB-T, 27°C
	Burkholderia dolosa	LMG18941	
	Burkholderia gladioli	ATCC33664	TB-T, 27°C
	Burkholderia glathei	ATCC29195^T, WD1	TSB, 37°C[c]
	Burkholderia glumae	NCPPB3708, ATCC33617^T	TB-T, 27°C
	Burkholderia graminis	WD 2	TB-T, 27°C
	Burkholderia multivorans	LMG13010^T	TB-T, 27°C
	Burkholderia phenazinium	LMG2247^T	TB-T, 27°C
	Burkholderia plantarii	NCPPB3590, ATCC43733^T	TB-T, 27°C
	Burkholderia pyrrocinia	ATCC15958^T	TSB, 37°C
	Burkholderia stabilis	LMG14294^T	TB-T, 27°C
	Burkholderia vietnamiensis	LMG10929^T	TB-T, 27°C
	Alcaligenes faecalis	A1501[d]	10% TSB, 27°C
	Alcaligenes sp.	Isolate[d]	10% TSB, 27°C
	Delftia acidovorans	Q3-4-6-9[d]	10% TSB, 27°C
	Ralstonia eutropha	815[d]	LB, 27°C
	Ralstonia solanacearum	IPO1609[d]	LB, 27°C
	Variovorax paradoxus	Q2-5-27-9	10% TSB, 27°C
-Proteobacteria	*Agrobacterium radiobacter*	Isolate[d]	LB, 27°C
	Rizobium leguminosarum bv. trifolii	ANV794[d]	10% TSB, 27°C
	Rizobium meliloti	L530[d]	LB, 27°C
	Sphingomonas chlorophenolica	ATCC33790	TSA, 27°C[e]
	Xanthobacter autotrophicus	GJ 10[d]	NB, 27°C[f]
-Proteobacteria	*Acinetobacter calcoaceticus*	BD413j[d]	LB, 27°C
	Enterobacter agglomerans	Isolate[d]	LB, 27°C
	Enterobacter cloaceae	BE1[d]	LB, 27°C
	Pseudomonas aeruginosa	PAO25[d]	LB, 27°C
	Pseudomonas chlororaphis	PC8[d]	LB, 27°C
	Pseudomonas cichorii	PC170[d]	LB, 27°C
	Pseudomonas corrugata	PD704[d]	LB, 27°C
	Pseudomonas fluorescens	R2f[d]	LB, 27°C
	Pseudomonas glycinea	Pg1[d]	LB, 27°C
	Pseudomonas putida	UWC1[d]	LB, 27°C
	Pseudomonas stutzeri	JM303[d]	LB, 27°C
	Pseudomonas syringae	Isolate[d]	LB, 27°C
	Stenotrophomonas maltophilia	Pd1484[d]	10% TSB, 27°C
Actinomycetes	*Mycobacterium chlorophenolicum*	PCP-1[d]	DSM, 27°C
	Streptomyces griseus	ISP5236[d]	TSBy, 27°C[g]
Gram-positive bacteria	*Bacillus cereus*	FoTc-30[d]	LB, 27°C
	Bacillus subtillis	168 TrpC2[d]	LB, 27°C
	Listeria innocua	ALM105[d]	LBg, 27°C[h]
	Paenibacillus azotofixans	ATCC35681	TBN, 27°C

[a] For explanations of TB-T, LB, DSM and TBN see references 27, 40, 46 and 41, respectively.
[b] Strain P2 was obtained from the culture collection, Cluster MIBU, Plant Research International, Wageningen, The Netherlands.
[c] TSB, Trypticase soy broth containing (per liter) 17 g of pancreatic digested casein peptone, 3 g of papaic digested soybean meal, 5 g of NaCl, 2.5 g of K_2HPO_4, 2.5 g of dextrose (pH 7.3).
[d] Strain obtained from the culture collection, Cluster MIBU, Plant Research International, Wageningen, The Netherlands.
[e] TSA, tryptone soya agar containing (per litre) 15 g of tryptone, 5 g of soya peptone, 5 g of NaCl, 15 g of agar, (pH 7.3).
[f] NB, nutrient broth containing (per litre) 3 g of Bacto beef extract (Difco), 5 g of Bacto peptone (Difco) (pH 7.3).
[g] TSBy, Tripticase soy broth supplemented with 5% yeast extract.
[h] LBg, Lauria-Bertani medium supplemented with 5 g of glucose per litre.

DNA extraction

Genomic DNA from all bacterial strains (Table 1) was extracted by the method described by Duineveld *et al*. (20). DNA extraction from bulk and rhizosphere soils were performed using the UltraClean™ soil DNA isolation kit (Mo Bio Laboratories, BIOzymTC, Landgraaf, The Netherlands) according to the protocol described by the supplier, except that the cells were disrupted by bead beating (60 sec) in a Braun's cell homogeniser (Braun, Melsungen, Germany). The bead-beating step was included to ensure maximal cell lysis without severe shearing of the DNA (46).

Primer design

16S rDNA sequences belonging to the genus *Burkholderia* were retrieved from GenBank (National Centre for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/) and aligned using Clustal_X (43). After the alignment, the sequences were manually searched for homologous regions specific for this genus. The selected regions were further analysed by BLAST (2) to search for homologous nucleotide sequences in GenBank. This procedure was repeated until the desired specificity for the genus *Burkholderia* was reached. After determining the optimal sequence for the forward (Burk3 - 5' CTGCGAAAGCCGGAT 3') and reverse (BurkR - 5' TGCCATACTCTAGCYYGC 3') primers, which included changing the second nucleotide on the forward primer from G to T to avoid the formation of secondary structures, a GC-clamp (5' CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG 3') (28) was attached to the 5'end of the forward primer to allow its use in a DGGE system.

PCR

Amplification of 16S rDNA from genomic DNA was performed in 50 µl reaction mixtures containing 1 µl of DNA (5-10 ng), 200 µmol l^{1} of each deoxyribonucleoside triphosphate, 400 nmol l^{-1} of each primer, 1× *TaqPlus* Precision buffer (Stratagene, Leusden, The Netherlands) and 2 U of *TaqPlus* Precision polymerase mixture (Stratagene, Leusden, The Netherlands). Amplification from soil or rhizosphere DNA extracts using a direct PCR was performed in 50 µl reaction mixtures containing 5 to 10 ng of target DNA, 10 mmol l^{-1} Tris-HCl (pH 8.3), 10 mmol l^{-1} KCl, 3.75 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each deoxyribonucleoside triphosphate, 400 nmol l^{-1} of each primer (GC-clamped Burk3 and BurkR), 1% (v/v) formamide, 0.25 µg of T4 gene 32 protein (Boehringer, Mannheim, Germany) and 5 U of AmpliTaq DNA polymerase, Stoffel fragment (Perkin Elmer, Nieuwerkerk, The Netherlands). The amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands). Before the start of the reaction, the temperature was maintained at 95°C for 4 min. To enhance the specificity of the reaction, a touchdown PCR was carried out as follows. The annealing temperature was initially 62°C and decreased by 1°C every fifth cycle until 60°C, after which 25 additional cycles were carried out at 58°C. Denaturing was performed at 94°C for 1 min, primer annealing was achieved at the temperatures described above (90 sec) and primer extension occurred at 72°C (2 min). After the thermal cycling, a final extension step was performed at 72°C for 10 min, followed by cooling to 10°C. Nested PCR consisted of running a first PCR with primer Burk3 in combination with universal eubacterial primer R1378 (28), using the PCR conditions described by Rosado *et al.* (38). The

products from the first PCR were diluted 1:1000 and used as template in the second PCR, which was performed with primers Burk3 (GC-clamped) and BurkR, as described above for genomic DNA. The PCR products, expected to be approximately 500 bp, were analysed by electrophoresis in 1.5% (w/v) agarose gel in 0.5× TBE buffer (40). When needed, products were stored at-20°C for further DGGE analysis.

Denaturing gradient gel electrophoresis

DGGE analysis was performed using the phorU2 system (Ingeny, Leiden, The Netherlands), according to the methodology described by Rosado *et al.* (38), except that gradients of 50-60% denaturants were used and run at a constant voltage of 100 V for 15 hours. After electrophoresis, the gels were stained with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and with the Silver Staining Kit (BIO-Rad, Veenendaal, The Netherlands). The Molecular Analyst software (version 1.61, BIO-Rad, Veenendaal, The Netherlands) was used to analyse the DGGE profiles. Tolerance with respect to band positions was set at 0.8%. Cluster analysis was done within the Molecular Analyst software, using the unweighted pair group method with mathematical averages (UPGMA). The correlation was calculated using the Dice coefficient of similarity. A relatedness tree was produced with the algorithm of the Molecular Analyst software.

PCR-DGGE system sensitivity

To evaluate the sensitivity of the PCR-DGGE system with soil DNA, a mixture of *Burkholderia* species (*B. andropogonis* ATCC19311, *B. caribiensis* LMG18531, *B. dolosa* LMG18941 and *B. multivorans* LMG13010) was added to 50g portions of non-sterile Wildekamp soil, in three cell densities (5×10^3, 5×10^4 and 5×10^5 cells of each strain per g of soil). In control pots, sterile water was added to the soil. All treatments were done in duplicate. After overnight incubation at room temperature, soil DNA was extracted as described above. The sensitivity of the PCR-DGGE method was evaluated using both direct and nested PCR, and further analysed by DGGE.

Soil clones and sequence analyses

PCR products generated with DNA extracted from bulk soil and grass rhizosphere samples were purified using the High Pure PCR product purification kit (Boehringer, Mannheim, Germany). Products were then cloned into the pGEM-T easy vector, using *Escherichia coli* strain JM109 for transformation, according to the procedure given by the manufacturer (Promega Benelux, Leiden, The Netherlands). Clones were randomly selected, grown, and after plasmid extraction by the Wizard Plus SV miniprep DNA purification system (Promega, Benelux, Leiden, The Netherlands), used as templates in PCR reactions to produce products for controls in agarose gels. Soil clones producing PCR fragments with the appropriate size were then subjected to sequencing using an ABI prism automatic sequencer (Greenomics, Plant Research International, Wageningen, The Netherlands). Sequence identities were obtained by BLAST analyses (2).

Sequence alignments

Sequences recovered from the GenBank/EMBL database or generated in this work were aligned by using Clustal_X (43). Phylogenetic trees were constructed by the neighbor-joining method (39) based on distance estimation calculated by the method of Jukes and Cantor (30). This analysis was performed with the TREECON program, version 1.3b (Yves van de Peer, Department of Biochemistry, University of Antwerp, Antwerp, Belgium).

Nucleotide sequence accession number

The sequences generated in this study were deposited in GenBank under the accession numbers AF407341 to AF407358.

Results and discussion

Primer design

A comparison of 16S rDNA from 19 different *Burkholderia* sequences and 19 non-*Burkholderia* sequences obtained from GenBank gave rise to one region that was potentially specific for all *Burkholderia* sequences analysed (Fig. 1). A forward primer region (15 mer) was selected based on this region and analysed for specificity for the genus *Burkholderia*, using all 16S rDNA sequences deposited in GenBank, estimated to represent more than 10,000 different sequences, via BLAST. The results obtained showed that out of the 97 hits, 51% belonged to the genus *Burkholderia*, 38% to unculturable clones or as-yet-unidentified bacteria and 11% to other genera, such as *Pandoraea* (6%), *Ralstonia* (1%), *Thiothrix* (3%) and *Lautropia* (1%). Subsequently, all 16S rDNA sequences from strains classified as *Burkholderia* were recovered from the database and, among those containing the primer region (178 sequences), 92% showed complete homology with the primer sequence, the remainder differing by insertions or deletions at the 3'end of the primer.

The 16S rDNA sequences of several of the non-*Burkholderia* species which gave a hit in the BLAST assay were included in additional alignments to search for a region to be used as reverse primer. This analysis revealed a consensus region at position 646-663 [*E. coli* numbering, (9)] common only to the genus *Burkholderia*, despite some variation in the third and fourth bases at the 3' end (T-to-C conversions) (Fig 1). A BLAST search was performed with this putative reverse primer sequence, including all C-T variations observed in *Burkholderia* spp. at the third and fourth nucleotides (CC, CT, TT; position 660-661). The BLAST report revealed that sequences containing nucleotides CT at position 660 and 661 were widespread among all *Burkholderia* species, representing 65 out of 145 (45%) *Burkholderia* 16S rDNA sequences available in the database and containing that region. Sequences containing the nucleotide motifs CC and TT were less dominant, occurring in 43 (30%) and 23 (16%) *Burkholderia* 16S rDNA sequences, respectively. Only 9% of the remaining *Burkholderia* 16S rDNA sequences showed low homology to the 3'end of the reverse primer sequence. Moreover, the BLAST search also identified 28 sequences from non-culturable or unidentified bacteria, which might represent *Burkholderia* sequences. A few sequences belonging to other genera were also detected.

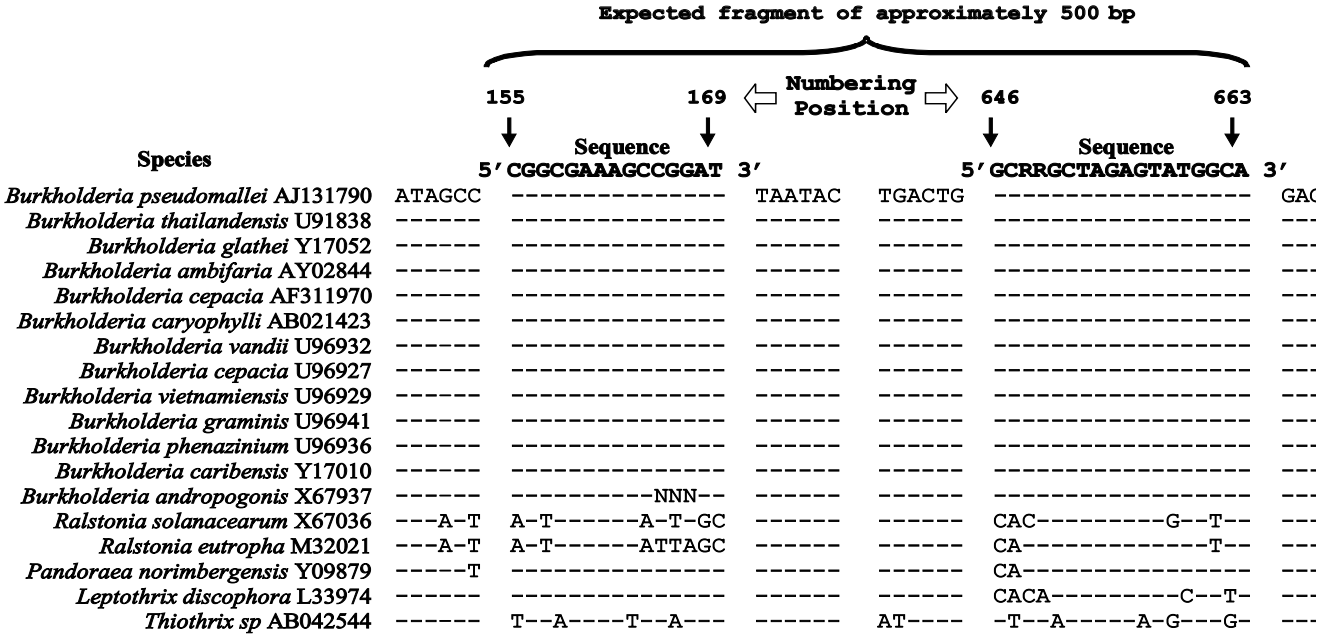

Figure 1. Alignment of 16S rDNA sequences from *Burkholderia* and non-*Burkholderia* species, corresponding to the region amplified by the *Burkholderia* specific primers. The numbering position corresponds to *E. coli* 16S rDNA sequence (9). Dashes indicate nucleotides that were identical to the nucleotides in the sequence at the top, which correspond to the 16S rDNA region homologous to the *Burkholderia* specific primers in the same DNA strand. N = A, T, C, or G; R = A or G.

However, since those sequences were not detected in the BLAST output obtained for the forward primer, the specificity of the primer system was not affected.

In order to evaluate the position of the *Burkholderia* species that had displayed low homology (LH) with one or both primers, a cluster analysis of full 16S rDNA sequences from a range of *Burkholderia* species was performed (data not shown). The tree obtained showed that the LH sequences clustered in distinct groups, quite apart from the other *Burkholderia* species. One cluster comprised sequences from *B. kururiensis* (52), *Candidatus B. kirkii* (47), three nitrogen-fixing *Burkholderia* species, *B. tuberum* (48), *B. unamae* (12), *B. tropica* (37), another as-yet-undescribed closely affiliated nitrogen-fixing species, tentatively denoted *B. brasiliensis*, and from three strains identified as *Burkholderia* sp. (accession numbers AF262932, AF074712 and AF074711). Another separate branch with LH *Burkholderia* sequences encompassed two putative *Burkholderia* sp. (X92188 and AJ011509) together with *Pandoraea norimbergensis* (Y09879), which has recently been removed from the genus *Burkholderia* (15). Finally, other LH sequences from strains identified as *B. cepacia* (AF244133) and *Burkholderia* sp. (AB011287, AY005032, U76088 and AY005039) were also apart from the main *Burkholderia* cluster.

Thus, the analysis of all sequences from the database reported to belong to the genus *Burkholderia* showed that only a minority of sequences (15 out of 145) had low homology with either one of the primers developed in this study. However, phylogenetic analysis showed that six of these might actually belong to genera outside of *Burkholderia*.

Lastly, the selected forward (Burk3) and reverse (BurkR) primer regions were checked for possible secondary structures that could prevent their annealing to the target region during the PCR reaction. Due to the formation of a strong hairpin structure in the forward primer, the second base at the 5´ end, a guanidine, was replaced by a thymidine (Fig.1). Although this change reduced the identity of the forward primer with the *Burkholderia* 16S rDNA sequences, it did not affect the specificity of the primers.

Sensitivity and specificity of the PCR-DGGE system for Burkholderia *spp*

The specificity of the PCR-DGGE system was tested with pure culture DNA from 14 *Burkholderia* species and 30 non-*Burkholderia* species as templates (Table 1). Products of the appropriate size (i.e. 500 bp) were detected with all strains of the *Burkholderia* species tested, but not with any of the non-*Burkholderia* species. This indicated that this primer pair displayed 100% specificity for species within the genus *Burkholderia*.

The specificity of the primers was also confirmed by sequence analyses of randomly chosen soil-derived clones (Fig. 3). All 18 clones sequenced were identified as affiliated with *Burkholderia* species, with percentages of similarity higher than 95%, and often higher than 97% (it has been suggested that 97% similarity is a level that can be used to define species).

The sensitivity of the PCR-DGGE method was evaluated with DNA extracted from a mixture of four *Burkholderia* species, before and after incorporation of the cells in soil. The content of inoculated cells was 5×10^3, 5×10^4 and 5×10^5 cells g^{-1} soil. The detection limit of the direct PCR-DGGE system in soil was high (5×10^5 cells g^{-1} soil) and in order to increase the

sensitivity, a nested PCR, in which the clamped primer is only applied in the second PCR, was used. In this case, the detection limit was lowered to 5×10^3 cells per g of soil (data not shown). The nested PCR increased the sensitivity of the method but did not interfered with the specificity, since the DGGE patterns obtained by both methods, nested or direct PCR, were equivalent (data not shown). The increase in sensitivity due to the use of a nested PCR is expected, especially when the target organism is present in an environment containing compounds that might inhibit the PCR reaction, such as plant derived compounds (33). Although the soil used in this study was not an organic soil, its organic matter content was high enough to affect the PCR reaction when the direct approach was applied. Due to the presence of potential inhibitors, the nested approach is more convenient for the sensitive detection of *Burkholderia* communities in soil samples.

PCR-DGGE analysis

PCR-DGGE analyses on the basis of genomic DNA of various *Burkholderia* strains showed that there was sufficient difference in migration of the amplicons to discriminate between the majority of the *Burkholderia* strains listed in Table 1 (Fig. 2). Products of different strains of the same *Burkholderia* species displayed the same electrophoretic mobility, except for two strains of *B. caribiensis* (Fig. 2A) and several strains of *B. cepacia* (Fig. 2B). On the other hand, the region amplified by the specific primers failed to distinguish *B. plantarii* from *B. gladioli*, as well as *B. cepacia* genomovar I and *B. vietnamiensis* due to their similar electrophoretic mobility. Sequence alignments showed that the species that could not be differentiated by DGGE shared a very high similarity in the 16S rDNA region amplified by the primers (99.2% to 99.4%). However, sequence analysis of DGGE bands can be used to differentiate between the species, and sequencing in combination with DGGE is now routinely applied in several laboratories (35).

To evaluate if the patterns obtained for each strain would be reproducible in a complex community, the DNA from four strains (*B. andropogonis* LMG6872, *B. multivorans* LMG13010 and *B. cepacia* ATCC25416 and *B. dolosa* LMG18941) was mixed in a 1:1:1:1 proportion and used as template in a PCR reaction. The DGGE profiles obtained were in line with the profiles obtained for each strain separately (data not shown). In addition, the intensity of the bands corresponding to each strain was equal, showing that there was no preferential amplification. Some *Burkholderia* species produced DGGE patterns comprising more than one band (Fig. 2), which could be explained either by the use of a degenerated reverse primer or by the presence of different 16S rDNA operons in one cell. To assess whether the use of a degenerated primer was the cause of the multiple bands, genomic DNA from all *Burkholderia* species (Table 1) was used as the template in PCR reactions with each one of the three possible reverse primers separately. Each strain tested gave strong PCR products with only one of the three reverse primers. DGGE analyses of these PCR products revealed that the patterns obtained with non-degenerated primers (only one sequence) were similar to those obtained with the degenerated primers (mixture of three sequences) (data not shown). The similarity among DGGE patterns obtained with degenerated and non-degenerated primers suggested that the multiple number of bands could not be explained by the use of a combination of three sequences as a reverse primer. Another plausible explanation for the multiple number of bands is the fact that bacterial species have multiple rRNA genes, which might display microheterogeneity

among the different copies. According to Klappenbach *et al.* (32), the number of rRNA operons per bacterial genome can vary from 1 to 15. This probably reflects ecological strategies of bacteria, such as the rate at which some bacteria respond to nutritional changes (upshift) in the environment. The *B. cepacia* genome was estimated to contain a maximum of 6 rRNA operons (Ribosomal RNA Operon Copy Number Database [http://rrndb.cme.msu.edu/rrndb/servlet/controlller]), but this estimation was based on a limited number of strains. Indeed, the multiple number of bands detected in some species with non-degenerated primers indicates that these species have multiple 16S rDNA operons, comprising different sequences in the fragment amplified by PCR. Based on this hypothesis, the number of bands found via PCR-DGGE can well be higher than the number of actual species present in *Burkholderia* communities. The fact that one organism might be represented by more than one band, and that one band might correspond to more than one organism suggest that the number of bands in DGGE profiles will not provide an accurate estimation of "richness". Therefore, diversity indices obtained through analysis of DGGE gels must be evaluated carefully. However, the DGGE profiles can certainly be used to detect shifts in the *Burkholderia* communities due to different environmental conditions and/or over time.

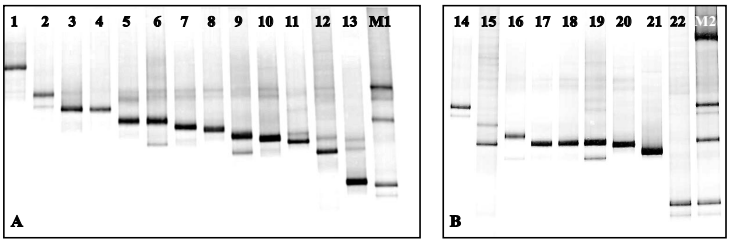

Figure 2. DGGE pattern of 16S rDNA fragments of *Burkholderia* species (A) and the *B. cepacia* complex (B) generated via PCR with *Burkholderia* specific primers (positions 155 to 663 *E. coli* numbering) in a denaturing gradient of 50 to 60%. Lanes: *B. glathei* WD1 (lane 1), *B. multivorans* LMG13010 (lane 2 and 14), *B. plantarii* NCPPB3590 (lane 3), *B. gladioli* ATCC33664 (lane 4), *B. pyrrocinia* ATCC15958 (lane 5), *B. stabilis* LMG14294 (lane 6 and 16), *B. vietnamiensis* LMG10929 (lane 7 and 18), *B. phenazinium* LMG2247 (lane 8), *B. caribiensis* WD3 (lane 9), *B. glumae* NCPPB 3708 (lane 10), *B. graminis* WD2 (lane 11), *B. caribiensis* LMG18531 (lane 12), *B. caryophylli* NCPPB353 (lane 13), *B. cenocepacia* LMG16656 genomovar III (lane 15), *B. cepacia* ATCC25416 genomovar I (lane 17), *B. cepacia* NCPPB945 (lane 19), *B. cepacia* P2 (lane 20), *B. cepacia* IPO1718 (lane 21), *B. dolosa* LMG18941 genomovar VI (lane 22). Lane M1, *Burkholderia* marker (from top to bottom): *B. multivorans* LMG13010, *B. cepacia* ATCC25416 and *B. dolosa* LMG18941. Lane M2, *Burkholderia* marker (from top to bottom): *B. andropogonis* LMG6872, *B. multivorans* LMG13010, *B. cepacia* ATCC25416 and *B. dolosa* LMG18941.

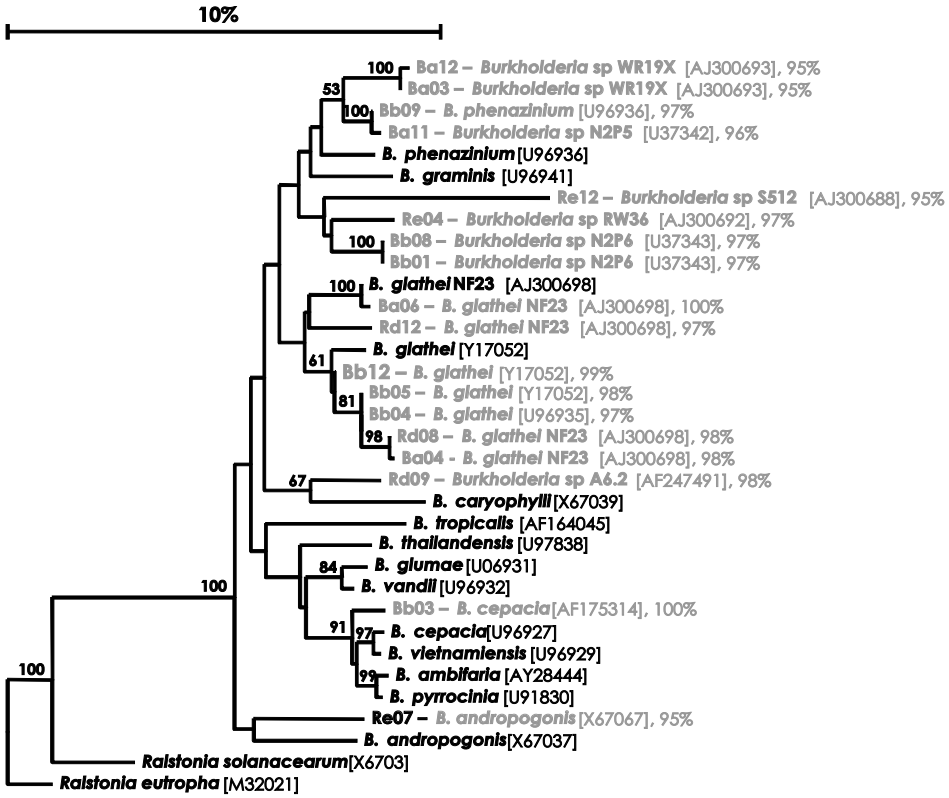

Figure 3. Phylogenetic tree showing the relationship between some *Burkholderia* species and soil-derived clones. The tree was constructed based on the fragment amplified by the *Burkholderia* specific primers [from positions 155 to 663 of the 16S rDNA, *E. coli* numbering, (9)], using the neighbour-joining method (39). A bootstrap analysis was performed with 100 repetitions, and only values above 40 are shown. The GenBank accession number for each strain is shown in brackets. The most closely related bacterial sequence related to each clone and the percentages of identity are shown after the clone code. Soil clones Ba03, Ba12, Bb01, Bb04 and Bb05 also showed similarity with unculturable eubacterium WD2116 (accession number AJ292648), with 96%, 96%, 98%, 98% and 98% of identity, respectively. Soil clone Bb08 displayed 98% of identity with unculturable eubacterium WD2120 (AJ292661); soil clone Rd12 showed 99% of identity with unculturable eubacterium WD263 (AJ262641) and soil clone Re07 displayed 96% similarity with unculturable eubacterium WD211 (AJ292651). Clone code: B, bulk soil; R, rhizosphere soil; "a" and "d", plot 47; "b" and "e", plot 31.

