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Bacterial community associated with the rhizosphere of wheat: interactions with arbuscular mycorrhizal fungi and selection of plant growth promoting rhizobacteria for the increase of wheat growth and soil health in Indian marginal rainfed fields

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IMPRIMATUR POUR LA THESE

Bacterial community associated to the rhizosphere of wheat : interactions with arbuscular mycorrhizal fungi and selection of plant growth promoting rhizobacteria for the increase of wheat growth and soil health in Indian marginal rainfed fields

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Pour Anne

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Summary

The studies presented in this thesis are integrated in the SA-7 project of the Indo-Swiss Collaboration in Biotechnology aiming at developping new biotechnologies such as the use of plant growth promoting rhizobacteria (PGPR) bio-inoculants in association with arbuscular mycorrhizal fungi (AMF) for improving plant growth and soil health in marginal rain-fed regions of India. However, a major setback in developing a large-scale use of PGPR bio-inoculations in low-input farming systems is due to the variability and inconsistency of the PGPR plant growth effect not only between laboratory studies and field applications but also between different fields. The interactions between the microflora and the plant and within the rhizosphere communities are complex and need to be clarified before successfully using PGPR and AMF dual inoculations. Moreover, a successful introduction of effective PGPR strains in association with AMF in the fields required not only evidence of the establishment of the inoculants in the rhizosphere *in situ* but also that the strains did not have any deleterious effect on AMF development and if possible stimulate fungal growth. The objective of this thesis was then to improve our knowledge on the interactions between wheat, rhizobacteria and AMF in the mycorrhizosphere in order to define criteria for the selection of PGPR strains in view of a PGPR/AMF dual inoculation in Indian wheat fields. First, microcosm systems were set-up to obtain mycorrhizosphere, AMF-free rhizosphere and root-free hyphosphere zones in order to examine the effects of AMF on the rhizobacterial community in the wheat mycorrhizosphere. The results showed that the bacterial community structure was more influenced by the type of rhizospheric fraction (non rhizospheric soil, rhizospheric soil and rhizoplane/endorrhizosphere), the plant age and the plant specie than by the presence of AMF. Nevertheless, specific populations were either inhibited or stimulated in the presence of AMF. No correlation was observed between the hyphal length and the bacterial community structure but this latter was affected indirectly as the presence of AM hyphae in the non rhizospheric soil modified the soil pH. In addition, there was a strong increase in the proportion of phosphate solubilizing bacteria in AMF related zones probably resulting from soluble phosphorus depletion in consequence to AMF phosphorus uptake. Secondly, spores of the arbuscular mycorrhizal fungi *Glomus geosporum* and *G. constrictum* were harvested from single spore derived pot cultures with either *Plantago lanceolata* or *Hieracium pilosella* as host plants to determine if specific bacterial populations were associated with AMF spores. The bacterial communities associated with the spores were more influenced by the AMF than by the host plant. The majority of the bacterial sequences that were common to both *G. geosporum* and *G. constrictum* spores were affiliated to taxonomic groups known to degrade biopolymers. These bacteria were probably feeding on the spore's outer hyaline layer. The third part of the study examined how PGPR strains directly

affected AMF growth in the hyphosphere. An *in vitro* device, consisting of a two-compartmental Petri plate system using Ri T-DNA transformed clover roots permitting the separation of the hyphosphere from the mycorrhizosphere, was designed and tested. Even though the PGPR strains tested were all DAPG producers, their effects on the AMF development varied from inhibition to improvement of the hyphal biomass or spore production. Interestingly, there was a positive mutualistic interaction between *Pseudomonas synxantha* R81 and *Glomus intraradices* that could be explained by the bacterial feeding on fungal proteins, providing a carbon source for the bacteria and a recycled N source for the fungus. For the fourth part of the study, we had to ensure that before applying the selected PGPR strains *P. jessenii* R62 and *P. synxantha* R81 in the fields, they were able to colonize the rhizosphere *in situ*. They were marked with the green fluorescent protein before testing them in greenhouse pot experiments. R62gfp had colonized the root at a later stage than R81gfp, explaining why the PGP effect of R62gfp was delayed. There was a continuous decrease of the R81gfp strain throughout the wheat growth period when bio-inoculated alone in the rhizosphere. However, when R81gfp was associated with two other PGPR strains in a consortium, it remained detectable even at the maturity wheat growth stage. Both gfp strains were located in the upper part of the root but R81gfp was also detected near the root elongation zone. The fifth part was undertaken in the fields to confirm the positive interactions between AMF and the PGPR strains R62 and R81 and to assess the changes in the wheat bacterial rhizospheric community with respect to field conditions, plant age and PGPR/AMF bio-inoculation. As compared to the bacterial community of the rhizoplane/endorrhizosphere, the bacterial community of the root-adhering rhizospheric soil was more influenced by the field conditions such as an increase in fertilizer input. The bacterial community structure was also dependent on the plant's growth stage. In addition, the type of PGPR consortium had a greater impact on the bacterial community structure than the mycorrhizal colonization. The treatment composed of R62/R81 and an indigenous AMF consortium had not only a positive but also in some aspects, a synergistic effect on plant development. Finally, indirect and direct effects of AMF on the bacterial community in the mycorrhizosphere and hyphosphere, the concept of mycorrhizosphere competence and proposals to increase the effectiveness of bio-inoculations in low-input systems are discussed.

Table of contents

1 General Introduction

1.1 ISCB SA-7 project: “Diversity and functions of free-living and associated rhizobacteria in wheat rhizosphere and their influence on soil quality and productivity

1.1.1 The goals of ISCB

The studies presented in this thesis are integrated in a project of the Indo-Swiss collaboration in biotechnology (ISCB). Founded in the beginning of the 1980s, the research collaboration between Swiss and Indian institutions in various areas of biotechnology aimed at the establishment of sustainable research partnerships and competences in which technology transfers play an essential role. The cost of the ISCB program is shared between the Swiss Agency for Development and Cooperation (SDC) and the Department of Biotechnology (DBT, Government of India), in accordance with a bilateral agreement. The program phase in which we were integrated dealt with establishment of sustainable partnerships taking into account economical, social and ecological issues. These include the development of environment-friendly technologies potentially leading to enhancement of production and productivity of wheat and pulses in rainfed areas (fig. 1).

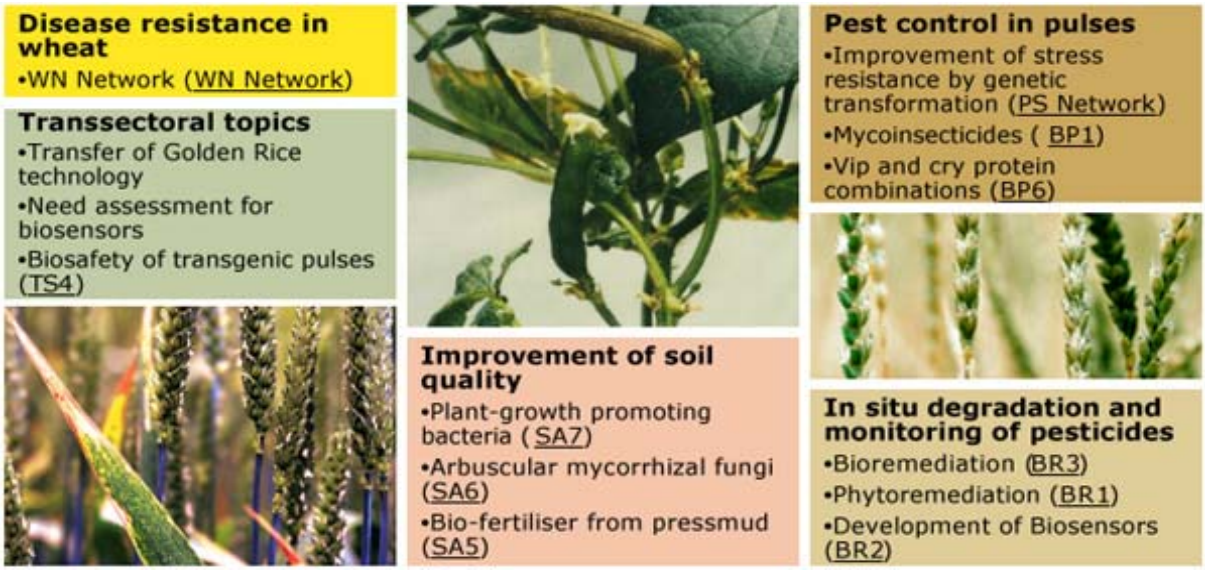

Fig.1. Broad outline of the ISCB projects during our program phase (source: http://www.biotech.biol.ethz.ch/india, accessed in 2001)

1.1.2 SA-7 project goals and partners

The main goals of our project were to develop new biotechnologies such as the use of PGPR bio-inoculants for improving plant growth and soil health in marginal rain-fed regions of India that have limited access to artificial fertilizer inputs. This project aimed to understand plant-rhizobacterial interactions in the wheat rhizosphere with special regards to PGPR (Plant Growth Promoting Rhizobacteria) strains isolation and characterisation and to the interactions between rhizobacteria and arbuscular mycorrhizal fungi (AMF).

The ISCB SA-7 project in the LAMUN was headed by Prof. Michel Aragno and the scientific coordination was carried out by Dr Pierre Rossi from April 2000 to December 2002 and by David Roesti from January 2003 to August 2004. Apart from this thesis, two diploma students participated in the project studies. The diploma works of Noam Shani and Gwenaël Imfeld were entitled respectively "*2,4-diacetylphloroglucinol (DAPG) producers in the rhizosphere of wheat: development of probes and primers to assess their presence and abundance in Indian rice-wheat crop rotations*", and "*Diversity of the phlD gene pool and bacterial community analysis of the wheat rhizosphere in the middle Indo-Gangetic plain (Uttar Pradesh, India)*". Their results have been submitted to Current Science India (Annex 2). Technical assistance was provided by Noémie Duvanel. Nathalie Fromin provided advising for issues on the rhizosphere during the whole project period.

Our ISCB SA-7 Indian project partners were guided by Prof. Bhavdish N. Johri, head of the Microbiology Department of the Govind Ballabh Pant University of Agriculture and Technology in Pantnagar (state of Uttaranchal, India). Many scientists from Pantnagar collaborated actively with us: Dr Anil Sharma; Dr Pankaj Mishra; Dr Rachna Gaur; Dr Kawal Jeet; Dr Shilpi Mittal; Supriya Sharma; Deepti Dwivedi; Dr Anita Sharma and Dr Reeta Goel). The Indian partners' work was devoted to the more applied aspects of the project dealing with PGPR isolation, characterisation and testing.

1.1.3 Collaborations

Many of the studies realised for this thesis and for the project were performed not only in collaboration with the Indian partners of the SA-7 project but also with the partners from the SA-6 project entitled "Introduction of arbuscular mycorrhizal fungi as bio-fertilizers and soil-structure stabilisers for sustainable agriculture". The Swiss partners of the SA-6 project belonged to the Arbuscular Mycorrhizal Fungi group of the Botany Institut of the University of Basel headed by Prof. Andres Wiemken: Dr Fritz Oehl; Dr Dirk Redecker and Kurt Ineichen. The Indian partners of the SA-6 project belonged to the Centre for Mycorrhizal Research

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Other scientists or technicians from the LAMUN and other institutes that actively collaborated in this thesis were: LAMUN: Dr Jérôme Hamelin; Dr Sonia Tarnawski; Marylline Jossi; Ludovic Roussel-Delif; Laurent Locatelli; Marie-Laure Heusler; Nicole Jeanneret. Laboratoire d'Ecologie végétale, University of Neuchâtel : Florian Kohler. Laboratoire de Géologie, University of Neuchâtel : Prof. Eric Verecchia and Olivier Braissant. Institut de Mathématiques, University Of Neuchâtel : Dr J. Moret. Laboratoire de Biochimie, University Of Neuchâtel : Ricardo Flückiger. Inst. für Planzenbiologie, ETH-Zürich : Prof. Défago and Dr Alban Ramette. Institut de Microbiologie Fondamentale, University of Lausanne: Prof. D. Haas and Eric Baehler. Université Claude Bernard, Lyon 1: Franck Poly

1.2 Context of the study

1.2.1 Rice-wheat cropping system in South Asia

Wheat is the most widely cultivated cereal grain, occupying about 17% (220 million hectares in 1994) of the total cultivated land in the world (Pingali, 1999). The crop is Asia's second most important staple food (after rice) and supplies about one-fifth of the total requirements of food of the developed countries (CGIAR, 1992). The Indo-Gangetic Plains, where approximately 85% of the rice-wheat system is practiced in South Asia, is composed of the Indus (areas in Pakistan and parts of Punjab and Haryana in India) and the Gangetic Plains (Uttar Pradesh, Bihar, and West Bengal in India). The remaining 15% is in the Himachal Pradesh, Madhya Pradesh, and South Eastern India and in the hills of Nepal (Timsina and Connor, 2001). In 1995, the areas planted with rice and wheat crops in India were 43 and 25 million ha, respectively (fig.2). Nearly 25% of the area under rice and 40% of the area under wheat are currently cropped in rice-wheat rotations (Abrol, 1999). In this system, rice is grown in the kharif (rainy) season and followed by wheat in the rabi (winter) season.

A large variety of cropping patterns is found in the rice-wheat systems, especially in rainfed areas, where an additional crop is often grown. For example, in the Eastern part of the Upper-Gangetic Plains (most of Uttar Pradesh and parts of Bihar and Nepal) rice is grown during the monsoon and post-monsoon period, when the lowlands are naturally flooded for a period of variable duration, usually from June/July to October/November. In contrast, wheat is grown during mid-to-late winter to early spring usually from November/December to March. In this area, crops such as mungbean (*Vigna unguiculata*), dhaincha (*Sesbania spp*.), jute

(*Corchorius spp*.) or maize (*Zea mays*) are planted just after wheat or before rice (Timsina and Connor, 2001).

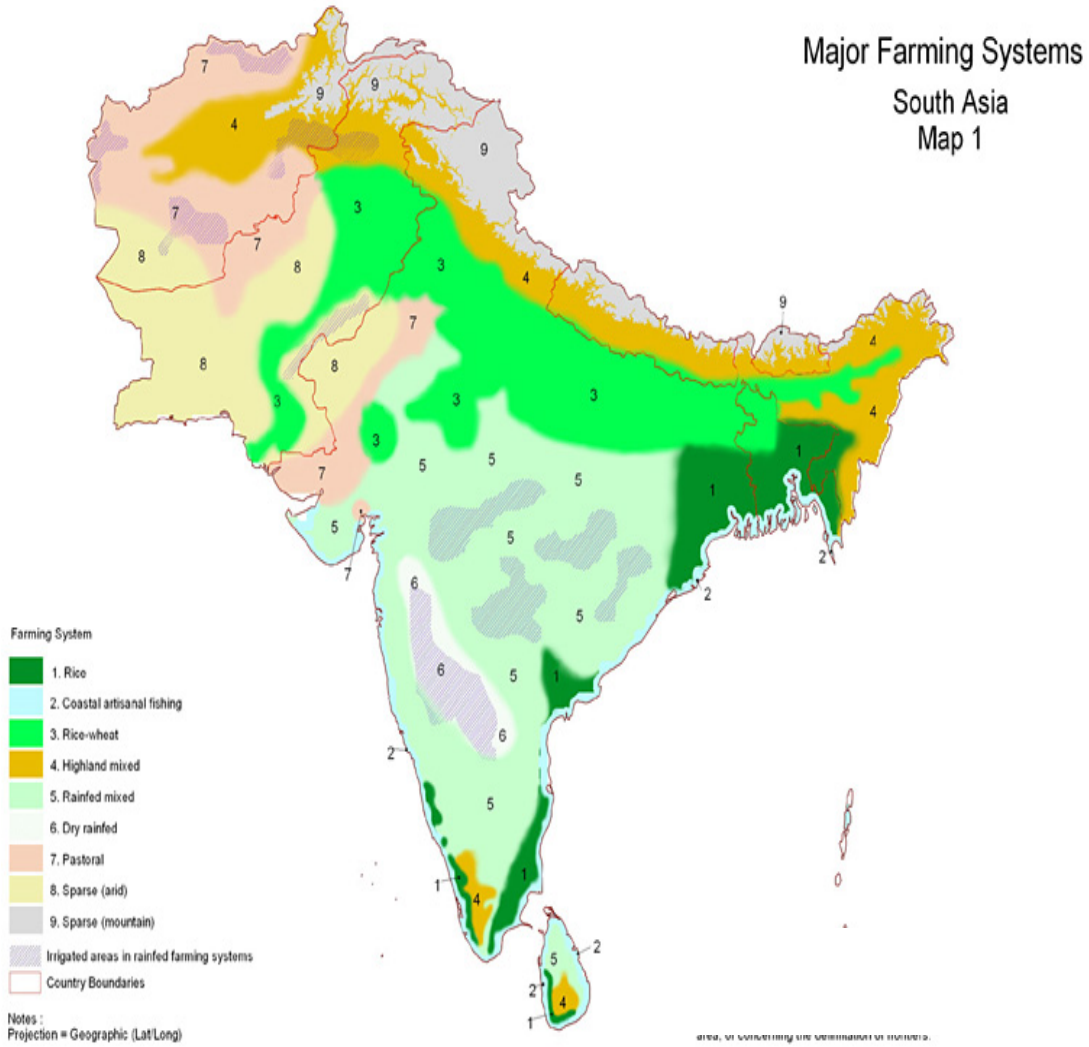

Fig. 2. Map of the main agricultural systems in South Asia (source: www.fao.org, accessed in 2002).

1.2.2 The Green Revolution and recent declines in wheat yields

The Indian "Green Revolution", initiated in the 1960s by Mrs Indira Gandhi, has increased dramatically crop yield. These improvements were due to the following combination of factors (Abrol, 1999):

- an expansion of irrigated area by harnessing surface and groundwater
- the introduction and spread of dwarf-photoperiod-insensitive high yielding varieties of rice and wheat
- the increased use of inputs including fertilisers and plant protection chemicals
- the greatly expanded and strengthened research and extension services
- the overall agricultural support policies.

The last decade has witnessed certain trends emerging both from the Indian farmers and the agricultural scientists. They made agricultural practices more efficient from an ecological and social point of view while increasing productivity and profitability, thus improving farmers' livelihood and reducing poverty (RWC, 2002). These trends are part of the called "Second Green Revolution".

Demand for rice and wheat will grow at 2,5% per year over the next 20 years (Hobbs and Gupta, 2001). However, repeated transition from anoxic to oxic conditions during the rice-wheat rotation, the flooding during the rice season as well as high input cropping practices have impaired the sustainability of the system. In some regions, the gains in food grain production have stagnated or even declined in recent years for both rice and wheat crops (Dawe and Dobermann,1999). Causes of decline may include changes in biochemical and physical composition of the soil organic matter, a depletion in bio-availability of soil nutrients, a scarcity of surface water or groundwater as well as poor water quality (salinity), and the buildup of pests (Ladha et al., 2000; Abrol, 1999; Timsina and Connor, 2001).

1.2.3 Marginal rainfed fields

Several districts of the Indian Indo-Gangetic plains (West Bengal, North Bihar, parts of Uttar Pradesh and near the foothills of the Himalayas, refer to fig. 3) are constituted of rainfed systems that have a limited development of the irrigation infrastructure and unreliable supply of irrigation water (Ladha et al., 2000). The wheat yields in these rainfed regions are in average twice as less as compared to the well irrigated regions (2t/ha instead of 4t/ha) (Ladha et al., 2000). On the total numbers of rice-wheat farming households in Uttar Pradesh, 72% have marginal (<0,25ha), 22% have small (0,26-0,5 ha) and 6% have medium sized (0,5-1,0 ha) landholdings (Ladha et al., 2000). The prices of rice and wheat have declined steadily over the last 30 years and partial removals of subsidies have put stress on the economy of average and marginal farmers impeding, a rapid introduction of sustainable agricultural practices (Hobbs et al., 2000). Increasing agricultural productivity, combined with reduction of inorganic fertilisers and pesticides, constitutes an important parameter in order to improve the standard of living in rainfed farming areas. The use of biotechnology will be required not only to improve the crop characteristics but also the soil fertility.

1.2.4 Study sites of the ISCB SA-7 project

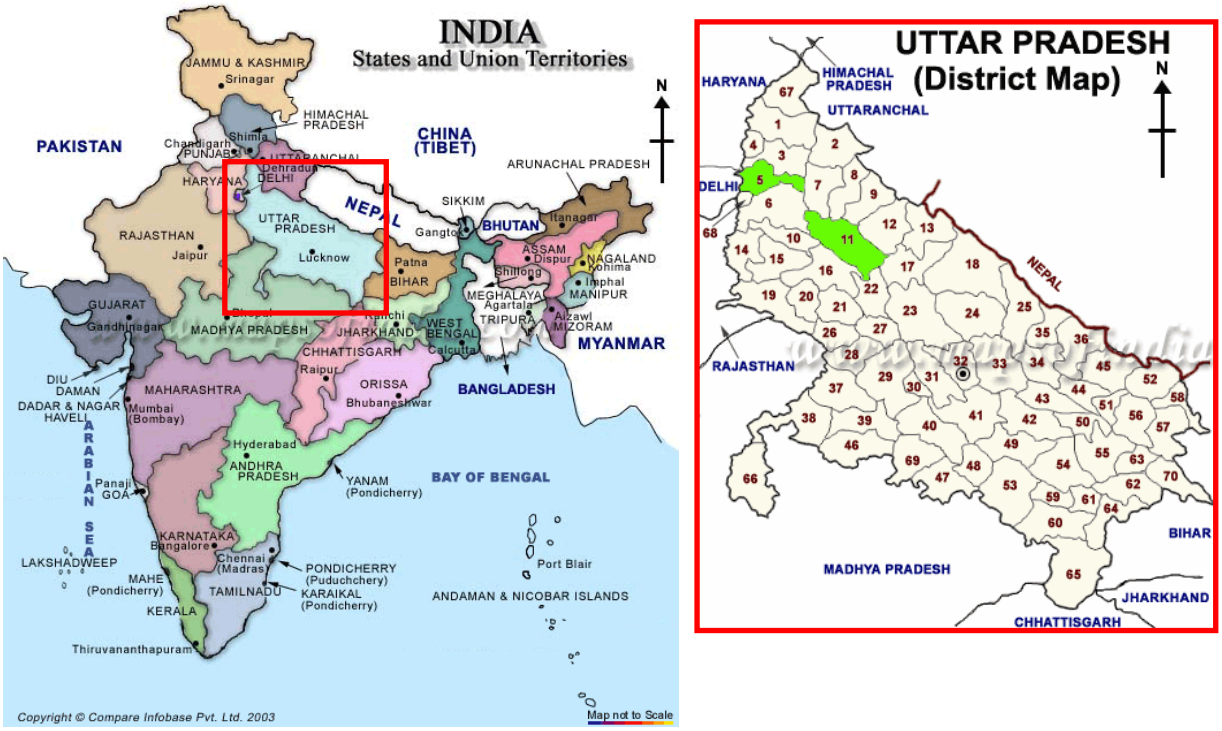

Fig.3 State map of India and Uttar Pradesh districts map. The experimental field sites presented in this thesis are outlined in the districts map. 5 = Ghaziabad district; 11 = Budaun district (source: mapsofindia.com, accessed in 2004)

The fields studied for the ISCB project SA-7 were located in the Uttar Pradesh (UP) state, India (fig.3). The first one is located in Bhavnipur village (Budaun District latitude 28,02 N, longitude 79,10 E) and the second, near Ghaziabad city (Ghaziabad District latitude 28,40 N, longitude 77,28 E). The study site in Budaun was rainfed; characterized by twenty years of rice-wheat rotation and by a basic irrigation system. In Ghaziabad, two fields also characterized by twenty years of rice-wheat rotation were selected. They differed only in the agricultural practices, one being a conventionally tilled plain field and the other a three-year-old practiced raised bed field. For wheat cropping, standard agronomic practices were followed, such as regular sowing, irrigation and weeding. For more details on the fields, please refer to chapter 6 and annex 2. The same wheat variety (UP 2338) was cultured in these fields.

1.3 The model plant: wheat

1.3.1 Characteristics

The model plant for the SA-7 ISCB project was wheat (*Triticum aestivum*) that belongs to the order *Poales* (*Glumiflorae*), family *Poaceae* (*Gramineae*), tribe *Triticaceae*, genus *Triticum* (Zeller, 1985; Körber-Grohne, 1988). *T. aestivum* is an hexaploid wheat species (2n = 42, genome AABBDD, Körber Grohne, 1988). Plants are annual with spring or winter forms and are cereals of temperate climates. The minimum temperature for germination is 3 to 4°C and above 14°C for flowering (Körber Grohne, 1988). The harvested grain (caryopsis) contains approximately 60% carbohydrates (starch), 10 to 16% proteins, 2% fat and 13% water (Hömmö and Pulli, 1993). The wheat variety used in this study was UP 2338, originating from Uttar Pradesh and recommended for irrigated or rainfed conditions.

1.3.2 Wheat growth stages

The wheat growth is typically described by the Feekes scale and the Zadoks scale (see fig.4).