Analysis of soil bacterial populations

Analysis of the sequences of 18 randomly-picked clones obtained from grassland-derived DNA revealed that all showed a high similarity to sequences typical for species of the genus *Burkholderia* (Fig. 3). These results confirmed that the primer set is likely to be fully specific

for the genus *Burkholderia*. The most abundant species to which similarity was found among the soil clones was *B. glathei*, which was detected as closest hit in seven different clones. Similarity to *B. phenazinium* and *B. andropogonis* was also detected, albeit in only one clone each. Although the remaining soil clones could not be identified at the species level, their relationship to *Burkholderia* species could be affirmed by phylogenetic analyses. A phylogenetic tree based on the 16S rDNA region amplified by the primers showed that three clones were closely related to *B. phenazinium*, one was related to *B. caryophylli* and one to species belonging to the *B. cepacia* complex (Fig. 3). Four different soil clones formed a separate cluster, close to the cluster formed by *B. phenazinium* and *B. graminis*. Two clones belonging to the latter cluster showed high similarity with *Burkholderia* sp. isolate N2P6, a strain that revealed to be closely related to *B. fungorum* and *B. caledonica*, two recently described species (16). Interestingly, almost half of the clones were thus included in this branch of the phylogenetic tree which contains species known by their ability to produce antimicrobial compounds, such as *B. phenazinium*, and to degrade xenobiotic compounds, such as *Burkholderia* sp. strain N2P6 (16) (Fig. 3).

DGGE profiles of total *Burkholderia* populations in bulk and rhizosphere soils from grassland were complex, comprising between 13 and 20 bands for each sample (Fig. 4A). The analysis of the DGGE profiles generated a dendrogram which showed a clear grouping of the samples in two clusters, one composed by the two bulk soil samples and the other one by the two rhizosphere soil samples (Fig. 4B). Therefore, this analysis demonstrated a clear influence of grass roots on the structure of the *Burkholderia* populations. As some strong bands could be

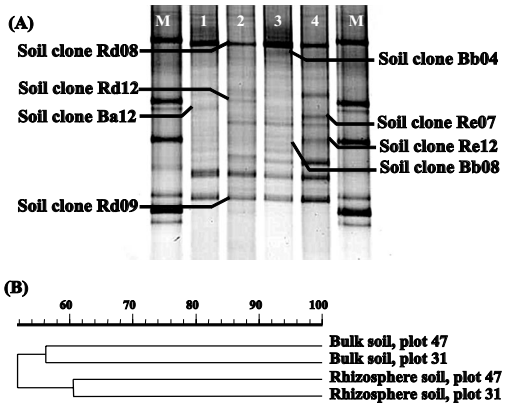

Figure 4. Comparison of DGGE patterns from bulk and rhizosphere soil community in a grassland field. Samples were taken in two different plots at the same location. (A) DGGE pattern: bulk soil, plot 47 (lane 1); rhizosphere soil, plot 47 (lane 2); bulk soil, plot 31 (lane 3); and rhizosphere soil, plot 31 (lane 4). Lane M, *Burkholderia* marker containing (from top to bottom) *B. andropogonis* LMG6872, *B. multivorans* LMG13010, *B. cepacia* ATCC25416, *and B. dolosa* LMG18941. (B) UPGMA clustering representing the similarity between the microbial communities obtained by *Burkholderia* specific PCR-DGGE.

detected in all samples, the main difference among samples was found by analysing the weaker bands. A comparison between the DGGE profiles directly obtained with soil DNA and the soil clones allowed the presumptive identification of some of the bands. Two strong bands present in all samples were thus identified as related to *B. glathei* (clones Rd08 and Bb04), and as related to *Burkholderia* sp. A6.2 (clone Rd09), a species closely related to *B. caryophylli*. In addition, several bands that were present in only one plot could also be identified. Thus, two bands detected only in the rhizosphere soil of plot 31 were identified as related to *B. andropogonis* (clone Re07) and *Burkholderia* sp. S512 (clone Re12) (Fig. 4A).

DGGE analyses of the PCR products from both pure culture and soil DNA revealed that this technique was useful for evaluating the *Burkholderia* diversity in soil samples. This is an advantage in relation to the methods used to date, which rely on the evaluation of specific groups within the genus *Burkholderia*, such as the *B. cepacia* group (5, 24). PCR-DGGE proved to be a powerful tool to detect the dominant members of the *Burkholderia* community since it combined the sensitivity and specificity of the genus specific PCR with the direct screening of the dominant sequences, visualised on the basis of sequence divergence, via DGGE. Using this system, an effect of the grass rhizosphere on the selection of specific groups of *Burkholderia* species could be observed. Since this effect occurs due the presence of root released compounds, changes in the composition of these compounds are likely to induce changes in the rhizosphere populations. In fact, different crops can induce shifts in diversity by selecting different bacterial communities in their rhizosphere (42). Therefore, agricultural practices can induce changes in microbial diversity and these changes presumably lead to a change in the ecological roles of *Burkholderia* spp. The PCR-DGGE system described here is now being applied to study the effect of crop rotation on the diversity of *Burkholderia* populations, in particular, assessing measures leading to an increase of the presumably beneficial (plant growth promoting or antagonistic) *Burkholderia* species. PCR-DGGE targeting specific groups of microorganisms will, thus, be a useful monitoring tool in the prediction of the effects of agricultural practices on microbial communities in soil.

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Chapter 3

Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history

Abstract

The assessment of *Burkholderia* diversity in agricultural areas is important, considering the potential use of this genus for agronomical and environmental applications. Therefore, the aim of this work was to ascertain how plant species and land use management drive the diversity of the genus *Burkholderia.* In a greenhouse experiment, different crops, *i.e.* maize, oat, barley and grass were planted, in pots containing soil with different land use history, *i.e.* maize monoculture, crop rotation and permanent grassland, for three consecutive growth cycles. The diversity of *Burkholderia* spp. in the rhizosphere soil was assessed by genus-specific PCR-DGGE and analysed by canonical correspondence analysis (CCA). CCA ordination plots showed that previous land use was the main factor affecting the composition of the *Burkholderia* community. Although most variation in *Burkholderia* community structure was observed between permanent grassland and agricultural field, differences between crop rotation and maize monoculture were also observed. Plant species affected *Burkholderia* community structure to a lesser extent than did land use history. Similarities were observed between *Burkholderia* populations associated with maize and grass on the one hand, and those of barley and oats, on the other hand. Additionally, CCA ordination plots demonstrated that these two groups (maize/grass vs. barley/oats) showed a negative correlation. Identification of bands from the DGGE patterns demonstrated that the species correlated with the environmental variables were mainly affiliated with other *Burkholderia* species that are commonly isolated from soil, in particular *B. glathei*, *B. caledonica*, *B. hospita* and *B. caribensis.*

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Introduction

The genus *Burkholderia* was created in 1992, when Yabucchi (45) reclassified *Pseudomonas* species belonging to rRNA group II. Since then, this genus has undergone many changes. It now comprises over 30 species, including the *B. cepacia* complex, which consists of 9 so-called genomovars (7). Many species have potential for agricultural or environmental purposes, such as biological control, bioremediation, atmospheric nitrogen fixation and plant growth stimulation (7, 11, 12, 42). Moreover, the ability to colonise the rhizosphere of plants such as maize, wheat, rice, grass, oat, lupine and coffee at high population densities might expand the potential application of these microorganisms (3, 9, 12, 43). Despite the great agronomic potential of *Burkholderia* spp., there is general concern about the environment functioning as a source of human-pathogenic organisms, mainly after *B. cenocepacia* (genomovar III), which is associated with the "cepacia syndrome" in cystic fibrosis (CF) patients, was found as a common plant-associated bacterium (3). Although this finding highlighted the importance of assessing the diversity of *Burkholderia* species in rhizosphere soil, most of the present ecological knowledge is based on *B. cepacia* populations isolated from the rhizosphere of just one plant species, maize (5, 10, 13). Only recently, also the diversity of *Burkholderia* community associated to woodland rhizosphere was assessed (30). However, these reports are based on culture-dependent techniques, which are likely to underestimate the natural bacterial population (25). In order to overcome this problem, we developed a method, based on PCR-DGGE, which allows the direct analysis of the diversity of *Burkholderia* species in environmental samples (33).

Plant roots play important roles in shaping microbial communities in soil by releasing a wide range of compounds. Although root-released products comprise an important pool of organic compounds for soil microorganisms, their composition and quality can vary according to plant species, soil type and plant development stage (37, 44). Due to this variation in exudation, different plant species growing in the same soil type are known to select divergent bacterial communities (21, 22, 44). However, when analysing the microbial community associated with the same plant species growing in different soil types, soil type might exert a great influence on microbial diversity (8, 44).

In view of the fact that the plant has a large impact on microbial diversity, one might expect agricultural management to play an important role as well. Indeed, many agricultural practices, such as crop rotation, continuous cropping and tillage, induce changes in the microbial communities in soil (2, 22, 43), which may persist long after the management practice took place (4). Although agricultural practices induce general changes in soil microbial communities, specific microbial groups may respond differently. Clegg *et al*. (6) showed that the application of inorganic nitrogen had a significant impact on eubacterial and Actinomycete community structures, whereas soil drainage significantly affected the community structures of Actinomycetes and pseudomonads. In addition, continuous wheat cropping affected the community structure of pseudomonads, such that an increase in the population of antibiotic-producing *Pseudomonas* spp induced the natural suppression of Take-all disease in wheat (29). Similarly, the establishment of apple orchards in a field where wheat had previously been grown led to an decrease of soil suppressiveness against *Rhizoctonia solani*, which was correlated with a decrease of *B. cepacia* and *P. putida* populations (24).

Considering the fact that agricultural management and plant species affect soil microbial communities, the main objective of this work was to get a better understanding of how land use and crop species, *i.e.* maize, oat, grass and barley affect the diversity of the genus *Burkholderia*. In addition, this study aimed to address which *Burkholderia* species are selected by specific crops and which factor (plant species or land use) had a greater influence on the soil-borne populations. To assess the diversity of *Burkholderia* species in soil, a PCR-DGGE system with primers specific for this genus was applied (33), allowing the evaluation of the total *Burkholderia* population, including the non-culturable fraction.

Material and methods

Microcosm experiment

In order to evaluate the effect of different plant species on the diversity of *Burkholderia* species, a pot experiment in the greenhouse was designed. The treatments consisted of four plant species: maize (*Zea mays* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and grass (commercial mix, containing *Lolium perenne* as the main species), planted in replicate pots containing soil with three different land use histories.

Soil

The soil used in this experiment was collected from different locations (according to land use) in a field (Wildekamp) located in Wageningen, the Netherlands. The soil was a loamy sand (3% clay, 10% silt, and 87% sand), with about 2% of organic matter and pH 4.8. This site was composed originally of a long-term (>50 years) permanent grassland (G) field that was partially converted to agricultural land (A) about 20 years ago, the latter being used mainly for crop rotation. In 2000, different treatments were established in both areas (G and A) before the growing season, using triplicate (10 x 10 m) plots per treatment in a randomized block design. The treatments comprised 4-year crop rotation (oat, maize, barley and potato), monoculture of maize and grassland. For the greenhouse experiment, we decided to focus mainly on the differences due to the conversion from permanent grassland (G) to arable land under crop rotation (A-R) and under maize monoculture (A-M). Therefore, at the end of the growing season of 2001, soil was collected from each triplicate plot of the treatments G, A-R and A-M. At that moment, the plots related to the crop rotation treatment had only had the first two crops (oat and maize). From this point on, the treatments mentioned above will be referred to as land use history.

Experimental design

Soil collected from each triplicate plot of the three different land use histories (G, A-R and A-M) was sieved and homogenised separately and approximately 600 g (500 ml) was then transferred to pots in the greenhouse. Pre-germinated seeds (three) of each plant species (oat, maize and barley) were transferred to the pots, in 3 replicates per crop. For the pots containing grass, approximately 300 mg of non-germinated seeds were used. As controls, two pots per land use history were kept fallow.

In order to enhance the rhizosphere effect of each plant species, at the end of the first growth cycle the plants were removed, the soil contained in each pot was homogenised separately and new seedlings (corresponding to the previous plant species) were transferred to soil in the pots. This procedure was repeated one more time, thus producing samples from the second and third growth cycles (Fig. 1).

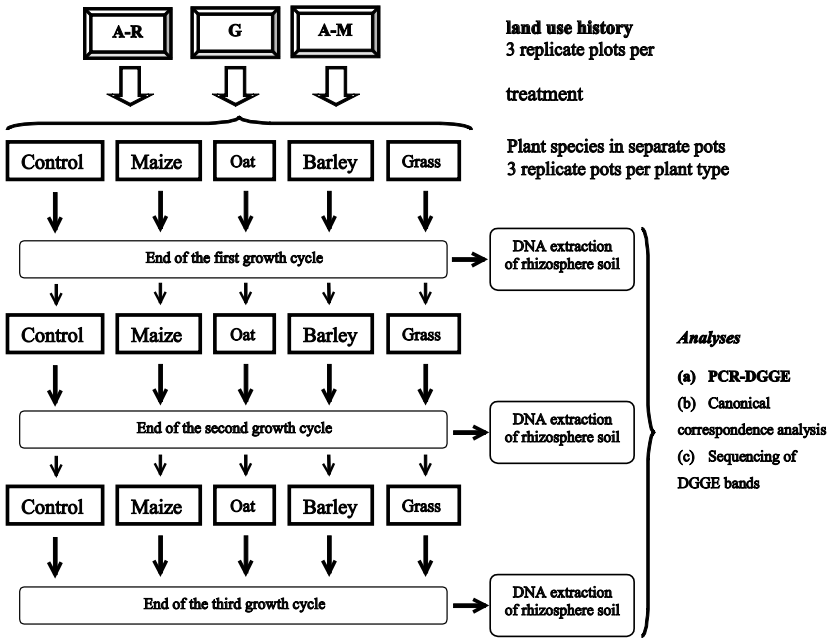

Figure 1. Schematic representation of the microcosm experiment. Soil collected from a field plots with different cropping history were distributed in pots, where four plant species were planted. At the end of the growth cycles, the plants were removed from the pots and rhizosphere soil was collected for further DNA extraction. Subsequently, the bulk soil from each pot was mixed separately and new seedlings from the previous plant species were transferred to the pot. A-R, arable land under crop rotation; A-M, arable land under maize monoculture; G, permanent grassland.

Rhizosphere soil sampling

At the end of each growth cycle (approximately 4 months after sowing), plants were removed from the pots and the roots were shaken gently, removing the loosely adhering soil. Twenty grams of roots containing the tightly-adhering soil (rhizosphere soil) were transferred to Erlenmeyer flasks containing 90 ml of sterile sodium pyrophosphate (0.1% NaPP) and gravel (10 g). After shaking the flasks for 30 min at 180 rpm, 2 ml of solution containing rhizosphere soil were taken for DNA extraction.

DNA extraction

DNA from rhizosphere soil was extracted using a MO BIO UltraClean soil DNA isolation kit (Mo Bio Laboratories, BIOzymTC, Landgraaf, The Netherlands). Briefly, 0.5 ml of sodium pyrophosphate solution containing rhizosphere DNA and 50 mg of glass beads (≤106 microns) were added to the microtubes and cells were lysed by beat beating for 60 sec in a cell disrupter (Hybaid Ribolyser, Hybaid, Middlesex, United Kingdom), in order to achieve maximal cell lysis. After the bead-beating step, DNA was extracted according to the protocol described by the supplier.

PCR amplification of partial 16S rDNA of **Burkholderia**

Amplification of 16S rRNA genes from rhizosphere soil DNA was done by using primers specific for the genus *Burkholderia*, in a semi-nested PCR, according to the methodology described by Salles *et al*. (33). Briefly, the PCR procedure consisted of a first PCR with primer Burk3 in combination with universal eubacterial primer R1378 (19), using the PCR conditions described by Rosado *et al*. (31). The products from the first PCR were diluted 1:1,000 and used as the template in the second PCR, which was performed with primers Burk3 (GC clamped) and BurkR, as follows: 50-µl reaction mixtures containing 1 µl of DNA (5to 10 ng), 200 µmol l^{-1} of each deoxyribonucleoside triphosphate, 400 nmol l^{-1} of each primer, 1× *TaqPlus* Precision buffer (Stratagene, Leusden, The Netherlands), and 2 U of *TaqPlus* Precision polymerase mixture (Stratagene). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands) (33) and the PCR products, expected to be approximately 500 bp long, were analysed by electrophoresis in a 1.5% (wt/vol) agarose gel in 0.5× TBE buffer (34). When necessary, products were stored at -20°C before they were used for DGGE analysis.

DGGE

DGGE analysis was performed by using the phorU2 system (Ingeny, Leiden, The Netherlands) and the method described by Salles *et al*. (33). After electrophoresis, the gels were stained with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and then photographed and digitalised using Imago compact apparatus (B&L System, Maarssen, The Netherlands).

Banding pattern analysis and statistics

DGGE banding patterns were analysed by the Molecular Analyst software (version 1.61; Bio-Rad, Veenendaal, The Netherlands). In order to compensate for internal distortions during electrophoresis, gels were aligned by using an external reference pattern. The pattern was composed of pooled PCR products from four *Burkholderia* species, loaded in at least four different lanes distributed along the gel. Subsequently, subtraction of non-linear background was achieved by using the rolling disk mechanism with intensity 8. To complete the gel analysis, identification and quantification of the bands present in each lane was performed by setting tolerance and optimisation at 0.75%. A table containing the calculated surface and position of each band was then exported to Excel, where band surface was converted to the relative intensity of the band per lane, with values between 0 and 1. The relative intensity was obtained by

dividing the surface of the band by the sum of the surfaces for all the bands within the lane, thus eliminating the variation in band intensity caused by differences in the amount of PCR product loaded on gel.

To perform the statistical analysis of the DGGE profiles versus the environmental variables, canonical correspondence analysis (CCA) was chosen, as it explains the structure of a "species" data table (in this case, band intensities) by environmental variables, assuming a unimodal distribution of species (39). For that purpose, community structures based on the relative intensity of each band were analysed by performing CCA (CANOCO 4.5; Biometris, Wageningen, The Netherlands). Community similarities were graphed by using ordination plots with scaling focused on inter-sample difference (23). The ordination plot of species and environmental variables is characterised by a biplot that approximates the weighed averages of each species with respect to each of the environmental variables. Thus, the ordination diagram represents not only a pattern of community distribution, but also the main features of the distribution of species along the environmental variables (39). Although classes of nominal environmental variables are more often symbolised by a point at the centroid (the weighed average), they can also be represented by arrows (39). Hence, to facilitate the interpretation of the ordination plots, the nominal environmental variables "plant species" were represented by arrows. Both the length and the slope of the vector are significant parameters, long vectors forming smaller angles with an ordination axis are more strongly correlated with that ordination axis (38). In addition, the angle between the vectors provides an approximation of the correlation. Consequently, vectors pointing in the same direction are positively correlated and those pointing in opposite directions are negatively correlated (39). The nominal variables "land use history" or "growth cycle" were represented by centroids and its position determines the relationship of these variables with either of the ordination axes (38). In order to investigate the statistical significance, a Monte Carlo permutation test based on 499 random permutations was used, assuming the null hypothesis that species data are unrelated to environmental data and the alternative hypothesis that the species respond to the environment.

Soil clones and sequence analyses

DGGE bands, which were correlated with environmental variables, were selected for sequence analysis. After cutting the inner part of the DGGE band, the DNA was eluted in 20 µl of sterile Milli-Q water and subsequently used as a template in PCR (primers Burk3 and BurkR), as described earlier in this section for the PCR in the nested approach. The reamplified PCR products were purified with a "High Pure" PCR product purification kit (Boehringer, Mannheim, Germany) and cloned into the pGEM-T easy vector, which was used to transform *Escherichia coli* strain JM109, according to the procedure recommended by the manufacturer (Promega Benelux, Leiden, The Netherlands). Plasmid extraction from randomly selected colonies was performed with the Wizard Plus SV miniprep DNA purification system (Promega Benelux). Plasmids containing the inserts corresponding to the cut DGGE bands were sequenced in an ABI Prism automatic sequencer (BaseClear B.V., Leiden, The Netherlands) and the identity of the sequences was determined by BLAST analyses (1).

Sequence alignment

Sequences generated in this study or recovered from the GenBank/EMBL database were aligned by using Clustal_X (41), considering only the 16S rDNA partial sequence covered by the *Burkholderia* specific primers (33). Phylogenetic trees were constructed by the neighbor-joining method (32) based on distance estimation calculated by the method of Jukes and Cantor (20). This analysis was performed with the TREECON program, version 1.3b (Yves van de Peer, Department of Biochemistry, University of Antwerp, Antwerp, Belgium).

Nucleotide sequence accession numbers

The sequences generated in this study have been deposited in the GenBank database under accession numbers AY571292 to AY571305.

Results

DGGE analysis and identification of bands

The number of bands in the DGGE profiles varied mainly along the growth cycles. Figure 2 shows that the number of bands in A-R increased from an average of 14.2 in the first growth cycle to 18.4 in the third. A-M and G contained in the third growth cycle averages of 18.4 and 19 bands per lane (Fig. 2), while the averages in the first growth cycle were 14.7 and 16.6 bands, respectively (data not shown). In all the samples, the DGGE patterns after the first growth cycle comprised bands which were limited to an area in the middle of the gel (around 55% denaturant), whereas in the last growth cycle bands were distributed over the whole gel (roughly between denaturant concentrations of 50-60%), indicating a change in the structure of the *Burkholderia* communities (Fig. 2). Additionally, the numbers of bands in patterns from fallow pots (control) were lower than those in patterns from pots containing plants, irrespective of the land use history or growth cycle (Fig. 2). Although no difference was observed in the average number of bands among plant species or land use history within the same growth cycle, the *Burkholderia* communities associated with G were more even than those associated with A-M and A-R, containing fewer dominant bands (Fig. 2). Moreover, an effect of different plant species could be observed by differences in the intensity of bands.

CCA allows not only the interpretation of DGGE profiles in relation to environmental variables, but also correlates species (band position) to those environmental variables. Therefore, after analysing the ordination plots, 15 band positions were selected on the basis of their association with some of the treatments. Bands corresponding with these positions were thus identified in different samples, excised from the gel and cloned. Bands corresponding to five out of 15 band positions could not be amplified or cloned and therefore could not be identified. From the remaining 10 band positions, 43 clones were obtained (around four per band position) and identified by sequencing. Five out of the 43 sequences were considered to be chimeric and removed from the analyses. All clones were affiliated to *Burkholderia* species, with identities varying from 97 to 100%, confirming the specificity of the PCR system. DGGE analysis of the clones revealed that 32% migrated to a different position on the gel than the band they originated from. Although these clones were affiliated to *Burkholderia* species, they were discarded from

Table 1. Sequence analysis of bands excised from DGGE and their relationship to environmental variables.

Band no. (GenBank accession no.)	Position in CCA plot[a]	Environ-mental variable[b]	Most closely related bacterial sequence[c]	% Identity	Accession no. of related sequence	Reference
1 (AY571292)	A27	G	Uncultured eubacterium WD232	99%	AJ292667	(28)
			Burkholderia sp. OY715	99%	AJ300696	(30)
2 (AY571302)	A29	A-R	*Burkholderia* sp. OY715	99%	AJ300696	(30)
3 (AY571296)	A30	G	Uncultured bacterial clone F2-41	97%	AY096172	Y. Ding, W. B. Whitman, K. Das and J. R. Kastner., unpublished data
			B. terricola	97%	AY040362	(18)
4 (AY571294)	A31	A-M	*Burkholderia* sp. OY715	100%	AJ300696	(30)
5 (AY571293)	A36	A-R	Uncultured earthworm cast bacterium	99%	AY154615	D. R. Singleton, P. F. Hendrix, D. C. Coleman, and W. B. Whitman, unpublished data
6 (AY571303)	A36	A-R	Uncultured earthworm cast bacterium	99%	AY154615	Singleton et al., unpublished data
7 (AY571295)	A40	G	Uncultured earthworm cast bacterium	98%	AY154615	Singleton et al., unpublished data
8 (AY571300)	B4	Maize	*Burkholderia* sp. NF23	97%	AJ300698	(30)
9 (AY571304)	B4	Maize	*Burkholderia* sp. P18G1120	97%	AF214131	(35)
10 (AY571299)	B4	Maize	*Burkholderia* sp. NF23	98%	AJ300698	(30)
11 (AY571298)	B26	Grass	Uncultured *Burkholderia* sp. clone Ba04	97%	AF407355	(33)
			Burkholderia sp. NF23	97%	AJ300698	(30)
12 (AY571297)	B32	Grass	*B. hospita*	98%	AY040365	(18)
13 (AY571301)	B40	Barley	Uncultured earthworm cast bacterium	99%	AY154615	Singleton et al., unpublished data
14 (AY571305)	B47	Oat	Uncultured earthworm cast bacterium	97%	AY154615	Singleton et al., unpublished data

[a] Species (band) position in CCA ordination plots; species followed by the same letter were analysed in the same gel;
[b] Environmental variables: A-R, arable land under crop rotation; A-M, arable land under maize monoculture; G, permanent grassland;
[c] Only one clone per band was listed. Clones, which closest hit was an unculturable organism, had the first hit with species identification also mentioned, except for AY154615, that showed low identity (<90%) too any culturable organism.

the analysis. Table 1 shows the list of the 10 band positions and respective clones considered in the analysis. Only one sequence per band position is shown in the Table 1, since the similarity between them (sequences per band position) varied from 99.8 to 100%. Bands present at the same position, but obtained from different samples, reassuringly showed sequence similarity between them of above 99.2% (30) but, surprisingly, did not always show the same bacterial sequence as closest hit in the database (bands 8, 9 and 10; Table 1; Fig.2). On the other hand, some bands located in different positions in the gel were identified as affiliated with the same organism in the database; this holds for bands present at the bottom of the gel (Table 1; Fig. 2). The percentage of similarity between these latter sequences ranged from 97.4% to 99.2%, which might indicate that organisms were hit that possess several ribosomal RNA operons with sequence microheterogeneity.

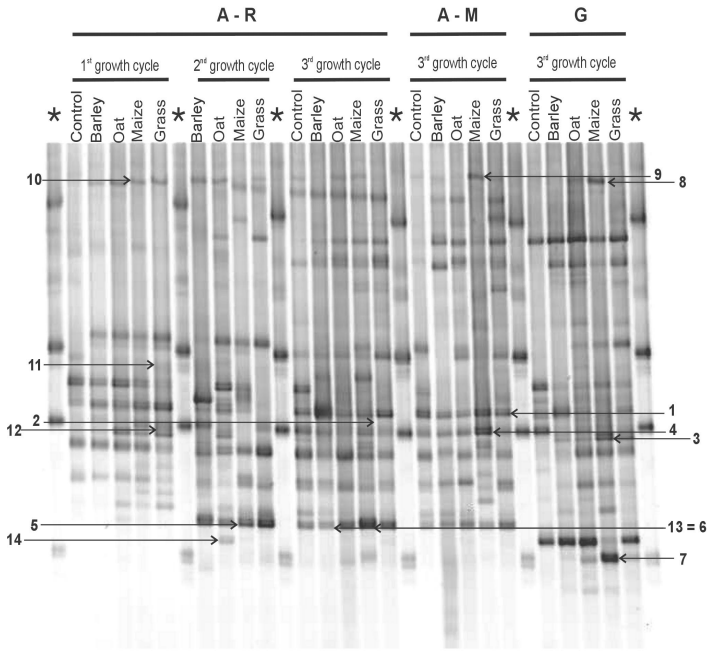

Figure 2. DGGE pattern of *Burkholderia* community associated to the rhizosphere of barley, oat, maize and grass, grown in soils with different land use history. A-R, arable land under crop rotation; A-M, arable land under maize monoculture; G, permanent grassland. Control lanes represent pots without plants (bulk soil). "*", *Burkholderia* marker containing (from top to bottom) *B. andropogonis* LMG6872, *B. multivorans* LMG13010, *B. cepacia* ATCC25416, and *B. dolosa* LMG18941. The arrows indicate bands identified by sequencing (Table 1).

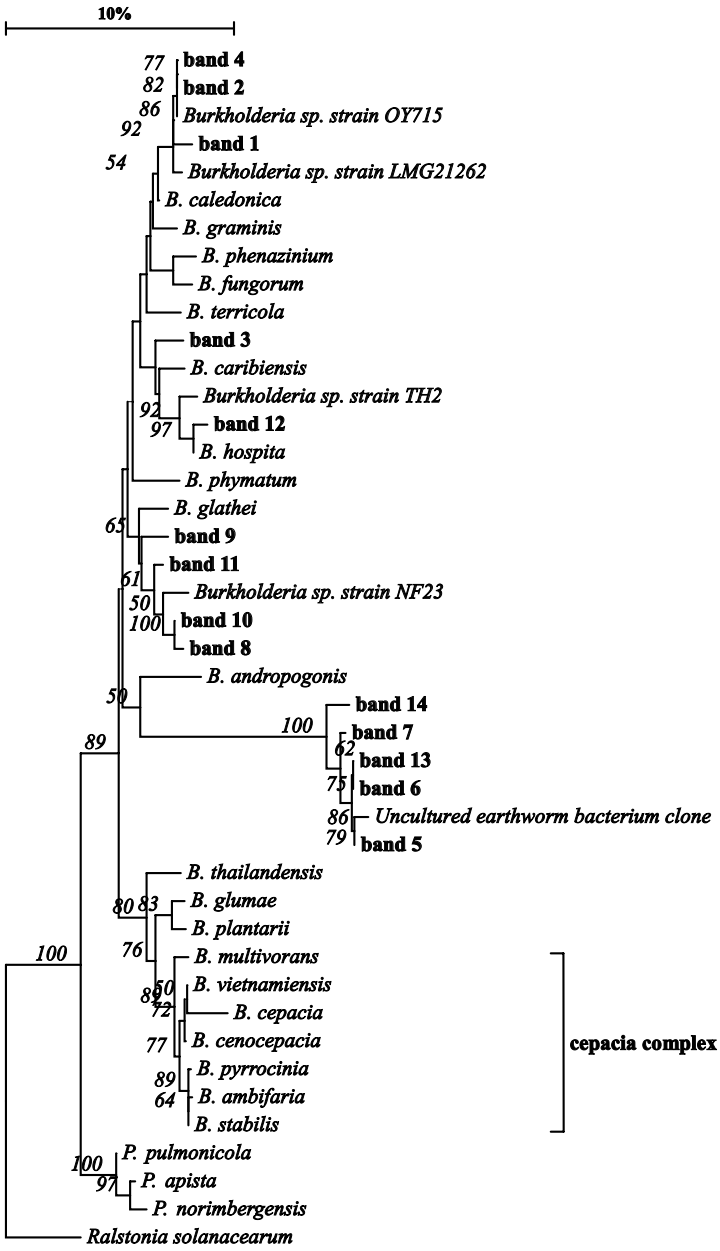

Figure 3. Phylogenetic tree showing the relationship between some *Burkholderia* species and bands excised from DGGE. The tree was constructed based on the fragment amplified by the *Burkholderia* specific primers (33), using the neighbour-joining method (32). A bootstrap analysis was performed with 100 repetitions, and only values above 50 are shown. A description of the bands is listed on Table1.

In order to determine the distribution of these clones within the genus *Burkholderia*, a phylogenetic tree was constructed, based on the region amplified by the primers (Fig. 3). Clones representing the 10 band positions were distributed within three branches of the *Burkholderia* phylogenetic tree, clustering apart from those belonging to the "cepacia complex" (Fig. 3). Two main groups of discriminating clones were distributed close to *B. glathei* (four clones) and to *B. caledonica* (three clones). Another main group of clones was affiliated with an unculturable (or uncultured) earthworm cast bacterium (five clones). The remaining two clones were affiliated with *B. hospita* or *B. caribiensis* (Fig. 3). All species displaying high similarity with the clones were originally isolated from soil.

Plant species effect

To eliminate artefacts associated with DGGE image analysis on the basis of different gels, the effect of plant species was analysed for each land use history treatment separately, giving rise to three different ordination plots (Fig. 4A-C). In addition, we used soil originally from one field plot, which was representative for the triplicate plots. In all ordination plots, the control samples (pots without plants) clearly clustered distantly from the other (planted) samples (data not shown). The controls were then excluded from the analyses to facilitate the visualisation of the treatment effects. For the same reason, we opted for ordination plots consisting of biplots of environmental variables and samples (DGGE lanes) instead of species (DGGE bands).

Figure 4 (A-C) shows that samples generally clustered according to growth cycle rather than plant species. The ordination plots corresponding to arable land A-M and A-R (Fig. 4A and B) showed that growth cycle was a highly significant explanatory variable ($P \leq 0.05$), consistently separating the samples along the first (most important) ordination axis. In A-M, oat and barley were the only plant species that were significant as explanatory variables, being correlated to the first and second axes respectively (Fig. 4A). Even though maize was not significant as explanatory variable ($P > 0.05$), maize and oat were negatively correlated as inferred by their opposition in the ordination plots. The same configuration was detected for barley and grass (Fig. 4A). A similar pattern for plant species distribution was observed for A-R (Fig. 4B), where positive correlation between maize and grass on the one hand, or oat and barley on the other hand were noticed (Fig. 4B). However, in A-R, none of these ordinations showed a significant *P* value ($P < 0.05$), all clustering close to the origin of the plot. In addition, the explanatory variable growth cycle was mainly separated along the first axis whereas plant species were distributed along the second axis (Fig. 4B). In the ordination plot representing the land use history G (Fig. 4C), growth cycle was again the main explanatory variable, being spread along the first axis. However, growth cycle 2 was not significant by *P* value estimation. In addition, it is noteworthy that the distribution of the samples followed a different pattern in G as compared to A-R and A-M, being scattered for the former and occurring concentrated around the centroids of growth cycle in the latter (Fig. 4). Maize and grass were significant as explanatory variables, being explained by the second axis (Fig. 4C). Again, these two explanatory variables were positively correlated with each other and negatively correlated with oat and barley (Fig. 4C). The positive correlation between maize and grass versus oat and barley was also observed when the DGGE profiles were compared by cluster analysis, considering the presence or absence of bands only (data not shown)

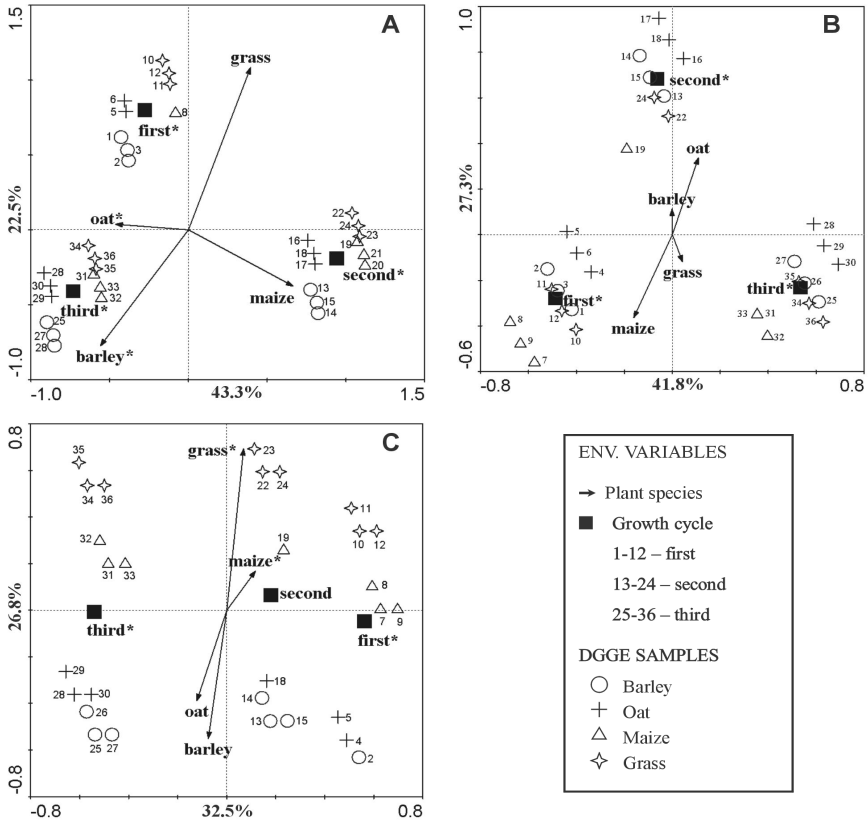

Figure 4. Ordination plots of *Burkholderia* communities associated with the rhizosphere of maize, barley, oat and grass, in soils with different land use history: arable land under maize monoculture (A), arable land under crop rotation (B) and permanent grassland (C). The plots were generated by canonical correspondence analysis of DGGE profiles. All environmental variables are shown, but only those marked with "*" were significant (P<0.05). Values on the axes indicate the percentage of total variation explained by each axis.

Land use history effect

To evaluate the effect of land use history on the *Burkholderia* communities, samples collected after the last growth cycle were chosen for the analyses. Thus, we generated biplots of environmental variables (land use history) and species (DGGE bands). In the rhizosphere soil samples taken from maize (Fig. 5A), it was observed that the three replicate G plots clustered together, separating from the A-M and A-R plots along the first axis. The second axis, though, explained the difference between the two treatments within the arable land, i.e. crop rotation (A-R) and maize monoculture (A-M). However, although the replicate samples belonging to the same land use history tended to group together, for A-R and A-M replicate plots there was always one outlier. Land use history was a significant explanatory variable, with all treatments being significant, except for two A-R replicate plots (Fig. 5A).

In order to check if a similar pattern was observed for other plant species, the same analysis was performed for oat. Largely, similar results were obtained, however the distinction between the treatments A-M and A-R was not clear (Fig. 5B). Oat was selected over the other plants since its effect was negatively correlated with maize. This analysis was not performed for grass and barley due to their similarity to maize and oat, respectively.

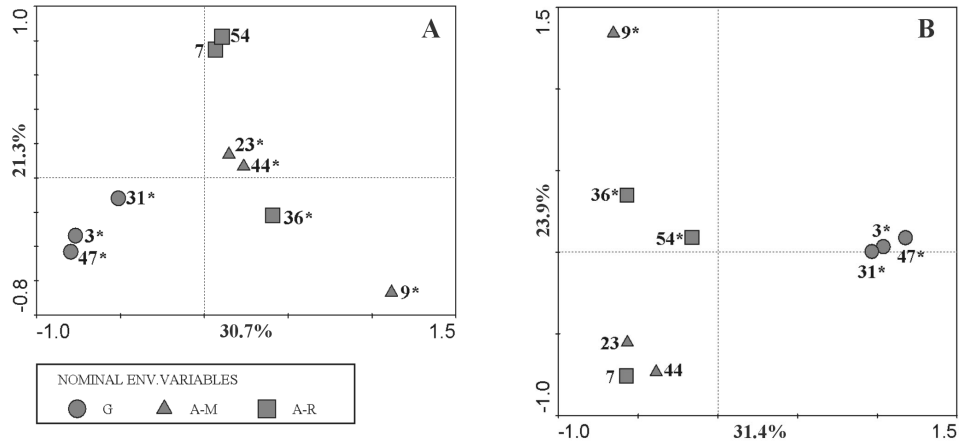

Figure 5. Ordination plots of *Burkholderia* communities associated with the rhizosphere of maize (A) and oat (B), in soils with different land use history. The plots were generated by canonical correspondence analysis of DGGE profiles. The numbers after the nominal environmental variable correspond to the identification of each one of the three replicate plots where the soil was originated from. All environmental variables are shown, but only those assigned with "*" were significant (P<0.05). Values on the axes indicate the percentage of total variation explained by each axis.G, permanent grassland; A-M, arable land converted to maize monoculture; A-R, arable land converted to crop rotaion.

To verify which environmental variable (plant species versus previous land use) had a greater effect on the community structure of *Burkholderia* species in soil, samples obtained after the third growth cycle, from all plant species, and belonging to one representative plot for each land use history treatment, were analysed. The results showed that land use history had a greater effect on the *Burkholderia* community structure than plant species, being largely responsible for the distribution of the samples in the ordination plot (data not shown). In addition, all land use history treatments and barley as the plant species were significant as explanatory variables. The distribution of plant species along the ordination plot followed the same pattern as described above for plant species effect (data not shown). Similar results were obtained when all plant growth cycles were included in the analysis, confirming that land use history effect had the greatest influence on *Burkholderia* community structure (data not shown)

Discussion

Due to the relevance of the genus *Burkholderia* in respect of its application for agronomic purposes versus its pathogenicity towards cystic fibrosis patients, it is important to assess *Burkholderia* diversity in agricultural areas. Therefore, the aim of this work was to ascertain which *Burkholderia* species in rhizosphere soil are selected by specific crops and which effect (current plant species or previous land use) has the largest influence on *Burkholderia* populations in soil. To assess the total *Burkholderia* community structure in the rhizosphere samples, a PCR-DGGE system specific for determining the diversity of types within this genus was used (33).

PCR-DGGE has been used in molecular ecology for about a decade now (27), and its efficacy in analysing microbial communities has greatly improved, as (i) primer systems for narrow taxonomic groups have been, and still are being, developed (16, 17, 19, 33), and (ii) different statistical strategies have been applied to analyse DGGE fingerprinting data (for a review, see (14). The most common way to perform analyses of DGGE profiles is by UPGMA based clustering, which identifies samples with similar patterns based on the presence or absence of bands (33), but does not take into account the band intensities or the correlation between banding patterns and environmental variables. This type of correlation can be achieved by using ordination methods, which are vastly used in macro-ecology (40) and have been recently applied to DGGE fingerprinting analysis (23, 26). However, care should be taken in selecting the most appropriate statistical procedure for ordination of the molecular profiles, and the underlying theoretical model should be carefully assessed (14). Multivariate analysis such as canonical correspondence analysis (CCA) can be applied to link changes in communities to changes in the environment, correlating the community structure with explanatory variables, which can be evaluated by statistical tests. The applicability of multivariate analysis of DGGE patterns was recently confirmed by Muylaert *et al*. (26), who monitored bacterial community composition in four eutrophic lakes. By using relative band intensities instead of presence/absence data matrices, it was possible to find a better correlation between bacterial community composition and explanatory variables than when using the latter method. In addition, using an artificial data set to which potential sources of error associated with PCR-DGGE analysis were introduced, similar results were obtained (26). After applying CCA to analyse the *Burkholderia* community structure revealed by PCR-DGGE, we were able ascertain that (long-term) land use history had a greater effect on this community than (short-term) plant species, even after three sequential growth cycles in pots.

Grassland and agricultural land subjected to rotation cropping represent two types of land use, each one having a distinct effect on both microbial communities and soil properties. Indeed, our results showed that the soil collected from the arable land plot, which was turned into arable land by cultivation of a part of the permanent grassland around 20 years ago, had a *Burkholderia* community structure that differed from that in the permanent grassland plot. Most likely, this was related to the clear differences between both soil management regimes. A comparison of different land use (soil management) regimes revealed that factors such as microbial biomass, pH and management factors were highly correlated with differences in microbial community composition (38). These results supported the hypothesis that soil disturbance as a result of cultivation rather than plant species alone distinguishes the microbial

communities of arable fields from those of grasslands (38). Furthermore, the influence of soil agricultural history might persist long after changes in land management have been made (4). Although the time necessary to overcome the persisting effects of land use history might vary, depending on the type of conversion (A to G or G to A), we could observe changes due to agricultural practices, mainly in the arable land, where A-R and A-M clustered only partially together. However, the distribution of these two clusters depended on the plant species the rhizosphere soil samples were taken from and maize exerted an extra effect in separating the land use history treatments. This could be explained by the fact that the continuous growth of maize for three cycles in the A-M pots represented a prolongation of the land use management (monoculture of maize).

The effect of plant species could be observed by analysing land use history treatments separately. Although these environmental variables did not always explain the distribution of the samples, the ordination plots indicated two groups of positively correlated crops, one composed of maize and grass and the other of barley and oat. Moreover, these two groups showed a negative correlation between each other. In addition, the difference observed between samples obtained from control (fallow pots) and planted plots using CCA analysis indicated that plant species had indeed an effect on *Burkholderia* populations, either by increasing the diversity or by affecting the evenness of the samples.

Interestingly, there was a distinct growth cycle effect, which could be observed by a rise in the numbers of bands from the first to the third growth cycle, regardless of plant species or land use history. This increase in diversity with growth cycle number could be explained by the fact that the pots were kept for a total of one year under constant greenhouse conditions, which might have been optimally selective for specific organisms. Since the bands that started to appear or became more intense were mainly present at the bottom of the gel, *Burkholderia* species with higher G+C % were apparently stimulated. Some of these bands were identified and showed high similarity to an unculturable or uncultured 16S ribosomal RNA gene clone isolated from the cast of *Lumbricus rubellus* (36). However, although earthworm casts are composed primarily of soil organisms (15), it is not clear whether this clone is specifically associated with earthworms, since it was isolated only once (D. R. Singleton, personal communication).

When comparing the growth cycle within different land use histories, we observed a more striking effect in the arable land treatments (A-M and A-R) than in grassland (G), in which the samples did not show the same tendency to cluster. A plausible explanation would be that the permanent grassland, due to its long land use history, would have a more stable and even community that would be recalcitrant to (drastic) changes. In the treatments originating from arable land (A-R and A-M), shifts in the *Burkholderia* community structure after the three plant growth cycles occurred in a more drastic manner, indicating that areas under this agricultural management regime are more amenable to changes.

The use of CCA proved to be an effective tool in evaluating how *Burkholderia* communities respond to changes in land use management and how the communities change according to different plant types. Furthermore, by plotting species (DGGE bands) and environmental variables, it was possible to identify band species that were correlated to certain treatments. However, as each DGGE band might harbour more sequence species, we cannot

provide substantial data on the actual diversity of the community. Based on this study, we concluded that although species belonging to the "*B. cepacia* complex" might be present in the rhizosphere soil, they seem to be less influenced by agricultural practices, since from 10 selected bands responding to changes in crop and land use management only typical soil *Burkholderia* species were found. These results were in agreement with our previous work (33) in which the analysis of randomly selected soil clones showed that most clustered close to species with biocontrol and bioremediation abilities. This trend does not seem to be correlated to the origin of the soil, since similar results were found by Richardson *et al*. (30) after assessing the diversity of *Burkholderia* isolates from woodland rhizosphere environments.

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Chapter 4

The diversity of culturable *Burkholderia* species in soil is driven by changes in agricultural management

Abstract

In order to assess the diversity of the culturable *Burkholderia* populations in rhizosphere and bulk soil and to evaluate how different agricultural management regimes and land use history affect this diversity, four treatments were evaluated: permanent grassland; grassland converted into maize monoculture; arable land and arable land converted into grassland. *Burkholderia* isolates obtained from PCAT medium were grouped in 47 clusters using 16S ribosomal RNA gene based PCR-DGGE combined with BOX genomic fingerprinting (DGGE-BOX). The distribution of the isolates in the DGGE-BOX clusters was used to calculate the Shannon diversity index per treatment. Interestingly, we observed that the *Burkholderia* diversity was affected by changes in the agricultural management, since the highest diversity was observed in permanent grassland and in continuous arable land. In addition, the diversity tended to be higher in the rhizosphere than in the corresponding bulk soil. The use of species abundance models indicated that rhizosphere communities had more even distributions than communities collected from the bulk soil. Identification of isolates revealed that only 2% of these belonged to the *B. cepacia* complex and that the majority was assigned to either (1) new *Burkholderia* species or (2) *Burkholderia* species that had originally been isolated from soil. Isolates classified as *B. hospita*, *B. caledonica* and *Burkholderia* sp. 'R-23316' and 'R-23326' were found mainly in the arable land while isolates belonging to *Burkholderia* sp. 'R-23336' and *B. phytofirmans* were associated with the grassland area. Another potentially new *Burkholderia* species, 'R-23330', was found in both areas, in close association with the maize rhizosphere.

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Introduction

The genus *Burkholderia* was created in 1992 (66) and since then, the list of species has changed several times; some species were removed from the genus (66), some were later re-classified as *Burkholderia* (25, 65) and some newly described species were added (62). In addition, in 1997 a polyphasic taxonomic study revealed that isolates identified as *B. cepacia* did not represent a single species, but a complex composed of several phenotypically similar species denoted genomovars (62). Currently, the *B. cepacia* complex comprises nine species or genomovars, i.e. *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (genomovars I to IX, respectively) which, along with approximately 24 other species, constitute the whole genus (5, 14).

In the agricultural sector, the genus *Burkholderia* became popular as a result of the biocontrol abilities of some species (6, 28). Additional beneficial traits, such as atmospheric nitrogen fixation (7, 20, 21, 38) and plant growth promotion (57), and more recently, its applicability for bioremediation (37), increased the relevance of this genus for environmental purposes. Similarly, in the clinical sector, the genus *Burkholderia* has gained importance in the last decade due to the pathogenicity of several species belonging to the *B. cepacia* complex for patients with cystic fibrosis (CF) (35). The high virulence observed for certain species, mainly *B. cenocepacia* and *B. multivorans* (51), combined with the fact that *B. cenocepacia* has been found as a common plant-associated bacterium, isolated from rhizosphere and plant tissues (2), increased the concern that the environment might be a source of pathogenic strains (32). However, due to the lack of genetic markers of transmissibility or virulence, any distinction between environmental and clinical strains, or between pathogenic and non-pathogenic strains, remains, so far, impossible (41). Based on these facts, several biocontrol strains recently had their risk assessment modified by the U.S. Environmental Protection Agency (41).

Burkholderia species have a broad natural distribution, occurring commonly in soil (1), water (64) and in association with plants (2, 3, 38), fungi (31) and animals (58). However, little is known about the diversity of this group of organisms in these settings. In addition, for the reasons mentioned earlier, most of the attention has concentrated on the *B. cepacia* complex. Furthermore, since species belonging to the *B. cepacia* complex are found in close association with maize roots, representing up to 3.6% of the culturable bacterial population (18), diversity studies have focused mainly on *B. cepacia* complex strains isolated from this plant (3, 8, 15, 22). For instance, Chiarini *et al.* (8) showed that the diversity of *B. cepacia* complex isolates associated with maize, increased during plant growth, with the highest values being obtained in the middle and terminal portions of roots from mature plants. When *B. cepacia* isolates were obtained from different maize cultivars growing in different soil types, it was noticed that soil type was the main factor affecting the diversity of root-associated *B. cepacia* community (15). In another survey performed in maize fields with different management history, mainly *B. cepacia*, *B. cenocepacia*, *B. vietnamiensis* and *B. ambifaria* were isolated (22). When different fields were compared (low versus high input), the highest degree of genomovar diversity was obtained from a field characterised by a low-input regime, to which only manure had been added (22). In addition, Dalmastri *et al.* observed that the diversity of maize-associated *B. cepacia* isolates differed from that of clinical samples (16). The majority of isolates recovered

was found to belong to *B. ambifaria*, while the remainder was distributed among *B. cenocepacia*, *B. pyrrocinia* and *B. cepacia* (16).

The impact of agricultural management on soil microbial community structure has been evaluated using different approaches. The hypothesis that land use history and agricultural management produce a unique soil environment was evaluated by Steenwerth *et al.* (52) who correlated physical and chemical soil factors, management factors and vegetation type with microbial community structure, as measured by phospholipid ester-linked fatty acid (PLFA) profiles. Higher values of soil C, N and microbial biomass and lower pH values occurred in grassland that in cultivated soils and in fact, land use type could be identified by the soil microbial community (52). By using culture-based techniques, Lupwayi *et al.* observed that conservation tillage and legume-based crop rotation supported a higher diversity of the soil microbial community than tillage and wheat monoculture (33). Although changes in soil microbial diversity occur, not all microorganisms respond the same way. For instance, Clegg *et al.* showed that grassland management regime affects specific bacterial groups in soil differently (9).

In a previous study, we observed that the diversity of the *Burkholderia* community, as determined by PCR-DGGE analysis of DNA from the rhizospheres of different plants, was affected mainly by land use history (47). Differences in the *Burkholderia* community structure due to agricultural management and plant species occurred to a lower extent as a result of the dominating influence of the history of agricultural use of the area (47). The effect of land use history on the diversity of the total *Burkholderia* community, the restricted information on the diversity of the culturable *Burkholderia* population and the increasing concern that human-pathogenic species might have originated from the soil environment raised two important questions: (1) what is the impact of agricultural management on the diversity of culturable *Burkholderia* species; and (2) which species are correlated with the different management regimes?

In order to answer these questions, rhizosphere and bulk soil samples, taken from field areas under different agricultural management, were plated on semi-selective medium and a large number of isolates was obtained. The isolates were clustered according to their combined DGGE-BOX patterns and the data obtained were used to evaluate the diversity of the culturable *Burkholderia* populations and the distribution of species over the treatments.

Material and Methods

Isolation of Burkholderia *strains from soil*

To evaluate the diversity of *Burkholderia* species in soil under different agricultural management regimes, soil samples were taken from an experimental field located in the vicinity of Wageningen, the Netherlands. Briefly, this site was originally composed of a long-term (>50 years) permanent grassland (G) field that had been partially converted to agricultural land (A) around 24 years ago. In 2000, an experiment was established in both areas (G and A), where a range of treatments differing in agricultural management were established in triplicate plots (10×10m) (59). For this study, two treatments were chosen: one consisting of monoculture

of maize (*Zea mays* L.) and another one of permanent grassland (commercial mix, containing *Lolium perenne* as the main plant species). The maize plots received annual fertilisation, were tilled at 20 cm, and control of weeds was done mechanically, whereas the grassland plots were not fertilised or tilled. Grassland plots were sown at the beginning of the experiment (in the arable land) and the grass was cut once a year. Therefore, according to land use history and agricultural management, four treatments could be distinguished: (i) permanent grassland (which was kept as grassland) (G); (ii) an area under continuous agriculture, represented by arable land under maize monoculture (A-M); (iii) arable land converted to grassland (A-G); and (iv) permanent grassland which was turned into an agricultural field under maize monoculture (G-M).

In September 2003, after four growth cycles in the field (2000, 2001, 2002, 2003), soil samples were obtained from the four treatments. Bulk soil was obtained by combining 100 sub-samples, taken from the surface of each 10×10 m plot with a soil core sampler (3×10 cm, diameter × height). Rhizosphere soil was obtained by harvesting five maize plants, or grass, from five different locations per plot and gently shaking the roots in order to remove the loosely adhering soil. Twenty grams of roots with tightly-adhering soil (rhizosphere soil) or 10 g bulk soil were transferred, in separate, to Erlenmeyer flasks containing 90 ml of sterile sodium pyrophosphate (0.1% NaPP in water) and gravel (10 g). After shaking the flasks for 30 min at 180 rev min^{-1}, a series of 10-fold dilutions in 0.85% NaCl was prepared and plated onto PCAT medium (composition: azelaic acid 2 g l^{-1}, tryptamine 0.2 g l^{-1}, $MgSO_4.7H_2O$ 0.1 g l^{-1}, KH_2PO_4 4 g l^{-1}, K_2HPO_4 4 g l^{-1}, yeast extract 0.02 g l^{-1}, agar 15 g l^{-1}; pH 5.7) (4) supplemented with 100 mg l^{-1} Delvocid (DSM, Delft, The Netherlands). Plates were incubated at 27ºC for 7 days, when approximately 30 colonies per replicate plot (15 colonies from bulk soil and 15 from rhizosphere soil) were transferred to new PCAT plates. After purification via re-growth of separate colonies on new PCAT plates, isolates were stored at -80ºC as cell suspensions in Trypticase Soya broth (46) containing 20% glycerol.

DNA extraction

DNA was obtained from the *Burkholderia* isolates by resuspending one colony per strain in a microcentrifuge tube containing 20 µl of sterile Milli-Q water. Cells were lysed by keeping the suspensions at 95ºC for 10 min, followed by cooling on ice for 10 min. Before performing the lysis, five mg of Chelex-100 resin (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) was added to the microtubes to prevent inhibition of PCR amplification, given that compounds released by lysing cells are chelated by the resin and therefore removed from the suspension, by centrifugation at 1200 rev min^{-1} for 5 minutes. The microcentrifuge tubes containing the crude lysates were stored at -20ºC.

PCR-DGGE analysis

Amplification of 16S rRNA genes of the *Burkholderia* strains was performed using the primers specific for the genus *Burkholderia* (Burk3-GC/BurkR) in a direct PCR, according to the methodology described by Salles *et al.* (46). DGGE analysis was performed by using the PhorU2 system (Ingeny, Leiden, The Netherlands), in which gels with denaturing gradients of 50 to 60% of denaturant were run for 15 h at 100 V (46). After electrophoresis, the gels were

stained with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and then photographed and digitised using the Imago compact apparatus (B&L System, Maarssen, The Netherlands).

BOX fingerprinting

Genomic fingerprintings of *Burkholderia* isolates was performed by PCR with primer BOX-A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). Briefly, 1 µl of cell lysate was added to 24 µl of a mix containing 600 µmol l^{1} of each deoxyribonucleoside triphosphate, 3.75 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris-HCl pH 8.3, 10 mmol l^{-1} KCl, 500 nmol l^{-1} of primer, and 2.5 U of *Taq* DNA polymerase Stoffel fragment (Perkin-Elmer). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands). The thermal cycling program consisted of an initial denaturing step (95ºC for 7 min), followed by 30 cycles, each consisting of 30 s at 90ºC, 1 min at 95ºC, 1 min at 52ºC and 8 min at 65ºC. After the thermal cycling, there was a final extension step consisting of 65°C for 16 min, followed by cooling to 10ºC. The PCR products, expected to vary from 2 kb to 100 bp, were analysed by electrophoresis in 1.5% (wt/vol) agarose gel in 0.5×TBE buffer (48). Isolates that were not successfully amplified with the BOX A1R primer were subjected to a second new PCR reaction in which a higher or lower volume of crude cell lysate was added.