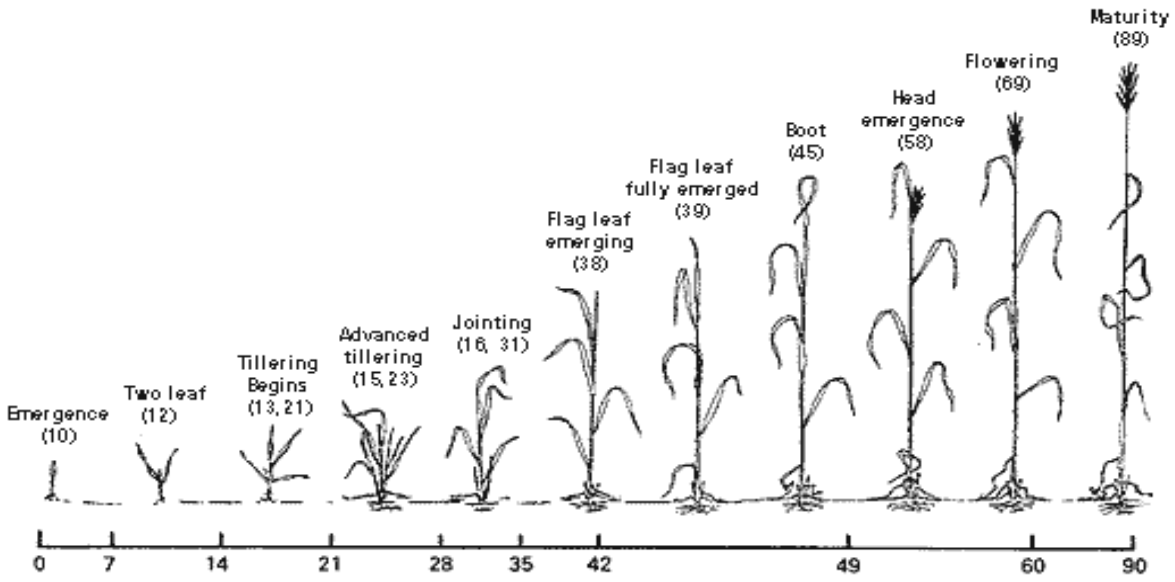

Fig. 4. Wheat growth stages according to the Zadoks scale (source : www.usask.ca/agriculture/plantssci, accessed in 2004).

The main stages, according to Large (1954), are:

- Germination: starts with the uptake of water (imbibition). Coleoptile, radicle and first three seminal roots are produced.
- Seedling: appearance of the first leaf, crown of the plant becomes distinct
- Emergence: plant emerges from the soil
- Two-leaf: two leaves are formed
- Tillering: apparition of tillers
- Stem elongation or jointing: node formation, elongation of internode regions, flag leaf appears
- Head emergence: head appears
- Flowering or anthesis: pollinisation and fertilization occurs during this period, embryo appears after fertilization
- Milk: kernel formation starts
- Dough: kernel formation completes
- Ripening: seed looses moisture
- Maturity: ripe for cutting

1.3.3 Wheat root development

The wheat root system develops as following (Weaver, 1926, fig. 5): upon germination of the grain, the primary root takes the lead but very soon, two other roots appear on opposite sides of the first. Others roots may be added and together they constitute the primary root system. In some cases, there may be as many as eight roots. Early in the development of the plant, roots of the secondary root system grow from nodes above the primary one. The first whorl of roots of the secondary root system always develops within a few cm of the soil surface. The number of roots increases somewhat in proportion to the number of tillers. The mature root network is composed of a vast network of rebranched laterals occupying a volume of soil extending approximately 25 cm on all sides of the plant and to a depth of 60 to 100 cm. The total number of roots varies from 20 to 25 according to the number of tillers.

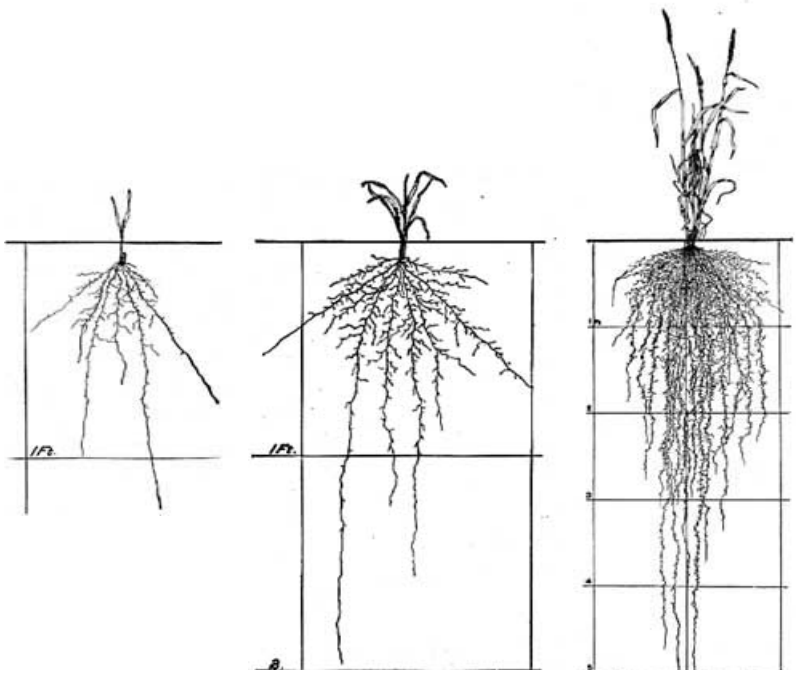

Fig.5. Sequence of wheat root growth at two-leaf, tillering and flowering growth stages (source : Weaver, 1926).

1.4. The living soil

Soil is a structured, heterogenous and discontinuous system, generally poor in nutrients and energy sources (Nannipierri, 2003). It is composed of organic and inorganic matrices formed by the combined action of biotic and abiotic processes (Liesack et al., 1997; Gobat et al., 2004). Organic carbon found in the soil is mainly plant derived (plant remains and rhizodeposition, see 1.5.3). A carbon source can come from plant remains that are degraded by the soil macro and microflora into organic matter through the process of humification (Kuzyakov and Domanski, 2000; Gobat et al., 2004). The soil organic matter (SOM) is composed of non or partially degraded litter and the humus fraction (Gobat et al., 2004). SOM accounts for as much as one third of the cation exchange capacity of surface soils and is responsible for stability of soil aggregates (Brady, 1990).

Fig.6: Bacterial cells and microcolonies in an agricultural soil (source: Winogradsky, 1949)

Eighty to ninety percent of the reactions in soils are mediated by microbes (Coleman and Crossley, 1996). In agroecosystems, bacteria are responsible for diverse metabolic functions that affect soil fertility and plant health including nutrient cycling, organic matter formation and decomposition, soil structure and plant growth promotion (Kennedy, 1999). The presence of microrganisms in the soil will depend on the number and volume of available microhabitats and bacterial activity to the amounts of available metabolic substrates found in those microhabitats (Stotsky, 1997; Nannipieri, 2003). The mineral composition, salinity, pH, nutrient availability, organic input, temperature and water content determine which ecological niches are available (Liesack et al., 1997). These soil properties in turn depend not only on the fauna and vegetation but also on the geographical, geological, hydrological, climate, and anthropogenic influences (Liesack et al., 1997). Soil contains many different microhabitats thus increasing the bacterial diversity. Indeed, several thousand bacterial species can be found in one g soil (Torsvik et al., 1990). The bacterial predation by bacteriophages, protozoans or nematodes enables to re-mineralise the nutrients immobilised by the bacterial

biomass (Ingham et al., 1985; Grifiths and Bardgett, 1997). Zones in the soils where microbial activity is increased are defined as hot spots (Sextone et al., 1985). In soils, the rhizosphere is probably the greatest of hot spots and offers different habitats and more resources for soil microorganisms than the bulk soil (Kuzyakov, 2002).

1.5. The rhizosphere

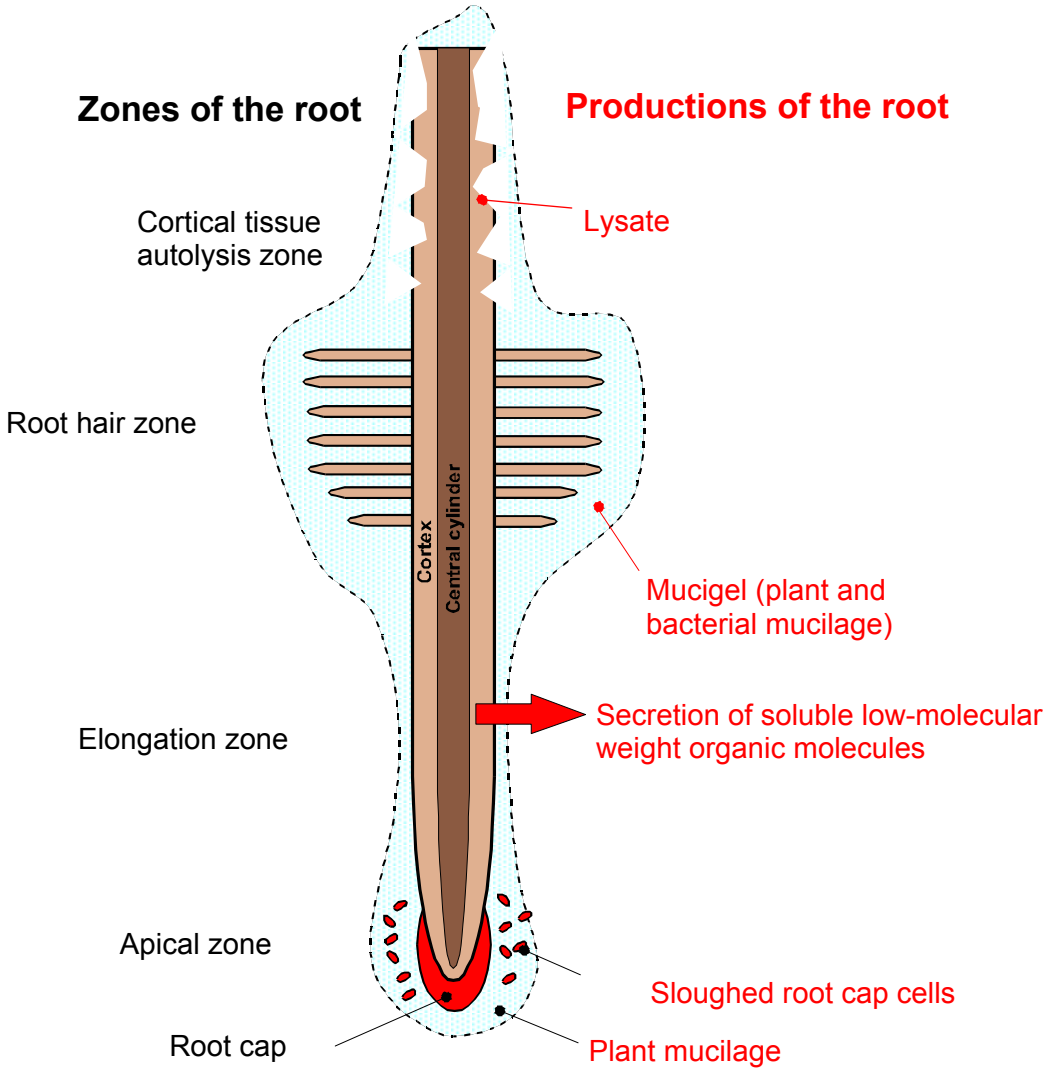

Fig. 7 Diagram of the root (source : Michel Aragno)

1.5.1 Definition of the rhizosphere

The rhizosphere *sensu lato* is now generally defined as the volume of soil under the influence of the root as well as the root itself (Hiltner, 1904; Darrah, 1991; Lynch, 1990). Three rhizosphere fractions can be distinguished (Gobat et al., 2004): the endorhizosphere (interior of the root), the rhizoplane (surface of the root) and the rhizospheric soil that adheres to the root when the root system is shaken manually. Finally, the volume of soil which is not influenced by the root is defined as non rhizospheric soil or bulk soil (Gobat et al., 2004). In our studies, three fractions were considered (see fig.8): non rhizospheric soil, (that detaches from the root when the plant is shaken); rhizospheric soil (fraction of soil that remains attached to the root); and rhizoplane/endorrhizosphere (washed roots).

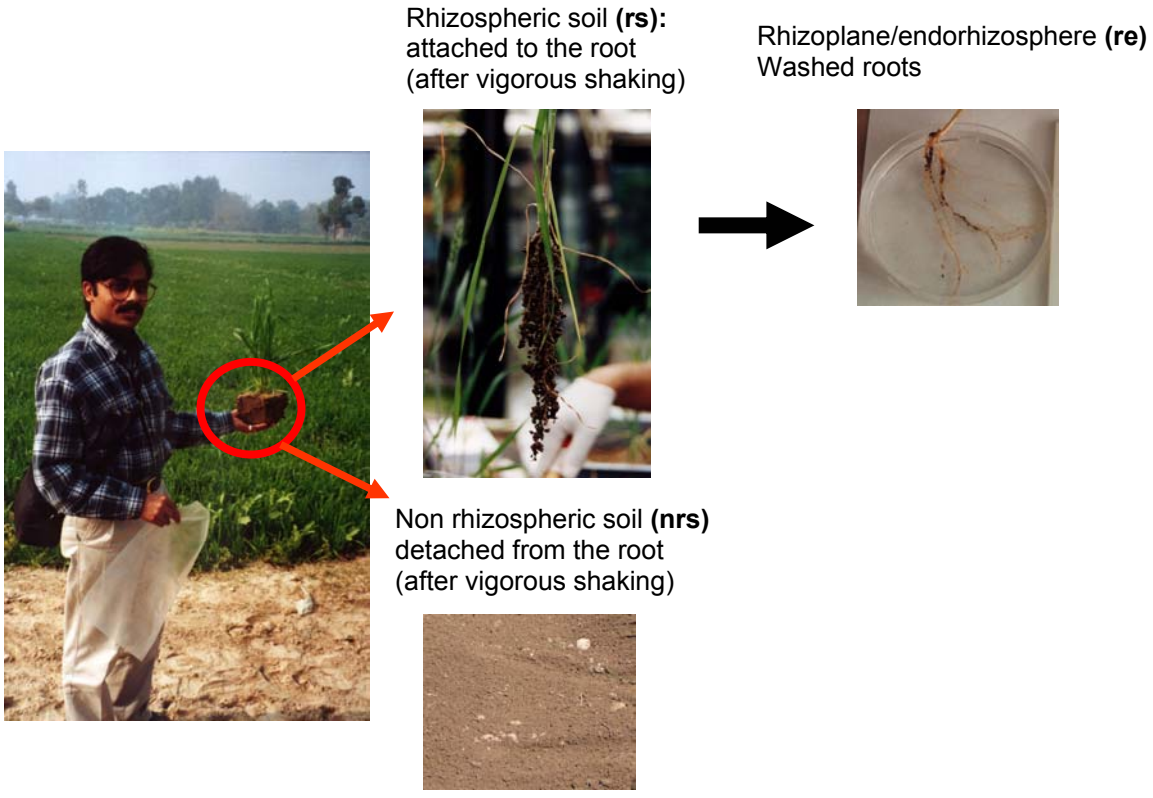

Fig.8. Three fractions of the rhizosphere analysed in our studies (photos: David Roesti).

1.5.2 Effect of the root activity on its environment

The root activity can modify the physico-chemical properties of its environing soil which in turn affects the soil microflora in different ways: Water and soil nutrient absorption induces a modification in the soil pH and redox potential as well as a nutrient stress on the environing microbial communities (Marschner et al., 1987; Frostegard et al., 1993; Baath and Anderson, 2003; Leong et al., 1986; Lemenceau et al., 1998; Clays-Josserand et al., 1995). The O_2 and CO_2 partial pressure varies in function of root respiration. There is a negative O_2 gradient from the root surface to it's surrounding soil (Højberg and Sørensen, 1993). As O_2 level is lower in vicinity to the root, anaerobic microbial processes such as denitrification could be favoured (Ghiglione et al., 2000). The rhizodeposition is however the most favourable process for the microbial community.

1.5.3 The rhizodeposition

The rhizodeposition is the release of organic or inorganic root products in the soil (Gobat et al., 2004). The rhizodeposition can be influenced by many biotic and abiotic factors of plant and soil, summarized in fig. 9. The rhizodeposition corresponds to 15-60% (30% in wheat) of the total photosynthetic production of the plant and conveys an important carbon and energetic source towards the microorganisms of the rhizosphere (Curl and Truelove, 1986; Darrah, 1996; Lynch and Whipps, 1990, Marschner, 1995). It comprises sloughed of cells and secreted mucilage as well as soluble exudates either secreted or as a result of cell lysis (Sørensen, 1997, see fig. 7).

Two types of mucilage are mainly produced by the root epidermal cells: neutral polysaccharides in the root cap, acting as a lubricant when the root advances in the soil (Lynch, 1990; Sørensen, 1997) and polygalacturonic acids in the root hair zone to protect against dessication by forming a matrix for water absorption/transport between epidermal cells and soil particles (Lynch, 1990; Gobat et el., 2004). The root cap cells are sloughed off in the soil by mechanical shear due to the speed of root elongation. Therefore, the root cap is devoid of microorganisms and the mucilage around it is only of plant origin (Sørensen, 1997). However, around the other parts of the roots, the mucilage is composed not only of root epidermal cells secretions but also of exopolysacharides produced by microorganisms (Rovira and Davey, 1971). It is also a site for microbial attachment to the root (Sørensen, 1997).

The root exudates constitute the major part of the rhizodeposition and are mainly composed of soluble low-molecular weight substances such as carbohydrate monomers, amino acids, organic acids, phytosiderophores, flavonoïdes, plant hormones and vitamins (Farrar et al., 2003; Lynch and Whipps, 1990; Kuzyakov and Demin, 1998). Five to ten percent of fixed C

in photosynthesis is lost by root exudation in soil (Jones et al., 2004). There are two classes of exudates: exudates which are lost as a result of passive diffusion, basal exudation, representing 3-5% of fixed C in photosynthesis (Pinton et al., 2001) and exudates which are released for a specific purpose and over which the plant exert a control by the opening of membrane pores (Jones et al., 2004). A large diffusion gradient is maintained as a result of constant removal in the soil by biotic and abiotic processes, for example microbial uptake (Kuzyakov et al., 2003), and sorption (van Hees et al., 2002). Moreover, root exudation is not a one-way transfer of C as a number of exudates components can be taken back into the root and this C influx is directed by the plant (Farrar et al., 2003; Jones and Darrah, 1994).

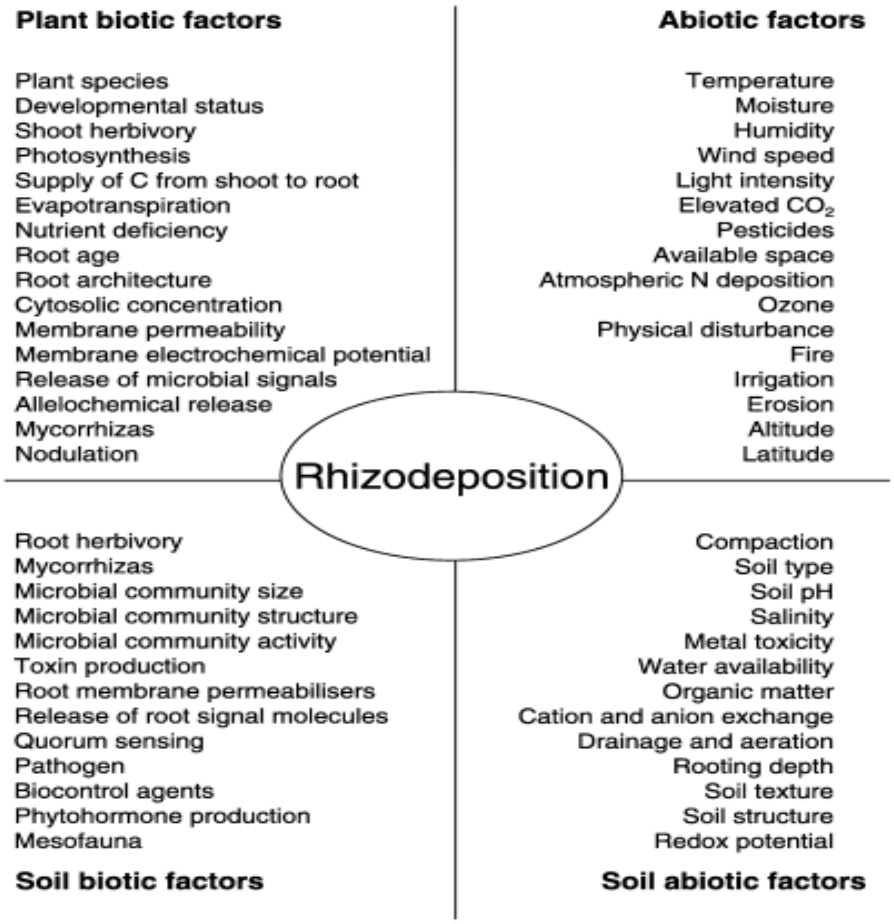

Fig. 9: Schematic representation of the biotic and abiotic factors of plant and soil that influence rhizodeposition (source : Jones et al., 2004).

This release of numerous organic compounds in the rhizosphere affects the rhizobacterial community as reflected by an increase of the bacterial biomass and of its turnover through increased predation, and a decrease of its diversity (Whipps, 2001; Marilley and Aragno, 1999; Ingham et al., 1985).

1.5.4 Rhizosphere microbial communities

Rhizosphere microbial communities can significantly influence phytopathogens development (Nehl et al., 1997; Glick, 1995), nutrient acquisition (Lynch, 1990), heavy metal resistance (Bradley et al., 1981), and ecological fitness of plants (Parker, 1995). Moreover, root-induced microbial activity increases the bacterial mucilage production thus modifying the soil structure by the formation of aggregates (Forster, 1990). The bacteria that are adapted to the rhizospheric living conditions and that are found in the rhizosphere are called rhizobacteria. Rhizobacteria can affect the plant development either negatively (deleterious rhizobacteria) or positively (plant growth promoting rhizobacteria). Deleterious rhizobacteria have been defined as minor pathogens that affect plant by their metabolites without parasiting plant tissues (Schippers et al., 1987). These rhizobacteria can produce phytotoxins, pectinolytic enzymes and/or phytohormones and compete with the plant or with the beneficial microorganisms for the uptake and metabolisation of nutrients (Suslow and Schrot, 1982; Nehl et al., 1997). Beneficial microorganisms in the rhizosphere comprise plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) described below.

1.6 Plant Growth Promoting Rhizobacteria

1.6.1 PGPR in agronomy

Plant growth promoting rhizobacteria have first been used for agricultural purposes in the former Soviet Union and India in the early 20th century and are now being tested worldwide (Lucy et al., 2004). The benefits for plant growth, consecutively to the addition of PGPR, include increases in: germination rates; root growth; yield (including grain); leaf area; biocontrol; chlorophyll content; hydraulic activity; tolerance to drought; shoot and root weights (Lucy et al., 2004). PGPR are of two general types: those that form a symbiotic relationship with the plant such as the nitrogen-fixing *Rhizobium* spp. and those that are free-living (Kloepper et al. 1980, Glick, 1995). The effectiveness of rhizosphere colonization and plant growth by PGPR depends on numerous factors in the agroecosystem. Among these, soil type, climatic conditions and fertilisation level plays an important role in root colonization (Kloepper et al., 1980; Okon and Labandera-Gonzales, 1994; Dobbelaere et al., 2001). In addition, soil pH and moisture are crucial for ultimate attachment and spread of the microbes (Burr et al., 1978).

1.6.2 Plant growth properties

The PGPR are beneficial to the plant via nutrient acquisition, biocontrol, plant hormone-like production and induction of systemic resistance (summarized in table 1).

Table 1: Plant growth promoting properties of PGPR

Plant growth promoting properties	Benefical action	References
Symbiotic or associative atmospheric fixation of atmospheric N_2	Increases plant N content or productivity	Ladha and Reddy, 2003; Dobbelaere et al., 2003; Okon and Labandera-Gonzalez, 1994
Production of bacterial siderophores	Provides iron for the plants, liberates phosphate from Fe-P compounds in acidic soils and inhibits fungal pathogens by sequestering iron	Crowley et al., 1988; Rodriguez and Fraga, 1999; Lemanceau and Alabouvette, 1993
Production of organic acids	Inorganic phosphate solubilization in neutral to alkaline soils	Rodriguez and Fraga, 1999; Kim et al., 1997; Richardson, 2001
Production of phosphatases	Mineralization of organic phosphorus	Rodriguez and Fraga, 1999
Antibiotic production (phenazine, pyoluteorin, 2,4-diacetylphloroglucinol, pyrolnitrin)	Inhibits fungal pathogens	Maurhofer et al., 1992 ; Keel et al., 1992; Walsh et al., 2001; Chin-A-Woeng et al., 2003; Dowling et al., 1994; Weller, 1988; Cook et al., 1995; Tomashow et al., 1988
Production of hydrogen cyanide	Inhibits fungal pathogens	Voisard et al., 1989; Ramette et al., 2003
Production of extracellular chitinase, laminarinase and β-1,3 glucanase	Damage or lysis of pathogenic fungal cells	Lim et al., 1991; Fridlender et al., 1993
Production of phytohormones (e.g. indole-3-acetic acid)	Increases root growth, increases number of secondary roots and root hairs	Steenhoudt and Vanderleyden, 2000; Barbiery and Galli, 1993
Production of ACC deaminase	Reduces ethylene concentration in the root thus increasing root elongation	Glick, 1995
Production of elicitors (e.g. salycilic acid)	Induction of plant systemic resistance	Maurhofer et al., 1998; van Loon et al., 1998

1.6.3 Rhizosphere competence

Successful colonization of the rhizosphere environment by microbial inoculants depends on their rhizosphere competence (Weller, 1988). Indeed, the bacterial strain needs to possess particular traits such as chemotaxis towards root exudates, compounds mediating attachment (adhesins, fimbriae, pilli, cell surface proteins and polysaccharides) and a capacity to metabolise root exudates compounds (Walsh et al., 2001; Chin-A-Woeng and Lugtenberg, 2004). Because they possess numerous root competence traits, the bacteria of the genus *Pseudomonas* are used as models of rhizobacteria (Sørensen et al., 2001). They constitute up to 10% of the culturable rhizospheric microflora (Kragelund et al., 1996; Tarnawski et al., 2003) and are used extensively as PGPR (Lucy et al., 2004).