Banding pattern analysis

Digitised gel images obtained from DGGE analysis and BOX fingerprinting were converted, normalised and analysed separately with the Molecular Analyst software (version 1.61; Bio-Rad, Veenendaal, The Netherlands) (43). In order to achieve perfect normalisation of the gels, markers were loaded in at least 3 positions, in both DGGE and BOX gels. The marker used for DGGE gels consisted of partial 16S rRNA genes from four *Burkholderia* species as described (36). For normalising the BOX fingerprints, the molecular mass marker was used (Invitrogen BV, Breda, The Netherlands). In order to validate the normalisation procedure, identical profiles were run in different gels. After normalisation of each gel separately, the fingerprints were combined considering the DGGE profile as the "master" gel. The similarity between the combined DGGE-BOX patterns was calculated using the Pearson or product-moment correlation coefficient, which is insensitive to the relative concentration of bands and to differences in the overall intensity of the profile (43). Cluster analysis was performed by applying the unweighted pair-group method using arithmetic averages (UPGMA) algorithm to the similarity matrix.

Identification of isolates

Species-level identification of *Burkholderia* isolates was achieved using a polyphasic approach. First, all isolates were examined using a *recA* based PCR test to identify putative *B. cepacia* complex bacteria (36). Isolates belonging to the *B. cepacia* complex were further identified using *Hae*III-RFLP analysis of the resulting amplicons (36). All non-*B. cepacia* complex isolates were subsequently examined using comparative whole-cell protein electrophoresis as described before (14, 42). Isolates were identified by comparing their whole-cell protein electrophoretic profiles with those present in a database comprising reference

profiles of all known *Burkholderia* species (14, 63); alternatively, several isolates were grouped into putative novel *Burkholderia* species because of the lack of similarity of their whole-cell protein profiles towards those of reference strains of all current *Burkholderia* species.

The exact phylogenetic position of representative isolates of putative novel species was determined by cloning and sequencing their 16S rRNA genes. Amplification of the 16S rRNA genes from representative strains was performed with primers 27F (5'-AGAGTTTGATC[CA]TGGCTCAG-3') and 1378R (5'-CGGTGTGTACAAGGCCCG GGAACG-3'), in 50 µl reaction mixtures containing 1 µl of cell lysate, 350 µmol l^{1} of each deoxyribonucleoside triphosphate, 300 nmol l^{-1} of each primer, 1× *TaqPlus* Precision buffer (Stratagene, Leusden, The Netherlands), and 2U of *TaqPlus* Precision polymerase mixture (Stratagene). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc. Tilburg, The Netherlands). The thermal cycling program consisted of an initial denaturing step (95°C for 2 min), followed by 35 cycles, each consisting of 30 s at 94ºC, 30 s at 60ºC, and 2 min at 72ºC. After the thermal cycling, there was a final extension step consisting of 72ºC for 7 min, followed by cooling to 10ºC. The PCR products were analysed by electrophoresis in a 1% (wt/vol) agarose gel in 0.5× TBE buffer (48). PCR products were then cloned in the pGEM-T easy vector, which was used to transform *Escherichia coli* strain JM109, according to the procedure recommended by the manufacturer (Promega Benelux, Leiden, The Netherlands). Plasmid extraction from randomly selected colonies was performed with the Wizard Plus SV miniprep DNA purification system (Promega Benelux). Sequencing was performed by using a ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands).

Sequence alignment

Sequences generated in this study or recovered from the GenBank/EMBL database were aligned by using Clustal_X (54), considering 1380 bp of the 16S rRNA gene. Phylogenetic trees were constructed by the neighbor-joining method (45) based on distance estimation calculated by the method of Jukes and Cantor (30). This analysis was performed with the TREECON program, version 1.3b (Yves van de Peer, Department of Biochemistry, University of Antwerp, Antwerp, Belgium).

Diversity measurements and statistical analysis

The cut-off value used to determine the level of similarity between DGGE-BOX clusters was tested by assessing its Discriminatory index (DI) (13). DI determines the probability that two unrelated strains are placed into different groups and is calculated by using the Simpson index of diversity, which takes into account the number of clusters defined and their relative frequencies (13).

The Shannon diversity index was determined as $H' = -\Sigma p_i \ln p_i$, where p_i is the proportion of individuals found in the ith DGGE-BOX cluster (34). To test for significant differences between the Shannon diversity index obtained for each sample, a *t* test was performed according to the methodology described by Magurran (34), as follows:

$$t = \frac{H'_1 - H'_2}{(VarH'_1 + VarH'_2)^{1/2}}$$

and the variance of *H'* was calculated using the equation:

$$VarH' = \frac{\sum p_i(\ln p_i)^2 - (\sum p_i \ln p_i)^2}{N} + \frac{S-1}{2N^2}$$

where *N* is the total number of individuals and *S* is the total number of DGGE-BOX clusters. The degrees of freedom were calculated according to the formula

$$df = \frac{(VarH'_1 + VarH'_2)^2}{(VarH'_1)^2/N_1 + (VarH'_2)^2/N_2}$$

where N_1 and N_2 are the total number of individuals in samples 1 and 2, respectively (34).

Species abundance models were calculated according to the formulas described by Hill *et al.* (29). The observed data were distributed among 5 abundance classes, divided according to the number of isolates per cluster, with upper boundaries of 2.5, 4.5, 8.5, 16.5 and isolates per cluster. The observed distribution was then compared with the expected distribution obtained from the models by using a goodness-of-fit test (χ^2) (32). Low χ^2 values (non-significant) indicate a good agreement between the observed and the expected data, i.e. that the model fits the distribution of the data (32).

Results

Enumeration and isolation of Burkholderia *strains*

The population densities of populations recovered from the semi-selective PCAT medium are shown in Figure 1. Significant differences were observed between bulk and rhizosphere soils, the latter showing populations approximately at 100 times higher densities than the former, except for A-G. The highest densities were observed in the rhizospheres of maize grown in the arable land (A-M) and in those of grass collected from the permanent grassland (G). A total of 344 colonies from both bulk and rhizosphere soil (around 30 per treatment, 15 from bulk and 15 from rhizosphere) were recovered from PCAT plates. From these, 287 isolates (83.4%) were positive for the 16S rDNA PCR with *Burkholderia* genus-specific primers.

DGGE-BOX banding pattern analysis

In order to characterise the culturable *Burkholderia* community in the different treatments, all 287 isolates were further characterised by BOX-PCR. The majority of the 287 *Burkholderia* isolates (89%) were amplifiable with the BOX A1R primer, whereas the remainder (11%) was not further analysed. The BOX fingerprints of the 254 *Burkholderia* isolates were compared and grouped according to their similarity, yielding a set of 158 isolates that represented all BOX types. These selected isolates were further characterised by 16S rDNA based PCR-

DGGE. Given that the PCR-DGGE method separates the isolates at approximately species level (46) while the BOX pattern has a resolution at strain level, the DGGE profile was considered the master pattern. The dendrogram generated by the combined DGGE-BOX pattern was divided in two main groups at 32% of similarity. One group contained most of the DGGE-BOX clusters, while the second group contained the *Burkholderia* isolates that had a lower G+C content in the 16S rRNA gene region amplified by the primers. Between 40-50% similarity, secondary and tertiary branches were formed for both groups and at 74%, the cut-off similarity value was set, generating 47 different DGGE-BOX clusters (Fig. 2) at a DI of 0.999.

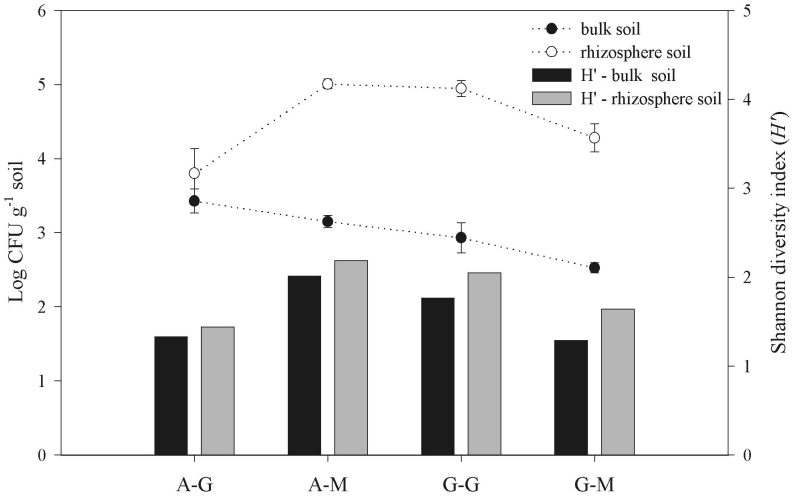

Figure 1. Mean density of the *Burkholderia* population isolated on the semi-selective media PCAT, from bulk (●) and rhizosphere (○) soil collected from Arable land (A) and Grassland field (G), cultivated with maize (M) and grass (G). Mean value and standard error are shown. Bars indicate the Shannon diversity index (*H'*) based on the number of DGGE-BOX types obtained from bulk (black bars) and rhizosphere (grey bars) *Burkholderia* soil populations.

Identification of the isolates

A total of 87 isolates representing the 47 DGGE-BOX clusters (1-5 isolates per cluster) was identified at the species level. Table 1 presents an overview of identification results from the total of 40 DGGE-BOX clusters assigned to one *Burkholderia* species, along with the method used for identification. The 40 clusters were distributed over 13 *Burkholderia* species, from which 8 represented putative novel species, referred to below as *Burkholderia* sp. 'R-23336', *Burkholderia* sp. 'R-23316', *Burkholderia* sp. 'R-23326', *Burkholderia* sp. 'R-23330', *Burkholderia* sp. 'R-23356', *Burkholderia* sp. 'R-23315', *Burkholderia* sp. 'R-23342' and *Burkholderia* sp. 'R-23355'. Figure 3 shows the whole-cell protein profiles of isolates representing the main groups detected in the present study, along with profiles of *Burkholderia* type or reference strains belonging to the same species, or, for the putative novel species, belonging to their nearest phylogenetic neighbours. A phylogenetic tree based on the 16S

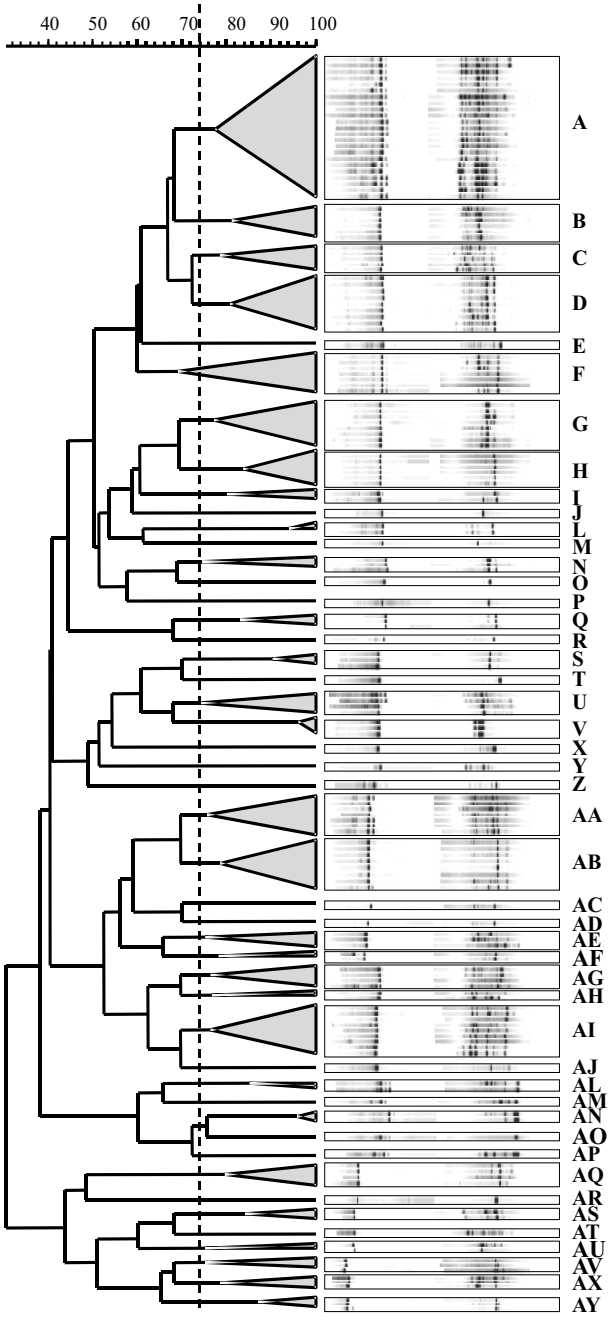

Figure 2. Combined DGGE-BOX dendrogram, showing the distribution of *Burkholderia* isolates. Similarity was based on product-moment correlation coefficient (43). Cluster analysis was performed by applying UPGMA algorithm to the similarity matrix.

rRNA gene is shown in Figure 4, indicating the position of some representative isolates described in Table 1 in the genus *Burkholderia*.

The species most frequently isolated was *B. hospita* (27), representing 28% of the isolates, which were distributed over seven DGGE-BOX clusters. Interestingly, 98.6% of the *B. hospita* isolates were recovered from the arable land carrying maize or grass (A-M and A-G) (Table 1). Twenty-two percent of the *Burkholderia* isolates were identified as *Burkholderia* sp. 'R-23336' and about 95% of these isolates were obtained from the plots with grassland history (G and G-M). In fact, this group clearly correlated with permanent grassland (G), which contained 88% of the isolates. In addition, this was the most diverse *Burkholderia* species, being represented by 12 DGGE-BOX clusters. Another abundant group of isolates (19%), spread over five clusters, was identified as *B. phytofirmans*, a new *Burkholderia* species originally isolated from onion infected with arbuscular mycorrhiza (49). This species was also mainly isolated from the plots with grassland history (85%) and the highest number of isolates was recovered from grassland converted into maize monoculture (G-M, 70%). *Burkholderia* sp. 'R- 23330', corresponding to a single cluster containing 9% of the isolates, was highly prevalent in the maize rhizosphere and contained isolates from plots currently under maize, but with different land use history (A-M and G-M). *Burkholderia* sp. 'R-23316' represented 5% of the isolates, spread over four clusters, and was mainly recovered from the arable land, currently under different crops (A-G and A-M). Two percent of the isolates, which were distributed over four clusters, were assigned to *B. caledonica* (12). These isolates were mainly obtained from the rhizosphere of maize plants collected from the arable field (A-M). *Burkholderia* sp. 'R-23326' (2% of the isolates) was mainly isolated from the bulk soil of the arable area under maize monoculture (A-M) and was represented by clusters AQ. Cluster V contained 1% of the isolates, which were obtained from permanent grassland (G) and identified as *Burkholderia* sp. 'R-23356'. Cluster AS was identified as *Burkholderia* sp. 'R-23315' and represented by 1% of the isolates, mostly obtained from rhizosphere soil of the arable land. Cluster R encompassed one isolate, called *Burkholderia* sp. 'R-23355', which was isolated from the permanent grassland (G). Both clusters X and Z were represented by one isolate each, obtained from the bulk and rhizosphere soil of arable land under maize monoculture (A-M), respectively. The isolate from cluster X belonged to a novel *Burkholderia* species described by Goris et al. (27). The latter novel species is referred to below as *Burkholderia* sp. 'LMG 21262'. The isolate from cluster Z was assigned to *Burkholderia* sp. 'R-23342'. Cluster AM, which also comprised one isolate recovered from the rhizosphere of maize growing in the grassland plot (G-M), was assigned to *B. pyrrocinia* (61).

Although the majority of the DGGE-BOX clusters were assigned at species level (Table 1), seven clusters comprised isolates belonging to more than one *Burkholderia* species (data not included in Table 1). Cluster N comprised four isolates obtained from the rhizosphere of the permanent grass (G). Three of these were assigned to *B. phytofirmans* and one to *Burkholderia* sp. 'R-23336'. Similarly, cluster AH was represented by two rhizosphere isolates recovered from the area under permanent grassland (G), which were identified as *B. phytofirmans* and *Burkholderia* sp. 'R-23336'. The isolates grouped in cluster U were identified as *B. caledonica* (two isolates) and *B. hospita* (four isolates) and were recovered from the rhizosphere of maize or grass, grown in the arable land (A-M and A-G). Cluster AG comprised four isolates: two were identified as *B. caledonica* and one as *B. xenovorans* (26). The fourth isolate was identified as belonging to the *B. cepacia* complex by protein profile, while

Table 1. Distribution of the identified *Burkholderia* species per cluster. Only clusters assigned to one species are shown.

Species	No of isolates	Cluster	No of isolates per treatment [a]				Method [b]	Reference
			A-G	A-M	G	G-M		
B. hospita	71	A	6(0)	28(9)	-	1(0)	2 and 3	(27)
		AL	-	6(6)	-	-	2 and 3	
		AN	7(7)	-	-	-	2	
		AO	2(2)	-	-	-	2	
		AP	2(2)	-	-	-	2	
		F	18(18)	-	-	-	2 and 3	
		Y	-	1(0)	-	-	2	
Burkholderia sp. 'R-23336'	57	C	-	-	5(0)	-	2	This study
		D	-	-	16(0)	-	2 and 3	
		E	-	-	1(0)	-	2	
		G	-	1(0)	8(7)	2(0)	2 and 3	
		H	-	-	10(10)	-	2	
		I	-	-	-	2(1)	2	
		J	-	-	1(1)	-	2	
		L	-	1(0)	2(0)	-	2	
		M	-	-	1(1)	-	2	
		O	-	1(0)	-	-	2	
		P	-	-	2(2)	-	2	
		Q	-	-	4(0)	-	2	
B. phytofirmans	47	AA	2(0)	1(0)	-	9(2)	2 and 3	(49)
		AB	-	1(1)	1(1)	13(13)	2 and 3	
		AI	-	3(1)	5(3)	4(1)	2	
		AT	-	-	1(0)	-	2	
		AJ	-	-	-	7(7)	3	
Burkholderia sp. 'R-23330'	22	B	-	4(0)	-	18(0)	2 and 3	This study
Burkholderia sp. 'R-23316'	13	AV	1(1)	1(1)	-	1(1)	2	This study
		AX	3(0)	1(1)	-	-	2 and 3	
		AY	1(1)	4(4)	-	-	2 and 3	
		AR	-	-	1(1)	-	2	
B. caledonica	6	AC	1(1)	-	-	-	2	(12)
		AD	-	1(1)	-	-	2	
		S	-	3(0)	-	-	2 and 3	
		T	-	1(0)	-	-	2	
Burkholderia sp. 'R-23326'	4	AQ	-	4(3)	-	-	2 and 3	This study
Burkholderia sp. 'R-23356'	3	V	-	-	3(0)	-	2 and 3	This study
Burkholderia sp. 'R-23315'	2	AS	1(0)	1(0)	-	-	2 and 3	This study
Burkholderia sp. 'R-23355'	1	R	-	-	1(0)	-	2 and 3	This study
Burkholderia sp. 'LMG 21262'	1	X	-	1(1)	-	-	2 and 3	(27)
Burkholderia sp. 'R-23342'	1	Z	-	1(0)	-	-	2	This study
B. pyrrocinia	1	AM	-	-	-	1(0)	1 and 3	(65)

[a] Number in brackets indicates isolates obtained from bulk soil.

[b] Methods used for identification: 1 , RFLP of the recA gene; 2, comparison of the whole protein profiles; 3, sequencing of the partial 16S rRNA gene.

sequencing analysis of the 16S rRNA gene indicated 97% similarity with *Burkholderia* sp. strain RP007 (accession number AF061872; Laurie, A.D. and Lloyd-Jones, G., unpublished). These were all obtained from the rhizosphere of maize, growing in the arable land (A-M; *B. caledonica* and *B. xenovorans*) or in the grassland (G). Four isolates collected from the maize rhizosphere (G-M) represented cluster AE. Of these, one was identified as *B. phytofirmans* and the remaining three as belonging to *B. cepacia* complex. The latter strains were characterised by a novel *Hae*III-*recA* restriction profile and therefore, their precise taxonomic status within the *B. cepacia* complex requires further taxonomic analyses. Cluster AF comprised three isolates from the rhizosphere of maize growing in the arable land (A-M). Two of these were identified as *B. hospita*, while the third one was identified as belonging to the *B. cepacia* complex by protein profile. Further taxonomic work is required to address the status of this isolate, since amplification of the *recA* gene was not successful. According to the partial sequencing of the 16S rRNA gene, this isolate was most similar to *B. vietnamiensis* strain LMG 10929 (accession number AF097534) (56). Cluster AU comprised two isolates, identified as *Burkholderia* sp. 'R-23352' and 'R-23321', which were isolated from bulk soil of permanent grassland and arable land under maize monoculture, respectively.

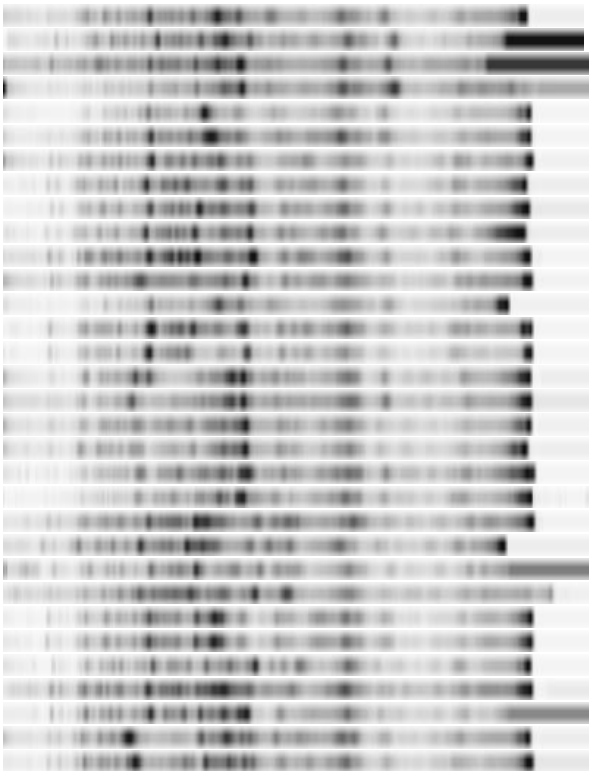

Figure 3. Whole-protein profiles (SDS-PAGE) of the *Burkholderia* species isolated in this study and reference *Burkholderia* species.

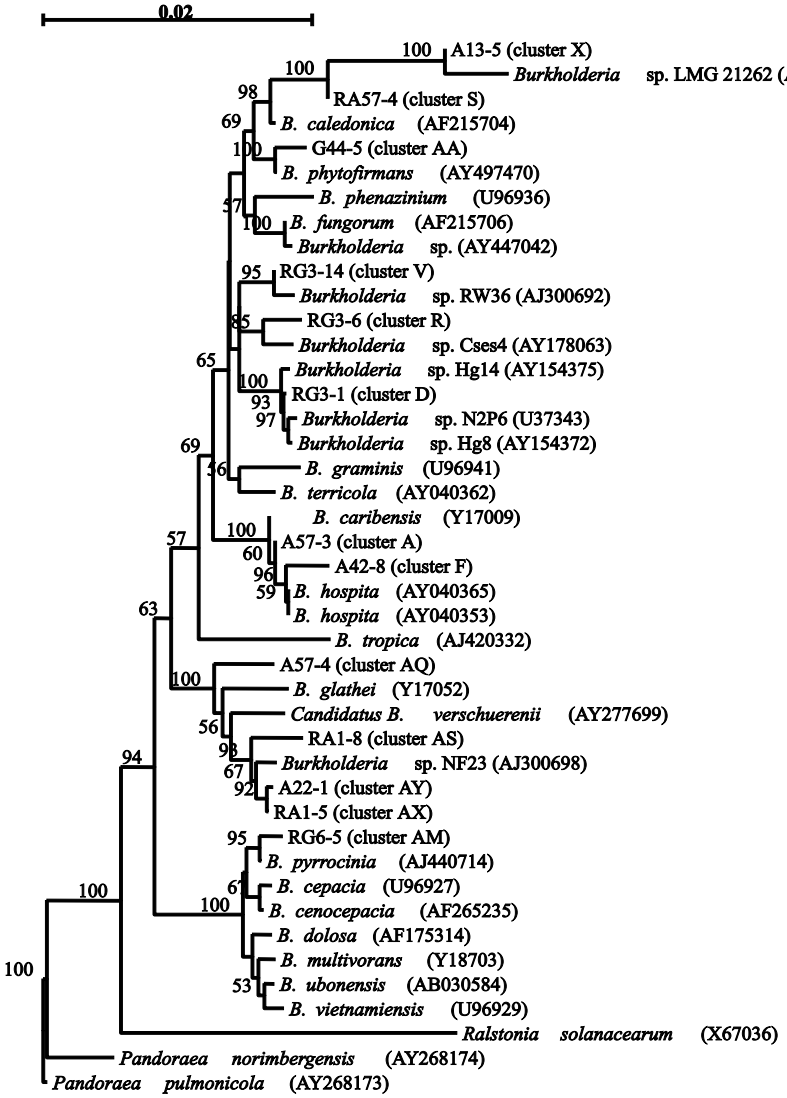

Figure 4. Phylogenetic tree showing the relationship between some *Burkholderia* species and the isolates described on Table 1. The tree was constructed based on 1384bp of the 16S rRNA gene, using the neighbour-joining method (45). A bootstrap analysis was performed with 100 repetitions, and only values above 50 are shown. The accession number of each strain is enclosed in brackets.

Diversity measurements

The distribution of the *Burkholderia* strains in the 47 clusters defined by DGGE-BOX fingerprinting was used to evaluate the diversity of the community as related to the treatments. Figure 1 shows that the Shannon diversity index (*H'*) for bulk and rhizosphere soils displayed the same trend, with the highest values being obtained for arable land under maize monoculture (A-M) and the lowest for arable land converted to grassland (A-G). In addition, the Shannon diversity index obtained for the *Burkholderia* community associated with rhizosphere soil tended to be higher than for that of the bulk soil, however this difference was significant ($P = 0.01$) only for the soil collected from the areas with grassland history (G and G-M) (Fig. 1). The *Burkholderia* communities associated with the rhizosphere of grass collected from the permanent grassland (G) and of maize growing in the arable land (A-M) were the most diverse according to the Shannon diversity index ($P = 0.001$) (Fig. 1). When comparing the *Burkholderia* communities of the bulk soils, the lowest diversity indices were found in the arable land converted to grassland (A-G) and grassland converted to maize monoculture (G-M) ($P = 0.001$) (Fig. 1).

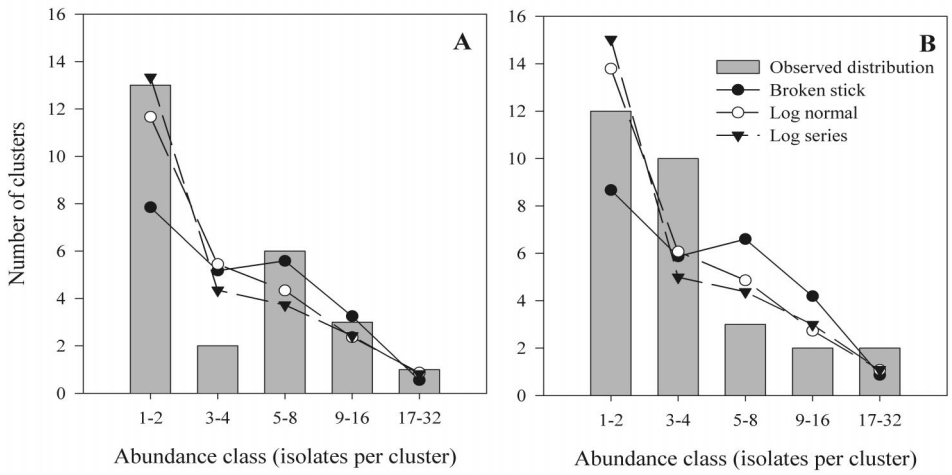

Figure 5. Observed and predicted distributions of the DGGE-BOX clusters in bulk (A) and rhizosphere soil (B). Species abundance classes were calculated according to the formulas described by Hill *et al*. (29).

To compare the effects of the plant rhizospheres on the diversity of *Burkholderia* communities, all isolates obtained from rhizosphere or bulk soil samples were pooled, generating two data sets (bulk and rhizosphere soil) that were divided in five abundance classes (29). Three species abundance models, ranging downward in equitability from "broken stick" to "log series" (29) were tested and compared with the observed distributions. Although some differences in the distribution could be observed between the rhizosphere and the bulk soil community (mainly in the intermediate classes) (Fig. 5), all models fitted the observed distribution to some extent, for both data sets. The distribution of the bulk soil community could be described by the broken stick model, the truncated log normal and the log series

model with probabilities of $P = 0.30$, 0.50 and 0.70, respectively. The rhizosphere community data could be described by these models with probabilities of $P = 0.10$, 0.30 and 0.20, the bulk and rhizosphere soil, respectively

Discussion

The genus *Burkholderia* occupies a wide variety of environmental niches and occurs naturally in soil, water and the rhizosphere of plants. Within this extremely versatile group of bacteria, certain species are able to cause plant disease, while others are typically used as biocontrol agents. Some are able to remediate areas polluted by man, by degrading toxic compounds; others are severe (opportunistic) pathogens for people with cystic fibrosis. Although *Burkholderia* is an intriguing genus, little is known about the diversity of *Burkholderia* species in different habitats or even how these organisms respond to changes in the environment. Given that different *Burkholderia* species are consistently found in the bulk soil or in association with plant roots, one would expect that different species respond differently to changes in agricultural practices. Indeed, in a microcosm experiment, it was observed that different land use history was correlated with different patterns in *Burkholderia* community structure (47), confirming other findings that agricultural practices play an important role in determining soil microbial community structure (24, 59). But how does the culturable *Burkholderia* population respond to different agricultural practices?

Overall, the culturable fraction of the soil microbial community often represents only 0.5-10% of the total discernible population (55), although this small fraction clearly remains important (39). However, in respect of the genus *Burkholderia*, the fraction of the soil community that is culturable on plates is unknown. Furthermore, the concern that the (soil) environment might serve as a source of pathogenic strains belonging to the *B. cepacia* complex (32) shows the importance of assessing the diversity of the genus *Burkholderia* also at the culturable level. Therefore, in order to evaluate the culturable *Burkholderia* community in soils under different agricultural management, the community able to grow on the semi-selective PCAT medium was assessed. PCAT has been widely used for assessing the diversity of species belonging to the *B. cepacia* complex in the environment (16, 18, 40, 44). Although this medium had initially been developed for the isolation of *B. cepacia*, most *Burkholderia* species are able to grow on it (65). The identification, at species level, of 87 representative isolates distributed among the 47 DGGE-BOX clusters revealed that, surprisingly, only six (2%) belonged to the *B. cepacia* complex. The fraction of isolates recovered from PCAT that belongs to the *B. cepacia* complex is known to be variable and dependent on the environmental source. It can range from 70% (18) to 74% (53) when roots are blended prior to dilution and plating, to below detection when bulk soil from a maize field was taken as sample (40). These differences may be attributed to the fact that, when macerated roots are used for dilution, not only the rhizosphere community is being analysed, but also the rhizoplane and the endophythic communities. By strictly sampling the bulk and rhizosphere soil, Pallud *et al.* (40) observed that the fraction of colonies growing on PCAT belonging to the genus *Burkholderia* varied between 35% to 86%, respectively, as determined by the use of *Burkholderia* genus-specific probes. Similar to this result, we observed that 89% of the colonies isolated from the rhizosphere of maize belonged to the genus *Burkholderia* and that this percentage was lower in the bulk soil (72%). The percentage of

isolates belonging to *Burkholderia* observed in the bulk soil was higher then the one observed by Pallud *et al.*, who also noticed that two thirds of the colonies growing on PCAT did not have a "*Burkholderia*-like" morphology (40). Heterogeneity in colony morphology was not observed in our study, as all colonies obtained on PCAT were similar to what DiCello *et al.* described as a "*Burkholderia*-like" colony morphology (18). Since the soil used by Pallud *et al.* was a glacial terrace, it is reasonable to assume that the variation observed in the community associated with the bulk soil is due to differences in soil type. These results indicate that PCAT is suitable for the enumeration and isolation of *Burkholderia* species, but not specifically for species from the *B.* cepacia complex. However, since the list of species belonging to the genus *Burkholderia* has been altered several times since the work performed by Viallard *et al.* (65), with new species added annually, more studies are necessary to verify which *Burkholderia* species are able or not to grow on PCAT medium.

Rhizosphere bacterial communities seem to have a higher level of culturability than bulk soil communities due to the high substrate availability observed around the roots (17). Indeed, the *Burkholderia* population densities present in the rhizosphere were 100-fold higher than in the bulk soil in all treatments, except A-G. This result is in agreement with those of Pallud *et al.*, who estimated that the genus *Burkholderia* represents 2% and 16% of the total bacterial communities of bulk and rhizosphere soil, respectively (40). Due to the differences in cell densities between the *Burkholderia* communities associated with rhizosphere and bulk soils, we decided to evaluate the distribution of the clusters within each of the two sub-sets (bulk and rhizosphere soil) of the *Burkholderia* community, by fitting the observed abundance to species abundance models. Such models depict diversity by the distribution of abundances in a community (29). The community is then considered to be more, or less, even according to the model that best fits the observed distribution. However, when small data sets are used, it can be difficult to detect differences between observed and expected distributions, resulting in the fitting, to similar extents, of all the models to the same data set (34). Nevertheless, abundance models still address the distribution of the population and can be recommended even when coverage is low (29). Most likely as a result of the size of our data set and the low number of abundance classes, we were not able to clearly specify the model that best fitted the data set. However, the probability (*P*) of the expected log series distribution being different from the distribution of the bulk soil population was < 30%, which indicated that the bulk soil community approximated the log series distribution. The log series abundance model represents the least equitable distribution tested and it tends to occur when one or few factors dominate the ecology of the community (34) and few groups are abundant. In the case of the bulk soil, the factors determining community composition could be the agricultural practices applied to the fields. In the rhizosphere soil, however, the model that best fitted the distribution of the *Burkholderia* community was the truncated log-normal model, although again with a low probability ($P <$ 30%). The log-normal distribution may originate from a community that has been affected by random variation of many independent factors (29). In our case, these random factors could be correlated with differences in quality and quantity of root exudation, in addition to agricultural management. According to the species abundance models, the *Burkholderia* community associated with the rhizosphere soil (log-normal distribution) was more even than the bulk soil community (log series).

In the present study we used a polyphasic strategy to identify *Burkholderia* isolates to the species level, in order to assess whether the clustering based on the DGGE-BOX approach

was suitable to determine the *Burkholderia* diversity. Several studies of the genus *Burkholderia* confirmed the correlation between the whole-cell protein profile similarity and DNA-DNA hybridisation, the latter being the standard for species delineation in bacterial taxonomy (10-12, 26, 27, 60, 62, 63). Only for some of the *B. cepacia* complex species which are very closely related, whole-cell protein profiles are too similar to allow unequivocal species differentiation (14) and, therefore, alternative molecular approaches have been elaborated (36). Whole-cell protein profiling, however, offers the additional advantage to allow the assignment of multiple isolates to the same new *Burkholderia* species. Phylogenetic analysis of representative isolates of such new taxa subsequently reveals the nearest phylogenetic neighbours to be included in formal taxonomic studies for the proposal of novel species. The identification results showed that isolates of most of the DGGE-BOX clusters could be identified at species level. Forty-one percent of the isolates were distributed over eight potentially new *Burkholderia* species, which, in general, were closely related to species commonly isolated from the environment. However, a full taxonomic characterisation of these species is beyond the scope of the present study. The *Burkholderia* species that could be identified at species level were common soil species, exhibiting different degrees of interaction with plants. In addition, only six isolates were assigned to the *B. cepacia* complex.

After evaluating the distribution of the *Burkholderia* species among the treatments, we observed that they could be divided in two groups, according to the land use history of the area. *Burkholderia* isolates identified as *B. hospita* or *Burkholderia* sp. 'R-23316' seemed to be well adapted to changes in agricultural management as they occurred in both arable land under maize monoculture and arable land converted to grassland. Since *B. hospita* has originally been isolated from agricultural soil as a transconjugant that had received plasmids pJP4 or pEMT1 (27), its plasticity may be due to its ability to acquire plasmids from other soil bacteria. *B. caledonica* and *Burkholderia* sp. 'R-23326' occurred mainly in the arable land under maize monoculture, although the former was mainly found in association with maize roots , while the latter was also found in the bulk soil. This result is in agreement with the original report of *B. caledonica*, which was first isolated from rhizosphere soil in Scotland (12). The isolates obtained from grassland were mainly distributed among two species, *Burkholderia* sp. 'R-23336' and *B. phytofirmans*. The former isolates were highly linked to the permanent grassland whereas the latter was found mainly in the grassland converted to maize monoculture (G-M). *B. phytofirmans* has been recently described as a powerful plant-growth promoting bacterium (49). However, additional tests should be performed in order to verify if plant-growth promotion is a common trait within this species. The only organisms that might be correlated with agricultural management regime were *Burkholderia* sp. 'R-23330'. This taxon was highly associated with the maize rhizosphere, occurring in maize planted both in former grassland (G-M) and in arable land (A-M).

It is generally accepted that the rhizosphere community is a subset of the bulk soil community and is, therefore, less complex (19). In this study, the diversity of the culturable *Burkholderia* communities associated with the treatments (estimated by comparing the Shannon diversity index) was consistently higher in the rhizosphere than in the bulk soil, irrespective of land use history or agricultural management. However, since only the culturable fraction of the *Burkholderia* community was assessed, one can speculate that the higher diversity observed in the rhizosphere was due to a positive effect of plant roots on the ability of the isolates to grow on the selective media used. Furthermore, comparison of the Shannon diversity indices

of the culturable *Burkholderia* communities associated with the treatments showed that the highest indices were obtained for the communities in arable land under maize monoculture (A-M) and permanent grassland (G). Interestingly, these results revealed that the *Burkholderia* diversity at the culturable level was affected mainly by changes in the agricultural management. The treatments with lower diversity indices were those subjected to a new agricultural management (A-G and G-M), suggesting that the conversion of arable land to grassland and vice versa led to a decrease in diversity. This result indicates that the impact of agricultural practices (fertilisation, tillage, etc.) applied to certain agricultural management regimes, can be greater than the crop used in that management. Moreover, the changes in soil management may have induced the selective outgrowth of specific *Burkholderia* types or species, decreasing the evenness and species abundance. Different plant species can select different portions of the microbial community (23, 50), but care should be taken when comparing plants growing in soils with different land use history or soils that went through recent changes in management. If we had considered only the arable land, we would have concluded that the *Burkholderia* community associated with maize (maize monoculture) was more diverse than the one associated with grass (grassland). On the other hand, by sampling only the plots derived from the permanent grassland, the opposite conclusion would have been achieved.

In conclusion, the diversity of the culturable *Burkholderia* community was mainly affected by the changes in the agricultural management, regardless of the direction of this change (towards more exploitative or more conservative management). Moreover, the culturable *Burkholderia* community associated with the rhizosphere was more even than the one associated with the bulk soil. The identification of the isolates showed that only 2% of these were assigned to the *B. cepacia* complex and that the majority of the species was assigned to either new *Burkholderia* species or to *Burkholderia* species commonly found in the soil.

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Chapter 5

Effect of agricultural management regimes on *Burkholderia* community structure in soil

Abstract

The main objective of this study was to determine the *Burkholderia* community structure associated with areas under different agricultural management and to evaluate to which extent this community structure would be affected by changes in agricultural management. Two fields with distinct soil history (arable land and permanent grassland) were exposed to three agricultural management regimes (crop rotation, maize monoculture, and grassland). By using a culture-independent approach, it was possible to observe that the conversion of *Burkholderia* communities typical from permanent grassland to that of arable land after four consecutive years. However, the time needed to achieve the reverse transition, i. e. converting the *Burkholderia* community associated with arable land to that of grassland, was beyond the duration of the field experiment. In addition, by applying principal response curves (PRC) the direction and extent of the conversion from grassland to arable land (maize monoculture and to crop rotation) were determined. Hence, the results suggested that agricultural practices, such as fertilisation and tillage, were more effective in changing the *Burkholderia* community structure than agricultural management regime. To determine the effect of agricultural management on the *Burkholderia* population with biocontrol abilities, the culturable fraction of the *Burkholderia* community was assessed. The areas under permanent grassland and grassland converted to maize monoculture had the highest percentages of *Burkholderia* strains with antagonistic activity against *Rhizoctonia solani* AG-3, mainly *B. pyrrocinia* and *Burkholderia* sp. 'R-23336'. The isolation frequency of antagonistic isolates from arable land was extremely low. Our results indicate that (changes in) agricultural management, mainly crop rotation, affect the frequency of isolation of antagonistic *Burkholderia* strains and that grassland represents a reservoir of *Burkholderia* species with great potential for agricultural applications.

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Introduction

Microbial communities play important roles in many soil processes by regulating nutrient cycling and organic matter decomposition, and by maintaining soil structure. Due to the importance of soil microorganisms for these key soil processes, many studies have been performed to determine the ecological factors regulating microbial community structure across temporal and spatial scales and the impact of different plant species on microbial diversity. The effect of environmental factors on microbial diversity has recently become a key research topic in the light of concerns about the preservation of biological diversity of soils. Most, most studies have been related to the effects of different soil properties on microbial communities (34). However, over the last 5 years, the impact of human activity, including agricultural practices, on the diversity of soil microorganisms and the consequences for soil processes became a more important issue (3, 6, 16, 22, 44).

Grassland is by far the most extensively studied soil system and the effect of management regimes on soil microbial communities associated with it has been assessed by a range of different techniques, such as direct ribosome isolation, PCR-DGGE, phospholipid fatty acid profiles (PLFA) and community level physiological profiling (CLPP) (3, 6, 16, 32, 44). Gradients in soil fertility have been shown to influence soil microbial community structure, and PCR-DGGE profiles showed that the total bacterial community in so called improved grassland soils was less even than that in unimproved grassland (32). Moreover, Clegg *et al.* showed that inorganic nitrogen fertiliser had a significant impact on bacterial and actinomycete community structures in grassland by analysing PCR-DGGE profiles of selected bacterial groups, as well as by PLFA profiling (6). By applying the latter method, Grayston *et al.* showed that bacteria dominated the improved grasslands while fungi were favoured in the unimproved sites, which also showed the highest microbial biomass (16).

Grassland and cultivated soils correspond to two distinct land use types that have different effects on soil characteristics and microbial community structures. Grassland soils tend to have higher contents of carbon, nitrogen and microbial biomass than cultivated soils (44). Steenwerth *et al.* observed that the impact of management inputs, such as fertiliser, herbicide and irrigation, could also be observed in cultivated areas with different land use history, where distinct microbial communities were associated with the different land use types (44). In addition, these authors observed that grassland soil sites, which had been last tilled from 3 to up to 33 years before being kept as grassland, were still different from permanent grassland. These data confirmed the results obtained by Buckley and Schmidt (3), who showed that the recovery of soil microbial communities from the effects of cultivation may require at least decades (44).

The possibility of changing the microbial community structure by applying different agricultural practices can be seen as a valuable tool to assist in the biological control of soil-borne pathogens. As an alternative to introducing antagonistic isolates, which usually originate from a different environment and may, thus, not be well adapted to the target environment, it may be possible to stimulate the indigenous antagonistic microflora. For instance, natural suppression of Take-all disease is induced in fields under monoculture of wheat, which has been associated with the presence of antibiotic-producing *Pseudomonas* spp. (36). Similarly, Mazzola *et al.* (31) found that the ability of the resident microflora to suppress *Rhizoctonia solani* in apple orchards was greater when the orchard had been established in a field where

wheat had previously been growing. In addition, a decrease in suppressiveness was correlated with a decrease of *Burkholderia cepacia* and *P. putida* populations, indicating that these microorganisms might be involved in disease suppression.

Species belonging to the genus *Burkholderia* have been identified as biocontrol agents of many plant-pathogenic fungi, such as *Pythium aphanidermatum*, *Pythium ultimun*, *Fusarium* sp., *Phytophthora capsici*, *Botrytis cinerea* and *Rhizoctonia solani* (5, 18, 20, 28); (1). Furthermore, a strain identified as a *Burkholderia* sp. was also able to inhibit the growth of bacteria, pathogenic yeasts and protozoa (5). The ability to suppress plant disease was observed in many different crops, such as corn, sweet corn, cotton, pea, tomato and pepper. In some cases, increased crop yield was observed even in the absence of the pathogen (18, 20, 28). For most of the studies mentioned above, the mechanisms involved in disease suppression are unknown. However, the antibiotic compounds phenazine and pyrrolnitrin have been isolated from *B. phenazinium*, *B. pyrrocinia* and *B. cepacia* NB-1. In addition, a novel antifungal lipopeptide has been identified from *B. cepacia* strain BC11 (10, 25). Although both phenazine and pyrrolnitrin play important roles in disease suppression by *Pseudomonas* species, the confirmation that antibiosis was the primary mechanism involved in the biocontrol by *Burkholderia* species was obtained only for strains *B. cepacia* AMMDR1 and BC-11, by using antibiotic production-deficient mutants (19, 25).

The genus *Burkholderia* represents a group of versatile organisms that are commonly found in soil, water and in association with plants. In addition to the biocontrol properties, some *Burkholderia* species are also useful for bioremediation and plant growth promotion (30, 47). Moreover, the ability to fix atmospheric nitrogen, which was initially observed only for *B. vietnamiensis* (46) has been extended to other species, including *B. tuberum*, *B. phymatum* and *B. caribensis*, which were also able to nodulate tropical legume plants (11, 49). The whole genus *Burkholderia* currently comprises about 34 species, nine of them belonging to the so-called *B. cepacia* complex (4, 9, 37, 43). This complex is characterised by a group of phenotypically similar species or genomovars that have been isolated not only from the environment, but also from clinical samples, mainly from cystic fibrosis (CF) patients (9). The impact of lung colonisation by *B. cepacia* complex strains on the survival of CF patients has led to stricter safety precautions. Hence, despite their great potential for environmental purposes, some biocontrol strains recently had their risk assessment restricted by the U. S. Environmental Protection Agency (35).

In the light of the vast possibilities to exploit the genus *Burkholderia* for biological applications, the main objective of this study was to determine the diversity of *Burkholderia* species in field soil under different agricultural management regimes. *Burkholderia* diversity was assessed by applying a PCR-DGGE system, with primers targeting a region of the 16S rRNA gene specific for the genus *Burkholderia* (39). In addition to this culture-independent approach, the *Burkholderia* community able to grow on a semi-selective medium was screened by dual culture assay for antagonistic activity against *R. solani* AG3. By applying this culture-dependent approach, our aim was to characterise antagonistic *Burkholderia* strains and to determine the effect of agricultural management on this subset of the *Burkholderia* community.

Material and methods

Experimental field

In order to compare the effect of different agricultural management regimes on soil microbial populations, an experiment was set up in a field (Wildekamp), located in the vicinity of Wageningen, The Netherlands. This field consisted initially of >50 year old permanent grassland, which was partially converted into agricultural land under crop rotation about 24 years ago. An experiment including both areas was started in 2000, and grassland (G) and arable land (A) were considered to represent field plots with different land use history. The experiment comprised three main treatments differing in agricultural management: grassland, monoculture of maize and 4-year crop rotation (oat, maize, barley and potato). Each main treatment was established in triplicate plots (10×10m), in both grassland and arable land. The plots under maize monoculture and crop rotation were fertilised and ploughed (10-cm depth) annually and weed control was done manually. The grassland plots did not receive any management, except for mowing of the grass every year. Hence, six different treatments were established in total, according to land use history and agricultural management regimes (Table 1).

Table 1. Description of the treatments according to their land use history and crop management, including the date when they were sampled.

Name	Description	Sampling dates
G	Permanent grassland which was kept as grassland	May, Sep, Nov'00; Sep'01; Sep'02; Sep'03
G-M	Permanent grassland converted into arable land under maize monoculture	Sep'01; Sep'02; Sep'03
G-R	Permanent grassland converted into arable land under crop rotation	Sep'01; Sep'02; Sep'03
A-R	Arable land which was kept as arable land under rotation	Sep'01; Sep'02; Sep'03
A-M	Arable land which was converted from crop rotation into maize monoculture	Sep'01; Sep'02; Sep'03
A-G	Arable land which was converted into grassland	Sep'02; Sep'03

Soil sampling

Soil samples were taken once every year at the end of the growing season, just before harvesting, except for 2000 (permanent grassland), when three samples were taken along the year (Table 1). Bulk soil was obtained by combining 100 sub-samples, taken from the surface layers of each plot with a soil core sampler (3×10 cm, diameter × height) and which were thoroughly homogenized. Rhizosphere soil was obtained by harvesting five plants, or grass, from five different locations per plot and gently shaking the roots in order to remove the loosely adhering soil. Twenty grams of roots containing the tightly-adhering soil (5–10 g of rhizosphere soil) or 10 g bulk soil were transferred to Erlenmeyer flasks containing 90 ml of sterile sodium pyrophosphate (0.1% NaPP) and gravel (10 g). The flasks were shaken for 30

min at 180 rpm and the sodium pyrophosphate solutions containing the soil samples were used for further analysis.

Total Burkholderia *community*

In order to assess the total (both the culturable and non-culturable) *Burkholderia* communities, a molecular method based on the amplification of the 16S rRNA gene from soil DNA followed by DGGE analysis was used.

DNA extraction: DNA from rhizosphere and bulk soil was extracted using an Ultra Clean soil DNA isolation kit (Mo Bio Laboratories, BIOzymTC, Landgraaf, The Netherlands). In order to achieve maximal cell lysis, a bead-beating step was included. Therefore, 50 mg of glass beads (≤106 microns) and 0.5 ml of soil slurry in sodium pyrophosphate were added to microcentrifuge tubes provided in the kit, which were then placed in a cell disrupter (Hybaid Ribolyser, Hybaid, Middlesex, United Kingdom) for 60 sec. After the bead-beating step, DNA was extracted according to the protocol described by the supplier.

PCR-DGGE analysis: Amplification of 16S rRNA genes from soil DNA was performed using primers specific for the genus *Burkholderia* (Burk3-GC/BurkR) in a semi-nested PCR (41). DGGE analysis was performed according to the methodology described by Salles *et al.* (39). After staining the gels with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands), they were photographed and digitised using an Imago compact apparatus (B&L System, Maarssen, The Netherlands).

Banding pattern analysis: DGGE gels were analysed by using the Molecular Analyst software (version 1.61; Bio-Rad, Veenendaal, The Netherlands). After compensating for internal distortions during electrophoresis by aligning the patterns with an external reference pattern (41), DGGE gels were analysed according to the methodology described by Salles *et al.* (41), taking into account the relative intensity of the bands.

Multivariate analysis: In order to correlate *Burkholderia* community structure to differences in agricultural management, a set of multivariate techniques was applied to the matrix containing the relative intensity values of the DGGE bands, using CANOCO (CANOCO 4.5; Biometris, Wageningen, The Netherlands). The goal of multivariate analysis as an ordination technique is to arrange sample points in space in such a way that the attributed axes corresponds to the greatest variability in the community composition. The distribution of samples and species (in our case, bands on the DGGE) is then visualised using an ordination diagram, which is interpreted following the basic idea that the degree of proximity indicates the degree of similarity (27).

The analyses were performed with log transformed data and replicate plots were treated as covariables. Sampling time was considered as a quantitative environmental variable and agricultural management regime and crop type in the rotation system were considered as nominal environmental variables (in the case of indirect analysis, these variables were projected into the ordination space). In order to check the heterogeneity of the data set, detrending correspondence analysis (DCA) was applied, using detrending by segments as the selected method. Data sets with gradient length shorter than 3.0 were considered to be homogeneous while those with a gradient length longer than 4.0 were considered to be heterogeneous (27). For redundancy analysis (RDA), the scaling was focused in inter-species correlations with the

species scores divided by the standard deviation. Centering by species was performed and neither centering nor standardisation by norm was chosen for samples. For canonical correspondence analysis (CCA), the scaling of ordination scores was focused on inter-species distance, using a biplot-scaling type.

To test the significance of the relationship of community response with environmental variables, the Monte Carlo permutation test was performed (499 permutations), considering the null hypothesis that species composition is independent of the environmental variables included. The permutation was restricted by split plot design, considering bulk and rhizosphere soil samples as split plots, and blocks were defined by the covariables. In the permutation scheme, at whole-plot level permutation was performed according to time series, whereas at split-plot level no permutation was allowed.

Principal response curve (PRC) analysis was applied according to Lepš and Šmilauer (27), using the scores of environmental variables obtained from RDA as vertical scores of PRC curves. Briefly, RDA was performed with scaling focusing on inter-sample distances and species scores were not post-transformed. Sampling times were used as covariables (qualitative variable) and the interactions between the treatments and sampling time represented the environmental variables. Permutations were restricted to a split-plot design, considering time as split-plots. In the permutation scheme, the whole-plots were freely exchangeable, but no permutation was allowed at split-plot level.

Identification of DGGE bands: DGGE bands that, according to the multivariate analysis, were correlated with the agricultural management regimes, were selected for sequence analysis. Identified DGGE bands were cut from the gel, eluted and cloned according to the procedure described by Salles *et al.* (41). Due to difficulties in re-amplifying some DGGE bands, an alternative approach was also used. After amplification of soil DNA with the primers Burk3 and BurkR as described by Salles *et al.* (39), the PCR product was purified with a "High Pure" PCR product purification kit (Boehringer, Mannheim, Germany). The purified PCR fragment were cloned into the pGEM-T easy vector, which was used to transform *Escherichia coli* strain JM109, according to the procedure recommended by the manufacturer (Promega Benelux, Leiden, The Netherlands). After checking for the presence of the right insert by PCR with primers Burk3-GC and BurkR (39), screening was performed by loading the clone-derived PCR products and their respective soil-derived PCR product on DGGE gels. All clones were sequenced in an ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands). The identity of the sequences was determined by BLAST analyses (2).

Culturable **Burkholderia** *community*

The assessment of the culturable fraction of the *Burkholderia* community was obtained by plating the soil samples on semi-selective (TB-T) agar medium (17).

Isolation of Burkholderia strains from soil: After shaking the Erlenmeyer flasks containing bulk or rhizosphere soil samples, a 10-fold dilution series in 0.85% NaCl was prepared and plated onto TB-T medium (composition, per litre: 2 g of glucose, 1 g of L-asparagine, 1 g of $NaHCO_3$, 500 mg of KH_2PO_4, 100 mg $MgSO_4.7H_2O$, 50 mg of trypan blue, 20 g of agar and 20 mg of tetracycline; pH 5.5), supplemented with 100 mg l^{-1} Delvocid (DSM, Delft, The Netherlands), in order to suppress fungal growth. Plates were incubated for

5 days at 27ºC, when colony forming units were counted to determine the population sizes (CFU/g soil). Then, an average of 32 colonies (16 colonies from bulk soil and 16 from rhizosphere soil) per plot per sampling was subcultured onto new TB-T plates (without Tetracycline).

Screening for antagonistic activity: Isolates obtained from TB-T plates were hecked for antagonism against *Rhizoctonia solani* AG-3 in an *in vitro* dual-culture assay. The *in vitro* test was performed by transferring purified colonies (4 colonies per plate) to 25% Potato dextrose agar plates containing a plug of *R. solani* AG-3 in the centre. Plates were incubated at 25ºC and inhibition zones were measured after 6 days. Isolates showing antagonistic activity towards *R. solani* were stored at -80ºC in Trypticase Soya broth (39) containing 20% glycerol.

Identification of antagonistic strains: The identification of the antagonistic isolates was achieved after applying a series of methods based on PCR. The templates for the PCR reactions were obtained by lysing cells from antagonistic isolates according to the methodology described by Salles *et al.* (40). The first method consisted of a PCR reaction with the genus-specific primers (Burk3/BurkR) targeting the 16S rRNA gene (39). Isolates which were confirmed to belong to the genus *Burkholderia* were then subjected to PCR with BOX A1R primers, according to the methodology described by Salles *et al.* (40), in order to identify identical isolates. Isolates showing different BOX patterns (cut-off value 100%) were then identified by various methods. Most isolates were assigned to species by direct sequencing of the 16S rRNA gene region amplified by the *Burkholderia*-specific primers (Burk3/BurkR) (39), as described before. Three isolates were identified based on other methods, mainly RLFP analysis of the *recA* gene or whole-cell protein profiling. The procedure consisted of performing initially a PCR targeting the *recA* gene, which amplifies only species belonging to the *B. cepacia* complex (29). Positive isolates were further identified at species level by *Hae*III-RFLP analysis of the PCR-amplified *recA* gene fragment (29). Non-*B. cepacia* complex isolates were subsequently examined using comparative whole-cell protein electrophoresis as described by Vandamme *et al.* (50) and were identified by comparing their protein profiles with those of known *Burkholderia* species (50).