1.7 Arbuscular mycorrhizal fungi

1.7.1 AMF in agronomy

The symbiotic arbuscular mycorrhizal fungi (AMF) have a key role in providing a sufficient level of nutrients to the crop in low input farming systems in order to maintain a sufficient productivity (Atkinson et al., 2002). Beneficial effects of AMF in agriculture comprise a better plant nutrition and hydric stress resistance (Smith and Read, 1997), biological control against pathogens (Azcón-Aguilar and Barea, 1996) and improvement of the soil structure (Miller and Jastrow, 1990).

1.7.2 An obligatory symbiotic fungus

AMF are aseptate, obligatory symbiotic fungi of the order Glomales (Zygomycotina) and colonise almost 90% of all plants in the biosphere (Smith and Read, 1997). AMF have three important components (fig.10): the root itself which provides reduced carbon to the fungus, the fungal structures within the cortical cells of the root (arbuscules providing a considerable increase in the contact surface area between the fungus and the plant's cytoplasm) and an extraradical mycelium in the soil that enables to uptake water and nutrients (Smith and Read, 1997). Some AM fungi also produce vesicles, which are structures believed to function as storage organs (Smith and Read, 1997).

The AMF inoculum is composed of spores, infected root fragments or hyphae. AMF spores provide a long-term reservoir of inoculum, have thick resistant walls, contain up to several thousand nuclei and are the only AMF propagules that can be identified to the specie level (Smith and Read, 1997). Vegetative hyphal growth can also be an important way of

propagation (Olsson et al. 2002) and hyphae survive at least 6 months in dry soil (Smith and Read, 1997). However, an extensive mycelium is not formed unless successful colonization of a root system occurs as they cannot utilise carbon compounds from the soil. Root exudates (flavonoids, volatiles, water soluble and hydrophobic compounds) can stimulate the spore germination, hyphal growth and branching thus helping the fungus in making contact with the root (Gianninazzi-Pearson et al., 1989; Giovannetti et al., 1993; Bécard et al., 1992; Vierheilig et al., 1998). In addition, the exudates production is higher in P-stressed plants (Nagahashi and Douds, 2000). Fungal biomass associated with roots has been estimated between 3% and 20% of root weight. Estimates usually do not include external hyphae or spores and the proportion of photosynthates used by the fungi can reach 26%, making them a considerable C sink (Johnson et al, 2002; Smith and Read, 1997).

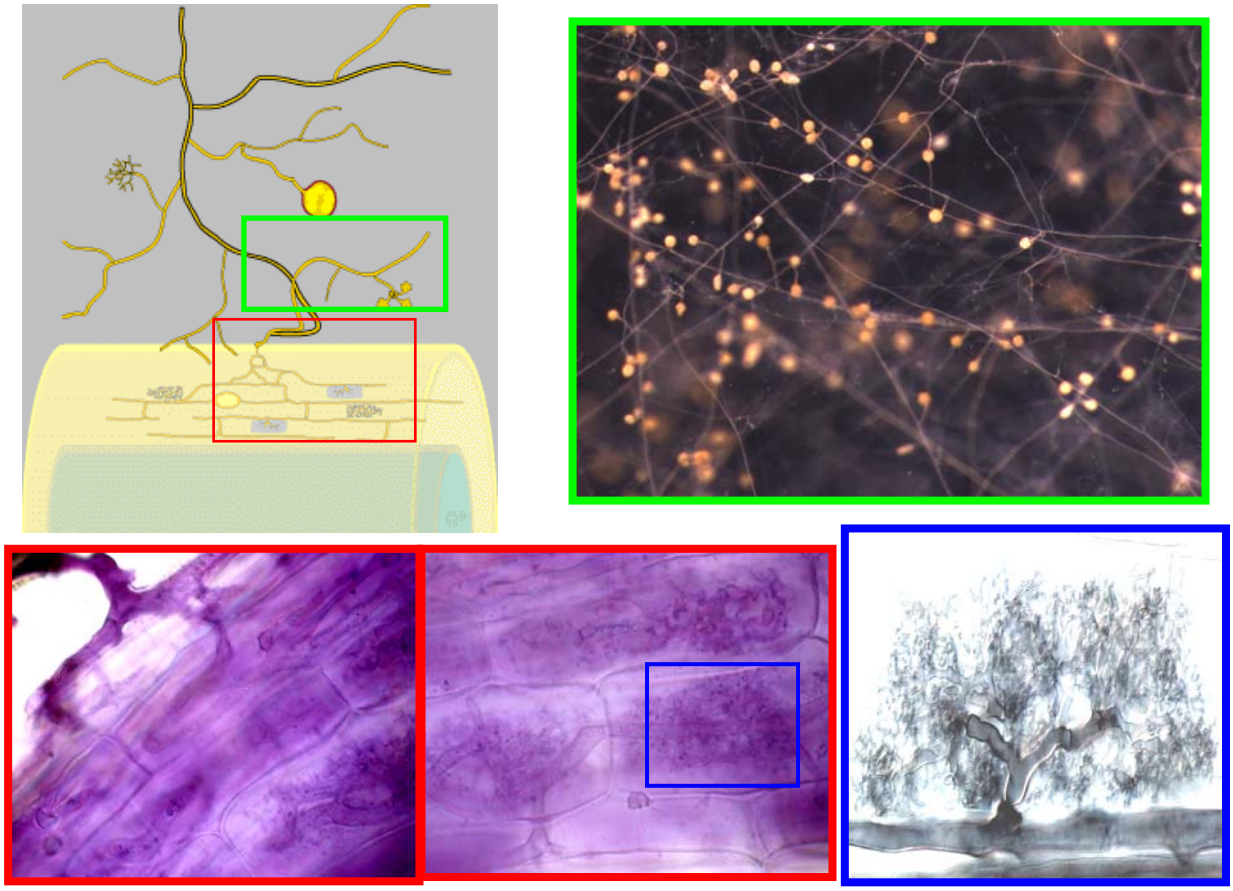

Fig.10. Components of the arbuscular myccorhizal fungi. Outlined in red (photo D. Roesti): the AMF penetrates the root cortical cells and form arbuscules that increase the surface of contact between the root cells cytoplasma and the fungus. Outlined in blue: arbuscule visualised with confocal laser scanning microscopy (source: http://www.ffp.csiro.au/research/mycorrhiza acessed in 2004). Outlined in green (photo D. Roesti): extraradical hyphae with formation of spores.

1.7.3 Transfer of nutrients from soil to the plant by AMF

The development of external mycelium enhances the plant's nutrient absorption. Indeed, under conditions of low nutrient availability, the hyphae can absorb nutrients from soil beyond the zones depleted by the roots. So, they increase the effectiveness with which the soil volume is exploited. Furthermore, the soil pores that can be penetrated by hyphae are perhaps an order of magnitude smaller than those available to roots (Smith and Read, 1997). In plants with well established infection, each cm of root length is associated with 0,5-1,5 m of extraradical hyphae (Harley, 1989). AMF have their more important effect on P nutrition. Not only is P required by both symbionts in relatively large amounts but it is poorly mobile in soil. It occurs in very low concentrations in the soil, being rapidly fixed as Fe, Al or Ca phosphates. Available P is uptaken from the soil by the fungal mycelium of AMF and is translocated within the hyphae to the intraradical fungal structures within the roots (Smith and Read, 1997). Other soil nutrients uptaken and transported to the plant by AMF include ammonium (NH_4^+), nitrate (NO_3^-) and micronutrients Zn or Cu (Smith and Read, 1997).

1.7.4 The mycorrhizosphere and the hyphosphere

The rhizosphere definition stated in paragraph 1.5.1 can then be extended to include the fungal component of the symbiosis, resulting in the term ''mycorrhizosphere''. The mycorrhizosphere is defined as the zone under the joint influence of the root and fungal hyphae (Linderman, 1992). The extraradical hyphae can penetrate the soil fractions that are not affected by the roots. This zone under the influence of AM hyphae only is defined as the hyphosphere (Andrade et al., 1997; Gryndler, 2000). AMF and bacteria can interact in the mycorrhizosphere and the hyphosphere as shown below.

1.8 AMF-bacterial interactions

1.8.1 Effect of AMF on its environment

As shown in table 2, different effects of AMF on the bacterial community of the mycorrhizosphere and hyphosphere were reported. The mycorrhizal infection modifies the rhizosphere functioning by changing the mineral composition and hormonal balance of the plant (Barea et al., 2002b), but also changes the root architecture by increasing root branching and apice size (Berta et al., 2002). The C allocation patterns of the plant are modified as more assimilates are transferred to the roots resulting from the carbon demand of the fungus (Schwab et al., 1984), and mycorrhizal roots have a higher respiration rate

(Kucey and Paul, 1982). In addition, root exudation could decrease or exudate composition could change in the presence of AMF (Marschner et al.,1997; Bansal and Mukerji, 1994). In the surrounding soil, extraradical hyphae not only change the soil's structure and physical properties such as aggregation (Wright and Upadhyaya, 1998; Andrade et al., 1998b; Miller and Jastrow, 1990) but also compete for nutrients (Ravnskov et al., 1999). They could also serve as a carbon source for microbial populations either by bacterial feeding on senescent hyphae or by the release of fungal exudates (Andrade et al., 1997; Andrade, 2004; Barea et al., 2002b).

1.8.2 Interactions between AMF and PGPR

Citernesi et al. (1996) found that bacteria from 17 year old *G. mosseae* pot cultures were actively antagonistic against the growth of *Fusarium* and *Phytophtora*, to fungal soil-borne root pathogens, suggesting to integrate the use of AM fungi and their associated bacteria in the biological control of soil borne pathogens. Moreover, some PGPR are known to induce a higher beneficial effect on the plant when co-inoculated with AMF. They include phosphate solubilizing bacteria or PSB (Toro et al., 1997; Barea et al., 2002c), nodule forming N_2-fixing *Rhizobia* or free-living *Azospirillum* spp. (Barea et al., 1996; Biro et al., 2000) and *Pseudomonas* spp. (Vàzquez et al., 2000; Barea et al., 1998). A synergistic effect of AMF and PGPR on plant growth could result either from a stimulation of bacterial PGP activity from the AMF or by a stimulation of fungal growth by the bacteria. Indeed, some bacteria can stimulate fungal growth.

1.8.3 Mycorrhiza helper bacteria

So-called mycorrhiza helper bacteria or MHB (Garbaye, 1994) are beneficial to ectomycorrhizae (Frey-Klett et al., 1997) or to endomycorrhizae (Gryndler et al., 2000). Their beneficial effects on the AMF growth are exerted not only by improving the mycorrhizal root colonisation or stimulating hyphal growth but also by favouring AMF spore germination (Garbaye, 1994; Gryndler et al., 2000). Among the mycorrhiza helper mechanisms, Garbaye (1994) hypothesised a modification of rhizospheric soil (change in pH or ion complexing by bacterial siderophores) and an increase in: root receptivity (plant hormone-like or cell wall softening enzymes production); root-fungus recognition; fungal growth and germination of fungal propagules (production of organic or amino acids, vitamins, carbon dioxide, etc…).

Table 2: Literature review comparing AMF and none AMF treatments in different systems

Plant host	AM Fungi/ bacterial co-inoculant	Main effect	Source
Zea mays (maize)	*Glomus mosseae, Glomus fasciculatum*	Increase of autotrophic ammonium oxidizers, decrease of ammonifying and denitrifying microorganisms in non rhizospheric soil of AMF^+ pots	Amora-Lazcano et al., 1998
Sorghum bicolor (sorghum)	*Glomus mosseae, Glomus etunicatum, Glomus intraradices*	Development of AM mycelium in soil had little influence on the composition of the microflora in the hyphosphere, AM root colonization positively related with bacterial numbers in the hyphosphere and with the presence of *Pseudomonas* in the rhizosphere.	Andrade et al., 1997
Sorghum bicolor (sorghum)	*Glomus mosseae, Alcaligenes eutrophus, Arthrobacter globiformis*	Fluorescent pseudomonad numbers increased in the order Mycorrhizosphere> rhizosphere> hyphosphere> non-rhizospheric-AMF soil.	Andrade et al., 1998a
Cucumis sativus (cucumber)	*Glomus fasciculatum*	AMF decreased the rate of bacterial DNA synthesis and the bacterial biomass, and changed the spatial pattern of bacterial growth	Christensen and Jakobsen, 1993
Zea mays (maize)	*Glomus mosseae*	Total microbial community similar in mycorrhizosphere and rhizosphere but proportion of Fe- or Mn-reducers was 20 to 30 times lower in the mycorrhizosphere	Kothari et al., 1991
Cucumis sativus (cucumber)	*Glomus intraradices*	No significant difference in total bacterial number between AMF+ and AMF- treatments. Small effect of AMF on the bacterial community structure : major differences were observed for a few bacterial species such as *Paenibacillus* spp.	Mansfeld-Giese et al., 2002
Zea mays (maize)	*Glomus mosseae, Glomus intraradices*	AM infection changed the bacterial community DGGE profiles in the rhizosphere and the differences increased with time. Two fungal conditions had similar bacterial communities after 4 weeks, differed after 7 weeks.	Marschner et al., 2001
Zea mays (maize)	*Glomus mosseae, Glomus intraradices*	Mycorrhizal colonisation changed the bacterial community DGGE profiles on the root and in the non-rhizospheric soil.	Marschner and Baumann, 2003
Zea mays (maize) *Trifolium subterraneum (subterranean clover)*	*Glomus fasciculatum*	Total bacterial counts increased in rhizoplane of AMF^+ plants. More facultative anaerobic bacteria, less fluorescent pseudomonads, *Streptomyces* spp and chitinase-producing actinomycetes decreased in rhizospheric soil of AMF plants	Meyer and Linderman, 1986b
Cucumis sativus (cucumber)	*Glomus invermaium, Glomus caledonium*	Bacterial numbers not affected by the AM mycelium. Bacterial PLFAs not affected by the presence of mycorrhiza	Olsson et al., 1996
Zea mays (maize)	*Glomus mosseae*	In the rhizosphere soil, the total microbial population was higher in AMF treatments, but the proportion of Mn-reducing microbial populations was 5x lower	Posta et al., 1994
Glycine max (soybean)	*Glomus etunicatum, Glomus mosseae, Gigaspora rosea*	Differences in Gram negative or Gram positive bacteria between AMF^+ and AMF^- treatments. Decrease of nodulation in AMF plants	Schreiner et al., 1997
Panicum maximum (guinea grass)	*Glomus fasciculatum* *Gigaspora margarita* *Acaulaspora laevis* *Sclerocystis dusii*	Total bacterial populations and nitrogen fixing bacterial numbers and Gram negative bacteria were significantly higher in 3 AMF treatments and urea hydrolysers increased in the 4 AMF treatments	Secilia and Bagyaraj, 1987
Trifolium subterraneum (subterranean clover) *Zea mays (maize)* *Allium porrum (leek)*	*Glomus intraradices*	AM colonisation had a low impact on bacterial activity in the mycorrhizosphere and affected differently bacterial numbers depending on the plant species. Only small effects of AM colonisation were detected with the PLFA technique and no effect was seen with Biolog.	Soderberg et al., 2002
Zea mays (maize)	*Glomus mosseae, Glomus deserticola,* Natural *AMF* *Azospirillum brasilense, Pseudomonas fluorescens, Trichoderma harzianum*	Mycorrhizal colonization induced qualitative changes in the bacterial community depending on the inoculant combination involved.	Vazquez et al., 2000

1.9 Bacterial diversity in agroecosystems

1.9.1 What is bacterial diversity?

Bacterial diversity generally refers to the genetic diversity, i.e. the amount and distribution of genetic information, within the bacterial communities. Diversity is a function of two components: 1. the total number of species present (species richness) and 2. the distribution of individuals among those species (evenness) (Margalef, 1968). Diversity indices characterise the species composition of the community at a given site and a given time (Legendre and Legendre, 1998). One of the most popular formulas for species diversity is the Shannon index (Shannon and Weaver, 1963).

$H' = -\sum_{i=1}^{q} pi \log pi$

For a given number of species, H' is maximum when the organisms are equally distributed among the q species. H' is lower when there is a stronger dominance of one or a few species. q is the number of species. pi is the relative proportion of species.

A diversity index cannot indicate the total makeup of a community. For example, two communities may have the same diversity index value but one may comprise a low evenness and a high richness and the other a high evenness and a low richness (Kennedy, 1999). In addition to the bacterial diversity, the bacterial community structure is related to its species composition and their relative abundance (Marschner et al., 2004). Perturbations can modify the structure of the community i.e. some species will be more abundant, others will disappear or remain stable. In studies that encompass several communities, species diversity or structure may be compared to environmental variables such as climate, pollution, physico-chemical parameters, plant species, etc… (Legendre and Legendre, 1998; Fromin et al., 2002 in Annex 1).

1.9.2 Importance of bacterial diversity in agroecosystems

The diversity of microorganisms in agroecosystems is critical to the maintenance of good soil health because they are involved in many important soil processes (Borneman et al., 1996). Moreover, species diversity can give rise to ecosystem stability through the ability of species or functional groups it contains to respond differentially and in a compensatory fashion to perturbations in the soil environment (Sturz and Christie, 2003). The bacterial community diversity or structure can be used as an indicator of these perturbations or disturbances in the agroecosystems. Disturbances could be caused by the presence of a plant (see paragraph 1.5) or changes in agronomic practices such as type of amendment (Workneh and van Bruggen, 1994; Kennedy et al., 2004; Marschner et al., 2004), reduced or no-tillage

(Ibeweke et al., 1998; Drijber et al., 2000; Höflich et al., 1999), irrigation system (Crrechio et al., 2004), monocropping (Cook, 1981), or crop rotation (Lupwayi et al., 1998; Larkin, 2003).

1.9.3 How to assess the bacterial diversity

The traditional method for the determination of microbial diversity consisted in identifying the culturable organisms in a soil system to the species level and use the taxonomic differences to measure diversity (Alexander, 1977). However, only a small fraction of the soil bacteria (1-10%) are cultivable (Nannipieri, 2003) and taxonomic differences based on physiological characteristics of isolated strains are not sufficiently discriminant (Torsvik et al., 1994). To overcome the biasis induced by culturability, fatty acid analysis have been used to study diversity (Zelles et al., 1995). Whole soil fatty acid methyl ester analysis (WSFAME) enables to examine the microbial community structure by assessing the lipid components from both live and dead microorganisms (Zelles et al., 1994). Phospholipid fatty acid analysis (PLFA) enables to estimate the structure of the living microbial community (Zelles et al., 1995; Zelles, 1999). However, even if fatty acid profiling provides information on certain microbial groups it does not permit detection at the specie level (Haack et al., 1994). Therefore, direct molecular approaches have to be used to obtain a broader image of the bacterial community in soil. Cloning and sequencing of the bacterial 16S ribosomal DNA can allow assess the bacterial diversity in soils with a high degree of discrimination (Borneman et al., 1996). Nevertheless, the cloning and sequencing techniques are time and resource consuming and many samples cannot be treated without a tedious procedure. Molecular fingerprinting techniques have then been developed to permit the simultaneous analysis of numerous samples. Amongst these techniques, denaturing gel electrophoresis (DGE) analysis of the 16S rDNA gene permits fingerprinting of the dominant bacteria of a given sample (Muyzer et al., 1993). The detection of populations representing as little as 0,1-1% of the total target organisms is feasible. The DGE patterns represent the relative abundance of the detectable bacterial populations and can relate to the biological structure of the bacterial community. For a more detailed explanation of the use of DGE fingerprinting in the environment, refer to Annex 1 of this thesis.

1.10 Objectives of the thesis

As mentioned in paragraph 1.1.2, the main goals of the ISCB projects SA6 and SA-7 were to develop new biotechnologies such as the use of PGPR and AMF bio-inoculants for improving plant growth and soil health in marginal rain-fed regions of India. However, a major setback in developing a large-scale use of PGPR bio-inoculations in low-input farming systems is due to the variability and inconsistency of the PGPR plant growth effect not only between laboratory studies and field applications but also between different fields. The interactions between the microflora and the plant and within the rhizosphere communities are complex and need to be clarified before successfully using PGPR and AMF dual inoculations. Studying the interactions between wheat, rhizobacteria and AMF should then help to determine criteria for a successful application of PGPR and AMF dual inoculations in low-input farming systems in Indian rainfed fields. Moreover, a successful introduction of effective PGPR strains in association with AMF in the fields required not only evidence of the establishment of the inoculants in the rhizosphere *in situ* but also that the strains did not have any deleterious effect on AMF development and if possible stimulate fungal growth.

The goal of this thesis was then to improve our knowledge on the interactions between wheat, rhizobacteria and AMF in the mycorrhizosphere in order to define criteria for the selection of PGPR strains in view of a PGPR and AMF dual inoculation in Indian wheat fields. To this end, its main objectives were:

1. Assessing the effect of AMF on the total and active rhizobacteria as well as on several PGPR functional guilds.

2. Determining if specific bacterial populations were associated with AMF spores.

3. Investigate the influence of specific PGPR strains on the mycorrhizal spread and development in the hyphosphere enabling to select putative mycorrhiza helper bacteria.

4. Before introducing selected PGPR strains as bioinoculants in field trials, test their root colonization ability.

5. Testing for the first time in field conditions selected PGPR strains as well as AMF bioinoculants confirming the positive interactions between PGPR strains and AMF. In parallel, studying the dynamics of the bacterial community according to the wheat growth stage, the field conditions and the AMF/PGPR co-inoculations.

2 Assessing the effect of AMF on the bacterial community

The first objective was to assess the effect of AMF on the bacterial community in the wheat mycorrhizosphere. This chapter comprises two studies:

- In the first study (chapter 2.1), we have built a multi-compartmented microcosm system establishing AMF and AMF-free conditions in the wheat rhizosphere to determine the effect of AMF on the bacterial community structure and on several PGPR functional guilds. In order to get a broader image of the bacterial community structure, the molecular fingerprinting technique DGGE was used and the fingerprints were analysed with numerical ecology statistics.
- In the second study (chapter 2.2), we have assessed the effect of AMF on the bacterial community structure not only taking into account the present but also the active populations. For this purpose, the analysis were not only performed on DNA-based DGGE profiles but also on RNA-based DGGE profiles. No other study yet reported has assessed the effect of the AMF on the active bacterial community molecular profiles.

2.1 Effect of a natural arbuscular mycorrhizal fungi inoculum on the bacterial community structure in the wheat mycorrhizosphere and in the hyphosphere

This chapter is a manuscript written by David Roesti[1*], Nathalie Fromin[1], Pierre Rossi[1], Fritz Oehl[2], Kurt Ineichen[2], Andres Wiemken[2] And Michel Aragno[1]. It has been submitted to FEMS Microbiology Ecology.

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2.1.1 Abstract

The goal of this study was to determine the impact of a natural arbuscular mycorrhizal fungi (AMF) inoculum on the bacterial community structure and on different plant-growth promoting rhizobacteria (PGPR) guilds in the wheat mycorrhizosphere and in the hyphosphere. An approach combining cultivable and molecular methods was used to assess the response of the bacterial community. In the experimental set-up, four zones of interest differing by their biological conditions were defined: the mycorrhizosphere, zone under the joint influence of roots and AMF hyphae, the rhizosphere, zone under the influence of the root, the hyphosphere, zone under the influence of AM hyphae and the soil control, containing only bulk soil. Three fractions were analysed at the flowering and maturity wheat growth stages: non-rhizospheric soil, rhizospheric soil, and rhizoplane/endorhizosphere. The bacterial community structure was predominantly influenced by the distance from the root and by the plant growth stage. Nevertheless, specific populations seemed either inhibited or stimulated in the presence of AMF. The influence of AMF on bacterial populations was probably indirect as no correlation was observed between the community structure and the hyphal length. The indirect influence of AMF on the bacterial community in the mycorrhizosphere was probably due to changes in root exudation rates or composition because of mycorrhizal root infection. Moreover, the presence of AM hyphae in the non rhizospheric soil affected the bacterial community structure indirectly by modifying the soil pH. Phosphate-solubilizing bacteria were strongly associated with the AMF in both non-rhizospheric soil and the mycorrhizosphere suggesting a preferential choice for phosphate-solubilizing bacteria in AMF-PGPR formulas used for low-P available agricultural soils.

2.1.2 Abbreviations

M	Mycorrhizosphere
R	Rhizosphere
H	Hyphosphere
C	Control
nrs	Non-rhizospheric soil
rs	Rhizospheric soil
re	Rhizoplane/endorrhizosphere
AMF	Arbuscular mycorrhizal fungi
PGPR	Plant growth promoting rhizobacteria
Sid+	Siderophore producing bacteria
PSB	Phosphate solubilizing bacteria
DGGE	Denaturing gradient gel electrophoresis
TMCB	Total mesophilic cultivable bacteria

2.1.3 Introduction

This study is integrated in a project of the Indo-Swiss collaboration in biotechnology (ISCB) whose main goals are to develop new biotechnologies such as the use of bio-inoculants for improving plant growth and soil health in marginal rain-fed regions of India. In Asia, the degradation of arable lands is a major threat to agricultural production (Pingali, 1997). In order to maintain economic crop production levels in a sustainable ecologically sound way, low input farming systems have to be developed (Bethlenfalvay and Linderman, 1992). In this context, the ability of arbuscular mycorrhizal fungi (AMF) to utilise organic forms of N and P in low-input organic farming becomes critical in order to maintain a sufficient productivity as the nutrient inputs in those systems come mainly from organic sources (Atkinson et al., 2002). Beneficial effects of AMF in agriculture also comprise a better plant nutrition and hydric stress resistance (Smith and Read, 1997), biological control against pathogens (Azcón-Aguilar and Barea, 1996) and better soil structure (Miller and Jastrow, 1990). Additionally, a comparison study between different field management practices in central Europe showed that an increase in land use intensity was correlated with a decrease in AMF speciess richness (Oehl et al., 2003).