Results

Total Burkholderia *community*

In order to evaluate the effect of different agricultural management regimes on the *Burkholderia* community structure, samples were taken from bulk and rhizosphere soils from each of the treatments, along a 4-year period. After DNA extraction, the 16S rRNA gene was amplified with *Burkholderia* specific primers and products were subsequently loaded on DGGE. The number of DGGE bands per sample varied from 6 to 16. The data from the DGGE patterns were divided in three data sets according to crop management regime (grassland, crop rotation and maize monoculture). By combining these 3 data sets, an extra one was created, which will be referred to as the agricultural management data set. Based on these four data sets, six analyses were performed, as listed in Table 2.

For each analysis, the first ordination technique applied was DCA, since it provides a basic overview of the compositional gradients of the data. The gradients were long for the

Table 2. Description of the analyses performed using multivariate techniques

Analysis	Species data used	Environmental variables	Covariables	Length of gradient [a]	Ordination technique	1st axis			
						Variability [b]	Correlation [c]	*F* [d]	*P* [e]
1	Agricultural management	G, G-M, G-R, A-G, A-M, A-R, time, bulk, rhizosphere	plot	3.872	CCA	7.7	0.954	7.680	0.002
2	Maize monoculture	A-M, G-M, time, bulk, rhizosphere	plot	4.495	CCA	11.8	0.916	4.004	0.008
3	Grassland	G, A-G, time, bulk, rhizosphere	plot	3.639	RDA	32.6	0.983	11.605	0.004
4	Crop rotation	G-R, A-R, potato, barley, maize, time, bulk , rhizosphere	plot	4.144	CCA	13.0	0.934	4.327	0.010
5	Crop rotation	potato, barley, maize, time, bulk, rhizosphere	G-R, A-R, plot	3.475	RDA	8.1	0.762	2.548	0.038
6	Plots from grassland area (G, G-M, G-R)	Interactions: G*time, G-M*time, G-R*time	time	3.547	RDA	21.2	0.975	6.176	0.0180

[a] The lengths of the gradient were determined by DCA analysis;
[b] Percentage of variability in species data explained by the first axis;
[c] Indicates the species-environment correlations for the first axis;
[d] *F* corresponds to the F-ratio for the first axis;
[e] *P* indicates the significance of the first axis, based on Monte Carlo permutation test (499 permutations)

analyses performed on agricultural management, crop rotation (one analysis) and maize monoculture (Table 2), indicating the heterogeneity of the data. Hence, we further used the assumption of unimodal distributions in these analyses. The analyses performed on partial crop rotation (analysis 5; Table 2) and grassland (Table 2) had gradient lengths between 3 and 4. In this case, both unimodal and linear distributions can be applied as models; we opted for the analysis based on linear distribution.

CCA analysis of the agricultural management data set indicated that the first axis was highly correlated with the species-environment data (Table 2; analysis 1). Moreover, the second axis showed the same degree of correlation (data not shown), suggesting that this data set was governed by more than one gradient. The constrained analysis of the data set corresponding to the agricultural management revealed that grassland (G) had the most distinct *Burkholderia* community structure, not grouping with any other environmental variable. In addition, agricultural management, irrespective of land use history of the soil, had a strong influence on the *Burkholderia* community structure (Fig. 1). The only exception was the arable land converted to grassland (A-G), which clustered together with the treatments under maize monoculture (A-M and G-M).

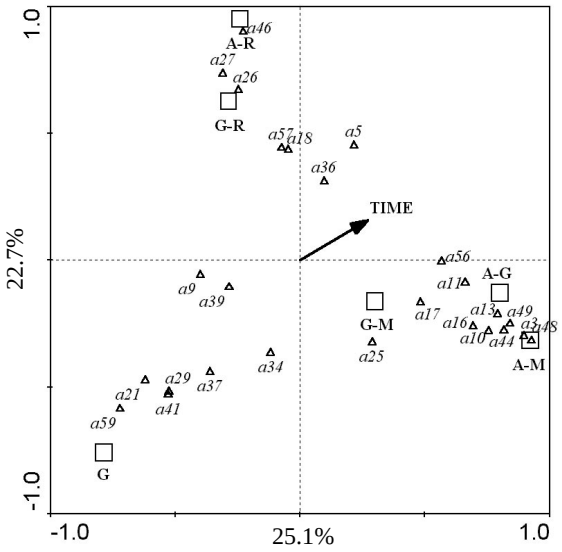

Figure 1. Ordination plot of *Burkholderia* communities associated with the bulk and rhizosphere soil, collected from areas under different agricultural management regimes. The ordination plot was generated by canonical correspondence analysis of DGGE profiles, using the settings described for analysis 1. Only environmental variables that were significant by Monte Carlo permutation test are shown. Values on the axes indicate the cumulative percentage of variance of species-environment relation. Triangles represent DGGE bands; only those with the highest fit with the axes are shown. Squares represent qualitative environmental variables: G, permanent grassland; G-M, grassland converted to maize monoculture; G-R, grassland converted to crop rotation; A-R, arable land under crop rotation; A-M, arable land under maize monoculture; A-G, arable land converted to grassland. Arrow represents the qualitative environmental variable, sampling time.

Constrained ordination techniques were applied to analyses 2, 3 and 4 with the purpose of examining each agricultural management regime separately. Again, we observed that the first axis was significant and correlated with the species-environment data. By creating ordination plots from these analyses, we found that the first axis was correlated with the land use history of the soil (A or G), as shown for the grassland data set (Fig. 2A). In addition, to evaluate the influence of each crop used for rotation on the *Burkholderia* community structure, a partial analysis was performed, in which the effect of land use history was removed (Table 2; analysis 5). Although there was a lower degree of correlation for the first axis, the comparison between crop rotation and partial analysis of crop rotation suggested that the lack of influence of land use history could explain the lower correlation of the first axis with the species-environment data observed for the partial analysis. For the grassland data set (Table 2; analysis 3), after plotting the environmental variables and samples, we observed that "time" positively influenced the numbers of bands per sample, as indicated by the isolines in figure 2A. On the other hand, when the partial crop rotation data set was used (Table 2; analysis 5), we observed that the main effect on the richness of the DGGE bands was the presence of maize, which was negatively correlated with the number of bands per sample (Fig. 2B).

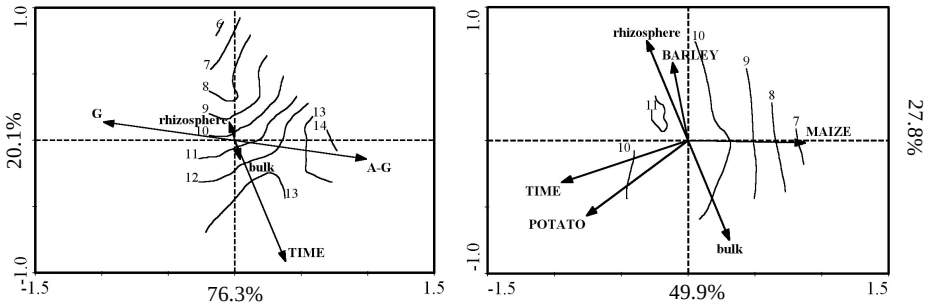

Figure 2. Ordination plot of *Burkholderia* communities associated with the bulk and rhizosphere soil, collected from areas under grassland (A) and crop rotation (B). The plot was generated by redundancy analysis of DGGE profiles, using the settings described for analysis 3 (A) and 5 (B). Isolines were created according to the position of samples (DGGE lanes) with same number of DGGE bands. The numbers in the isolines indicate the number of bands observed in the samples. Values on the axes indicate the cumulative percentage of variance of species-environment relation. Arrows indicate the environmental variables: G, permanent grassland; A-G, arable land converted to grassland; time, sampling times

To determine the impact of the conversion of grassland into arable land over time, another partial analysis was performed, by considering only the plots belonging to the "grassland area" (Table 2; analysis 6). After performing RDA analysis on this data set, the RDA scores were used to create a principal response curve (PRC), on which the effects of the treatments were plotted against time (Fig. 3). The PRC of this data set showed that the change in agricultural management regime (from grassland to arable land) had an effect on the *Burkholderia* population associated with the bulk and rhizosphere soil compartments. However, the dynamics

observed for the change from grassland to both crop rotation and monoculture of maize were quite similar, especially for the rhizosphere soil. This result suggested that the changes in *Burkholderia* community structure were more correlated with changes in agricultural practices (fertilisation and ploughing), implemented after the conversion from grassland to arable land, than with the agricultural management regime (Fig. 3).

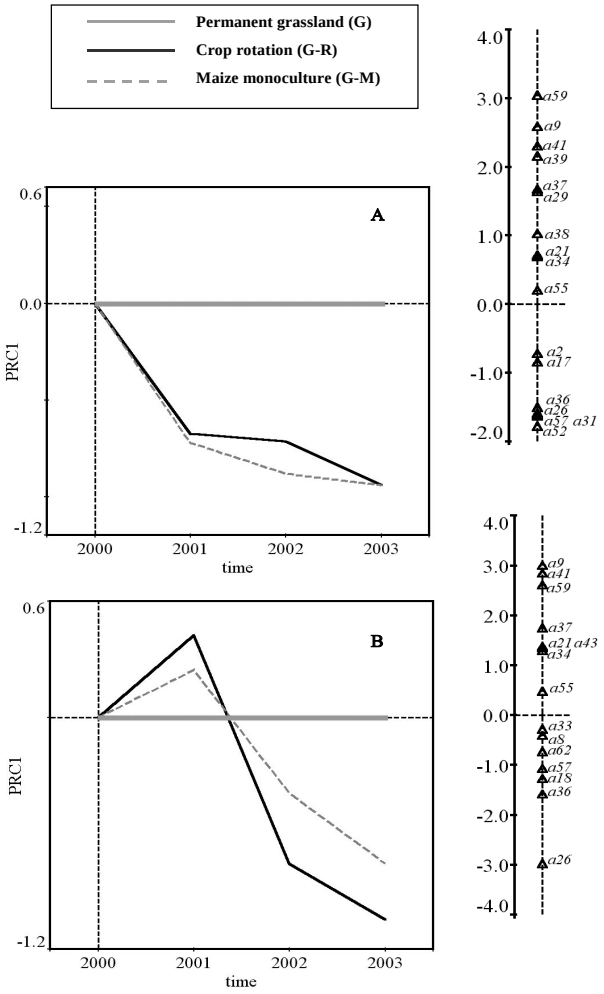

Figure 3. Principal response curves of the *Burkholderia* communities associated with (A) rhizosphere and (B) bulk soil indicating the effects of the conversion of grassland into arable land under maize monoculture or crop rotation over time. The plots were generated based on the settings used for analysis 6. Values deviating from the reference value of 0 indicate a treatment effect. The PRC diagram is supplemented by a one-dimension diagram showing the "species" (DGGE bands) scores on the corresponding RDA axis, the triangles represent DGGE bands.

Table 3. Identification of the *Burkholderia* isolates with antagonistic activity against *R. solani*.

Isolate/BOX group [a]	Treat. [b]	Similar isolates [c]	Identification [d]	Reference
T6RA24-7	A-G		*B. phytofirmans* (AY497470, 98%)	(43)
T1G3-17	G	T2RG31-29, T2G31-39	*B. pyrrocinia* [§]	(51)
T2G3-25	G		*Burkholderia* sp. Hg14 (AY154375, 97%)	(52)
T2G3-27	G		*B. phytofirmans* (AY497470, 98%)	(43)
T2G3-28	G		*Burkholderia* sp. Hg8 (AY154372, 100%)	(52)
T2G3-40	G		*Burkholderia* sp. Hg8 (AY154372, 99%)	(52)
T2G47-5	G	T2RG47-46	*Burkholderia* sp. Hg14 (AY154375, 99%)	(52)
T3G31-34	G		*Burkholderia* sp. Hg14 (AY154375, 97%)	(52)
T3G3-7	G		*Burkholderia* sp. Hg14 (AY154375, 97%)	(52)
T4RG31-11	G		*Burkholderia* sp. WR19X (AJ300693, 99%)	(38)
T4RG31-9	G		*Burkholderia* sp. WR19X (AJ300693, 98%)	(38)
T4RG47-12	G		*B. pyrrocinia* (AJ440714, 98%)	Storms, V.V.S., unpub. data
T4RG20-5	G-M	T4RG20-8, T4RG20-9	*B. graminis* K14 (AJ300687, 99%)	(38)
T4RG6-3	G-M	T4RG6-6	*B. pyrrocinia* (AJ440714, 99%)	Storms, V.V.S., unpub. data
T4RG6-9	G-M	T4G6-9, T4RG6-11, T4RG6-13, T4RG6-15, T4RG6-16, T5RG6-15	*B. pyrrocinia* [§]	(51)
T5RG44-3	G-M		*B. graminis* strain K14 (AJ300687, 99%)	(38)
T6RG6-6	G-M		*B. graminis* strain K14 (AJ300687, 97%)	(38)
T4RG28-3	G-R		*B. gladioli* (AY268167, 98%)	(14)
T4RG28-9	G-R		*B. gladioli* [¥]	(7)

[a] Isolates containing the letter R were obtained from rhizosphere soil.

[b] Indicates the treatments where the isolates were obtained from: A-G, arable land converted to grassland; G, permanent grassland; G-M, grassland converted to arable land under maize monoculture; G-R, grassland converted to arable land under crop rotation.

[c] Isolates sharing the same BOX pattern.

[d] Identification was based on sequencing of the 16S rRNA gene fragment amplified by primers Burk3/BurkR (39). Accession number and percentage of similarity with closest hit are shown in brackets.

[§] Identification based on RFLP of *recA* gene.

[¥] Identification based on protein profile.

Culturable Burkholderia *population*

In order to understand how different agricultural management regimes affect the population of *Burkholderia* strains with antagonistic activity against soil-borne pathogens, soil samples taken each year were plated on a semi-selective medium. To evaluate the selective power of the medium, in the first year of the experiment, 1154 isolates recovered from both rhizosphere and bulk soil samples collected from the grassland and arable land, were checked with the *Burkholderia*-specific primers. An average of 29% of the isolates was identified as *Burkholderia* species, and no differences were observed between the treatments. During the four-year experiment, a total of 2288 isolates (1152 for A, 1136 for G) was checked for antagonistic activity against *R. solani* AG-3. Those isolates exhibiting inhibition zones in the dual-culture assay were confirmed to belong to the genus *Burkholderia* by PCR with the genus-specific primers. From the 1152 isolates from arable land, 84 (7%) showed antagonistic activity against *R. solani* AG-3; however, after performing PCR with *Burkholderia* specific primers, only one of these 84 antagonists was confirmed to belong to the genus *Burkholderia*. In the grassland area, 9% of the isolates (103 out of the 1136 checked) had antagonistic properties. From these 103 antagonistic isolates, 30 were identified as *Burkholderia* spp. In order to compare the effect of land use history on the amount of antagonistic isolates and the number of antagonistic *Burkholderia* isolates, a Z-test for comparing two proportions was applied. The results showed that, although grassland and arable land did not differ in the amount of antagonistic isolates ($Z=1.55$), the proportion of antagonistic *Burkholderia* isolates obtained from grassland was significantly different ($P=0.001$) than the one from arable land.

The only antagonistic *Burkholderia* isolate obtained from arable land was isolated in 2003 from the rhizosphere of grass, growing in the plot that had been converted from arable land to grassland (A-G). Among the 30 antagonistic *Burkholderia* isolates obtained from grassland, 47% were obtained from the grassland converted to maize monoculture (G-M), 47% from the permanent grassland (G) and 6% from the grassland converted to crop rotation (G-R). In addition, 67% were obtained from the rhizosphere and 33% from bulk soil. Identification of the isolates was performed mainly by sequencing of the 16S rRNA gene amplified by *Burkholderia* specific primers, except for strains T1G3-17 and T4RG6-9, which were identified by RFLP analysis of the *recA* gene and strain T4RG28-9, which was identified by protein profile. The thirty-one antagonistic *Burkholderia* isolates were divided in 19 BOX patterns, which were identified as belonging to five different *Burkholderia* species (Table 3). *B. pyrrocinia* was the most frequently isolated species, representing 42% of the antagonistic isolates. Seven antagonistic isolates were closely related to the naphthalene-degrading *Burkholderia* strains (denoted Hg 8 and 14) isolated by Wilson *et al.* (52), from a contaminated area. Wilson *et al.* identified these strains, according to their 16S rRNA gene, as *B. phenazinium* (52), however protein profiles later revealed that they correspond to a new *Burkholderia* species (40). Five antagonistic isolates were identified as *B. graminis* strain K14 (38), which had originally been isolated from Egyptian soil where potato was planted. The remaining six antagonistic isolates were identified as *B. gladioli* (2 isolates from G-R), *Burkholderia* sp. WR19X (2 isolates from G) and *B. phytofirmans* (2 isolates from A-G and G).

Table 4. Identification of DGGE bands associated with the agricultural management regimes.

DGGE band code [a]	Treat. [b]	Agricultural management [c]	Most close related bacterial sequence [d]	Reference
a9	A-G	Grassland	*Burkholderia* sp NF23 (AJ300698, 99%)	(38)
a9	A-M	Grassland	*Burkholderia* sp NF23 (AJ300698, 98%)	(38)
a17	G-R	Maize monoculture	*B. phytofirmans* (AY497470, 99%)	(43)
a25	G-M	Maize monoculture	*Burkholderia* sp 418 (AY580068, 97%)	Y. Hetong, D. Bazhanov and C. Kai, unpub. data
a36	A-M	Crop rotation	B. caledonica *(AF215704, 100%)*	(8)
a36	A-R	Crop rotation	*B. caledonica* (AF215704, 99%)	(8)
a39	G	Grassland	*Burkholderia* sp UCT71 (AY178064, 99%)	(52)
a39	G	Grassland	*Burkholderia* sp UCT71 (AY178064, 99%)	(52)
a46	A-R	Crop rotation	*B. hospita* (AY040365, 98%)	(15)
a52	G-R	Crop rotation	*Burkholderia* sp Ellin155 (AF408997, 98%)	(42)

[a] DGGE bands, bands codes correspond to those used in Figures 1 and 3.

[b] Indicates the treatments where the DGGE bands were obtained from: A-G, arable land converted to grassland; A-M, arable land under maize monoculture; A-R, arable land under crop rotation; G, permanent grassland; G-M, grassland converted to arable land under maize monoculture; G-R, grassland converted to arable land under crop rotation.

[c] Indicates the agricultural management regimes to which the DGGE bands were associated with.

[d] Identification was based on sequencing of the 16S rRNA gene fragment amplified by primers Burk3/BurkR (39). Accession number and percentage of similarity with closest hit are shown in brackets

Relationship between agricultural management regime and* Burkholderia *species

According to the multivariate analysis, DGGE bands could be correlated with different agricultural management regimes, based on their presence or increase in intensity on the DGGE profiles. Therefore, several DGGE bands were sequenced, in order to identify the *Burkholderia* species affiliated with the treatments (Table 4). DGGE bands a17 and a25 correlated with maize monoculture and arable land converted to grassland (A-G), were affiliated with *B. phytofirmans* (43) and *Burkholderia* sp. 418 (Y. Hetong, D. Bazhanov and C. Kai, unpublished data), at similarity levels of 99 and 97%, respectively. DGGE bands a36, a46 and a52 were related to *B. caledonica* (99-100% similarity) (8), *B. hospita* (98% similarity) (15) and *Burkholderia* sp. Ellin155 (98% similarity) (42), respectively, and were correlated with crop rotation. DGGE bands associated with grassland were affiliated with *Burkholderia* sp. NF23 (DGGE band a9; 98-99% similarity) (38) and *Burkholderia* sp. UCT71 (DGGE band a39; 99% similarity) (52). In addition, PRC analysis showed that the latter two species (DGGE bands a9 and a39), among other unidentified ones, were affected by the conversion from grassland to arable land. The fact that these DGGE bands were located above the first axis, whereas the curves representing the grassland plots converted to arable land were below it, indicates that the species represented by these DGGE bands were more abundant in the grassland. Conversely, the plots converted to arable land became dominated by *Burkholderia* types related to *B. phytofirmans* (43), *Burkholderia* sp. 418 (Y. Hetong, D. Bazhanov and C. Kai, unpublished data), *B. caledonica* (8), *B. hospita* (15) and *Burkholderia* sp. Ellin155 (42) (DGGE bands a17, a25, a36, a46 and a52, respectively at similarity levels of 99%, 97%, 99-100%, 98%, 98%) and were correlated with crop rotation.

Discussion

To evaluate the dynamics of the *Burkholderia* community structure in the light of agricultural management regimes, ordination techniques were applied to the agricultural management data sets. Ordination is the collective term for techniques of multivariate analysis, which allows the distribution of treatments along the ordination axes on the basis of species data (in our case, data on the intensity of DGGE bands). Ordination techniques can be classified in two models of species response to environmental gradients, (i) linear and (ii) unimodal. The decision whether or not the species response is linear or unimodal can be obtained by estimating the heterogeneity in the species data, using the length of the community composition gradients, as calculated by DCA. Ordination techniques can further be divided in "constrained" (direct gradient analysis) and "unconstrained" (indirect gradient analysis), according to the presence or absence of environmental variables, respectively. Since we were interested in the effect of the different agricultural management regimes on the *Burkholderia* community structure, we opted for a method based on direct gradient analysis. These analyses revealed that the *Burkholderia* community structures could be grouped according to the agricultural management regime. The only exception was the arable land that had been converted to grassland (A-G), which was more correlated with the areas under maize monoculture (A-M and G-M) than with the permanent grassland (G). This demonstrates that the evolution of *Burkholderia* communities typical for an arable field under intense management to a community that is typical for a more "natural" area (grassland) was not achieved during the sampling period. Thus, it may take longer than four years for the community to adapt to the new situation. Indeed, Buckley and Schmidt (3) compared agricultural fields, which had been abandoned from agriculture for

different periods (and therefore were in different successional stages), to a historically cultivated field, and a field which had never been cultivated. Based on rRNA abundance, assessed by probes targeting different bacterial groups, these authors concluded that the field that had never been cultivated differed significantly from the historically cultivated ones. In addition, when comparing the abandoned fields in different successional stages, the field that had been abandoned for nine years was still rather similar to the historically cultivated areas (3). Only the field that had been abandoned for more than 45 years had a microbial community structure that was comparable to the fields that had never been used for agriculture (3). On the other hand, the effect of conversion from non-cultivated to cultivated soil on the *Burkholderia* community structure might become apparent in a shorter period of time. According to our data, samples obtained from the grassland area, which was converted to crop rotation four years before (G-R), showed a *Burkholderia* community structure very similar to that in the area that had been under crop rotation for at least 24 years (A-R). Similarly, the plots under maize monoculture (A-M and G-M) grouped together, in spite of the different land use history of the plots.

The fact that (historical) arable land and grassland plots clustered together according to the current agricultural management clearly indicates that crop rotation and maize monoculture were able to overcome land use history as the main determinant of *Burkholderia* community structure. Previously, in order to evaluate the effect of different crop types on *Burkholderia* diversity, we performed a microcosm experiment with soil from the same experimental field (41). The microcosm consisted of a cycling experiment, where three successions of the same crop were applied to soil samples collected in 2002. The results indicated that land use history had a great effect on the *Burkholderia* community structure. Even after growing the same plant species in pots for three consecutive growing cycles, it was not possible to nullify the land use history effect (41). The microcosm experiment was performed under controlled conditions, where the difference in crop types was the only variable, whereas in the field experiment the agricultural practices applied to the soil and the climate represented extra variables. Based on these facts, we hypothesised that the agricultural practices, such as fertilisation, tillage and mowing, were more effective in changing the *Burkholderia* community structure than the crop by itself. This hypothesis was confirmed by applying PRC to evaluate the directions and extent of the conversion of grassland into arable land. By comparing the control treatment (G) with the crop rotation and maize monoculture treatments, we observed that, although these two agricultural management regimes were distinct from each other, both showed the same trend in the PRC plot. Similar results were found by Steenwerth *et al.* (44), after evaluating microbial community composition, as measured by PLFA profiles, in nine different land use types. By applying multivariate analyses, these authors showed that management inputs, such as fertilisers, herbicides and irrigation, were associated with distinct microbial community structures in the different cultivated land uses (44).

RDA enables the use of non-standardised analyses, implying that the results reflect not only the differences due to the treatments, but also differences in relative species composition. By applying this method, we observed that the *Burkholderia* community structure changed over time in converted grassland plots (A-G), as an increase in the number of DGGE bands was correlated with the number of years after the conversion from arable land to grassland. Additionally, by observing the plots under crop rotation, it was found that, when compared with other crops used in the rotation management, maize led to a decrease in *Burkholderia*

diversity by selecting certain *Burkholderia* species. The great impact of maize on the soil microbial community is well known, and bacterial composition may show a gradient up to 2.2 mm from the root surface (24). It is possible that, by assuming that each DGGE band corresponds to one species and not taking into account the co-migration of bands or that one species might be represented by more than one band, the number of true species in a sample was underestimated in our study. Nevertheless, the differences in the number of bands indicate changes in the community structure, which in this case, was affected by conversion of arable land into grassland and maize monoculture.

The identification of the ten DGGE bands revealed that several *Burkholderia* species commonly isolated from soil were correlated with the agricultural management regimes, confirming our previous results from the microcosm experiment (41). Additionally, PRC analysis also showed that, on the one hand, *Burkholderia* sp. WF23, which is closely associated with *B. glathei* (38) and *Burkholderia* sp. UCT71, became less abundant in the grassland plots converted to agriculture. On the other hand, *B. hospita*, *B. caledonica* and *B. phytofirmans*, among other *Burkholderia* species identified only at genus level, had their abundance increased. These results are in agreement with our previous work, where the *Burkholderia* community associated with different agricultural management was assessed by culturing techniques (40). The identification of *Burkholderia* isolates showed that *B. hospita* and *B. caledonica* were found mainly in the plots with arable land history, whereas *B. phytofirmans* was more abundant in the grassland converted to maize monoculture (G-M) (40). Interestingly, DGGE band a34, which was also detected above the first axis in the PRC analysis, and therefore positively correlated with grassland, migrated on the DGGE gel at the same position as the amplicon of one of the antagonistic isolates identified as *B. pyrrocinia* (data not shown). However, further sequence analysis is necessary to confirm the identity of this DGGE band.

In the light of the potential application of *Burkholderia* species for the biological control of phytopathogens, the population able to grow on TB-T medium was assessed. The purpose was to select *Burkholderia* isolates with antagonistic activity against *R. solani* AG-3, an important pathogen of potato, among a range of crops. TB-T was chosen as selective medium due to its ability to recover *B. cepacia* from soil samples (17). Although its selective power may vary from 100% (45) to 6.4% (33), we observed that an average of 29% of isolates recovered from TB-T belonged to the genus *Burkholderia*. Interestingly, among the *Burkholderia* isolates with antagonistic activity against *R. solani* AG-3, we observed a large effect of land use history; in particular, most antagonistic *Burkholderia* species were obtained from the grassland area. The positive correlation between the prevalence of antagonistic *Burkholderia* strains and grassland was corroborated by the fact that the only antagonistic strain isolated from arable land was obtained from one of the plots converted to grassland (A-G). Moreover, the negative effect of crop rotation on the *Burkholderia* population antagonistic to *R. solani* AG-3 could already be observed two years after the conversion from grassland to arable land, since no antagonistic isolates were obtained from the G-R plots after 2001. Similar results were observed by Garbeva *et al.* in the same experimental field, when analysing the frequency of antagonistic *Pseudomonas* species (12). In addition, it has been shown that a higher level of suppressiveness towards *R. solani* AG-3 occurred in the permanent grassland (G) and grassland converted to arable land under maize monoculture (G-M) than in the arable land under rotation (48). Recently, the use of real-time PCR to measure the abundance of the *prnD* gene, which is one of the genes encoding the biosynthesis of the antibiotic pyrrolnitrin,

in the soil showed that the G and G-M plots had the highest densities of this gene (13). Pyrrolnitrin, which is produced by many bacterial genera, including at least two *Burkholderia* species, is effective against several bacteria and fungi, and its role in biological control has been demonstrated by using *Pseudomonas fluorescens* mutants defective in pyrrolnitrin production (21). The high frequency of isolation of *B. pyrrocinia*, one of the pyrrolnitrin-producing *Burkholderia* species, from the permanent grassland (G) and the grassland converted to maize monoculture (G-M) indicated the importance of these strains for the suppressiveness of these soils. When considering only the *Burkholderia* community, the mechanisms involved in the high disease suppression level observed for G and G-M might differ, since with the exception of *B. pyrrocinia*, these two plots differed in the composition of antagonistic *Burkholderia* isolates. In particular, high numbers of antagonistic isolates obtained from the permanent grassland (G) were identified as *Burkholderia* sp. Hg, while *B. graminis* was mainly found in the grassland plots converted to maize monoculture (G-M). Interestingly, *Burkholderia* sp. Hg is closely related to *B. phenazinium* (52), a species known for the production of the antibiotic phenazine. However, further tests are needed to evaluate if the production of phenazine is a common trait between these two *Burkholderia* species. The only two antagonistic isolates recovered from grassland plots converted to crop rotation (G-R) were identified as *B. gladioli*, which has been described as a chitinase-producing species (26). Chitinases are enzymes that degrade chitin, an important structural component of the cell wall of many fungi, including *R. solani* AG-3. Moreover, greenhouse experiments have shown that the biological control of *R. solani* can indeed be achieved by using chitinase-producing bacteria (23).