Plant growth promoting rhizobacteria (PGPR) are beneficial to the plant via nutrient acquisition (Ladha and Reddy, 2003; Dobbelaere et al., 2003; Rodriguez and Fraga, 1999), biocontrol (Walsh et al., 2001; Chin-A-Woeng et al., 2003), plant hormone-like production (Glick, 1995; Steenhoudt and Vanderleyden, 2000) and induction of systemic resistance (van Loon et al., 1998). AMF and PGPR strains could therefore be used as biofertilizers, biocontrol agents and soil stabilisers in low input sustainable agriculture. However, when selecting the AMF and PGPR co-inoculants, care should be taken not to add antagonistic microorganisms that would compete with one another, thus reducing the potential benefits for the plant. The study and comprehension of the interactions between beneficial organisms in the rhizosphere such as AMF and PGPR may therefore provide valuable data for the selection of microbial inoculants to improve plant growth in sustainable agricultural systems (Bethlenfalvay and Linderman, 1992).

Interestingly, some PGPR have a plant beneficial effect in synergy with AMF. They include phosphate solubilizing bacteria or PSB (Toro et al., 1997; Barea et al., 2002c), nodule-forming N_2-fixing Rhizobia or free-living *Azospirillum* spp. (Biro et al., 2000; Barea et al., 1996) and *Pseudomonas* spp. (Vàzquez et al., 2000; Barea et al., 1998). These PGPR populations could be beneficial to AMF either by enhancing mycorrhizal root colonisation or

stimulating hyphal growth. These so-called mycorrhiza helper bacteria or MHB (Garbaye, 1994) are beneficial to ectomycorrhizae (Frey-Klett et al., 1997) or to endomycorrhizae (Gryndler et al., 2000). Among the mycorrhiza helper mechanisms, Garbaye (1994) hypothesised a modification of rhizospheric soil (change in pH or ion complexing by bacterial siderophores) and an increase in root receptivity (plant hormone-like or cell wall softening enzymes production), in root-fungus recognition, in fungal growth (production of organic or amino acids, vitamins, carbon dioxide, etc…) and in germination of fungal propagules.

Effects of AMF on the rhizosphere and surrounding soil probably influence the bacterial community. Indeed, the mycorrhizal infection modifies the rhizosphere functioning by changing the mineral composition and hormonal balance of the plant (Barea et al., 2002b) but also changes the root architecture by increasing root branching and apice size (Berta et al., 2002). The C allocation patterns of the plant are modified as more assimilates are transferred to the roots resulting from the carbon demand of the fungus (Schwab et al., 1984) and mycorrhizal roots have a higher respiration rate (Kucey and Paul,, 1982). In addition, root exudation could decrease in the presence of AMF as shown by Marschner et al (1997). In the surrounding soil, extraradical hyphae not only change the soil structure and physical properties such as aggregation (Miller and Jastrow, 1990; Wright and Upadhyaya, 1998; Andrade et al., 1998b) but could serve as a carbon source for microbial populations either by bacterial feeding on senescent hyphae or by their release of exudates (Barea et al., 2002b).
To date, most of the studies regarding the effect of AMF on bacterial communities have been performed on cultivable micro-organisms. Only a small fraction (1-10%) of the total bacterial community is cultivable (Amann et al., 1995). Direct molecular-based approaches allow a more complete picture of the bacterial community. PCR-DGGE analysis on the 16S rDNA gene permits to fingerprint the dominant bacterial populations (detection of populations representing as little as 0,1-1% of the total target organisms) of a given sample (Muyzer et al., 1993; Fromin et al., 2002). Such fingerprinting methods do not allow to isolate beneficial bacterial strains for agricultural purposes but help to identify organisms related with AMF as shown by the detection of uncultivable *Tuber* associated symbionts (Barbieri et al., 2000).
In most reports dealing with the influence of AMF on the bacterial community, the inoculum was composed of one or two fungal strains. In the present study the mycorrhizal inoculum was composed of 18 different species present in an organic managed wheat field. The goal of this study was to determine the impact of a natural AMF inoculum on the bacterial community in the mycorrhizosphere and the hyphosphere. In order to limit the complexity of a field trial, the experiment was performed in microcosms. An approach combining cultivable and molecular methods was used to assess the response of the bacterial community.

2.1.4 Materials and Methods

Microcosm system design

Plants were grown in polypropylene boxes (20 x 30 x 20 cm, Plastic-Haus AG, Arlesheim, Switzerland) that were equipped at the bottom with a 20-mm thick drainage mat (Enkadrain ST, Schoellkopf AG, Zurich, Switzerland). Each box contained 9 L of a sandy loam soil (organic managed wheat field for 3 years after ploughing a calcareous grassland at the Botanical Garden Neuchâtel) and coarse quartz sand respectively (1:1, v/v) mix that was tyndallised at 80°C for 2 h twice at 24h interval. The soil/sand mix after tyndallisation had a pH of 8,24 and contained (mg kg^{-1}): NH_4-N, 18,5; NO_3-N, 16,9; $NaHCO_3$-extractable P, 9,95.

Four zones of interest differing by the biological conditions were defined (fig.1): the mycorrhizosphere (M), zone under the joint influence of roots and AM hyphae, the rhizosphere (R), zone strictly under the influence of the root, the hyphosphere (H), zone strictly under the influence of AM hyphae and the soil control (C), zone containing only bulk soil.

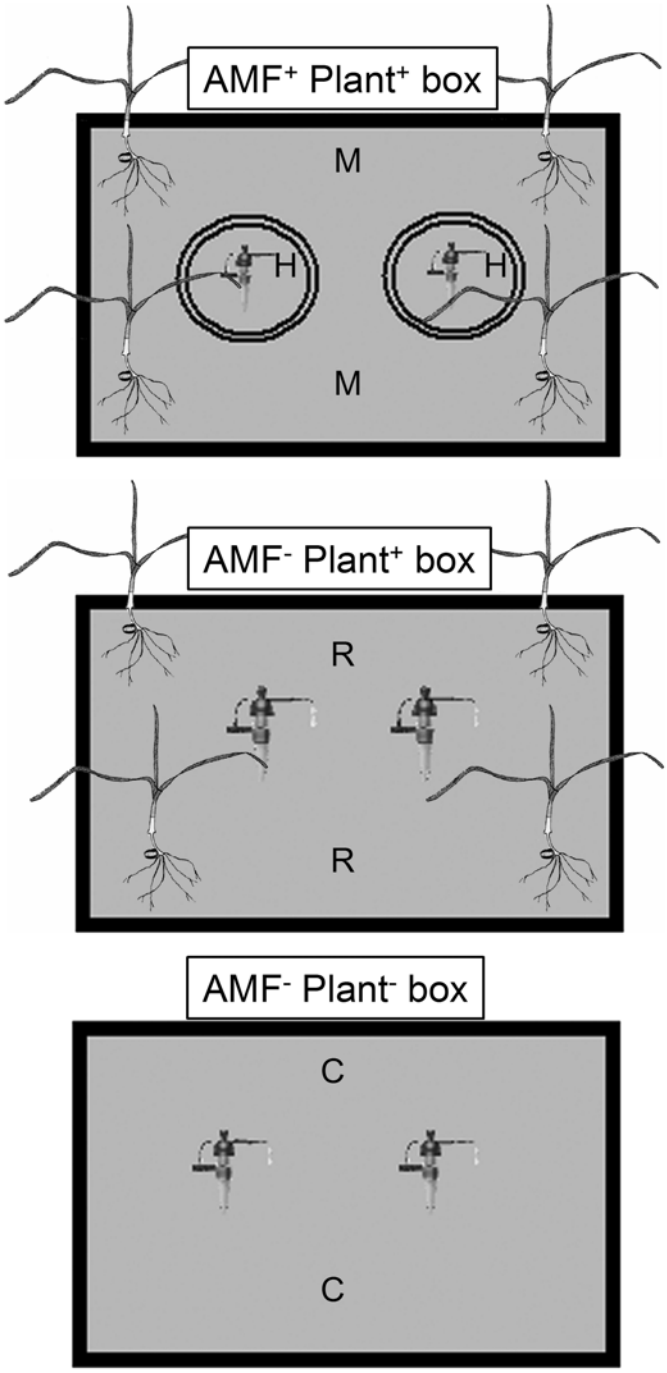

Fig.1. Microcosm box set-ups with the different zones of interest. AMF^+Plant^+ box, contains 4 wheat plants and AMF inoculum, M = mycorrhizosphere, H = hyphosphere. AMF^- $Plant^+$ box contains 4 wheat plants and no AMF, R= rhizosphere. AMF^- $Plant^-$ box containing only soil, C = soil control. In M and R, three fractions were analysed: nrs = non-rhizospheric soil; rs = rhizospheric soil; re = rhizoplane/endorrhizosphere. In H and C only the nrs was analysed.

Three different experimental conditions were set to study these zones (fig.1). The boxes in which 4 wheat plants (*Triticum aestivum L*. var. UP2338) were grown on each corner and that received mycorrhizal inoculum were designated AMF^+ $Plant^+$ boxes. They hosted the zones in relation with AMF: the mycorrhizosphere (M) and the hyphosphere (H). The hyphosphere zone was constructed as followed: two layers of 30 μm mesh nylon membranes were glued on each side of a 0,5 mm mesh nylon membrane used as mounting piece. The triple layer sheet was then folded circularly and glued in order to obtain a cylinder. The bottom part of the cylinder was sealed with a plastic sheet in order to prevent roots from passing in the compartment. Two cylinders were then placed in AMF^+Plant^+ boxes and filled with the soil matrix. The boxes in which 4 wheat plants were grown and which did not receive mycorrhizal inoculum were designated AMF^- $Plant^+$ boxes. They hosted the rhizosphere (R) zone. Finally, the boxes containing soil without plant nor AMF were designated AMF^- $Plant^-$ boxes and were used as soil control (C). Six replicate boxes were prepared for each experimental condition.

From the M and R zones of the plants root system, three different fractions were distinguished:

- non-rhizospheric soil (nrs): soil without root (soil particles that detached after vigorous shaking of the roots)
- root-adhering rhizospheric soil (rs): soil particles that remained attached to the root, and recovered by washing the roots in sterile 0.9% NaCl solution
- rhizoplane/endorhizosphere (re): washed roots without soil particles.

Additionally, for the H and C zones, only the nrs fraction was available.

Incubation conditions

Plants were grown from 1st June to 7th August in a temperate greenhouse at the Botanical Garden of Neuchâtel. Plants were watered using Blumat ceramic watering system (Weninger GmbH, Telfs, Austria) linked to 2 L polypropylene flasks (Merck Eurolab SA, France) containing sterile tap water. At 15 days, 2 L of an autoclaved nutrient solution ($Ca(NO_3)_2 \cdot 4H_2O$ 1mM; KH_2PO_4 0,1mM; K_2SO_4 0,75mM; $Mg(SO_4) \cdot 7H_2O$ 0,65mM; $FeCl_2 \cdot 4H_2O$ 0,1mM) replaced the tap water in order to prevent nutrient deficiency. No artificial sunlight was used but the temperature was controlled to remain between 25 and 30°C.

Biological materials

The mycorrhizal inoculum was composed of 25 g of non-sterilised wheat field soil. The AMF spore extraction and determination was undertaken according to Oehl et al. (2003). The

following species were found in the inoculum: *Glomus mosseae*, *G. geosporum*, *G. diaphanum*, *G. constrictum*, *G. etunicatum*, *G. versiforme*, *G. intraradices*, *G. fasciculatum*, *G. aureum*, *G. invermaium*, *G. microcarpum*, *G. sinuosum*, *G. sp. BR4*, *Paraglomus occultum-like*, *Entrophospora infrequens*, *Scutellospora calospora*, *S. fulgida*, *S. castanea.*

Wheat seeds were surface sterilised with calcium hypochlorite and H_2O_2, soaked in sterile water for 5-8 hours and pre-germinated on sterile Whatman paper. After 24 hours, the seedlings were selected according to their root length uniformity and 4 were sowed (depth ~2 cm) in each box. In the microcosm box containing the AM fungi (AMF^+Plant^+), the seedlings were placed on top of the mycorrhizal inoculum. A bacterial inoculum was prepared by suspending 200 g of the wheat field soil in sterile water and after the soil particles had sedimented, the supernatant was filtered firstly on a 30 μm mesh and then through a S&S filter paper (Schleicher & Schuell GmbH, Germany) to eliminate mycorrhizal spores and hyphae. 200 ml of the mycorrhizal-free bacterial suspension (1,5 x 10^6 CFU/ml, counted on modified Angle's medium, Tarnawski et al., 2003) was added in all the boxes.

Sampling procedure

All the samples were collected with sterilised instruments. The samples were taken at the flowering (40 days) and maturity (68 days) wheat growth stages (destructive sampling). Three boxes out of the six initial replicates of each experimental condition were used at the flowering stage for analysis and the remaining three at the maturity stage. The different zones were then obtained per sampling date as following: the mycorrhizosphere (M) was composed of the 12 plants with their root system and 100 g of non rhizospheric soil from each of the three AMF^+Plant^+ boxes. The rhizosphere (R) was composed of the 12 plants with their root system and 100 g of non rhizospheric soil from each of the three AMF^-Plant^+ boxes. The hyphosphere (H) comprised the soil of the 2 cylinders from each of the three AMF^+Plant^+ boxes. Finally the soil control (C) was composed of 100 g from each of the three AMF^-Plant^- boxes.

Each sample was then divided in homogenous parts. The first part was immediately used for the plant and mycorrhizal assays, available nitrogen and phosphorus measures as well as for bacterial counts. The second part was dried, sieved and grinded for further soil analysis and the third was stored at –80°C for consecutive DNA extraction.

Bacterial counts

Bacterial counts were performed on three sub-samples from each zone. The nrs suspension was prepared by adding 20 g of nrs in a glass bottle containing 100 ml of sterilised 0,9% NaCl solution. The bottle was then closed and shaken vigorously during 30 seconds to resuspend the soil particles. To separate the rs from the re fractions, the roots with the adhering soil were immerged into 0,9% NaCl solution and stirred. The washed roots were removed and the remaining suspension constituted the rs suspension. The roots were rinsed with 0,9% NaCl solution and dried on sterile Whatman paper (Merck AG) to remove the excess rinsing solution. 1 g of roots was crushed steriliy in 10 ml 0,9% NaCl solution using a mortar and pestle, constituting the re suspension. Nrs, rs and re suspensions were then serially diluted (1:10) in sterile 0,9% NaCl solution and 0,1 ml of the appropriate dilution was spread on agar medium Petri plates in triplicate. The following plating media were used for bacterial counts: modified Angle's medium (Tarnawski et al., 2003) for total mesophilic cultivable bacteria, modified PSB medium (Kim et al., 1997) for phosphate solubilizing bacteria ($Ca_3(PO4)_2$ was used as P source instead of hydroxyapatite on the upper layer of a double-layer plating medium), modified CAS medium (Schwyn and Neilands, 1987) for siderophore producing bacteria (containing Angle's solution 10x and 0,2% glucose as nutrient source) and modified S1 medium (Tarnawski et al., 2003) to select *Pseudomonas* strains. Colony forming units (CFUs) were counted after incubation at 24°C for 72 h and calculated per g dry soil or root.

Soil physico-chemical parameters

Physico-chemical parameters analysis were performed on three sub-samples from each zone. Fresh non-rhizospheric soil was immediately placed in an incubation chamber at 105°C for 48 h to measure the soil moisture. In parallel, it was also analysed for NO_3^- and NH_4^+ (modified Kjeldahl method in Allen et al., 1974) and for $NaHCO_3$ extractable phosphorus content using Olsen's method (Olsen et al., 1954). Dried, sieved (1 mm mesh) and grinded nrs was analysed for pH (measured after suspending 20 g of crushed soil in 50 ml deionized water), total nitrogen with Kjeldahl's method (Schinner et al., 1996) and organic carbon (Aubert, 1978).

Plant and mycorrhiza assays

The plant shoot biomass and seed weight was performed on each of the 12 plants per M or R zone. Mycorrhizal colonization assay was performed on three sub-samples from each zone. After harvesting, the plant shoots were weighed for fresh biomass and dried at 105°C during 24 h for dry biomass. At maturity, all the seeds were enumerated and weighed.

Mycorrhizal structures were stained with 0,05% w/v trypan blue after having cleared the roots with KOH 10% according to Brundrett et al. (1994). Mycorrhizal colonization percentage was evaluated by the grid-line intersect method (Giovanetti and Mosse, 1980). Hyphal length in the non- adhering soil was measured from 5 g of nrs sample by the gridline intersect method (Sylvia, 1992) modified by Andrade et al (1998b).

Bacterial community analysis

DNA extraction for the DGGE analysis was performed on two sub-samples from each zone. DNA from about 0,5 g of nrs, rs and re samples was extracted using the FastDNA Spin Kit for soil (Bio101, Vista, USA) according to the manufacturer's protocol using a bead beater (Fast-Prep, Model FP 120, Bio101). The DNA extracts were then stored at –20°C until further analysis. A double step PCR was used to amplify the V3 region of the bacterial 16S rDNA. The amplification reaction mix was composed of (final concentration): 1x Thermophilic DNA polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Gibco), 0.25 μM of each primer (Microsynth, Balgach, Switzerland) and 0.05 U/μl Taq polymerase (Promega). 0.1-1ng/μl (final concentration) of extracted DNA was used as template for a 20 μl reaction. Bacterial primers GM3f and GM4r were first used to amplify the 8 -1492 region of the 16S rDNA (Muyzer et al., 1995). The first PCR was carried out with an initial denaturation step at 94°C for 4 min, followed by a touchdown PCR of 11 cycles consisting of denaturation at 94°C for 30 sec, annealing from 56°C to 51°C for 30 sec, elongation at 74°C for 1 min. The touchdown was followed by 15 cycles comprising denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec and elongation at 74°C for 1 min. The process was completed by a final elongation step at 74°C for 10 min. Amplicons were diluted 10 times in sterile deionised water. Then the nested amplification was carried out with the universal primers 338f and 520r (Ovreas et al., 1997) that target the V3 region of the 16S rDNA gene. It comprised an initial denaturation step at 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 65°C for 30 sec with a touchdown rate from 65°C to 55°C during 10 cycles and elongation at 74°C for 1 min. A final elongation step at 74°C for 10 min completed the PCR.

A composite mix of different bacterial 16S rDNA fragments was added on each side of the DGGE gel as a reference DGGE pattern ordered as followed after migration: *Pseudomonas fluorescens* ATCC 27663, *Acidovorax facilis* DSM 550, *Bacillus subtilis* ATCC 14893, *Sphingomonas capsulata* DSM 30196, *Sinorhizobium meliloti* DSM 1981, *Aquaspirillum dispar* ATCC 27510 and *Arthrobacter globiformis* DSM 20124. DGGE was performed with a 8% (w/v) acryl-bisacrylamide gel (37,5:1, Qbiogene, Illkirch, France) with 30-60% linear urea/formamide (Fluka, Buchs, Switzerland, Qbiogene) denaturing gradient (100%

denaturant corresponds to 40% formamide + 7 M urea). 500 ng of the PCR product were electrophorated in 1x TAE buffer (Qbiogene, France) at 60°C with a constant voltage of 150 V during 5,5 hours using the BioRad D-Code Electrophoresis System (Bio-Rad Inc. California, USA). The gels were stained in the dark for 20 min in 0,01% Sybr Green I (Molecular Probes, Leiden, The Netherlands) in 1x TAE solution. The gels were photographed with the Multi-Analyst package (Bio-Rad Inc., California, USA). The DGGE fingerprints were normalised according to the reference patterns and were compared using the GelCompar software (Applied Maths, Kortrijk, Belgium). The amplified product of each sub-sample was loaded on two different DGGE gels. The banding pattern of each sub-sample took into account only the bands common to both DGGE patterns. DGGE banding patterns were then converted into a numerical matrix used in the statistical analysis. Each band was considered as corresponding to a single bacterial population and the band intensity was representative of the relative abundance of the population (Fromin et al., 2002). The bands whose average relative contribution was below 1% were discarded.

Selection and sequencing of AMF-associated DGGE bands

Bands only present or more intense in the mycorrhizosphere as compared to the rhizosphere in rs and re fractions were cut, cloned and sequenced with the following procedure: the selected bands were cut out and placed in a 1,5 ml Eppendorf tube containing 100 µl Tris-HCl 10 mM pH 7,5 and incubated at 4°C for 3 days. The supernatant was recovered in a new Eppendorf tube. One volume of iced isopropanol (-20°C) and 1/10 volume of sodium acetate 3 M were added and this mix was incubated at -20°C for 1 day. After centrifugation at 13'000 rpm at 4°C for 30 minutes, the supernatant was discarded. The pellet containing the DNA was washed with 1 volume of 100% ethanol and then centrifuged at 13'000 rpm for 30 minutes. The supernatant was completely removed and the pellet was air-dried for 15 minutes. DNA was resuspended in 50 µl Tris-HCl 10 mM pH 7,5. The V3 region of the DNA was then re-amplified according to the PCR protocol described above. Again, the amplified products were loaded on a DGGE gel to improve DNA yield and check band purity. If the band on this second gel matched the previously selected one, it was cut out, purified and re-amplified the same way. The amplified products were then purified with the *NUCLEOTRAP-CR kit* (Macherey-Nagel, Düren Germany) according to the manufacturer's protocol. The DNA fragments were ligated using the pGEM®-T Vector System (Promega), following the protocol of the manufacturer. Transformation was performed by electroporation using the *Bio-rad Gene Pulser XCell* and *PC module* into *E. coli XLI-Blue*. The transformed bacterial cells were then plated onto Luria–Bertani (LB) agar containing ampicillin (150 µg/ml), X-Gal (0,1 mM) and IPTG (0,2 mM). Plasmids were recovered from white colonies using the NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer's protocol. The

inserts were then amplified using a T7 labeled primer with the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies, Madison USA) and sequenced on a LI-COR 4000L by MWG Biotech (Ebersberg Germany). Three clones per band were sequenced and only the bands having similar sequences within these 3 clones were presented in the results. The percentage homology with existing 16S rDNA sequences in the *Genbank* database was determined using the BLASTt software (Altschul et al., 1997: http://www.ncbi.nlm.nih.gov). The sequences have been submitted to the EMBL Nucleotide Sequence Database at accession n° **AJ845024** to **AJ845032**.

Statistical analysis

Statistical significance for the differences between values for the plant and mycorrhiza data, physico-chemical parameters and bacterial counts was determined with the Student's t tests. Statistical significance of the difference between proportions of the tested functional guilds to total mesophilic cultivable bacteria was determined with the unpaired Wilcoxon rank sum test using the Splus software (Insightful Corporation, Seattle, USA).

Principal Component Analysis (PCA) was used to analyse the relations between the different samples with reference to their soil parameters. The zones correspond to the objects and the physico-chemical parameters to the descriptors (represented by the vectors) of the multivariate analysis. Since the descriptors were measured in different units, they were standardized before the analysis. The PCA was scaled as a correlation biplot.

To analyse the relations between the DGGE patterns of the different zones, correspondence analysis (CA) is a more suitable model than PCA. This ordination method is adapted to analyse presence/absence or abundance data tables and is well suited for populations with unimodal distribution along environmental gradients (Fromin et al., 2002). To obtain the CA, the data matrix was composed of rows of objects representing the zones and columns of species representing the DGGE band position along the vertical gel gradient. The relative abundance of a species in a zone corresponded to the DGGE band's relative intensity with regards to the sum of all band intensities in a pattern.

The ordination methods were applied on the basis of numerical data matrices converted using the program Progiciel R (Legendre and Vaudor, 1991). From the association matrix obtained, the characteristic values associated with the characteristic vectors were calculated using a multidimensional dispersion cloud of the data with the Canoco 4.0 software (Canoco 4.0, Microcomputer Power, Ithaca, USA). Finally, Mantel tests were performed with the Progiciel R to determine the correlation between soil parameters and the bacterial counts or DGGE numerical data matrices.

2.1.5 Results

Plant and AMF data

No significant influence of AMF was observed when comparing plant biomass of mycorrhizal plants (Mean ± standard deviation; 0,349 g ± 0,031) and none mycorrhizal plants (0,396 g ± 0,047) at the maturity stage. Nevertheless, the weight per seed was higher for the non-mycorrhizal plants (42,7 mg ± 8,3) as compared to mycorrhizal ones (30,7 mg ± 5,61). At the flowering stage, the AMF colonisation percentage of the roots was (49,1% ± 5,1) in the mycorrhizal plants and arbuscules were observed in the cortical cells of the root under the microscope. No AMF colonisation of the root was visible in non mycorrhizal plants. At the maturity stage, no arbuscule was visible in mycorrhizal plants but infection by AMF caused morphological changes in the wheat root (evidence of root cortical senescence, residual hyphae and spores) as observed by Liljeroth (1995) and Fester et al. (1999). The hyphal length (table 1) measured in the non AMF zones (R and C) was probably due to AMF hyphal residues in the soil matrix as well as saprophytic fungi. The hyphal length was also lower in the hyphosphere as compared to the mycorrhizosphere probably because it was more difficult for the hyphae to pass the double-layer membrane.

Physico-chemical parameters

In order to represent differences between the zones, the physico-chemical data were analysed by principal component analysis (fig.2). The first axis or principal component (PC1) represents the largest part of the original variance (43,4%) and the second axis (PC2) represents 42,2% of the variance. The vectors representing the NO_3^- and the NH_4^+ parameters are oriented in the same direction (positive correlation) but are in the opposite direction with regards to organic C (negative correlation). A negative correlation was also observed between the pH and the available phosphorus. The orthogonal projection of an object on a descriptor allows to approximate the correlation between that object and the descriptor. Globally, the maturity stage samples were positively associated with the pH value and negatively with the amount of available phosphorus. Moreover, the samples of the zones containing plants (M and R) were negatively associated with the organic carbon and total nitrogen as compared to the hyphosphere and control. Indeed, total N and organic C values were significantly lower at the maturity stage in plant-associated zones (M + R) as compared to the hyphosphere and soil control (table 1).

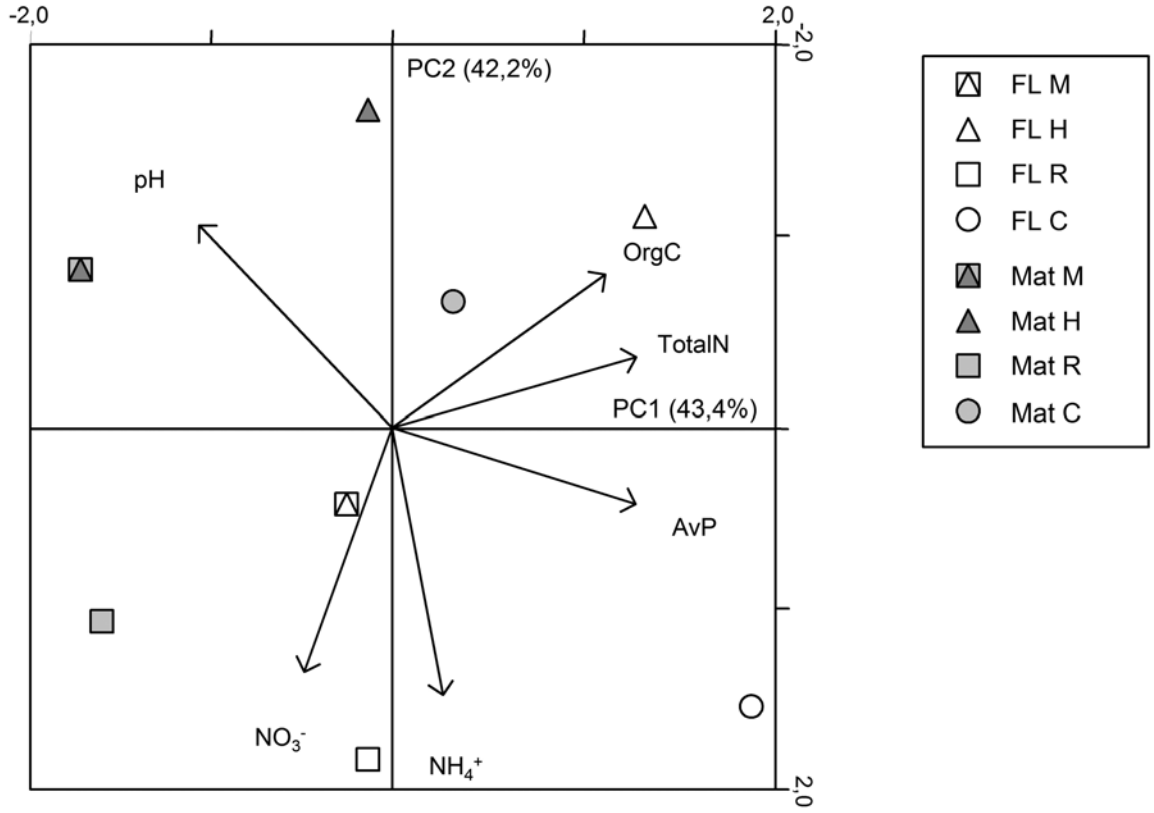

Fig.2. Ordination plot generated by principal component analysis representing the relationship between the zones and the physical-chemical parameters measured in the non rhizospheric soil. Description vectors correspond to: Av. P = available P; NH_4^+ = ammonium; NO_3^- = nitrate; Org. C = organic carbon; pH; total N = total nitrogen. Objects correspond to: M = mycorrhizosphere, H = Hyphosphere; R = rhizosphere; C = soil control, at two different wheat growth stages: Fl = flowering; Mat = maturity. Open symbols, flowering stage; grey symbols, maturity stage. Values on the axes indicate % of total variation explained by the axes. PC1= principal component axe 1; PC2 = principal component axe 2.

Table 1: Hyphal length, pH, nitrogen, organic C and available P measured in the different zones at flowering (fl) and maturity (mat) growth stages in non rhizospheric soil. M = mycorrhizosphere, H = Hyphosphere; R = rhizosphere; C = soil control. Values are indicated per g or kg of dry non rhizospheric soil. Values are means ± standard deviation of 3 sub-samples per zone. Column values with the same letter for a given growth stage were not significantly different according to student's t test ($p< 0,05$).

Zones	Hyphal length (m/g)	pH	Total N (mg/kg)	NH_4^+ (mg/kg)	NO_3^- (mg/kg)	Organic C (mg/kg)	Available P (mg/kg)
M fl	2,12±0,14c	8,01±0,04b	534,4±30,4a	10,25±0,81a	14,62±2,74b	2329,9±418,0b	7,02±0,57a
H fl	0,90±0,18b	7,98±0,03b	926,9±17,4d	5,51±3,60a	4,80±1,89a	3516,7±337,0c	7,76±0,58a
R fl	0,17±0,02a	7,85±0,02a	730,0±13,0b	21,18±5,41b	15,96±2,99b	925,8±184,7a	7,09±0,72a
C fl	0,19±0,02a	7,60±0,04a	807,3±39,8c	16,19±4,41b	7,70±0,28a	2138,0±678,0b	10,05±0,62b
M mat	2,15±0,20c	8,29±0,06b	437,7±21,7a	5,65±0,56a	6,02±1,29b	992,5±574,5a	5,73±1,08a
H mat	0,91±0,21b	8,30±0,04b	772,2±17,1b	4,28±1,08a	1,33±0,65a	2265,2±26,2b	6,17±0,42a
R mat	0,13±0,01a	8,02±0,03a	471,1±22,1a	13,04±1,37b	14,49±3,86b	407,2±260,8a	5,88±0,71a
C mat	0,11±0,03a	7,98±0,04a	863,4±19,7c	10,61±0,68b	3,33±2,56a	2131,0±423,2b	9,44±0,73b

The pH was significantly higher and the NH_4^+ significantly lower in the mycorrhizosphere and hyphosphere as compared to the rhizosphere and soil control at both growth stages (table 1). At the flowering stage, the concentration of NO_3^- was similar between plant-associated zones

(M and R) but was significantly lower in the hyphosphere and soil control (table 1). At the maturity stage however, the concentration of NO_3^- diminished also in the M zone. Finally, soil moisture was slightly lower in the root-containing soils (8-9% of the weight) than in the hyphosphere and soil control (12-14% of the weight), probably due to plant evapotranspiration.

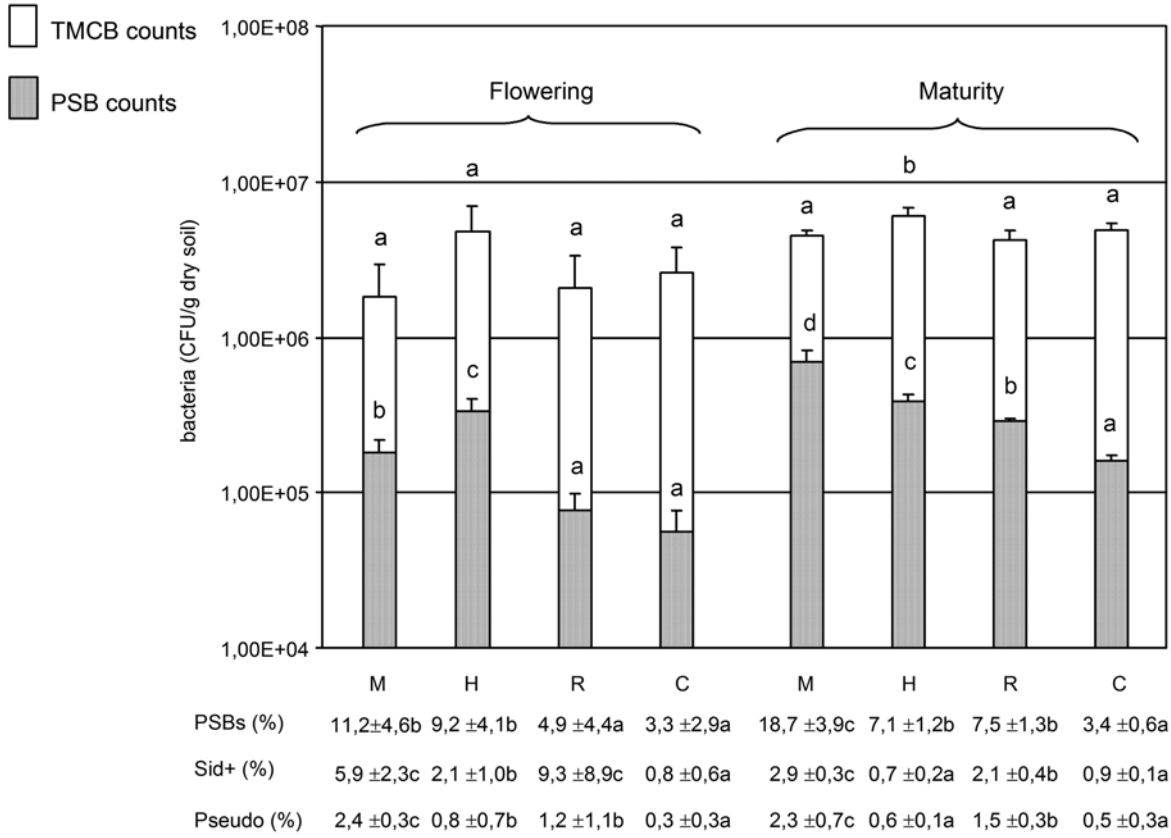

Fig.3. Total mesophilic cultivable bacteria (TMCB) and phosphate solubilizing bacteria (PSB) counts in nrs fraction for the different zones at flowering and maturity stages, M = mycorrhizosphere, H = Hyphosphere; R = rhizosphere; C = soil control. Numbers below the columns indicate percentage ratio of bacterial guild counts as compared to TMCB counts for PSB: phosphate solubilizing bacteria; Sid+: Siderophore producing bacteria; Pseudo: *Pseudomonas* populations. Values are indicated per g dry soil. Values are means ± standard deviation of 3 sub-samples per zone. Identical letters within a same growth stage indicate non-significantly different counts according to student's t test ($p < 0,05$) or non-significantly different proportions using the unpaired Wilcoxon rank sum test ($p<0,05$).

Bacterial counts

The total mesophilic cultivable bacteria (TMCB) counts were not significantly different between the various zones in the non rhizospheric soil except in the hyphosphere at the maturity stage where significantly higher counts were obtained (fig.3). In other fractions, TMCB counts were significantly lower in the mycorrhizosphere for rs (maturity stage) and re (both stages) (fig.4).

As well as TMCB, three different cultivable bacterial guilds were enumerated. A guild is a group of organisms sharing common characteristics (either taxonomic affiliation or a function) in a given habitat. The guilds studied were *Pseudomonas* spp. populations (Pseudo) because this genus contains many strains having interesting properties regarding plant growth promotion and rhizosphere colonization (Lucy et al., 2004), siderophore producing (Sid+) and phosphate solubilizing bacteria (PSB) for their role in phosphorus availability (Rodriguez and Fraga, 1999). These different bacterial guilds were expressed as their percentage to TMCB in a defined sample.

The proportion of siderophore producing bacteria and *Pseudomonas* spp. in the nrs fraction was significantly higher for root related zones (fig.3). This finding could indicate an effect exerted by the root on the different bacterial populations. PSB populations seemed to be strongly associated with AMF. Indeed, at the maturity stage, the proportion of PSB in the mycorrhizosphere reached 19 % and 26% in nrs and re fractions respectively (fig.3 and 4). In addition, the proportion of PSB in the nrs was significantly higher in the hyphosphere zone as compared to R and soil control at the flowering stage (fig.3). This result indicates a probable elective effect of AMF on PSB populations.

DGGE analysis

The DGGE pattern relates more to the bacterial community structure (i.e. to the relative abundance of the main bacterial populations) than to its total richness (Muyzer and Smalla, 1998). Correspondence analysis (CA) of the DGGE patterns for the nrs, rs and re fractions at flowering and maturity stages shows that the bacterial community structure of the different zones were mostly related to the fraction analysed (nrs, rs or re) and to a lower extent to the growth stage (flowering and maturity) (fig.5). The total inertia value of the CA indicates that the different patterns shared few common bands, i.e that the three fractions harbour very distinct bacterial populations.

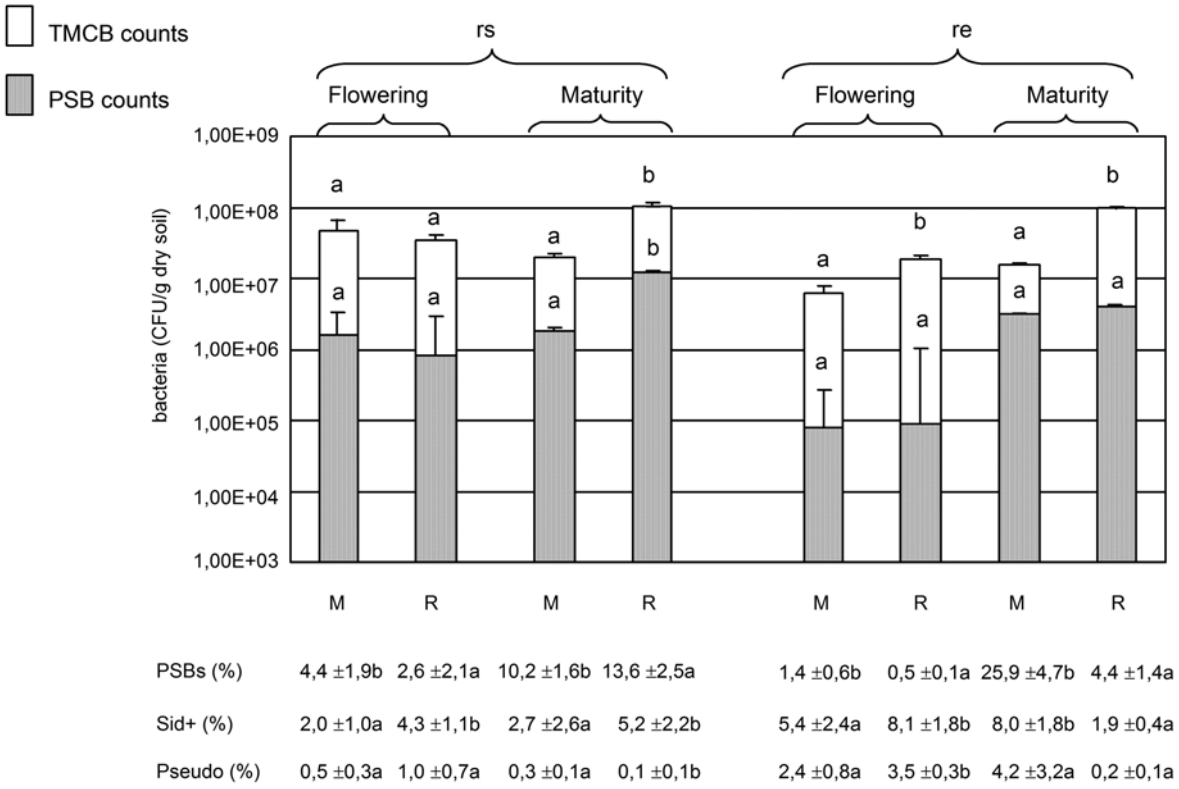

Fig.4. Total mesophilic cultivable bacteria (TMCB) and phosphate solubilizing bacteria (PSB) counts at the flowering and maturity growth stages in the rhizospheric soil (rs) and rhizoplane/endorhizosphere (re) fractions. M = mycorrhizosphere; R = rhizosphere. Numbers below the columns indicate percentage ratio of bacterial guild counts as compared to TMCB counts for PSB: phosphate solubilizing bacteria; Sid+: Siderophore producing bacteria; Pseudo: *Pseudomonas* populations. Values are means ± standard deviation of 3 sub-samples per zone. Identical letters within a same wheat growth stage and fraction indicate non-significantly different counts according to student's t test ($p < 0,05$) or non-significantly different proportions using the unpaired Wilcoxon rank sum test ($p<0,05$).

The pattern of the soil control differed from those of the other zones in the nrs especially at the flowering stage. The influence of the plant on its surrounding soil might be maximal at the flowering stage where the plant activity and root exudation was probably at their greatest level. Since the bacterial community of the soil control was not affected by the presence of the plant, the discrepancy between the DGGE patterns of this zone and the others was high. In addition, the DGGE patterns of the flowering and maturity stages of the soil control were very different. To explain such a difference we hypothesise that different T°C and humidity, affecting the development of the bacterial community, were occurring in the greenhouse between the flowering stage and the maturity stage.

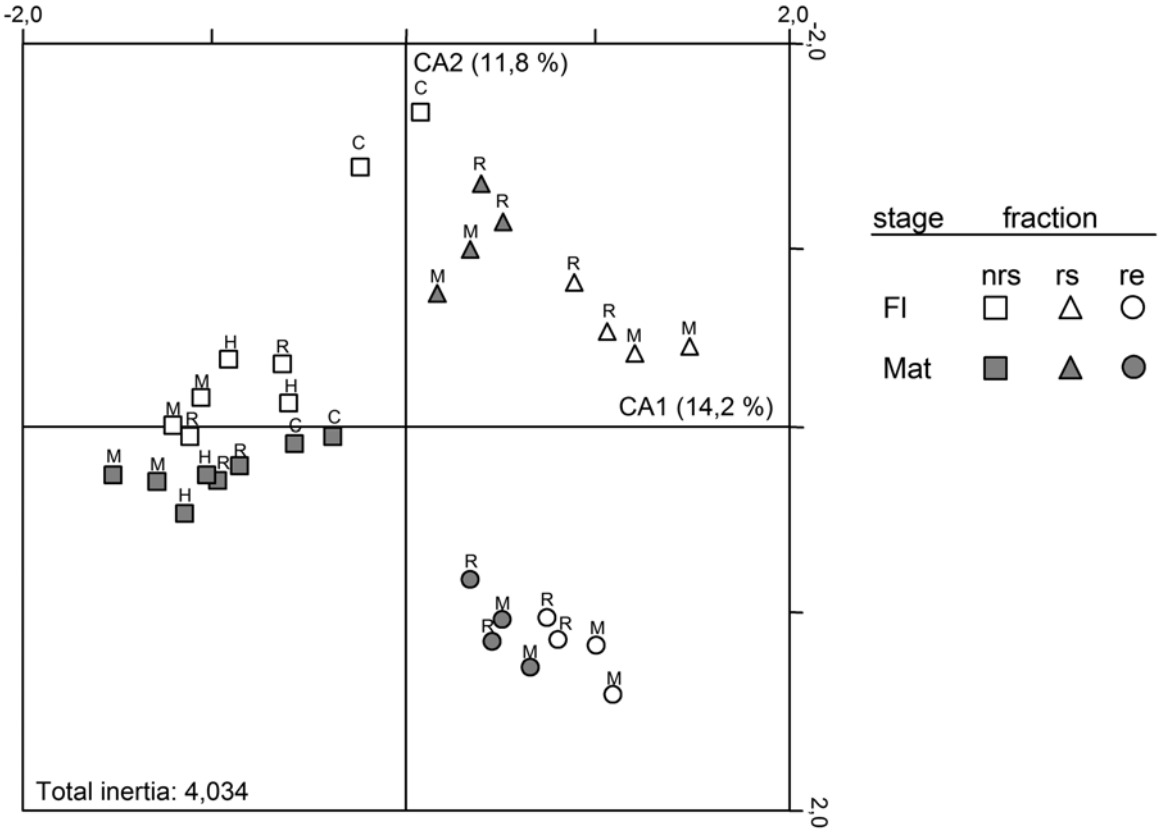

Fig.5. Ordination plot generated by correspondence analysis representing the relationship between the bacterial communities defined by the DGGE patterns of the different zones in the non-rhizospheric soil, rhizospheric soil and rhizoplane/endorrhizosphere. Two extraction replicates per zone have been integrated. M = mycorrhizosphere, H = Hyphosphere; R = rhizosphere; C = soil control. Pseudo: *Pseudomonas* populations. Values on the axes indicate % of total variation explained by the axes. CA1= correspondence analysis axe 1; CA2 = correspondence analysis axe 2.

Even though the main factor influencing the bacterial community structure seemed to be the root, several bands were only present or more intense in the AMF zones, indicating an effect of the AMF on specific bacterial populations, as shown on DGGE patterns of the mycorrhizosphere and rhizosphere samples at flowering and maturity (fig.6). Nine bands that were only detected or more intense in the mycorrhizosphere were cut and sequenced. The sequences corresponding to the selected bands were related to different bacterial phylogenetic groups such as Firmicutes, α and β-proteobacteria and most of all Cytophagales-Flexibacter-Bacteroides (table 2).

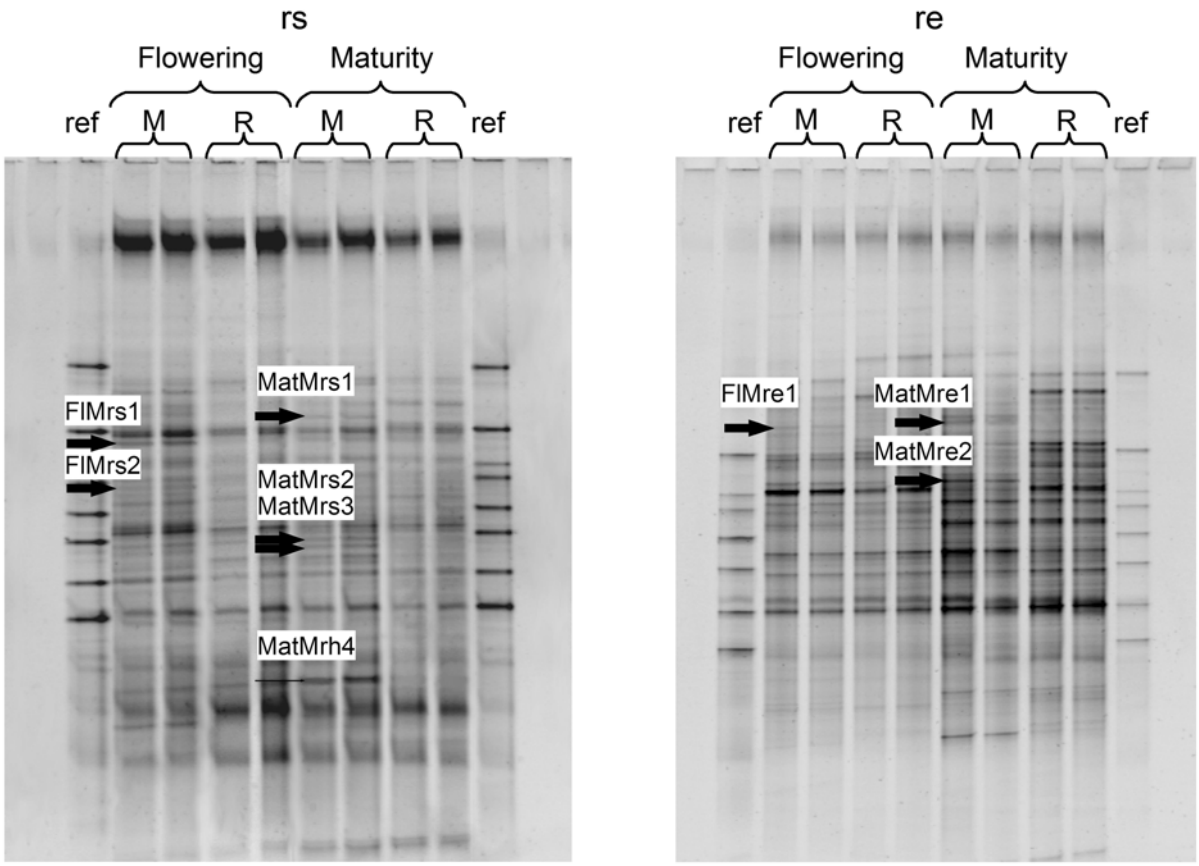

Fig.6. DGGE gels of the V3 region of 16S rDNA for bacterial communities at the flowering and maturity growth stages in the rhizospheric soil (rs) and rhizoplane/endorhizosphere (re) fractions, M = mycorrhizosphere; R = rhizosphere. ref = reference pattern. The amplified product of two sub-samples per zone was loaded on the gel. Bands cut and sequenced are indicated with an arrow and labelled.

Finally, in order to test which parameter influenced the most the bacterial community structure, soil parameters were combined with DGGE data and bacterial counts using Mantel tests. As the physico-chemical measures were only taken from nrs soils, only the DGGE banding patterns of this fraction were used. Mantel tests showed that only two parameters, the available P (p=0,014) and the pH (p=0,033) were significantly correlated with the bacterial DGGE patterns. No significant correlation was found between the bacterial counts and the physico-chemical parameters. Finally, the hyphal length was correlated neither with the DGGE patterns nor with the bacterial counts indicating that AMF do not have a direct effect on the bacterial community.

Table 2: Affiliation of the sequences retrieved from the DGGE bands with existing 16S rDNA sequences in the *Genbank* database determined using the BLASTt software. The sequences are deposited in the EMBL Nucleotide Sequence Database under accession n° **AJ845024** to **AJ845032**.