As observed by the analysis of the PCR-DGGE patterns of the total *Burkholderia* communities, this study showed that agricultural practices had a great impact on the *Burkholderia* community structures in the soil. After four years of a field experiment in which two areas with different land use history were subjected to three distinct agricultural management regimes, we were able to detect clear effects of the conversion of a more "natural" area (permanent grassland) into an arable field on the *Burkholderia* community structure. On the other hand, we observed that the time span needed to turn the *Burkholderia* community structure of an arable field into one typical of a "natural" (grassland) area was beyond the extent of the experiment. These results suggested that agricultural practices, such as fertilisation and tillage, are key factors in modifying the soil microbial community. By analysing the culturable fraction of the *Burkholderia* community, we observed that the frequency of isolation of strains with antagonistic activity against *R. solani* AG-3 was affected by changes in agricultural management, mainly the conversion from grassland to crop rotation. In addition, we observed that grassland represented a reservoir of *Burkholderia* species with great potential for agricultural applications.

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Chapter 6

General discussion

The genus *Burkholderia* is a key bacterial group in different ecosystems due to its ability to degrade pollutants, to produce antimicrobial compounds, to promote plant growth and/or to fix atmospheric nitrogen. It occupies a wide variety of niches and occurs naturally in soil, water and the rhizosphere of plants. Although *Burkholderia* is an intriguing genus, little is known about the diversity of *Burkholderia* species in different habitats or even how these organisms respond to changes in the environment.

Given that agricultural management can affect the soil microbial community, and in the light of the great applicability the genus *Burkholderia* holds for agriculture, the main aim of this thesis was to evaluate the effect of different agricultural management regimes on the *Burkholderia* community structure in soil. In order to achieve this aim, an experiment was set up in a field (Wildekamp), located in the vicinity of Wageningen, The Netherlands. This field consisted initially of over 50 year old permanent grassland, which was partially converted into agricultural land under crop rotation about 24 years ago. An experiment including both areas was started in the year 2000, and grassland (G) and arable land (A) were considered to represent fields with different land use history. The experiment comprised three main treatments differing in agricultural management regime: grassland, monoculture of maize and a four-year crop rotation (oat, maize, barley and potato). As a result, six treatments were established, according to both land use history and agricultural management regime: permanent grassland (G), permanent grassland converted to arable land under crop rotation (G-R), permanent grassland converted to arable land under maize monoculture (G-M), arable land under crop rotation (A-R), arable land under maize monoculture (A-M), and arable land converted to grassland (A-G). After starting the experiment, the first task was the development of a molecular method to allow the assessment of the *Burkholderia* community structure in environmental samples.

Molecular method to assess *Burkholderia* community structure in environmental samples

The assessment of *Burkholderia* species in environmental samples has traditionally been mainly based on analyses of isolates, in particular those of the *B. cepacia* complex associated with maize roots, using restriction fragment length polymorphism (RLFP) analyses of the *recA* gene or 16S rDNA based ARDRA – Amplified Ribosomal DNA Restriction Analysis (2, 6, 7, 10). However, none of the methods used, including the PCR based approaches, is applicable to direct evaluation of the diversity in the genus *Burkholderia* in natural settings. Therefore, a DGGE method was developed, on the basis of *Burkholderia*-specific PCR, to allow the direct analysis of the *Burkholderia* community structure in soils.

A primer set targeting the 16S rRNA gene was developed, which proved to be specific, virtually amplifying only targets from within the genus *Burkholderia*. DGGE analysis of genomic DNA from numerous *Burkholderia* strains showed that there were sufficient differences in the 16S rRNA gene fragment amplified by the primers to discriminate all 14 *Burkholderia* species tested, with the exceptions of *B. plantarii*/*B. gladioli* and *B. vietnamiensis*/*B. cepacia* (genomovar I). The DGGE analyses also revealed that some *Burkholderia* species produced a pattern comprising more than one band, suggesting the presence of multiple rRNA genes, which might display microheterogeneity among the different copies present. This is a common phenomenon in bacterial genomes, where the intergenomic sequence heterogeneity of the 16S rRNA genes may range from 98.7 to 100% similarity (5). However, the information about the number of rRNA gene copies per genome or the percentage similarity among these copies within the genus *Burkholderia* remains incomplete, as only two species have been fully analysed (5). Thus, diversity indices obtained by DGGE analysis must be evaluated carefully due to the presence of species that produce multiple bands on DGGE, in addition to a few species producing bands of similar migration. However, the DGGE profiles can certainly be used in a comparative fashion, e. g. to detect shifts in the *Burkholderia* community structures resulting from different environmental conditions and/or over time.

The DGGE profiles of the *Burkholderia* communities associated with bulk and rhizosphere soil collected from permanent grassland (G) confirmed the applicability of the PCR-DGGE method for the analysis of this community in environmental samples. By performing cluster analysis, it was possible to show that grass roots affected the structure of the *Burkholderia* communities, since all rhizosphere samples clustered apart from bulk soil samples. A small clone library was also analysed and sequence analysis of eighteen (randomly picked) clones confirmed the specificity of the primers, since all sequences were affiliated with species of the genus *Burkholderia*. Identification of the clones indicated the presence of species such as *B. glathei*, *B. andropogonis* and *B. phenazinium*. Although some clones could be identified only at the genus level, phylogenetic analysis revealed that they were similar to *Burkholderia* species known by their ability to produce antimicrobial compounds, such as *B. phenazinium*, and to degrade xenobiotic compounds, such as *Burkholderia* sp. strain N2P6 (4).

In conclusion, the analyses of the PCR products from both pure culture and soil DNA revealed that the PCR-DGGE technique developed was very useful to evaluate the *Burkholderia* diversity in environmental samples. In addition, the method proved to be a powerful tool for detecting the dominant members of the *Burkholderia* community, by combining the sensitivity and specificity of the genus-specific PCR with the screening of dominant sequences via DGGE and sequence analysis.

Effect of different crop species and land use history on the *Burkholderia* community structure in soil

Basically, the results of the studies on the effect of the grass rhizosphere on the *Burkholderia* community structure in soil were in accordance wit those obtained by other authors, who observed that plants, by varying the quality and quantity of exudates, were able to shape soil microbial communities (15, 17, 29). These facts highlighted the second research

question, which aimed to ascertain which *Burkholderia* species were selected by specific crops and which effect (current plant species or previous land use) had the greatest influence on *Burkholderia* populations in soil.

To answer these questions, we performed a microcosm experiment with soil collected in the experimental field, from permanent grassland (G), arable land under crop rotation (A-R) and arable land under maize monoculture (A-M). The microcosm experiment consisted of a cycling scheme, in which successions of the same crop were applied to the soil in pots. After extracting the DNA from the rhizosphere soil and applying the genus-specific PCR-DGGE system (chapter 2), the DGGE gels were analysed by multivariate analysis, taking into account the intensity of each of the DGGE bands. The suitability of multivariate techniques for the analysis of DGGE patterns was recently confirmed by Muylaert *et al*. (19). The main advantage of applying multivariate techniques such as canonical correspondence analysis (CCA) is that it allows linking changes in communities to changes in the environmental conditions. After applying CCA to analyse the *Burkholderia* community structures revealed by PCR-DGGE, we observed that land use history had a strong effect on the *Burkholderia* community structure. Even after growing the same plant species in pots for three consecutive growth cycles, it was not possible to overcome this land use history effect. This result suggests that the *Burkholderia* community structure is more recalcitrant to the changes imposed by the different plant species tested. Although the organic compounds released by the different crops vary, the nutritional versatility attributed to the genus *Burkholderia*, which is able metabolise a wide range of carbon compounds, might explain the diminutive response of the *Burkholderia* community to the different crops.

The effect of plant species could be observed by analysing the land use history treatments separately. Although these environmental variables did not always explain the distribution of the samples, the ordination plots indicated the occurrence of two groups of positively correlated responses determined by the crops, one composed of maize and grass and the other one of barley and oat. Moreover, these two groups showed a negative correlation between each other.

Unexpectedly, there was a distinct growth cycle effect, which could be observed by a rise in the numbers of bands from the first to the third growth cycle, irrespective of plant species or land use history. This increase in diversity with growth cycle number could be explained by the fact that the pots were kept for a total of one year under constant greenhouse conditions, which might have been optimally selective for specific organisms, which developed into PCR-detectable numbers over this time frame. Interestingly, these organisms consisted of *Burkholderia* species with rather higher G+C % (57.2 – 58.4 %), which showed high similarity to a 16S ribosomal RNA gene clone from an unculturable or uncultured organisms isolated from the cast of *Lumbricus rubellus* (24).

When comparing the growth cycle within different land use histories, we observed a more striking effect in the arable land treatments (A-M and A-R) than in the grassland (G), in which the patterns analysed did not show the same tendency to cluster. A plausible explanation would be that the permanent grassland, due to its long land use history, contained a better established, more stable and even *Burkholderia* community that would be recalcitrant to (drastic) changes. In the treatments originating from arable land (A-R and A-M), shifts in the *Burkholderia* community structures after the three plant growth cycles would occur more readily,

indicating that the microbial communities in areas under these agricultural management regimes are less stable and more sensitive to changes.

The identification of the DGGE bands related to the treatments showed the same trend found in our previous study, where most bands clustered close to species with biocontrol and bioremediation abilities. This trend, however, does not seem to be correlated to the origin of the soil or soil type, since similar results were found by Richardson *et al*. (22). After assessing the diversity of *Burkholderia* isolates from woodland rhizosphere environments, these authors observed that most of the isolates represent species closely related to those used for biological control or bioremediation (22). Moreover, it is unknown to what extent 16S rRNA gene fragments can serve as reporters of biocontrol or bioremediation capabilities, so any inference in this direction should be made with great caution.

In response to the second research question raised on chapter 1, current crop species had an effect on the *Burkholderia* community structure in soil. However, this effect was minor when compared to that of land use history, which could not be overcome even after three continuous growth cycles of the same crop.

Effect of agricultural management on the diversity of culturable *Burkholderia* populations

Agricultural practices play important roles in determining the microbial community structure of soil (11, 27). Indeed, in the microcosm experiment it was observed that different land use histories led to different patterns in the *Burkholderia* community structure in soil. This result is valid for the total *Burkholderia* community, but how does the culturable *Burkholderia* population respond to different agricultural practices? More specific, how do the *B. cepacia* complex species respond to these changes?

The culturable fraction of the soil bacterial community is known to often represent only 0.5-10% of the total discernible population (26). However, when the genus *Burkholderia* is regarded, the fraction of the total community that is culturable on plates is unknown. Moreover, the concern that the soil/plant environment might serve as a source of pathogenic strains belonging to the *B. cepacia* complex (16) highlights the importance of assessing the diversity of the genus *Burkholderia* in such habitats. Therefore, in order to evaluate the culturable *Burkholderia* community in soils under different agricultural management, four treatments were chosen: permanent grassland (G), arable land under maize monoculture (A-M), permanent grassland converted to arable land under maize monoculture (G-M) and arable land converted to grassland (A-G). The *Burkholderia* community was assessed by plating soil samples on the semi-selective PCAT medium initially developed to isolate *B. cepacia*. Isolates confirmed to belong to the genus *Burkholderia* were grouped using a combination of DGGE and BOX patterns, generating 47 clusters, which were further used for the diversity measurements.

In accordance with the results of Pallud *et al*. (20), it was observed that population densities in the rhizosphere were higher than those in the bulk soil, which could be due to the positive effect of the rhizosphere on the culturability of rhizosphere bacterial community (8). By applying species abundance models to the *Burkholderia* communities associated with rhizosphere and bulk soils, it was noticed that the bulk soil community approximated the log

series distribution. The log series abundance model represents the least equitable distribution tested and it tends to occur when only one or a few factors dominate the ecology of the community (18), which is dominated by a small number of species better adapted to these factors. In the rhizosphere soil, however, the model that best fitted the distribution of the *Burkholderia* community was the truncated log-normal model, although with a low probability ($P < 30\%$). This distribution was more even than the one explaining the bulk soil community, and may originate from a community that has been affected by random variation of numerous independent factors (14), such as differences in quality and quantity of root exudation, in addition to agricultural management. In a community characterised by the log-normal model, although one or few groups are abundant, intermediary groups represented by more than one individual start to emerge.

The diversity of the culturable *Burkholderia* communities associated with the treatments (estimated by comparing the Shannon diversity indices) was consistently higher in the rhizosphere than in the corresponding bulk soil sample, irrespective of land use history or crop management. The rhizosphere community is considered as a subset of the bulk soil community and is, therefore, less complex (9). However, since only the culturable fraction of the *Burkholderia* community was assessed, one can speculate that higher diversity observed in the rhizosphere was due to a positive effect of plant roots on the ability of the isolates to grow on selective media used. Furthermore, comparison of the Shannon diversity indices of the culturable *Burkholderia* communities associated with the treatments showed that the highest indices were obtained for the communities in arable land under maize monoculture (A-M) and permanent grassland (G). Interestingly, these results indicated that the *Burkholderia* diversity at the culturable level was affected by changes in agricultural management. The conversion of arable land to grassland and vice versa led to a decrease in diversity, indicating that the impact of soil management (fertilisation, ploughing) can be greater than that of crop type. Moreover, the changes in soil management may have induced the selective outgrowth of specific *Burkholderia* types, decreasing the evenness and species abundance. Different plant species are able to select different portions of the microbial community, but care should be taken when comparing plants growing in soils with different land use histories or soils that have gone through recent changes in management.

The identification of *Burkholderia* isolates showed that only one percent of the isolates belonged to the *B. cepacia* complex and that the majority was assigned to either (i) new *Burkholderia* species or (ii) *Burkholderia* species that had originally been isolated from soil. The putative new *Burkholderia* species were generally closely related to species commonly isolated from the environment and their definition, as novel species, has to await further work (P. Vandamme, pers. comm.). The isolates that could be assigned to species level were identified as common soil *Burkholderia* species that can exhibit different degrees of interaction with plants. In addition, some correlation between land use history and *Burkholderia* species was observed. Isolates classified as *B. hospita*, *B. caledonica* and *Burkholderia* sp. 'R-23316' and 'R-23326' were found mainly in the arable land, while isolates belonging to *Burkholderia* sp. 'R-23336' and *B. phytofirmans* were associated with the grassland area. The only species associated with crop rather than land use history was *Burkholderia* sp. 'R-23330', which is closely related to *B. fungorum*. This species was found in soil from both areas (G and A), in close association with the maize rhizosphere. The most abundant *Burkholderia* species were *B. hospita* (26%), *Burkholderia* sp. 'R-23336' (22%) and *B. phytofirmans* (19%), together

representing about two thirds of the isolates. *B. hospita*, which was originally isolated due to its ability to acquire plasmids from the environment (13), might use this trait in its adaptation to changes in the soil due to tillage and fertilisation, since it occurred only in the area of traditionally arable land. This species was also the most abundant one in the arable land converted to grassland (A-G), where it might have adapted to exploit the change in resources (due to the conversion in agricultural management) in a more efficient manner. In contrast, *Burkholderia* sp. 'R-23336' appears to be negatively affected by changes in agricultural management regime, since it was mainly isolated from the permanent grassland (G). In fact, the conversion from permanent grassland to arable land under maize monoculture (G-M) drastically reduced its abundance. *Burkholderia* sp. 'R-23336' seems to be more adapted to the soil conditions built up by the long-term grassland than by to the grass itself, since it could not be detected in the arable land converted to grassland (A-G). *B. phytofirmans* was the only species that could be recovered from all agricultural management regimes. Additionally, *B. phytofirmans* isolates were mainly obtained from rhizosphere soil (62%), confirming the original description of this species, considered to be a true plant-associated bacterium, able to establish both outside the root, in the rhizosphere, and inside the roots, as an endophytic population, in a range of plants (23). Moreover, the type strain *B. phytofirmans* PsJN, had an ACC deaminase activity which was 15 times higher than the amount described by Penrose and Glick (21) as sufficient to induce a plant growth response (23). The fact that plants inoculated with *B. phytofirmans* PsJN showed not only higher root biomass but also high levels of resistance to (low levels of) pathogens, indicates the potential use of this organism for agricultural purposes (1, 23).

The main conclusions that could be drawn from this study and the answers to the third research question (chapter 1) were: (i) the diversity of the culturable *Burkholderia* community was more affected by changes in the agricultural management regime than by crop type or land use history, regardless of the direction of this change (towards more exploitative or more conservative management); and (ii) a range of *Burkholderia* species could be related to agricultural management regimes and among them, *B. hospita* (arable land, A-G and A-M), *Burkholderia* sp. 'R-23336' (permanent grassland, G) and *B. phytofirmans* (grassland, G and G-M), were the most abundant ones. The effect agricultural management regimes had on the species belonging to the *B. cepacia* complex could not be measured since only 2% of the population isolated from the treatments belonged to this group. Although the PCAT medium was suitable for the isolation of *Burkholderia* species, it did not specifically select for species from the *B. cepacia* complex.

Effect of agricultural management on the *Burkholderia* community structure in soil

The aforementioned results clearly showed the influence of agricultural management on the culturable fraction of the *Burkholderia* population. In addition, by considering the total *Burkholderia* community, it was possible to show that land use history had great impact on the *Burkholderia* community structure, which could not be overcome in a growth-cycling microcosm experiment (chapter 3). However, what happened to the *Burkholderia* community structure in the field? Were the changes in agricultural management able to overcome the land use history? To answer these questions, a series of ordination techniques was applied to the data sets obtained

by PCR-DGGE of the field soil samples, in order to evaluate the dynamics of the *Burkholderia* community structure.

Canonical correspondence analysis (CCA) analysis indeed revealed that the *Burkholderia* community structures could be grouped according to (current) agricultural management. The only exception was the arable land that had been converted to grassland (A-G), which was more correlated with the areas under maize monoculture (A-M and G-M) than with the permanent grassland (G). This demonstrates that the shifts of *Burkholderia* communities as a response to a change from arable land to a more "natural" area (grassland) takes longer than the four years of this experiment. Indeed, this result is in agreement with data obtained by Buckley and Schmidt (3), who showed that microbial communities in a field that had been abandoned for nine years were still more similar to those of historically cultivated areas than to those in a field which had never been cultivated (3). On the other hand, the effect of conversion from "non-cultivated" (grass) to cultivated soil on the *Burkholderia* community structure might become apparent in a shorter period of time, as samples obtained from the grassland converted to crop rotation four years before (G-R) showed a *Burkholderia* community structure very similar to that in the soil that had been under crop rotation for at least 20 years (A-R). Similarly, the communities of plots under maize monoculture (A-M and G-M) grouped together, in spite of the different land use history of the plots. Furthermore, after applying principal response curve (PRC) analysis, it was possible to evaluate the directions and extent of the conversion of grassland into an arable land. The comparison between the control treatment (G) with the areas (former G) under crop rotation and maize monoculture showed that both G-M and G-R had the same trend in the PRC plot, even though they correspond to two distinct agricultural management regimes. The similarity in dynamics observed in the PRC plot indicated that, agricultural practices such as fertilisation and tillage, were more effective in changing the *Burkholderia* community structure than the crop type. This result is coherent, since both regimes (G-M and G-R) included a major overhaul of the soil (conversion from grassland to arable land) which may constitute a much stronger factor than just sowing a different plant. Similar results were found by Steenwerth *et al*. (25), who showed that agricultural management inputs, such as fertilisers, herbicides and irrigation, were correlated with distinct microbial community structures in the different cultivated land uses (25). In addition, this result could explain why it was not possible to overcome land use history in the experiment performed in the greenhouse. The microcosm consisted of a controlled experiment where the difference in crops was the only variable, whereas in the field experiment the agricultural practices applied to the soil represented an extra and dominant variable. Possibly, the use of conservation tillage could reduce the impact of the conversion from grassland into arable land.

Besides the analysis of the total *Burkholderia* community, the culturable faction of the population was assessed in the field soil samples, with the purpose of selecting *Burkholderia* isolates with antagonistic activity against *R. solani* AG3, an important pathogen of potato. Interestingly, isolates with biocontrol capacity were mainly obtained from the grassland area, indicating a large effect of land use history. Moreover, it has been shown that an enhanced level of suppressiveness towards *R. solani* AG3 occurred in the permanent grassland (G) and grassland converted to arable land under maize monoculture (G-M) than in the arable land (27). This result was correlated with higher densities of one of the genes, *prnD*, coding for the production of the antibiotic pyrrolnitrin (12, 27). The high frequency of isolation of *B. pyrrocinia*, one of the pyrrolnitrin-producing *Burkholderia* species, from the permanent

grassland (G) and grassland converted to maize monoculture (G-M) thus indicated the importance of these organisms in the suppressiveness of these soils to *R. solani* AG3. In addition, an interesting antagonistic *Burkholderia* species, *B. phytofirmans*, was isolated from permanent grassland (G) and arable land converted into grassland (A-G). The potential of this species was confirmed by Ati Barka *et al*. (1), who observed that grapevines inoculated with strain *B. phytofirmans* PsJN were resistant to the pathogen *Botrytis cinerea*. Even though the mechanisms involved in the antagonism towards the pathogen remains unknown, it was observed that in the presence of the bacterium, fungal growth was suppressed and hypha structure was modified (1). As described earlier (section 3), this species has a great potential for plant growth promotion due to the production of ACC-deaminase, and as suggested by Welbaun *et al*. (28), the management of ACC-deaminase-containing bacterial communities might offer a great opportunity to manipulate soil ethylene level in order to induce crop growth. However, further tests are necessary to confirm that the *B. phytofirmans* described in this thesis have also the ability to promote plant growth.

The significance of grassland as a source of beneficial *Burkholderia* species is remarkable, but yet unexplained. Therefore, grassland should be considered an important step in priming the soil. Soil priming is a new concept described by Welbaum *et al*. (28) as "setting the 'readiness' of a specific soil to receive a selected crop". The pathway to implement this concept might include a transition phase in between the primer phase and cropping phase, what can be used to adjust the effects of soil priming (30). In our case, maize monoculture could be used as a transition phase, since it had a positive effect on the population of *Burkholderia* species with potential for agricultural purposes. The economic viability of this concept certainly depends on the choice of the crop and the improvements in yields to be made in order to compensate for the unproductive period of soil priming (30). Since priming the soil with grassland should take at least 4 years, if the area had been under agriculture, other practices, such as zero tillage, should be applied in order to keep the beneficial influence of soil priming.

Concluding and addressing the fourth research question (chapter 1), the analysis of the DGGE patterns of the *Burkholderia* community showed that agricultural practices had a great impact on the *Burkholderia* community structure. After four years of field experiment in which two areas with different land use history were subjected to three distinct agricultural management regimes, it was possible to detect the effects of the conversion of a semi-natural area (permanent grassland) into an agricultural field (arable land) on the *Burkholderia* community structure. On the other hand, it was observed that the time span needed to turn an agricultural field into a grassland area with a stabilised *Burkholderia* community structure was beyond the duration of the field experiment. These results suggested that agricultural practices, such as fertilisation and tillage, which were not carried out in the permanent grassland, may play important roles in modulating the soil microbial community structures. The effect of agricultural management regime on *Burkholderia* isolates with biocontrol abilities was evaluated by analysing the culturable fraction of the *Burkholderia* community. It was possible to observe that the frequency of isolation of strains with antagonistic activity against *R. solani* AG3 was affected by (changes in) agricultural management, mainly the conversion to crop rotation. The areas under permanent grassland (G) and grassland converted to maize monoculture (G-M) had the highest numbers of antagonistic *Burkholderia* isolates, mainly *B. pyrrocinia* and *Burkholderia* sp. strain 'R-23336', whereas the isolation frequency of these was extremely low for arable land. These results, together with the occurrence of *B. phytofirmans* in the permanent grassland (G) and

arable land converted to grassland (A-G), indicate that grassland is a reservoir of *Burkholderia* species with great potential for agricultural applications.

Concluding remarks

The effect of agricultural management on the community structure of the genus *Burkholderia* in soil was evaluated by both culture-dependent and -independent methods. By applying the culture-independent approach, it was observed that the historical use of the soil had great impact on the *Burkholderia* community structure, and that the time span needed to overcome land use history varied with the type of conversion. On the one hand, shifts in the *Burkholderia* community structure as a response to the change from permanent grassland into arable land could be observed after four years. Although agricultural management regime, such as crop rotation or monoculture of maize, selected for different *Burkholderia* communities, the comparison of these with the community associated with the permanent grassland showed that the main changes in community structure were due to agricultural practices (fertilisation, tillage) and not agricultural management. On the other hand, the conversion of a community typical for arable land to one typical for grassland, could not be reached in four years. Although the *Burkholderia* community structure associated with the arable land converted to grassland changed over time, the four-year experiment was clearly not long enough to return this community structure to the one observed in the (original) permanent grassland.

By applying a culture-dependent approach, it was observed that the diversity of the genus *Burkholderia* was affected by changes in agricultural management, since the highest diversity indices were observed for the permanent grassland (54 years old) and the arable land under maize monoculture (24 years old). Conversely, the lowest diversity indices were observed in the recently (4 years old) converted field plots (A-G, arable land converted to grassland and G-M, grassland converted to maize monoculture). Interestingly, by comparing the results from the culture-dependent and culture-independent approach, it is clear that the conversion in agricultural management affected the culturable fraction of *Burkholderia* community to a greater extent than the total *Burkholderia* community. While maize monoculture had similar *Burkholderia* community structures in both areas (G-M and A-M), as determined by PCR-DGGE analysis, the isolates recovered from arable land under maize monoculture (A-M) were more diverse than those isolated from grassland converted to maize monoculture (G-M). The impact of changes in agricultural management on the culturable *Burkholderia* community is an interesting finding and should be taken into account when analysing bacterial communities obtained from soils that have gone through changes in agricultural management. The culture-dependent approach also indicated the preference of some *Burkholderia* species for certain agricultural management regimes. The effect of agricultural management on the selection of *Burkholderia* species was even more remarkable when considering the *Burkholderia* strains with biocontrol properties, which were mainly found in the permanent grassland and the grassland converted to maize monoculture.

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Glossary

ACC – 1-aminocyclopropane-1-carboxylate.

Agricultural management regimes – crop production systems applied to an agricultural area. Ex: crop rotation, monoculture, and grassland.

Agricultural practices – agronomic practices used to increase crop production. Ex: fertilization, tillage, drainage, and irrigation.

BNF – Biological nitrogen fixation

CA – Correspondence analysis.

CCA – Canonical correspondence analysis.

CF – Cystic fibrosis.

CLPP – Community level physiological profiling.

Conservation tillage – Any tillage practice that reduces the loss of soil and water as compared to (conventional) tillage. Ex: minimum tillage, zero tillage.

Constrained ordination – Method of gradient analysis on which the variability in species composition is explained by known environmental variables. Ex: CCA, RDA.

DGGE – Denaturing gradient gel electrophoresis

Direct gradient analysis – Statistical model in which both sets of species data and predictors are present.

Environmental variables – *see explanatory variables*

Explanatory variables – quantitative, semi-quantitative (scale) or qualitative (factors, binary variable) measurements or variables used in ordination technique to predict the primary data.

FAME – Fatty acid methyl ester analysis.

GFP – Green fluorescent protein.

IAA – Indole acetic acid.

Indirect gradient analysis – Statistical model in which sets of species data are present but

predictors are absent.

Linear distribution – Model of species response to environmental gradient that assumes that species abundance either increases or decreases with the environmental variable.

Minimum tillage – minimum amount of tillage required for seedbed preparation and plant establishment.

No till – *see zero tillage.*

Ordination technique – Is a technique of matrix approximation (as data are presented in the two-way matrix layout), of which the aim is to summarise multivariate data in a convenient way in scatter diagrams. It assumes that the occurrence of all species under consideration is determined by a few unknown environmental variables according to a response model.

PCA – Principal component analysis.

PCB – Polychlorinated biphenyls.

PLFA – Phospholipid fatty acid profiling.

PRC – Principal response curve.

Primary data – Matrix containing the records on a collection of observations (samples and species distribution per sample).

RDA – Redundancy analysis.

Sample – Sampling unit, each one comprising values for multiple species.

Species data – Species distribution in a specific sample.

TGGE – Temperature gradient gel electrophoresis

Tillage – The manipulation of soil into desired conditions by mechanical means.

Unconstrained ordination – Method of gradient analysis on which the variability in species distribution is explained by the ordination axes that represent the greatest variability in community composition. Ex: PCA, CA.

Unimodal distribution – Model of species response to environmental gradient which assumes that species has an optimum on the environmental gradient (bell-shaped curve).

UPGMA – Unweighted pair group method with mathematical averages.