Band	homology (%)	Closest related species	Genbank Accession n°
FlMrs1_**AJ845024**	92	*Flexibacter filiformis*	AB078049
FlMrs2_**AJ845025**	94	*Flexibacter tractuosus*	AB078076
MatMrs1_**AJ845026**	93	*Flexibacter japonensis*	AB078055
MatMrs2_**AJ845027**	91	*Clostridium neonatale*	AF275949
MatMrs3_**AJ845028**	96	*Ammoniphilus oxalaticus*	Y14579
MatMrs4_**AJ845029**	93	*Geobacillus toebii*	AY608982
FlMre1_**AJ845030**	96	*Flexibacter sanctii*	AB078068
MatMre1_**AJ845031**	98	*Erythrobacter aquimaris*	AY461443
MatMre2_**AJ845032**	94	*Pseudomonas saccharophila*	AF396932

2.1.6 Discussion

Plant and AMF data

The weight per seed was lower for the mycorrhizal plants indicating that AMF infection might have modified the carbon allocation patterns to the seed. Surprisingly, no difference was observed between AMF and non-AMF plant biomass despite a sufficient mycorrhizal colonization of the root and low levels of available nitrogen and phosphorus in the soil of the microcosms. Vázquez et al. (2000) observed that a natural AMF inoculum could even decrease maize growth, whereas single species inocula of *G. mosseae* and *G. deserticola* did not. As the natural inoculum in our experiment is composed of different AMF species, it is possible that the fungi able to colonize the most efficiently the wheat root had no effect on the plant growth. Indeed, the growth response of plants to different fungi depends on the identity of the plant and on the fungus involved in the interaction (Hart and Klironomos, 2002; van der Heijden, 2002). Even with one isolate, different host responses exist, ranging from parasitism to mutualism (Johnson et al., 1997). In this study, the hyphal length was quite low in comparison with the extraradical mycelium of AMF ranging from 1,1 to 54 m/g soil observed in different experiments (Giovanetti et al., 2002). The natural inoculum from our soil contained probably less hyphae or spores than an AMF inoculum from trap or axenic

cultures. Moreover, this experiment lasted only 68 days, perhaps letting not enough time for a proper mycorrhizal establishment occupying the complete box volume. In addition, although tyndallisation is less aggressive than autoclaving of the soil, toxic organic residues caused by the heating process could have inhibited hyphal growth to a certain extent.

Effect of the root

The presence of roots, acting either as a water or nutrient sink has a strong impact on the environment, by modifying the soil structure or the microbial community structure via root exudation or rhizodeposition (Sørensen, 1997). In our study, different factors showed an effect of the root on the surrounding soil. For example, levels of organic C and total N measured in the mycorrhizosphere and the rhizosphere were lower as compared to the hyphosphere and soil control. The lower level of total N in the plant-associated zones could be caused either by root uptake of nitrogen compounds or by an enhancement in microbial immobilization of N (Mahmood et al., 1997). In addition, available phosphorus decrease was more pronounced in the zones containing plant roots but no difference was observed between the mycorrhizosphere and the rhizosphere, indicating a high efficiency of root for the phosphorus uptake. A noticeable effect of the root on the bacterial community was perceptible in two observations. Firstly, proportions of siderophore producing bacteria and pseudomonads were higher in root-related zones such as the mycorrhizosphere and the rhizosphere as compared to the hyphosphere and soil control. Siderophore producing bacteria and pseudomonads are probably favoured near the rhizosphere due to their root competent traits (Marilley and Aragno, 1999; Latour et al., 2003). Secondly, correspondence analysis of the DGGE banding patterns revealed a grouping of the samples by fraction (nrs or rs or re) and by growth stage (flowering or maturity). This suggests that the root affected predominantly the bacterial community structure, either spatially (effect on nrs ≠ rs ≠ re) or temporally, probably by modifying or diminishing the amount and composition of root exudation (higher at flowering). This finding is in agreement with studies showing a strong modification of the bacterial community by the root in the rhizosphere (Marilley and Aragno, 1999; Yang and Crowley, 2000; Baudoin et al., 2002).

Effect of AMF

As previously described in the introduction, AMF can exert many different effects on their environment. In this study, the presence of AMF induced modifications in a number of soil parameters. At the flowering stage, total N was lower in the mycorrhizosphere than in the rhizosphere and was also lower in the hyphosphere as compared to soil control. This finding suggests a contribution of AMF to N plant nutrition as hyphae have access to N sources spatially unavailable to the plant (Smith and Read, 1997). The nitrogen uptake was also

revealed by the lower amounts of NH_4^+ and NO_3^- at the flowering and maturity stages in the mycorrhizosphere and the hyphosphere. Jakobsen et al. (2002) reviewed different studies showing that depletion of inorganic N in the presence of AMF in root-free soil ranged from 71 to 90%. In our study, the level of NH_4^+ was lower than those of NO_3^- in the presence of hyphae, probably because of a hyphal preference for the NH_4^+ which may be energetically more favorable when assimilated (Hawkins et al., 2000). Also, AM fungi might be more efficient in uptaking NH_4^+ as this ion is relatively non-mobile as compared to NO_3^- (Smith and Read, 1997). Available-P uptake by the AM hyphae might have taken place in the hyphosphere as available-P levels were lower as compared to soil control. This difference in ion uptake could in turn affect the pH as Bago and Azcón-Aguillar (1997) suggested. This was demonstrated in the present experiment by a higher pH value in the mycorrhizosphere and hyphosphere.

As explained above, the greatest influence on the bacterial community structure in this study was suggested to be exerted by the root. However, AMF could also influence specific bacterial populations as indicated by lower numbers of total mesophilic cultivable bacteria in the mycorrhizosphere (as compared to the rhizosphere) in re fraction at flowering and in rs and re fractions at maturity. Moreover, the proportions of different bacterial guilds analysed were affected by the AMF. This suggests either a competition for root exudates between AMF and bacteria or a reduction or change in root exudation composition leading to a modification of certain bacterial groups' density (Marschner et al., 1997; Christensen and Jakobsen, 1993). Indeed, changes caused by a mycorrhizal infection could either stimulate bacterial density (Andrade et al., 1998a; Meyer and Linderman, 1986a) or on the contrary decrease it (Christensen and Jakobsen, 1993).

The most pronounced effect of AMF on the cultivable bacterial composition was the strong increase in the proportion of phosphate solubilizing bacteria (PSB) in AMF related zones. This proportion even reached 25 % in the mycorrhizosphere re fraction at the maturity stage. PSB growth could be stimulated by the sparingly available phosphate close to mycorrhizal hyphae. Indeed, in soils with a pH between 7 and 8, the soluble phosphate ions (Pi) are taken up predominantly by the AMF and transported to the plant, thus the level of mineralised or solubilized Pi decreases dramatically in close vicinity of the hyphae (Smith and Read, 1997). P could become the limiting element for bacterial growth. However, PSB can produce organic acids that acidify the surrounding of the bacterial cells. The protons in excess could release Pi from calcium phosphates by proton substitution with calcium. Pi solubilized is subsequently uptaken by the PSB, fulfilling the bacterial P demand and enabling bacterial growth and division. The number of PSB cells increases then more than the other bacteria. Hence, the bacteria capable of solubilizing phosphate compounds by

producing organic anions as chelating agents have there a competitive advantage over other soil micro-organisms for the P source. In addition, excess solubilized Pi not taken up by the bacteria could be transported by the AMF hyphae to the plant. This phenomenon could be of importance in agricultural systems in low input soils, where a combined inoculation of AMF and PGPR strains capable of solubilizing phosphate could enhance plant growth synergistically. Indeed, Toro et al. (1997) showed that the microbiologically solubilised phosphate might be taken up by the mycorrhizal mycelium to the plant and improve plant P nutrition. Moreover, in Toro's experiment, the two bacterial PSB inoculants behaved as mycorrhiza-helper bacteria, promoting the establishment of both indigenous and introduced AM fungi in onion plants. A mutual interaction between these micro-organisms could then exist as the PSB first promote mycorrhiza establishment and the AMF could favour PSB competitivity with other soil or root micro-organisms.

When comparing DGGE banding patterns from AMF and none AMF zones, some bands appeared more intense or disappeared in the presence of the fungi, indicating an effect of AMF on specific bacterial populations. This finding is in accordance with the work of Mansfeld-Giese et al. (2002) who analysed 1367 colonies isolated from cucumber with and without *G. intraradices* using FAME profiles. They found that there was little influence of the fungi on the bacterial community but that certain population densities such as *Paenibacillus* were altered. In the present study, 9 DGGE bands more intense or present only with AMF root infection in the rs and re fractions were sequenced. As the 16S rDNA amplicon is short (see materials & methods), care should be taken when interpreting these results. Four out of these DGGE band sequences were affiliated to the CFB group and more specifically to the genus *Flexibacter*, two band sequences were affiliated to the firmicutes and the other ones to α and β *Proteobacteria*. Uncultured CFB bacteria have already been detected in the mycelium of *Tuber borchii* (Barbieri et al., 2000) but our band sequences did not cluster with these Tuber's endosymbionts. Some *Flexibacter* strains are known to degrade chitin (Larkin, 1989) and are also important producers of peptidases in the soils (Bach and Munch, 2000). They could then be feeding on the hyphae or even dead root tissue. α and β *Proteobacteria* were already detected as epibionts on the mantle surfaces on ectomycorrhizae of *Fagus sylvatica* (Mogge et al., 2000). Bacteria endosymbionts of *the Gigasporaceae* family, belonging to the genus *Burkholderia,* have also been observed (Bianciotto et al., 2000). Finally *Bacillus* species are known to stimulate plant growth when co-inoculated with AMF (Toro et al., 1997) and are well-known PSB bacteria (Rodriguez and Fraga, 1999). However, all these bacteria are common soil inhabitants (Borneman et al., 1996).

Indirect influence of AMF

From the results obtained in this study we can hypothesise that AMF did not influence directly the bacterial populations (i.e. the bacteria feeding on hyphae or on hyphal exudates). However, AMF can influence indirectly the bacterial community by modifying root exudation rates or composition (Kucey and Paul, 1982; Marschner et al., 1997). These changes in root exudation could explain the differences observed between the bacterial populations of the mycorrhizosphere and the rhizosphere in the rs and re fractions. In the non-rhizospheric soil, the bacterial community structure was probably influenced by the physico-chemical parameters of the soil. Indeed, the DGGE banding patterns were significantly correlated with the pH or the available P but not with the hyphal length. However, out of these parameters, the soil pH was the only one that was really different in AMF related zones (mycorrhizosphere and hyphosphere) as compared to the rhizosphere and soil control. Interestingly, the influence of pH-induced changes by AM colonization on the bacterial community structure has already been observed in the rhizosphere of split-root maize by Marschner and Baumann (2003). Bethlenfalvay et al. (1999) hypothesised a functional relationship between a higher soil pH value and water stable aggregate stability (e.g. low soil pH diminishes the bridging of clays and organic materials) and attributed a higher soil pH value in AMF treatments to the increased uptake of counter-balancing anions by the plants.

In conclusion, the use of a complex AMF inoculum in this study instead of an inoculum composed of only one or two AMF species enabled to use experimental conditions closer to field conditions. DGGE patterns of the different zones and fractions revealed that the bacterial community structure was predominantly dependent on the distance from the root and on the plant's growth stage. Nevertheless experiments combining molecular and culture-based techniques showed that AMF inoculation did influence specific bacterial populations but indirectly. In the mycorrhizosphere an indirect influence of AMF on the bacterial community could be exerted by changes in root exudation rates or composition due to mycorrhizal root infection. Several bacterial populations related to the Firmicutes, α and β-proteobacteria and Cytophagales-Flexibacter-Bacteroides were more abundant in the mycorrhizal roots as compared to mycorrhizal-free roots. In the non rhizospheric soil, the presence of AM hyphae caused a modification of the soil pH that affected indirectly the bacterial community structure. Another indirect effect of AMF on the bacterial community could result from the depletion of soluble phosphorus in the environment of the AM hyphae. This depletion could result in competitive advantage of phosphate-solubilizing bacteria over other soil micro-organisms for P source. Indeed, in this study, phosphate-solubilizing bacteria were higher in AMF-related zones. The findings suggest a preferential choice for a combined bio-inoculation of AMF and PGPR able to solubilize phosphorus in low-P available

agricultural soils. Such bio-inoculant formulas should improve the wheat's phosphorus nutrition in a sustainable way in agricultural sites that do not have access to proper fertilizer inputs such as the ones monitored by the ISCB in marginal rain-fed areas of India. Finally, this study helps to comprehend the mechanisms of AMF influence on the soil bacteria, i.e. effects primarily indirect exerted through modifications of the physico-chemical environment of the soil.

2.1.7 Acknowledgements

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2.2 Effect of an AMF inoculum on the active and present rhizobacterial community structure and diversity in the mycorrhizosphere of wheat and green gram

2.2.1 Introduction

The study presented in chapter 2.1 showed that a natural arbuscular mycorrhizal fungi (AMF) inoculum could affect the rhizobacterial community structure but in a lesser extent than the root. Other studies assessing modifications caused by AMF infection in the rhizobacterial community structure have been performed with fingerprinting techniques on the bacterial populations that were present in the rhizosphere but not necessarily active (Marschner et al., 2001; Marschner and Baumann, 2003; chapter 2.1). In order to analyse not only the present but also the active bacterial community structure, the fingerprints should be based on the 16S rRNA and not only on the 16S rDNA gene. Indeed, the ribosome content of cells depends on their activity level. Therefore profiles obtained after RT-PCR on environmental 16S rRNA are weighed according to the actual activity of related populations (Wagner, 1994). Ribosomal 16S RNA has to be extracted and reverse-transcripted into complementary DNA (cDNA). The cDNA is then amplified with the same primers as for total DNA for the DGGE analysis. The results presented in this chapter are part of a joint study headed by Dr Anil Sharma (Pantnagar University) between the Microbiology Department of the Pantnagar University, the TERI in New Delhi, the Botany Institute of the University of Basel and the LAMUN of the Neuchâtel University. The goal of this experiment was to study the impact of Indian and Swiss AMF on wheat (*Triticum aestivum* UP 2338) and greengram *(Vigna radiat*a, PM 3) growth with a PGPR co-inoculation considering tricalcium phosphate as P source. Greengram is a leguminous plant which is planted during the intercropping season after rice culture and whose seeds are used commonly as food. These pulses produce the protein-rich *dal* of the Indian diet. The LAMUN's objective, which is presented in this chapter, was to test the effect of two AMF treatments, one of Indian and the other of Swiss origin, on the total and active bacterial community structure in the mycorrhizosphere of wheat and green gram, taking into account the influence of the plant species.

2.2.2 Material and Methods

Microcosm system, AMF treatments and sampling

A three-chamber system was designed in order to separate the mycorrhizosphere from the hyphosphere (fig.1). The two root compartments on the extremities of the microcosms were separated with 30 µm nylon meshes preventing the roots from passing through. The root compartments contained 1 kg autoclaved substrate composed of a 1:1 v/v sieved loess (5 mm mesh) and quartzsand (K30). The middle chamber contained 450 g of the autoclaved substrate mixed with 90 mg of β-tri-calciumphosphate (TCP), reaching a final dose of 200 mg/kg TCP as used in a previous study (Ratti-Neelima et al., 2001). A bacterial community from an Indian marginal land soil was re-introduced as following: 292 g of this soil was suspended in 500 ml sterile water, sieved and filtered through filter paper. The suspension was then diluted to 2000 ml and added in the compartments (10 ml for each root compartment, 4,5 ml for middle chamber).

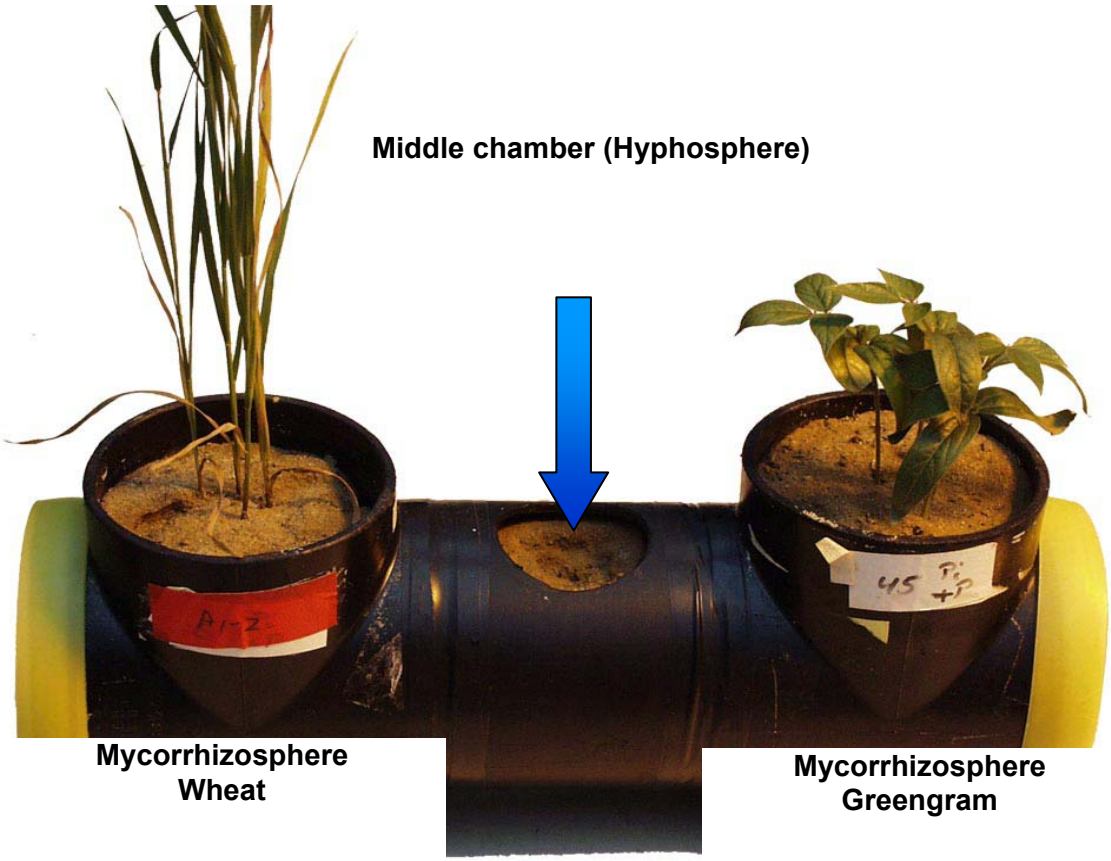

Fig.1. Three-chamber microcosm unit. The mycorrhizosphere compartments on both the extremities of the microcosm units contained 1 kg autoclaved substrate and were sown with wheat or greengram. The middle chamber, representing the hyphosphere, contained 450 g of the autoclaved substrate and was separated from the mycorrhizosphere with nylon meshes.

Two plants of Indian origin, wheat (*Triticum aestivum* var. UP 2338) and greengram *(Vigna radiat*a, var. PM 3), were sown (6 seeds) in each of the root compartments. A PGPR inoculum composed of three *Pseudomonas* strains from Pantnagar (R81+R62+R709), characterised in chapters 4 and 5, was applied to the seeds of both plants and in the middle chamber. The seed bacterization was realised by immersing the seeds in 0,8% NaCl solution containing 10^8-10^9 PGPR cells/ml for 30 minutes. The middle chamber received also 1 ml of the PGPR suspension.

The AMF treatments were composed of AMF strains from Indian (*G. etunicatum*, LL2) and Swiss (*G. mosseae*, BIB-ISCB18). 5 g of the respective AMF inoculum was introduced by making a hole in the centre of the root chamber and the bacterized seeds were placed over the inoculum.

Four microcosm units were used per treatment (Indian AMF, Swiss AMF and AMF-free control) and placed randomly on a table in the greenhouse of the Botany Institute in Basel. The temperatures were maintained to 16°C in the night and were at 24°C up to more than 30°C in the day. The plants were sampled after 45 days of growth, corresponding to the flowering stage of wheat. The plants were shaken to remove non-adhering soil. The roots were separated from the shoots sterily. They were weighed in sterile Petri dishes and then divided for the AMF root colonization analysis and DNA extraction. About 1 g of roots with root-adhering rhizospheric soil were placed in 2 sterile Eppendorf tubes, one for DNA and the other for RNA extraction (RNase-free sampling conditions). The tubes were then immersed in liquid nitrogen for the transport from Basel to Neuchâtel in order to prevent bacterial activity and RNA digestion by RNases. The tubes were finally stocked et –80°C in Neuchâtel.

DNA and RNA extraction

In this experiment, the mycorrhizosphere was composed of both the root-adhering soil and rhizoplane/endorrhizosphere. About 0,5 g of mycorrhizosphere sample was submitted to DNA extraction by the bead-beater technique (FastPrep FP120, SAVANT, BIO101, Carlsbad, USA) using a FastDNA spin kit for soil DNA extraction (BIO101) according to the manufacturer's protocol.

RNA was extracted according to Jossi et al. (in prep). From sampling up to cDNA synthesis, all the RNA handlings were performed in RNase-free conditions. Total RNA was extracted and purified using a combination of FastRNA™ tubes (Bio101) and RNeasy® Plant Kit (Qiagen AG, Basel). In each FastRNA™ tube containing about 100 to 500 mg of frozen sample, 450 µl of RLT Buffer (Qiagen) were added. The mixture was shaken for 10 s at 6

m/s using FastPrep™ cell disruptor. This step was repeated once after cooling the tubes for 5 min on ice. The tubes were then centrifugated for 5 min at 13000 g and the supernatant was loaded on QIAshredder Spin Columns (Qiagen) and then processed as recommended by the manufacturer. The final RNA extract was eluted with 100 µl of 10 mM Tris and a DNase treatment was added in order to remove any DNA contamination: RQ1 RNase-Free DNase (Promega), corresponding buffer and Stop Reaction solution were used according to the manufacturers protocol. The final RNA extracts were stored at –80°C before use.

Reverse transcription reactions of total RNA were performed using ImProm-II™ Reverse Transcription System (Promega) with random hexamer primers. 3,5 µl of RNA extract (from 55 to 70 ng depending of the sample) were mixed with 1 µl of primers (10 mM) and 0,5 µl of RNasin® Ribonuclease Inhibitor. This mixture was incubated at 70 °C for 5 min for RNA and primers optimal contact, then chilled on ice until the reverse transcription mix was added. This mix was then combined to (final concentrations): 1 X ImProm-II™ Reaction Buffer, 0,05 $U.\mu l^{-1}$ RNasin, 6,0 mM $MgCl_2$, 0,5 mM each dNTP, 5 % (vol/vol) ImProm-II™ Reverse Transcriptase and diethyl pyrocarbonate-treated nanopure water in a final volume of 20 µl. The reaction comprised annealing at 25 °C for 5 min, extension at 42 °C for one hour and inactivation of reverse transcriptase at 70 °C for 15 min. The resulting cDNAs were stored at -20 °C.

PCR and DGGE of DNA and cDNAs

The PCR and DGGE procedure as well as DGGE profile statistical analysis are similar to the description in chapter 2.1. The Shannon diversity index of the DGGE profiles was calculated as $-\Sigma$ pi $\log_2$(pi) where pi represents the relative abundance of a given population in the profile. For the ordination analysis on the DGGE profiles, The DNA and RNA data matrices were composed of rows of objects (samples) and columns of species (DGGE band relative intensity). In order to reduce the contribution of rare species in the ordination analyses, bands appearing only in one RNA or DNA profile were discarded.

Cloning and sequencing of DGGE bands

DGGE bands in the wheat RNA bacterial community profiles were excised cloned and sequenced as described in chapter 2.1. The resulting DNA fragments were sequenced by Macrogen Corp. South Korea. Three clones were sequenced per band and only the bands having similar sequences in two out of these three clones are presented in the results. The percentage of homology with existing 16S rDNA sequences in the *Genbank* database was determined using the BLASTt software (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov).

Statistical analysis

The data of the root and shoot dry weight as well as bacterial diversity were subjected to analysis of variance (ANOVA) and the means were compared with the least significant difference (LSD) test using the S-Plus software vers. 6.1 (Insightful Corp, USA). In order to test and quantify the contributions of various sets of explanatory variables on the DNA and RNA data matrices, Canonical Correspondence Analysis (CCA) was applied. The type of treatment and plant species were integrated as qualitative explanatory variables (centroïds). Variation partitioning analysis (Borcard et al., 1992) using a series of CCA enables to display the variability of patterns constrained by the factors of interest. Therefore this analysis was used to display the relative importance of the contributions type of treatment or plant species on the total (DNA) and active (RNA-based) bacterial community profiles. The significance of the result was tested with the Monte Carlo permutation test.

2.2.3 Results and discussion

The treatments did not affect strongly the diversity (H' of the Shannon index) of the total (DNA) and active (RNA) bacterial communities as compared to the control (fig.2). The diversity of the total bacterial populations diminished slightly in green gram as compared to wheat. Moreover, the diversity of the active communities was significantly higher in the Indian AMF treatment as compared to the control in greengram. Less bands were visible in the RNA profiles as compared to the DNA profiles (data not shown) resulting in a decrease of the bacterial diversity index. This result indicates that even if predominant bacterial populations are present in the mycorrhizosphere, they might not be active at the time of observation. Nevertheless, the rhizosphere or the mycorrhizosphere offers an environment where a high bacterial activity is observed as bacterial growth is stimulated by a constant supply in carbon compounds via the root exudates and remains dynamic due to protozoan grazing (Lynch, 1990). To corroborate this statement, Weisskopf et al. (2004) observed that the total and active bacterial communities were more different from each other in the soil fraction than in the root fraction suggesting that the bacterial community associated to the roots were generally more active.

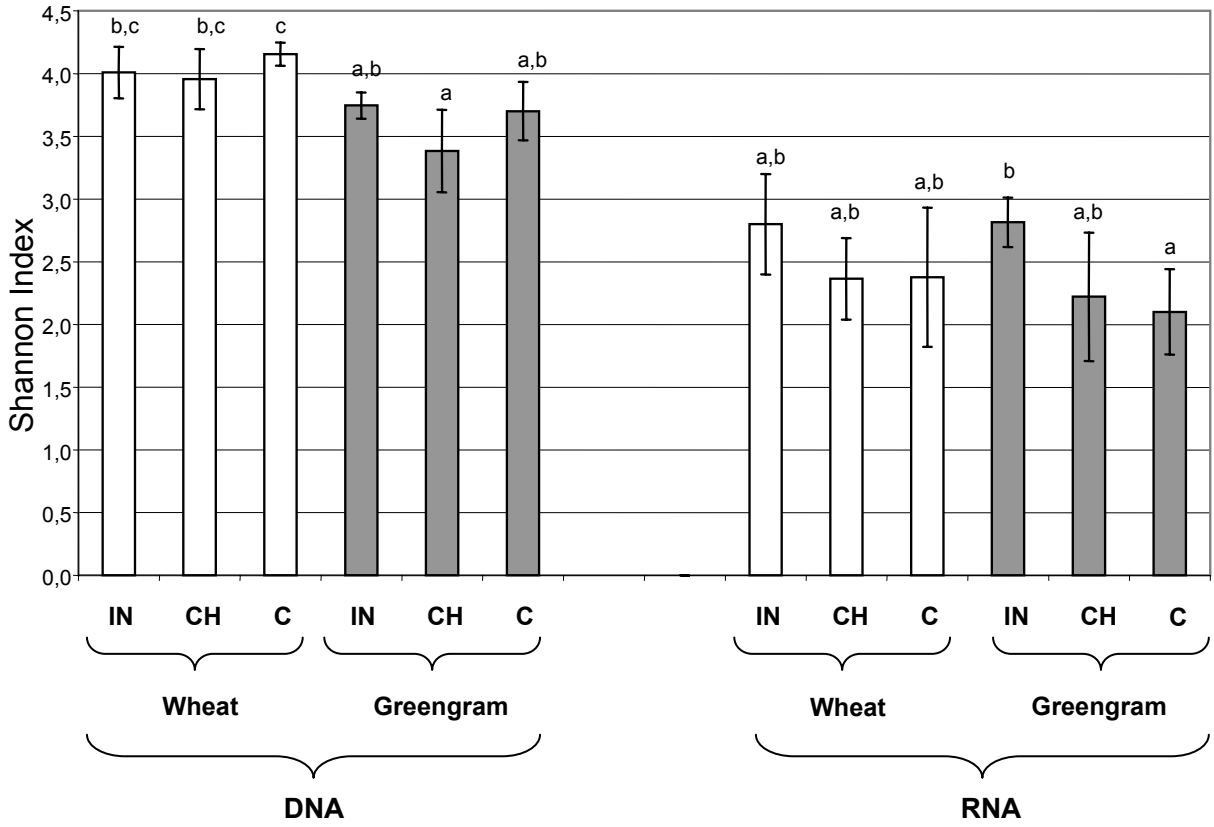

Fig. 2. Diversity of total and active bacterial communities. Shannon index was calculated as $-\Sigma$ pi $\log_2$(pi) where pi is the relative abundance of a given band in the profile. Values are means ± standard deviation of 3 treatment replicates. Identical letters indicate non-significantly different means between treatments in DNA or RNA profiles according to LSD test ($p < 0,05$, $n=3$). Column in blank = wheat; Column in grey = greengram. IN = Indian AMF treatment; CH = Swiss AMF treatment; C = AMF-free control

The correlation structure between the variables “bacterial community profiles”, “type of treatment” and “plant species” is summarised in fig. 3. Objects (DGGE profiles represented as squares, triangles and circles) close together are likely to have similar bacterial community profiles. Objects close to centroïds points (X in fig. 3) are bacterial community profiles that are likely to contain species (DGGE bands) that are found frequently (or more abundantly) in the conditions of the qualitative explanatory variables. Profiles of the AMF-free control and Swiss AMF treatment were more closely grouped together than with the Indian AMF treatment. This means that the bacterial community structure of the Indian AMF treatment was more different than the community structure of the Swiss AMF treatment or from the control. The absence of a significant effect of the Swiss AMF on the bacterial community structure was due to its incapacity to colonize the roots of the wheat and green gram. Indeed, no fungal structure was found in the roots treated with the Swiss AMF and contrarily to the Indian AMF treatment, the root and shoot dry weight of both plants remained similar to the control (table 1). Small variations in the bacterial community structure observed with this treatment could then probably be caused by the micro-organisms (bacteria, protozoans, nematodes) present in the AMF inoculum but not by a fungal presence. This

absence of fungal development probably resulted from too high greenhouse temperatures favouring the Indian strains that are used to grow in elevated T°C (Wiemken and Ineichen, personal communication). Indeed, soil temperature along with soil moisture will exert a major influence on mycorrhizal colonization of plants (Braunberger et al., 1997, Entry et al., 2002).

Table 1. Root and shoot dry weight and AMF root colonization percentage of wheat and greengram at the wheat flowering stage (Sharma and Ineichen, personal communication). Significantly different treatment means per plant are indicated with an asterix according to LSD test ($p<0,05$; $n = 4$).

Treatment	Plant	Root dry weight (g)	Shoot dry weight (g)	Root colonization (%)
Indian AMF	Wheat	0,39±0,08	1,40±0,14*	50
Swiss AMF	Wheat	0,14±0,05	0,54±0,11	0
Control	Wheat	0,14±0,04	0,54±0,06	0
Indian AMF	Green gram	0,08±0,02	0,95±0,12*	50
Swiss AMF	Green gram	0,10±0,06	0,72±0,11	0
Control	Green gram	0,08±0,05	0,62±0,08	0

We observed more variations between the replicates of the Green Gram DGGE profiles of the active populations than between those of the total populations. It is expected that variations in the rhizosphere environment would affect more intensely the metabolic activity of specific populations (faster response due to the production of ribosomes) than total populations (slower response due to the population growth). For example, Jossi et al. (in prep) observed that the active bacterial populations were more affected by an increase in atmospheric pCO_2 than the total bacterial populations in the rhizosphere of *Lollium perenne and Molinia coerulea*.

The plant species influenced the total bacterial community structure slightly more than the AMF treatment: the variance could be explained to 19,6% ($p=0,001$) by the plant species and 16,1% by the type of treatment ($p=0,003$) with no shared variance (fig. 3a). This result is in accordance with the study of Marschner and Baumann (2003) who found that the effect of AMF on the bacterial community structure was in part plant mediated and with our study in chapter 2.1 in which the bacterial community structure in the mycorrhizosphere was predominantly affected by the root. However, the plant effect on the active bacterial community was reduced as the plant species explained 11,7% of the variance and the type of treatment 15,8% with no shared variance. In addition, more DGGE bands were detected in the Indian AMF treatment as compared to the control in the active community profiles. This results implies that a higher number of bacterial populations were active in this treatment which is expressed by a higher diversity index (fig. 2). In consequence, the Indian AMF colonization has stimulated a higher number of bacterial populations possibly through a modification of the root exudation pattern or by bacterial feeding of fungal structures (see chapter 3; Barea et al., 2002b) or hyphal exudation (see chapter 4, Andrade, 2004).

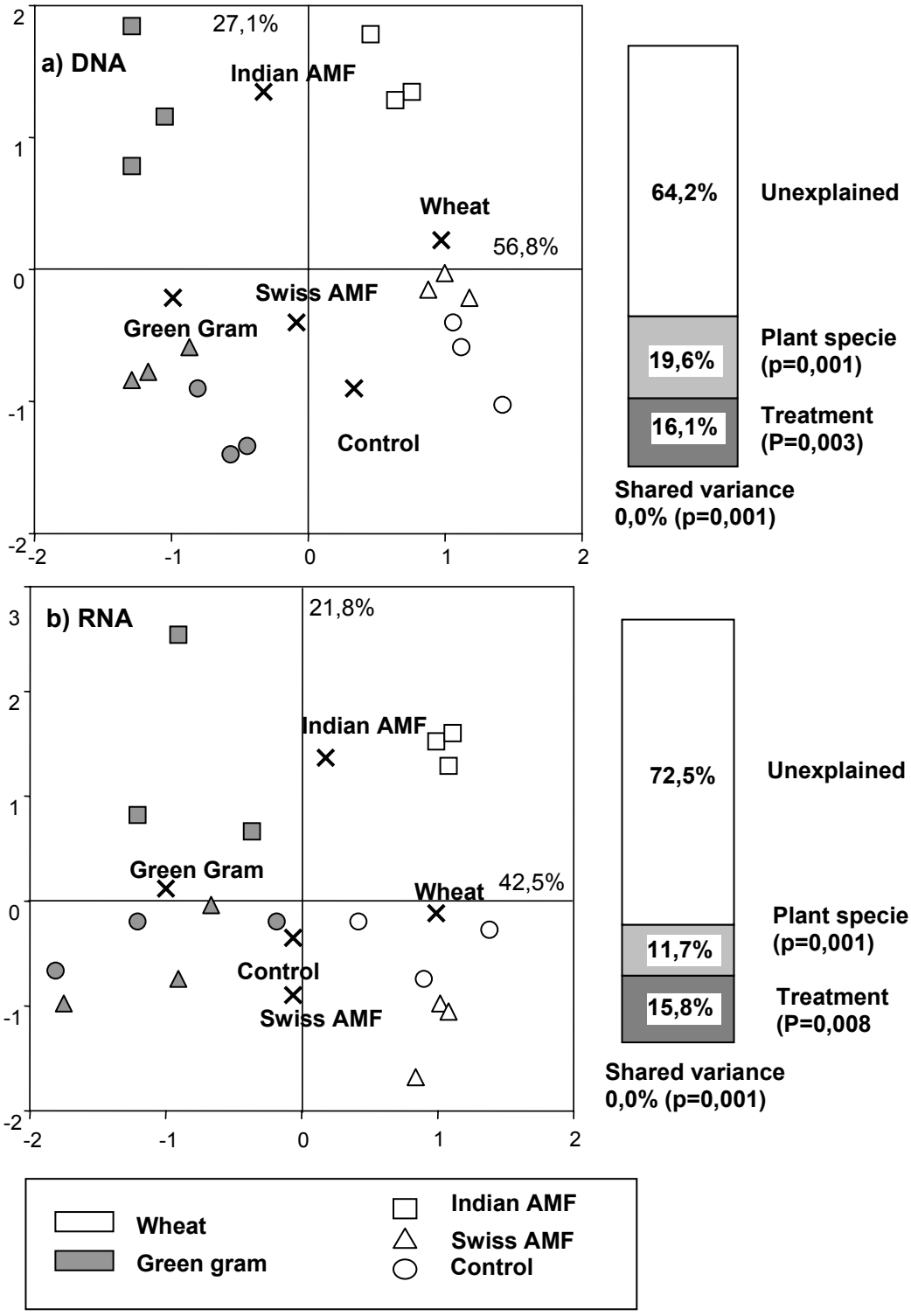

Fig. 3. Canonical correspondence analysis (CCA) of DGGE bacterial community constrained with the qualitative explanatory variables (centroids), plant specie and treatment. Values on the axes indicate % of total variation explained by the axes. The variance decomposition of the CCA of the bacterial community profiles is represented as a bar diagram. (a) Analysis of the community profiles of total populations (DNA-based profiles, 3 replicates per treatment): sum of all canonical eigenvalues, 0,722; total inertia, 2.019; Monte Carlo overall 999 permutation test, p = 0,001. (b) Analysis of the community profiles of the active populations (cDNA-based profiles, 3 replicates per treatment): sum of all canonical eigenvalues, 0.998; total inertia, 3,624; Monte Carlo overall 999 permutation test, p = 0,001.

Several bands from the active community profiles were cut and sequenced. Their location and affiliation is shown in table 2.

Table 2: Affiliation of the sequences retrieved from the DGGE bands in RNA profiles with existing 16S rDNA sequences in the *Genbank* database determined using the BLASTt software. "+" indicates in which profile the band is present.

	Indian AMF	**Swiss AMF**	**Control**	Species affiliation	homology	reference
W1	+			*Pseudomonas fluorescens*	197/197 (100%)	AY447046
W2	+			*Stenotrophomonas maltophilia*	193/197 (97%)	AY297751
W3	+	+		*Ralstonia pickettii*	197/197 (100%)	AF526914
W4		+		*Escherichia albertii*	197/197 (100%)	AJ634381
W6	+	+	+	*Citrobacter freundii*	196/197 (99%)	AJ233408

Two bands were affiliated to genera related to the Enterobacteriaceae family: *Escherichia* and *Citrobacter*. Their activity in the mycorrhizosphere is unusual as the normal habitat of these bacteria is the gut of animals. The enterobacteriaceae *Citrobacter,* found in all our treatments, utilizes citrate as carbon source, however wheat does not produce citrate. A source of citrate in the mycorrhizosphere could nonetheless occur from the P-solubilizing bacteria present in the mycorrhizosphere. The five bands sequenced are affiliated to bacteria that can possess fimbriae or pili, enabling them to initiate attachment to solid surfaces such as the root or fungal hyphae (Schlegel and Jannasch, 1999), which could explain why they would be favoured in this environment. The three other bands sequenced were afiliated to *Pseudomonas*, *Stenotrophomonas* and *Ralstonia* genera that are common rhizosphere inhabitants. Moreover, these genera are known to produce fungal cell-wall degrading enzymes such as chitinases which could explain why they are more active in the presence of AMF (Dunne et al., 1997; Nagarajkumar et al., 2004; Sutrisno et al., 2004). This finding is in accordance with the above statement that the presence of AMF might have increased the number of active populations by providing a new carbon source (fungal structure or exudates) for the bacterial metabolism.

3 Are specific bacterial populations associated with fungal structures ?

In chapters 2.1 and 2.2, we studied if bacteria of the mycorrhizosphere or hyphospere were affected by the presence of AMF. A direct interaction between the AMF and bacteria would be the bacterial feeding on fungal structures. The fungi could then provide for the bacteria a carbon source in addition to root exudates. A specialised group of bacteria could be adapted to the AMF structures by feeding on them or being attached on their surfaces. Our objective was then to determine if specific bacterial populations were associated with AMF structures. In order to avoid the step of cultivation and obtain a broader picture of the bacterial communities, we used PCR-DGGE analysis. Indeed, bacteria not cultivable on ordinary media could represent a significant part of the bacterial community living on AMF structures. Isolating AMF hyphae from the soil as rapidly and sterily as possible in order to get an instant snapshot of the bacterial community structure proved to be difficult. Moreover, a sufficient quantity of hyphae devoid of soil particles was necessary for the DNA extraction but hard to obtain from the microcosm systems. It was therefore decided to study the bacteria associated with another AMF structure that is easier to isolate in sufficient quantities i.e. the spores. We tried not only to investigate if specific bacterial populations were associated to the fungal spores but also to test whether root exudates or the fungal species influence more the bacterial community structure of the spores. This is the first study yet reported using molecular fingerprinting techniques to assess the most abundant bacterial populations related to the AMF spores.

This chapter is a manuscript entitled “Bacteria associated with spores of arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum”* that has been submitted to Applied and Environmental Microbiology.

Bacteria associated with spores of arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum*

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3.1. Abstract

Spores of the arbuscular mycorrhizal fungi (AMF) *Glomus geosporum* and *G. constrictum* were harvested from single spore derived pot cultures with either *Plantago lanceolata* or *Hieracium pilosella* as host plants. PCR-DGGE analysis revealed that the bacterial communities associated with the spores depended more on AMF than host plant identity. The composition of the bacterial populations associated to the spores could be predominantly influenced by a specific spore wall composition or AMF exudate over specific root exudates. The majority of the bacterial sequences that were common to both *G. geosporum* and *G. constrictum* spores were affiliated to taxonomic groups known to degrade biopolymers (*Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter* and *Pseudomonas*). Scanning electron microscopy of *G. geosporum* spores revealed that these bacteria are probably feeding on the outer hyaline spore layer. The process of maturation and eventual germination of the AMF spores might then benefit from the activity of the surface microorganisms degrading the outer hyaline wall layer.

3.2 Introduction

Arbuscular mycorrhizal fungi (AMF) play a key role in facilitating nutrient uptake of crops in low input farming systems, a prerequisite to maintain a sufficient productivity (Atkinson et al., 2002). AMF spores provide a long-term reservoir of inoculum and are the only AMF propagules that can be identified to the species level (Smith and Read, 1997). The spore wall of *G. geosporum* is composed of three layers: an outer hyaline layer that decays until it sloughs off leaving a granular surface, a laminated yellow-brown to orange-brown layer, and a more rigid layer often adherent to the second layer (INVAM). The thin hyaline layer is

composed mainly of chitin (Sbrana et al., 1995) and has been found to be often colonized by microorganisms in several *Glomus* species (Bonfante-Fasolo and Schubert, 1987). The *G. constrictum* spore wall is composed of only two layers, a decomposing outer hyaline layer absent in older spores and a rigid laminated orange-brown to reddish-black dark layer (INVAM).

An optimal colonization of plant roots depends on the survival and well-timed germination of AMF spores in the soil. This process can be altered by various abiotic and biotic factors, in particular by the association with soil microorganisms (Xavier and Germida, 2003). Indeed, some bacterial populations, called mycorrhiza helper bacteria, have beneficial effects on the AMF growth not only by improving mycorrhizal root colonisation or stimulating extraradical hyphal growth but also by facilitating AMF spore germination (Garbaye, 1994; Gryndler et al., 2000). This latter effect has been shown for actinomycetes (Mugnier and Mosse, 1987; Ames et al., 1989; Carpenter-Boggs et al., 1995), *Pseudomonas* and *Corynebacterium* (Mayo et al., 1986), or *Bacillus* spp (Xavier and Germida, 2003).

Bacteria associated with AMF spores colonize mainly the outer wall layer and rarely penetrate into the inner layers (Bonfante-Fasolo and Schubert, 1987; Maia et al., 1998; Walley and Germida, 1996; Filippi et al., 1998). Nevertheless, some bacteria have been found in the cytoplasm of AMF spores (MacDonald and Chandler, 1981; Bianciotto et al., 1996). The role of AMF spore-associated bacteria is not clear. They could stimulate spore germination by eroding spore walls (Maia et al., 1998; Filippi et al., 1998), by producing stimulatory compounds such as CO_2 and other volatiles (Carpenter-Boggs et al., 1995), by influencing AMF phosphorus metabolism (Ruiz-Locano and Bonfante, 2000), or by fixing N_2 (Minerdi et al., 2001).

Root exudation could enhance spore germination by stimulating the growth of AMF beneficial bacteria (Mayo et al., 1986). However, since the quantity and composition of exudates differ from one plant to another (Lynch and Whipps, 1990), different bacterial populations could be stimulated depending on their preference for distinct plant exudates.

In most of the previous studies on spore-associated microorganisms, the bacteria were isolated upon culturing. However, bacteria not cultivable on ordinary media could represent a significant part of the bacterial community associated with AMF spores. Indeed, only a small fraction (1-10%) of the total bacterial community is cultivable (Amann et al., 1995). Direct molecular approaches avoiding a step of cultivation gives a broader picture of bacterial communities. PCR-DGGE analysis of the 16S rDNA gene permits fingerprinting of the dominant bacteria of a given sample (Muyzer et al., 1993; Fromin et al., 2002). The detection

of populations representing as little as 0.1-1% of the total target organisms is feasible. In the present study, the bacterial community associated with spores of *Glomus geosporum* BEG 18 and *G. constrictum* BEG 19 was assessed with PCR-DGGE analysis. In order to find out whether specific root exudates or rather the fungal species determine the bacterial community structure, spores of the two *Glomus* species were harvested from both pot cultures with *Plantago lanceolata* (Plantaginaceae) and pot cultures with *Hieracium pilosella* (Asteraceae) as host plants.

3.3 Material and methods

Mycorrhizal inoculum

The AMF used in this study were *Glomus geosporum* (BEG 18) and *Glomus constrictum* (BEG 19), originating from the same calcareous grassland at Nenzlingen, Switzerland (van der Heijden et al., 1998). The single spore derived cultures were maintained by subculturing in pots under the same conditions using *Plantago lanceolata* and *Hieracium pilosella* as host plants. The seeds were purchased from FENACO (Winterthur, Switzerland). The growth substrate was TerraGreen/Sand/Loess 2:2:1 (TerraGreen: American aluminum oxide, oil dry US special, type III R, <0.125 mm, from Lobbe Umwelttechnik, Iserlohn, Germany Sand: Quartz d'Alsace, K30, from Kaltenhouse, France, Loess: from a local site, near Basel, Switzerland). The pots were cultivated in a greenhouse with ambient natural light and temperature conditions and irrigated with deionized water using an automated watering system (Tropf-Blumat; Weninger GmbH, Telfs, Austria).

Experimental set up

Twelve 1L plastic pots were filled with sterile substrate composed of Terragreen/quartz sand/loess (5:4:1) and moistened with water. In each pot, a small hole was drilled in which a tea spoon of mycorrhizal inoculum was placed. On top of the inoculum, a few seeds of *Hieracium pilosella* or *Plantago lanceolata* were sown which were covered with sterile quartz sand. The Tropf Blumat watering system was installed and the cultures were grown in a greenhouse in spring and summer under ambient conditions. The following four combinations of symbionts were cultivated in triplicates:

- *H. pilosella* either with *G. geosporum* or *G. constrictum*
- *P. lanceolata* either with *G. geosporum* or *G.constrictum*

Sampling

Six 15 ml soil cores were sampled from each pot after 170 days of growth in the case of *H. pilosella* and for 247 days in the case of *P. lanceolata* in order to obtain a sufficient amount of spores. The soil cores were wet-sieved through 250 and 63 μm meshes. The suspension of residues gained from the 63 μm mesh sieve was centrifuged at 900 x g for 2 min in a sterile density gradient with a 70% (w/v) sucrose layer at the bottom. The spores were collected from the gradient interphase, placed in glass plates and rinsed three times with sterile milli Q water. For the DNA extraction, 200 spores per pot were individually recovered under the stereomicroscope with a micropipette. For the scanning electron microscopy (SEM) analysis, the sampling procedure was repeated from the *G. geosporum/P. lanceolata* replicates to recover more than 100 spores.

DGGE analysis

DNA extraction for the DGGE analysis was performed with 200 spores per pot using the FastDNA Spin Kit for soil (Bio101, Vista, USA) according to the manufacturer's protocol and using a bead beater (Fast-Prep, Model FP 120, Bio101). A double step PCR was used to amplify the V3 region, a fragment of about 200 bp of the bacterial 16S rDNA, according to Weisskopf et al. (2004). A composite mix of different bacterial 16S rDNA fragments was added on each side of the DGGE gel as a reference DGGE pattern: *Pseudomonas fluorescens* ATCC 27663, *Acidovorax facilis* DSM 550, *Bacillus subtilis* ATCC 14893, *Sinorhizobium meliloti* DSM 1981 and *Aquaspirillum dispar* ATCC 27650. DGGE was performed using a 8% (w/v) acryl-bisacrylamide gel (37,5:1, Qbiogene, Illkirch, France) with 30-60% linear urea/formamide (Fluka, Buchs, Switzerland, Qbiogene) denaturing gradient (100% denaturant corresponds to 40% formamide + 7 M urea). 500 ng of the PCR product were electrophorated in 1x TAE buffer (Qbiogene, France) at 60°C with a constant voltage of 150 V during 5.5 hours using the BioRad D-Code Electrophoresis System (Bio-Rad Inc. California, USA). The gels were stained in the dark for 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden, The Netherlands) in 1x TAE solution. The gels were photographed with the Multi-Analyst package (Bio-Rad Inc., California, USA). The DGGE fingerprints were normalised according to the reference patterns and were compared using the GelCompar software (Applied Maths, Kortrijk, Belgium). DGGE banding patterns were then converted into a numerical matrix used in the statistical analysis. Each band was considered as corresponding to a single bacterial population and the band intensity was representative of the relative abundance of the population (Fromin et al., 2002). The bands whose average relative contribution was below 1% were discarded.

Band sequencing

The DGGE profiles were more similar among *H. pilosella* pot replicates. Therefore, 10 DGGE bands in the profile of one replicate of *H. pilosella / G.geosporum* and 10 more bands in the profile of one replicate of *H. pilosella / G. constrictum*. DNA was recovered and purified as following: the selected bands were cut out and placed in a 1.5 ml Eppendorf tube containing 100 μl Tris-HCl 10 mM pH 7.5 and incubated at 4°C for 3 days. The supernatant was then recovered in a new Eppendorf tube. One volume of iced isopropanol (-20°C) and 1/10 volume of sodium acetate 3 M were added and this mix was incubated at -20°C for 1 day. After centrifugation at 13'000 rpm at 4°C for 30 minutes, the supernatant was discarded. The pellet containing the DNA was washed with 1 volume of 100% ethanol and then centrifuged at 13'000 rpm for 30 minutes. The supernatant was completely removed and the pellet was air-dried for 15 minutes. The DNA was resuspended in 50 μl Tris-HCl 10 mM pH 7.5. The V3 region of the DNA was then re-amplified according to the PCR protocol described above. Again, the amplified products were loaded on a DGGE gel to improve DNA yield and check band purity. If the band on this second gel matched the previously selected one, it was cut out, purified and re-amplified the same way. The amplified products were then purified with the *NUCLEOTRAP-CR kit* (Macherey-Nagel, Düren Germany) according to the manufacturer's protocol. The DNA fragments were ligated using the pGEM®-T Vector System (Promega), following the protocol of the manufacturer. Transformation was performed by electroporation using the *Bio-rad Gene Pulser XCell* and *PC module* into *E. coli XLI-Blue*. The transformed bacterial cells were then plated onto Luria–Bertani (LB) agar containing ampicillin (150 μg/ml), X-Gal (0.1 mM) and IPTG (0.2 mM). Plasmids were recovered from white colonies using the NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer's protocol. The resulting DNA fragments were sequenced by Macrogen Corp. South Korea. Three clones per band were sequenced and only the bands having similar sequences in two out of these three clones are presented in the results. The 16S rDNA sequences were aligned using the ClustalX software (Thompson et al., 1997) and the phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) with the Njplot software (ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/njplot) (Perriere and Gouy, 1996). The topology of the distance tree was tested by resampling data with 100 bootstraps (Felsenstein, 1985) to provide confidence estimates for tree topologies.