Zero tillage – Cultivation technique in which the soil is disturbed only along the silt or holes into which the seeds are planted.

Summary

The genus *Burkholderia* represents an interesting group of bacteria that can be found in many different habitats, from soil to water, as well as in close association with plants, fungi, insects (ants) and other animals, including human beings. The degree of interaction apparently depends on the organism *Burkholderia* is associated with. In the case of fungi and ants, a symbiotic relationship has been established, and both hosts benefit from the presence of *Burkholderia*. In contrast, the association of *Burkholderia* with animals and humans can be deleterious to the host. The bacteria can act as pathogens, causing diseases known as 'glanders' and 'meloidosis', or as opportunistic pathogens in people with cystic fibrosis (mainly species belonging to the so called *B. cepacia* complex). Regarding *Burkholderia* and plants, the association is mainly beneficial, although a few species are phytopathogenic. Non-pathogenic *Burkholderia* species generally colonise the rhizosphere soil (soil closely adhered to plant roots), but some species are even found inside plant tissues. In return, *Burkholderia* species can stimulate plant growth, contribute to plant nutrition by fixing atmospheric nitrogen and protect plants from pathogens by producing antibiotic compounds. In the latter case, *Burkholderia* species act as biological control agents of soil-borne plant pathogens, reducing or eliminating the use of chemical pesticides in agriculture, which brings not only economical, but also ecological benefits. Overall, biological control can be achieved by introducing the biocontrol agent in the area where disease occurs. Usually, however, the introduced antagonistic species is not adapted to the new environment and therefore, is not able to overcome the indigenous population and colonise the plants efficiently. As an alternative, biological control can be accomplished by manipulating the soil and crops in such way that the community of antagonistic species already present in the soil is stimulated.

The main idea behind this thesis was to assess how the genus *Burkholderia* responds to agricultural management and which species would be selected by a particular management. By understanding the ecology of specific *Burkholderia* species, for instance those with biocontrol abilities, it would be possible to manipulate the soil microbial community by managing the system, in order to favour biocontrol species and, thus, increase the level of soil suppressiveness towards diseases.

Chapter 1 of this thesis describes the genus *Burkholderia* in terms of its distribution, diversity, occurrence and application. It also describes the consequence of anthropogenic disturbance for soil microbial diversity, the effect of agricultural practices and management regimes on microbial communities associated with grassland and arable land and the impact changes in agricultural management have on soil microbial community structure. This chapter also contains a brief description of multivariate analysis, which is considered to represent a tool to investigate microbial diversity. Chapter 1 finishes with the aim, research questions and outline of the thesis.

Chapter 2 deals with the development of a PCR-DGGE method that would allow the assessment of *Burkholderia* communities in soil. Therefore, the first step was to design primers specific for the genus *Burkholderia*, targeting the 16S ribosomal RNA (rRNA) gene. The specificity of the primers was evaluated *in silico* (using databases), *in vivo* (with genomic DNA from both *Burkholderia* and non-*Burkholderia* species) and by sequencing of the amplicons generated with DNA contained in the soil samples. In order to validate the method, DNA was extracted from the bulk and rhizosphere soils collected from two grassland plots. The PCR-DGGE analysis of these two plots revealed that differences in the *Burkholderia* diversity were mainly observed mainly between bulk and rhizosphere soil samples.

Once the molecular method had been developed and validated, the next step was to evaluate the effect that different crops and soils under different agricultural management would have on the *Burkholderia* community structure. Therefore, a microcosm experiment was set up and it is described in **chapter 3**. In this experiment, four different crops (maize, oat, barley and grass) were grown for three consecutive cycles in pots containing soil with different land use histories (maize monoculture, crop rotation and permanent grassland). After using a multivariate technique (CCA) to analyse the DGGE patterns from the *Burkholderia* communities, it was observed that the major factor affecting the composition of the *Burkholderia* community was land use history. Even after growing the same crop in the same pot for 3 growth cycles, it was not possible to overcome the effect of land use history. Only when the soils with different histories were analysed separately (one-by-one), the effect of crops could be seen. Hence, the *Burkholderia* populations associated with maize and grass on the one hand, and those of barley and oats on the other hand, were similar. In addition, it was observed that the *Burkholderia* community associated with these two groups (maize/grass vs. oats/barley) was negatively correlated.

With the purpose of determining the effect of land use history and agricultural management on *Burkholderia* community structures, soil samples were taken from an experimental research site. This site consisted of areas with distinct soil history (permanent grassland and arable land), in which various agricultural management regimes (crop rotation, monoculture of maize and grassland) were introduced. In **chapter 4**, the effect of these two factors (land use history and agricultural management regime) on the culturable *Burkholderia* populations was assessed (??). Two hundred and fifty four *Burkholderia* isolates were obtained from four treatments, i.e. permanent grassland, grassland converted to maize monoculture, arable land (maize monoculture) and arable land converted to grassland. The isolates were grouped according to their DGGE and BOX patterns in 47 clusters, and this distribution was then used to calculate ecological parameters. The Shannon diversity index showed that *Burkholderia* diversity was affected by changes in agricultural management, given that permanent grassland and continuous arable land showed the highest diversity. In addition, diversity tented to be higher in the rhizosphere than in the corresponding bulk soil. By applying species abundance models, it was observed that the *Burkholderia* communities associated with the rhizosphere were more evenly distributed than the communities collected from bulk soil. Identification of the isolates showed that the majority (98%) was assigned to either new *Burkholderia* species or *Burkholderia* species that had originally been isolated from soil, while the remaining 2% belonged to the *B. cepacia* complex.

In **chapter 5**, the influence of agricultural management on the total *Burkholderia* population was evaluated by multivariate analysis of the PCR-DGGE profiles obtained from the soil samples collected from the field experiment. It was possible to observe that after four years of field experiment the conversion of permanent grassland to arable land was achieved in respect of the *Burkholderia* community structure. However, the time needed to convert the *Burkholderia* community structure associated with arable land to that of permanent grassland was beyond the duration of the experiment. In addition, by analysing the direction and extent of the conversion from grassland to arable land (maize monoculture and crop rotation), it was observed that the agricultural practices (fertilisation and tillage) applied in these agricultural managements were more effective in changing the *Burkholderia* community structures than the agricultural management regime. In order to link agricultural management to a functional group within the genus *Burkholderia*, isolates with biocontrol abilities against the soil-borne fungus *Rhizoctonia solani* AG-3, an important potato pathogen, were obtained. The results suggested that (changes in) agricultural management, mainly crop rotation, affected the frequency of isolation of antagonistic *Burkholderia* strains and that permanent grassland represents a reservoir of *Burkholderia* species with great potential for agricultural applications.

Chapter 6 contains a summary of the work carried out for this thesis, together with the answer to the research questions raised in chapter 1 and possible alternatives to stimulate the population of antagonistic *Burkholderia* species and, therefore, increase the suppressiveness of soil against diseases.

In conclusion, the historical use of soil had a great effect on the *Burkholderia* community structure, and the time span needed to overcome land use history varied according to the type of conversion. Furthermore, the use of agricultural practices such as fertilisation and tillage played an important role in accelerating this process. When analysing the culturable population, it was noticed that the diversity within the genus *Burkholderia* was affected by changes in agricultural management, which was highest in the fields which had the same land use history for more than 24 years, i.e. continuous arable land and permanent grassland. In addition to the higher diversity, permanent grassland had a remarkable stimulatory effect on the *Burkholderia* species with biocontrol abilities.

Samenvatting

Het geslacht *Burkholderia* vertegenwoordigt een interessante groep bacteriën die in verschillende habitats voorkomen, i.e. van bodem tot water en vaak in nauw verband met planten, schimmels, insecten, hogere diersoorten en de mens. De mate van interactie hangt af van het organisme waarmee *Burkholderia* is geassocieerd. In het geval van bv. schimmels en mieren is er sprake van een symbiotische relatie waarbij genoemde gastheren profiteren van de aanwezigheid van *Burkholderia*. Maar in de relatie met dieren en mensen kunnen sommige *Burkholderia* soorten zich als schadelijke pathogenen gedragen en ziektes als 'glanders' en 'meloidosis veroorzaken'. Ook kan *Burkholderia* van het zogenaamde *cepacia* complex bij taaislijmziekte bij de mens betrokken zijn.

De associatie van *Burkholderia* met planten kan zowel in het voordeel van de plant alsook in haar nadeel zijn, dwz er kunnen ook plantpathogene interacties zijn. Niet-pathogene *Burkholderia* soorten koloniseren de plant vaak in de rhizosfeer (rondom wortelstelsels). Sommige soorten kunnen zich zelfs in de plant vestigen. In de laatste situatie kan *Burkholderia* de groei van de plant stimuleren, atmosferische stikstof binden en bescherming bieden aan de plant tegen pathogenen door de productie van antibiotica. Deze *Burkholderia* soorten kunnen daarmee een rol spelen bij de bescherming van het gewas, hetgeen een vorm van biologische ziektebestrijding biedt waarmee het gebruik van chemische beschermingsmiddelen in de landbouw teruggebracht of zelfs uitgesloten kan worden. Dit geeft, naast economische, ook ecologische voordelen. Biologische gewasbescherming kan dus bereikt worden door toevoeging van antagonistische organismen in een omgeving waar een ziekte heerst. Vaak zijn deze geïntroduceerde soorten niet aangepast aan de nieuwe leefomgeving en daarom niet in staat zich te vestigen binnen de al aanwezige microbiële gemeenschap, met als gevolg dat een potentiële beschermer de plant niet efficiënt kan koloniseren. Een ecologisch alternatief in de strijd tegen plantpathogenen is het stimuleren van de van nature al aanwezige antagonisten, via een uitgekiende bewerking van de bodem en het gewas.

In dit proefschrift wordt beschreven hoe *Burkholderia* soorten zich ontwikkelen bij verschillende landbouwstrategieën en welke soort geselecteerd kan worden door een bepaald gewas of bij een rotatie van gewassen. Het begrijpen van de ecologie van specifieke *Burkholderia* soorten en met name de antagonistische *Burkholderia* soorten, geeft de mogelijkheid om door het landgebruik deze soorten te stimuleren en hierdoor de intrinsieke weerstand van de bodem tegen ziektes te verhogen.

Hoofdstuk 1 van dit proefschrift beschrijft de soort *Burkholderia* in termen van zijn verspreiding, diversiteit, voorkomen en mogelijkheden. Vervolgens worden de gevolgen van anthropogene verstoringen op de microbiële diversiteit in de bodem beschreven en in het bijzonder het effect van landgebruik op microbiële gemeenschappen die geassocieerd zijn met grasland en akkerbouw en de gevolgen voor de microbiële gemeenschap in de bodem bij een verandering van landbouwstrategie. Hoofdstuk 1 geeft verder een korte beschrijving van

multivariant analyse voor de statistische beschrijving van de resultaten uit onderzoek naar microbiële diversiteit. Het hoofdstuk eindigt met het doel, de onderzoeksvragen en de opzet van dit proefschrift.

In **hoofdstuk 2** wordt de ontwikkeling van een PCR-DGGE methode voor de specifieke bepaling van *Burkholderia* gemeenschappen in de bodem beschreven. Hiervoor zijn primers ontworpen op basis van het 16S ribosomale RNA (rRNA) gen dat specifiek het DNA van bacteriesoorten binnen het geslacht *Burkholderia* amplificeert. De bruikbaarheid van de primers is geëvalueerd, *in silico* (op de databank met DNA sequenties), *in vitro* (met DNA van *Burkholderia* en *niet Burkholderia* soorten) en door middel van sequenties van PCR producten uit bodem DNA. Voor de validatie van de methode is bodem DNA geëxtraheerd uit de bulk en rhizosfeer van twee grasland plots. Er werden verschillen gevonden in de diversiteit van *Burkholderia* soorten tussen de bodemmonsters uit de bulk en rhizosfeer.

Na de ontwikkeling en validatie van de moleculaire methode is de invloed van landgebruik op de *Burkholderia* gemeenschap onderzocht in een microcosmos experiment in de kas. **Hoofdstuk 3** behandelt dit experiment, waarin vier gewassen (maïs, haver, gerst en gras) drie groeicycli doormaakten in potten met landbouwgrond van velden met een verschil in landgebruik, te weten: maïs in monocultuur, gewasrotatie en permanent grasland.

DGGE patronen van de *Burkholderia* gemeenschappen zijn geanalyseerd met behulp van de multivariant analyse CCA. Uit deze analyse bleek dat de belangrijkste factor met invloed op de samenstelling van deze gemeenschappen de gebruikshistorie van de grond was. Zelfs na drie groeicycli was de invloed van de landgebruikhistorie nog aanwezig. Alleen bij een analyse op basis van de herkomst van de grond werd de invloed van de verschillende gewassen aangetoond. Zo waren er overeenkomsten tussen de *Burkholderia* populaties bij maïs en gras en bleken de populaties bij haver en gerst vergelijkbaar. De twee sets planten waren negatief met elkaar gecorreleerd.

Voor het bepalen van de invloed van de landgebruikhistorie en het actuele landgebruik op de *Burkholderia* populatie, zijn bodemmonsters genomen op proefvelden met voorgaand gebruik als grasland of akkerbouw. Op deze velden zijn voor de periode van dit onderzoek de volgende drie regimes toegepast; een rotatie (haver, maïs, gerst, aardappel), maïs in monocultuur en grasland. In **hoofdstuk 4** zijn de effecten van het voorgaande en het actuele landgebruik op de aantoonbare *Burkholderia* soorten onderzocht. Er zijn tweehonderd en vierenvijftig *Burkholderia* isolaten geïsoleerd uit vier behandelingen: permanent grasland, grasland omgezet naar maïs in monocultuur, akkerbouwgrond met maïs in monocultuur en akkerbouwgrond omgezet naar permanent grasland. De isolaten zijn gegroepeerd in 47 clusters op basis van hun DGGE en BOX patronen. Deze clustering is vervolgens gebruikt bij het bepalen van de ecologische parameters. De ‘Shannon diversity index’ liet zien dat de diversiteit van *Burkholderia* in de bodem beïnvloed wordt door veranderingen in het landgebruik. Permanent grasland en de continue teelt van akkerbouwgewassen vertoonden de hoogste diversiteit. Daarnaast leek de diversiteit in de rhizosfeer hoger dan die in de bulkgrond. Statistische analyse volgens ‘species abundance modeling’ liet zien dat *Burkholderia* gemeenschappen in de rhizosfeer een gelijkmatiger verdeling lieten zien dan de gemeenschappen in de bulksfeer. Identificatie van de isolaten wees uit dat 98% behoort tot nog niet beschreven soorten, terwijl 2% toe te kennen was aan het *cepacia* complex.

In **hoofdstuk 5** zijn de effecten van de veranderingen in het landgebruik op de totale *Burkholderia* populatie geanalyseerd met een multivariant analyse op de PCR-DGGE profielen van grondmonsters uit het veldexperiment. De analyse toonde aan dat gedurende het vierjarige veldexperiment de structuur van de *Burkholderia* gemeenschap bij een conversie van grasland naar akkerbouw zich heeft gevormd naar de nieuwe omgeving. Dezelfde periode was echter niet voldoende voor het ontwikkelen van een *Burkholderia* gemeenschap, in een omzetting van bouwland naar grasland, die verwacht kon worden onder permanent grasland. Na de conversie van akkerbouw naar grasland bleef de *Burkholderia* gemeenschapsstructuur van akkerbouw zichtbaar. Uit de analyse bleek verder dat na de omzetting van permanent grasland naar akkerbouw (bij maïs in monocultuur en de rotatie), grondbewerking en bemesting meer effect hadden op de samenstelling van de *Burkholderia* gemeenschap dan het nieuwe landgebruik. In de hoop landgebruik te koppelen aan een stimulus van, een voor de landbouw belangrijke functionele groep van het geslacht *Burkholderia*, zijn isolaten geselecteerd die een remmend effect hadden op de bodemschimmel *Rhizoctonia solani* AG3, een bodem-gerelateerd pathogeen van aardappel. Uit de resultaten bleek dat de veranderingen in landgebruik (voornamelijk rotatie) invloed hadden op de frequentie waarmee antagonistische *Burkholderia* soorten geïsoleerd worden. In vergelijking daarmee bevatte permanent grasland een groot reservoir van deze *Burkholderia* soorten met mogelijkheden voor gewasbescherming.

Hoofdstuk 6 bevat naast een overzicht van het onderzoek beschreven in dit proefschrift, de antwoorden op de vragen uit hoofdstuk 1. Daarnaast worden mogelijkheden besproken voor het stimuleren van antagonistische *Burkholderia* soorten in de bodem, waarmee de weerstand van de bodem tegen (plant)pathogenen verhoogd kan worden.

De overall conclusie van deze studie is dat het voorgaande bodemgebruik een groot effect heeft op de structuur van de *Burkholderia* gemeenschap in de grond en dat de tijd die nodig is voor de ontwikkeling van de te verwachten *Burkholderia* gemeenschap afhangt van de doorgevoerde veranderingen in het landgebruik. Verder blijkt dat bewerking van de grond en bemesting een belangrijke rol spelen en het veranderingsproces versnellen. Analyse van de cultiveerbare *Burkholderia* soorten toonde aan dat de diversiteit binnen de *Burkholderia* gemeenschappen beïnvloed werd door de veranderingen in landgebruik. Bodems waar al meer dan 24 jaar grasland ligt of akkerbouw plaatsvindt hadden de hoogste diversiteit binnen de aanwezige *Burkholderia* gemeenschap. Naast hoge diversiteit had grasland een opmerkelijk stimulerend effect op *Burkholderia* soorten met gewasbeschermende eigenschappen tegen plantpathogenen.

Resumo

O gênero *Burkholderia* representa um interessante grupo de bactérias que pode ser encontrado em diferentes habitats, como solo, água, ou em associação com plantas, fungos, insetos (formigas) e outros animais, incluindo seres humanos. O nível de interação depende do organismo com o qual *Burkholderia* se associa. No caso dos fungos e formigas, existe uma interação simbiótica, onde ambos hospedeiros se beneficiam da presença dessas bactérias. Em contraste, a associação de *Burkholeria* com animais, pricipalmente seres humanos, é deletéria. Neste caso, bactérias do gênero *Burkholderia* agem como patógenos, causando doenças conhecidas como "glanders" e "meloidosis", ou como patógenos oportunistas em pessoas com fibrose cística (principalmente as espécies pertencentes ao complexo *B. cepacia*). Já a interação entre *Burkholderia* e plantas é na maioria das vezes benéfica, embora algumas espécies pertencentes a este gênero sejam fitopatogênicas. As espécies de *Burkholderia* não fitopatogências geralmente colonizam o solo fortemente aderido às raízes (solo rizosférico), apesar de algumas espécies também serem encontradas colonizando tecidos vegetais. Em troca, estas bactérias podem estimular o crescimento das plantas, contribuir para a nutrição vegetal através da fixação de nitrogênio atmosférico e proteger as plantas do ataque de fitopatógenos através da produção de antibióticos. Neste último caso, espécies do gênero *Burkholderia* agem como agentes de controle biológico de fitopatógenos de solo, reduzindo ou eliminando o uso de pesticidas na agricultura, o que acarreta benefícios não só econômicos, como também ecológicos. De modo geral, o controle biológico pode ser alcançado através da introdução do agente de controle biológico na área onde a doença ocorre. Porém, as espécies antagonistas introduzidas normalmente não estão adaptadas ao novo ambiente, o que as torna incapazes de competir com a microflora indígena e colonizar eficientemente as plantas. De forma alternativa, o controle biológico de doenças pode ser obtido através da manipulação do solo e cultivos agrícolas, de tal maneira que a comunidade de espécies antagonisticas presente no solo seja favorecida

O principal objetivo do presente trabalho foi avaliar como o gênero *Burkholderia* responde ao manejo agrícola e quais espécies são selecionadas quando um determinado manejo agrícola é empregado. O conhecimento da ecologia de certas espécies de *Burkholderia*, tornaria possível a manipulaçào da comunidade microbiana do solo através do manejo do sistema agrícola. O favorecimento das espécies com potencial para o controle biológico aumentaria então o nível de supressividade do solo em relação às doenças.

O **capítulo 1** dessa tese descreve o gênero *Burkholderia* com relação a sua distribuição, diversidade, ocorrência e aplicação. Também discute como distúrbios antropogênicos afetam a comunidade microbiana do solo; o efeito de práticas e manejos agrícolas nas comunidades microbianas associadas com pastagem e solos sob cultivo; e como mudanças no manejo agrícola afetam a estrutura da comunidade microbiana no solo. Esse capítulo contem também uma breve descrição sobre o uso de análise multivariada como ferramenta na investigação da

diversidade microbiana. O capítulo 1 termina com os objetivos, questões científicas e sumário da tese.

O **capítulo 2** descreve o desenvolvimento de um método baseado em PCR-DGGE, que permite acessar as comunidades de *Burkholderia* no solo. Para desenvolver esse método, o primeiro passo foi desenhar primers específicos para o gênero *Burkholderia*, tendo como alvo o gene 16S rRNA. A especificidade dos primers foi avaliada *in silico* (usando base de dados), *in vivo* (com DNA genômico obtido de diversas bactérias pertencentes ou não ao gênero *Burkholderia*), e através do sequenciamento dos amplicons obtidos após PCR com DNA extraído de amostras de solo. Com o intuito de validar o método, foi extraído DNA de amostras de solo e solo de rizosfera, coletados de duas áreas sob pastagem. A análise de PCR-DGGE dessas duas áreas revelou que as diferenças com relação a diversidade de *Burkholderia* foram observadas principalmente entre o solo e o solo associado com a rizosfera.

Após desenvolver e validar o método molecular, o passo seguinte foi avaliar o efeito que diferentes culturas e solos sob diferentes manejos agrícolas teriam na estrutura da comunidade de *Burkholderia*. Para tal, um experimento de microcosmo foi iniciado e está descrito no **capítulo 3**. Neste experimento, quatro culturas (milho, aveia, cevada e grama) foram cultivadas por três ciclos consecutivos em potes contendo solo obtido de áreas com diferentes históricos (monocultura de milho, rotação de culturas e pastagem permanente). Após usar uma técnica de análise multivariada (CCA) para analisar a comunidade de *Burkholderia* através dos padrões de DGGE, foi observado que o histórico da área era principal fator afetando a composição da comunidade de *Burkholderia* . Mesmo após cultivar a mesma cultura, no mesmo pote, por 3 ciclos de crescimento consecutivos, não foi possível superar o efeito da história agrícola do solo. Foi possível observar o efeito das diferentes culturas somente quando esses solos com diferentes históricos foram analisados separadamente. Foi observado então, que a população de *Burkhoderia* associada ao milho e a grama eram similares, assim como a população associada com aveia e a cevada. Além disso, observou-se uma correlação negativa entre a comunidade de *Burkholderia* associada a estes dois grupos (milho/grama versus aveia/cevada).

Com o intuito de determinar o efeito do histórico do solo e do manejo agrícola na comunidade de *Burkholderia*, foram coletadas amostras de solo de um campo experimental. Esse campo consistia de áreas com histórico de solos distintos (pastagem permanente e solo sob cultivo), onde diferentes manejos agrícolas foram introduzidos (rotação de culturas, monocultura de milho e pastagem). No **capítulo 4**, o efeito desses dois fatores (histórico do solo e manejo agrícola) sobre a população culturável de *Burkholderia* foi avaliado. Duzentos e cinqüenta e quatro isolados de *Burkholderia* foram obtidos de 4 tratamentos: pastagem permanente, pastagem convertida a monocultura de milho, solo sob cultivo contínuo (monocultura de milho) e solo sob cultivo que foi convertido a pastagem. Os isolados foram agrupados de acordo com seus padrões de DGGE e padrões de BOX-PCR em 47 clusters, e essa distribuição foi então usada para calcular medidas ecológicas. O índice de diversidade Shannon revelou que a diversidade de *Burkholderia* foi afetada por alterações no manejo agrícola, tendo em vista que a pastagem permanente e o solo sob cultivo contínuo apresentaram os maiores valores de diversidade. Ainda, foi observada uma tendência de maior diversidade nas amostras de solo de rizosfera do que no solo. Através da aplicação de modelos de abundância, foi observado que as comunidade de *Burkholderia* associadas à rizosfera se

mostravam mais uniformemente distribuídas do que a as comunidades associadas com o solo. A identificação dos isolados mostrou que a maioria (98%) pertencia a novas espécies do gênero *Burkholderia* ou a espécies de *Burkholderia* que foram originalmente isoladas de solo, enquanto somente 2% dos isolados pertenciam ao complexo *B. cepacia.*

No **capítulo 5**, a influência dos manejos agrícolas na população total de *Burkholderia* foi avaliada através da análise multivariada dos perfis de PCR-DGGE obtidos de amostras de solo coletadas no campo experimental. Com isso, observou-se que, , a conversão de pastagem permanente para solo sob cultivo, no que se refere à estrutura da comunidade de *Burkholderia* no solo, foi atingida após quatro anos de experimentação. Entretanto, o tempo necessário para converter a estrutura de comunidade de *Burkholderia* associada com o solo sob cultivo àquela associada com a pastagem permanente foi superior a duração do experimento. Além disso, através da análise da direção e da extensão da conversão de pastagem para área sob cultivo (monocultura de milho e rotação de cultura), foi possível observar que práticas agrícolas (adubação e aragem) usadas nestes manejos agrícolas, alteraram a estrutura da comunidade de *Burkholderia* mais eficientemente que o tipo de manejo agrícola. Com o intuito de associar manejo agrícola com um grupo funcional dentro do gênero *Burkholderia,* foram obtidos isolados com potencial para o controle biológico do fungo fitopatogênico *Rhizoctonia solani* AG3, um importante patógeno da cultura da batata. Os resultados indicaram que (mudanças no) manejo agrícola, principalmente rotação de culturas, afetam a freqüência de isolamento de estirpes antagonistas de *Burkholderia* e que a pastagem permanente possui uma reserva de espécies de *Burkholderia* com grande potencial para uso agrícola.

O **capítulo 6** contém o resumo do trabalho realizado durante essa tese, juntamente com as respostas às questões científicas levantadas no capítulo 1 e possíveis alternativas para estimular a população de espécies antagonistas de *Burkholderia* e, com isso, aumentar a supressividade do solo a doenças.

Os resultados obtidos no presente trabalho permitem concluir que o histórico de cultivo do solo tem um grande efeito sobre a estrutura da comunidade de *Burkholderia* e o período de tempo necessário para superar esse efeito varia em função do tipo de conversão. Adicionalmente, o uso de práticas agrícolas, tais como adubação e aragem, tem papel importante na aceleração do processo de conversão. Através da análise da população culturável de *Burkholderia*, observou-se que a diversidade deste gênero de bactérias era afetada principalmente por mudanças nos manejos agrícolas, sendo maior nas áreas que tinham o mesmo histórico por pelo menos 24 anos (solo sob cultivo contínuo e pastagem permanente). A pastagem permanente, além de apresentar alta diversidade, também teve um grande efeito na estimulação de espécies de *Burkholderia* com potencial para controle biológico.

Curriculum Vitae

Joana Falcão Salles was born on March 26th, 1971, in the "wonderful city" of Rio de Janeiro, Brazil, two blocks away from the sea. In 1988 she finished her secondary education at Colégio Notre Dame, Rio de Janeiro and in 1990 she started Biology at the Rural Federal University of Rio de Janeiro (UFRRJ), in Seropédica, few kilometres further away from the sea. In 1991 she transferred to the Agronomy course, which she graduated from in January 1995. During her undergraduate studies, from 1991 to 1995, Joana was granted scholarships to initiate her in the scientific world. For the first two years she worked at the department of citogenetics, UFRRJ and in 1993 she worked as trainee at Embrapa Agrobiologia (Brazilian Agricultural Research Corporation, National Centre for Agrobiology). After graduation, Joana continued at Embrapa Agrobiologia, first with a specialisation scholarship (1995) and later as member of the MSc program in Plant Science, UFRRJ. Her MSc thesis dealt with the development of a biocontrol agent of coleopteran insects in sugarcane, by introducing the *cry3A* gene from *Bacillus thuringiensis* in the diazotrophic bacteria *Gluconocetobacter dizotrophicus*. In July 1998 she obtained the MSc degree, under the supervision of Dr. José Ivo Baldani. In the following year, she was awarded a one-year fellowship to continue at Embrapa Agrobiologia, in a project evaluating the response of micropropagated sugarcane plants to inoculation with endophytic diazotrophic bacteria. During this period (April-August, 1999) she also worked as teacher at the Plant Physiology department, UFRRJ. In the end of 1999 she moved to Wageningen, the Netherlands, even more kilometres away from sea. In August 2000 she was granted a 4-year fellowship by CNPQ (Brazilian Council for Scientific and Technological Development) to carry out her Ph.D. thesis at Plant Research International, under the supervision of Prof. Dr. Dick van Elsas and Prof. Dr. Hans van Veen, within the research project that resulted in this thesis.