SEM analysis

The spores were fixed using 1% OsO_4 and air-dried. After coating with gold, the samples were examined with a Phillips XL 30 Scanning electron microscope (SEM) with an acceleration voltage of 10 kV.

Statistical analysis

To analyse the relations between the DGGE patterns of the different samples, correspondence analysis (CA) was used. This ordination method is adapted to analyse presence/absence or abundance data tables and is well suited for populations with unimodal distribution along environmental gradients (Fromin et al., 2002). To perform the CA, a data matrix was composed of rows of objects representing the culture condition replicate and columns of species representing a DGGE band position along the vertical gel gradient. The relative abundance of a species in a sample corresponded to the DGGE band's relative intensity with regard to the sum of all band intensities in a pattern. The CA was then applied on the basis of numerical data matrices converted using the program Progiciel R (Legendre and Vaudor, 1991). From the association matrix obtained, the characteristic values associated with the characteristic vectors were calculated using a multidimensional dispersion cloud of the data with the Canoco 4.0 software (Canoco 4.0, Microcomputer Power, Ithaca, USA). Variation partitioning analysis (Borcard et al., 1992) enables to display the variability of patterns constrained by the factors of interest. Therefore this analysis was used to display the contributions of an AMF species or plant species on the bacterial community profiles. The significance of the results was tested with the Monte Carlo permutation test. Variation partitioning analysis was performed with the software R (R Development Core Team, 2004).

Sequence submission

The DGGE band sequences were submitted to the EMBL Nucleotide Sequence Database at accession n° **AJ864379** to **AJ864393**.

3.4 Results

The homogeneity among replicates of DGGE patterns of bacterial communities associated with AMF spores was higher in cultures on *H. pilosella*, compared to those on *P. lanceolata*. This was obvious by optical observation (fig.1) and confirmed by correspondence analysis (fig.2). The distances among the samples of two different host plants within one AMF species were shorter than the distances among the samples of the two AMF strains with one plant species, indicating that the bacterial community was structured mainly by the AMF species (Fig.2). This observation is supported by variation partitioning analysis which revealed that the host plant explained 12.1% (p=0.001) of the variation of the bacterial DGGE patterns, that the AMF species explained 21.6% (p=0.001) of the variation and that there was no cross-variation (p=0.12). A strong proportion of bands were common to all the culture conditions (fig.3) showing that many bacterial populations were always associated with AMF

spores whatever the fungal species and the host plant. In addition, many of these common bands had a high relative intensity indicating that they were probably the most dominant populations on the AMF spores.

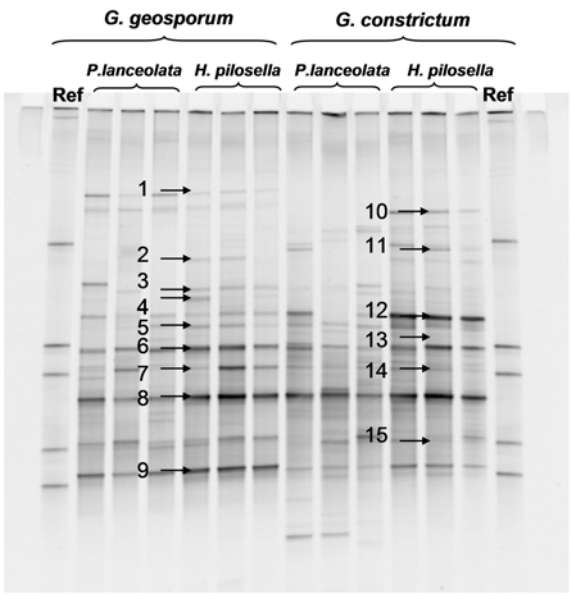

Fig.1. DGGE gels of the 16S rDNA V3 region for bacterial communities associated with the spores of *G. geosporum* and *G. constrictum*. Ref = reference pattern composed of five known bacterial sequences. The amplified product of each of the three replicates per culture condition was loaded on the gel. Bands cut and sequenced are indicated with an arrow and labelled 1 to 15.

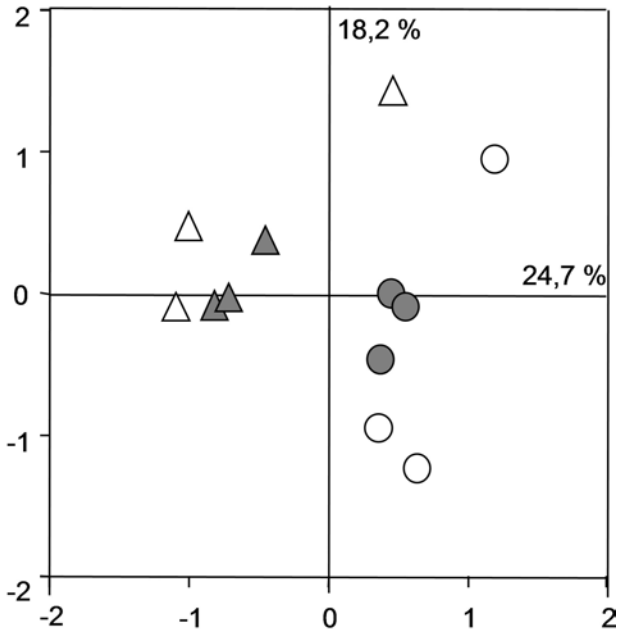

Fig.2. Ordination plot generated by correspondence analysis representing the relationships between AMF spore-associated to the AMF spores defined by the DGGE patterns. Three replicates per culture condition have been integrated. Circle: *G. geosporum*; triangle: *G. constrictum*; open symbols, *P. lanceolata*; grey symbol, *H. Pilosella*. Values on the axes indicate % of total variation explained by the axes. CA1= correspondence analysis axe 1; CA2 = correspondence analysis axe 2.

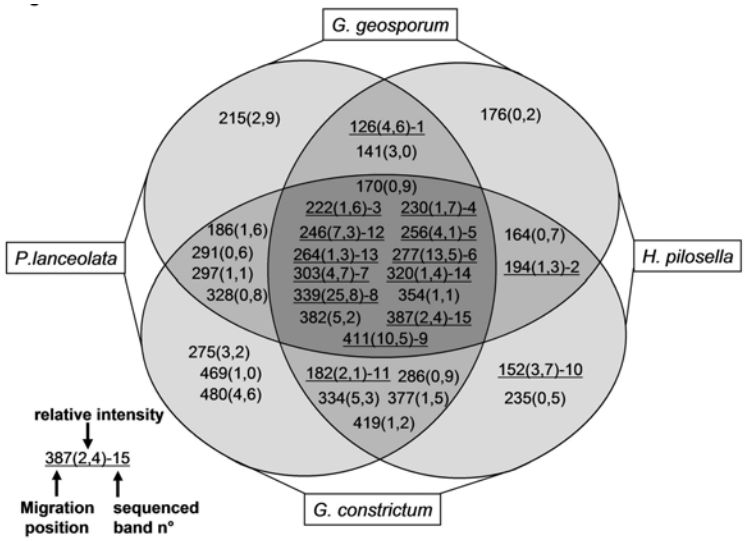

Figure.3. Distribution of the bands composing the DGGE profiles amongst the different culture conditions. The bands are named after their vertical position along the DGGE gel. The relative intensity as a mean value of each band intensity obtained for the corresponding culture conditions is indicated in brackets. Bands excised, cloned and sequenced are underlined and their assignation number is added

To determine their affiliation, eleven bands in common (bands n° 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, 1) and four bands belonging to particular culture conditions (bands n° 1, 2, 10, 11) were excised, cloned and sequenced.

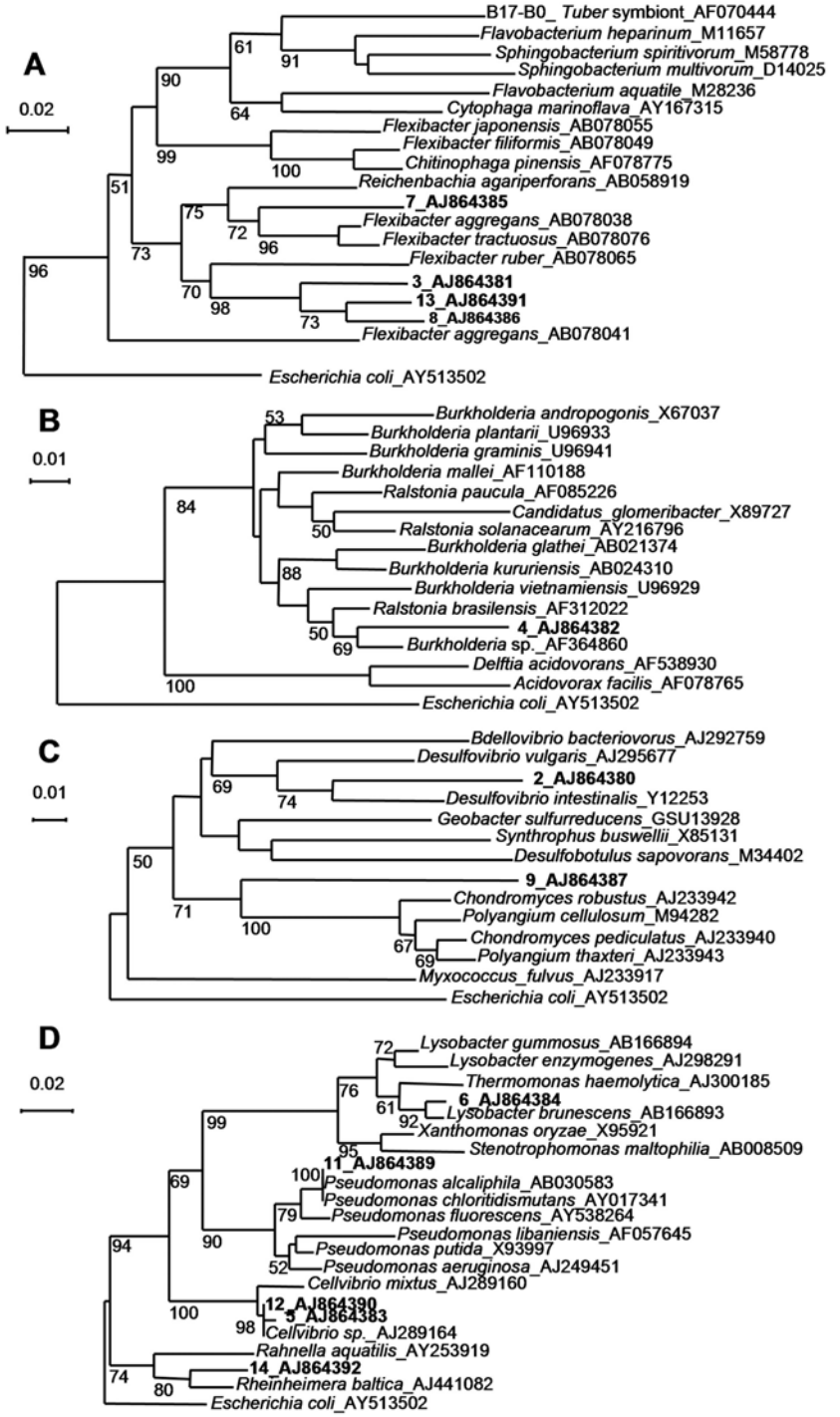

Fig.4. Affiliation of the sequences retrieved from the DGGE bands with existing 16S rDNA sequences using neighbour joining trees. Bootstrap values below 50 are not indicated. The sequences from the database are indicated in italic with their affiliation number. The sequenced bands are outlined in bold. A, Cytophaga-flexibacter-bacteroides group ; B, *beta-proteobacteria* ; C, *delta-proteobacteria* ; D, *gamma-proteobacteria*. The sequences are deposited in the EMBL Nucleotide Sequence Database under accession n° **AJ864379** to **AJ864393** .

Because of the higher homogeneity of the replicates, only DGGE bands obtained from *H. pilosella* cultures were selected. Band n°1_AJ864379 was only present with *G. geosporum* and affiliated to *Fibrobacteres* (data not shown), band n°2_AJ864380 was only present with *H. pilosella* and was related to the genus *Desulfovibrio* (C in fig.4), band n° 10_AJ864388 was only present in *G. constrictum* and *H. pilosella* cultures and was affiliated to *Fibrobacteres* (data not shown). Band n°11_AJ864389 was only present with *G. constrictum* and was affiliated to the genus *Pseudomonas* (D in fig.4). Three bands were present in all the culture conditions but with a relative abundance much higher in the case of *G. geosporum*: band n°6_AJ864384 related to the genus *Lysobacter* (D in fig.4), band n°7_AJ864385 related to the genus *Flexibacter* (A in fig.4), and band n°9_AJ864387 related to the genus *Chondromyces* (C in fig.4). Finally, eight bands were found in all culture conditions and with a similar relative abundance: bands n°3_AJ864381; 8_AJ864386, 13_AJ864391 related to the genus *Flexibacter* (A in fig.4); bands n°5_AJ864383 and 12_AJ864390, both related to the genus *Cellvibrio* (D in fig.4); band n°4_AJ864382 related to the genus *Burkholderia* (B in fig.4); band n°14_AJ864392 related to the genus *Rheinheimera* (D in fig.4); band n°15_AJ864393 related to *Cyanobacteria* (data not shown). Interestingly, most of the genera identified are bacteria that can hydrolyse biopolymers such as cellulose and chitin. These polymer-degrading bacteria represent probably the main populations contributing to the bacterial community associated with AMF spores. Indeed, when adding up the mean value of the relative intensity of all the band sequences related to biopolymer-degrading genera, they represent 60% of the overall intensity in *G. geosporum*/*P. lanceolata*, 84% in *G. geosporum*/*H.pilosella*, 53% in *G. constrictum*/*P.lanceolata* and 73% in *G. constrictum/H. pilosella* cultures.

The bacterial saprophytic activity was confirmed by scanning electron microscopy observations of *G. geosporum* spores showing that the spore's outer hyaline layer was strongly degraded and was probably substituted by mucilaginous products as suggested by Maia and Kimbrough (1998) (D and E in fig.5). Prior to the microscopical preparation, *G. geosporum* spores were divided into three maturity stages visible under the dissecting microscope: the youngest spore, light yellow-brown without or containing a few dark patches; medium-brown with many patches; dark-orange brown with many patches or one giant patch. A rough surface composed of the degraded and mucilated outer hyaline layer was present to different extents depending on the spore maturity stage. Out of 38 light-coloured spores observed, only 6 had a smooth surface, 18 were covered with a roughness up to half of the visible surface by SEM, and the surface of 14 was entirely rough (A in fig.5). Out of 24 medium brown spores, 16 were virtually smooth, 8 were covered with a rough material on

50% of the surface and eight were entirely rough (B in fig. 5). Finally out of 17 dark-coloured spores examined, 15 were entirely smooth and only 2 were slightly rough (C in fig.5). Differently-sized bacterial cells were present either in the sloughing hyaline layer or on the surface of the second, laminated wall layer (F in fig.5). Decaying material complicated the observation of bacterial cells because they appeared to be recovered with their own mucilage. On the smooth laminated surfaces, bacterial filaments were observed covered with mucilage products (G in fig.5). Different lysis zones were also located near the bacterial cells (F, H in fig.5). Many small-sized cells were also present (H and I in fig.5).

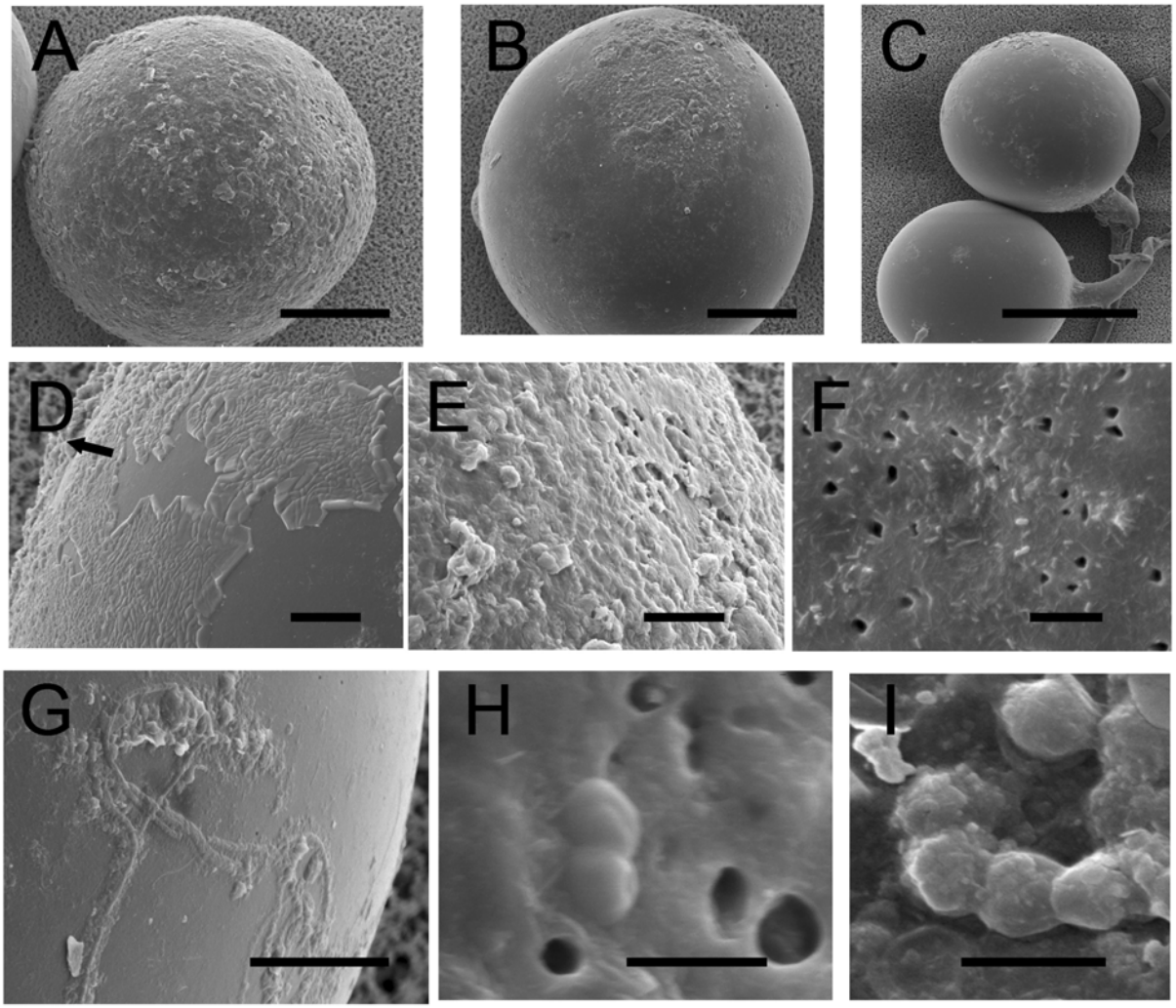

Fig.5. SEM images of the surface of *G. geosporum* spores. (A) light yellow-brown spore with its sloughed and degraded outer hyaline layer covering the whole surface; (B) medium-brown spore with a residual outer hyaline layer; (C) dark orange-brown spore that lost almost all its outer hyaline layer; (D) outer hyaline layer starting to "peel off" and being substituted by mucilaginous products (arrow); (E) mucilagenated outer hyaline layer; (F) Bacterial cells of various shapes adhering to the surface of the laminated layer, lysis zones (holes) in the spore wall; (G) filamentous bacterial cells adhering to the laminated layer covered with mucilaginous products; (H) Small-sized cells on the spore surface; (I) Small-sized cells covered with mucilaginous products. Bars (µm): A, B = 50; C = 100; D, E, F,G = 10; H, I = 1.

3.5 Discussion

The bacterial community associated with the *Glomus* spores was more influenced by the AMF identity (*G. geosporum* or *G. constrictum*) than by the host plant (*H. pilosella* or *P. lanceolata*). Despite the impact of the root on its surrounding environment and consequently on the microbial community, the plant did not predominantly affect the spore-associated bacterial community structure. Moreover, there was a good homogeneity within replicates. The AMF spores seem then to provide a microhabitat with particular conditions for the development of specific bacterial populations. The difference in composition of the spore wall or of hyphal exudates of these two *Glomus* species may have played a major role in the selection of bacterial populations living on the spore. In addition, the two *Glomus* were isolated from the same site (van der Heijden et al., 1998) and subcultured under the same conditions. The subculturing process may have enriched spore-associated bacterial populations adapted either to *G. geosporum* or to *G. constrictum* which could increase the discrepancies between the spore-associated bacterial community structures of the two fungi at the time of analysis.

Roughly, 1/3 of the DGGE bands, having some of the highest relative intensities, were found in profiles from all the culture conditions. As a whole, these bands represented more than 50% of the relative intensity of the entire profiles. These bands comprised sequences mainly affiliated to genera with hydrolytic representatives (*Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter* and *Pseudomonas*). These biopolymer-degrading bacteria are probably feeding on the outer hyaline layer of both species´ spores that is composed in majority of chitin, a straight chain polymer of N-acetylglucosamine (Sbrana et al., 1995). SEM observations of *G. geosporum* spores confirmed that the outer hyaline layer is gradually being degraded by microorganisms located on the spore surface. The light spores represent a more juvenile state in which the outer hyaline layer is at an early stage of degradation and the darker ones a more mature state as this hyaline layer was in most cases completely degraded. Bacterial cells were found either adhering to the laminated layer or embedded in the sloughing hyaline layer. Enzymatic vesicles have been observed in the outer layer of *G. mosseae* spores (Filippi et al., 1998). In our study, this bacterial lytic activity may explain the numerous holes near the bacterial cells on the spore surface.

Some of the bacteria were identified as *Burkholderia* spp.. Interestingly, bacteria-like organisms of the same genus were found in the cytoplasm of *Gigaspora margarita* (Bianciotto et al., 1996; Bianciotto et al., 2000). They harbour genes involved in nitrogen

fixation (Minerdi et al., 2001) or in phosphorus transport (Ruiz-Locano and Bonfante, 2000). The most dominant bacterial population we identified was constituted by the genus *Flexibacter*. This genus is well known for its capabilities to degrade biomacromolecules in various habitats (Reichenbach, 1999). Some *Flexibacter* species can form long threads up to 50 μm long (Reichenbach, 1999). In our study, SEM observations revealed that many long filaments were present on the spore surface. However, these filaments could also be actinobacteria. Actinobacteria were often found to be associated with AMF spores. For example, Mugnier and Mosse (1987) reported that *G. mosseae* spores germinated in vitro only in the presence of microorganisms including *Streptomyces orientalis*. Ames and co-workers (1989) found that out of 190 spores examined, 100 were colonized by one or more chitin-decomposing microorganism, 82% were colonized by actinomycetes, 17% by bacteria and 1% by fungi. Carpenter-Boggs et al. (1995) demonstrated a positive correlation between higher germination rate and the amount of production of geosmin, CO_2 and 2-methylisoborneol by the actinomycetes. In our study, none of the DGGE bands sequenced were affiliated with Actinobacteria. As not all the discrete bands of the DGGE profiles were sequenced, we may have missed this group of microorganisms. Moreover, the studies mentioned above have been performed with isolation techniques meaning that only a small fraction of microorganisms present on the spores were taken into account. It is probable that the more abundant-spore associated bacteria might not be cultivable. Many are perhaps in a viable but not cultivable state described by Mascher et al. (2000). The fact that numerous bacteria observed by SEM were small-sized, supports this hypothesis.

Several bands sequenced were affiliated with genera capable of cellulolytic activity. The presence of cellulolytic bacteria on the spore surface indicates that microorganisms attached to the spores may also decay plant material around them (e.g. cellulose from sloughed off cortical root cells). Gryndler et al (2002) have reported that an amendment of cellulose, if incubated in the soil for a long time, was able to increase the number of bacteria and saprophytic fungi in the soil as well as to stimulate AMF growth. These authors suggested that the AMF stimulation could result from an AMF uptake of nutrients released from the decomposing saprophytic microflora. The root exudates also provide the microorganisms with readily assimilable organic substrates (Lynch and Whipps, 1990). The presence of the plant roots could then stimulate the growth of the biopolymer-degrading populations who would in turn accelerate the decaying process of the fungal outer wall. Indeed, the outer hyaline layers are often the first component of the spore wall to be synthesized in juvenile spores and are rarely present on mature spores in the soil (INVAM). In addition, a strong presence of active biopolymer-degrading bacterial populations on the spore surface could favour spore germination by releasing nutrients and degrading toxic compounds or

molecules inhibiting germination. The process of maturation and eventual germination of the AMF spores might then benefit from the activity of the surface microorganisms degrading the outer hyaline layer.

3.6 Acknowledgments

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4 Mycorrhiza helper bacteria

In chapters 2 and 3, we studied the effect of AMF on the bacterial community. We will now focus on how bacteria affect AMF growth. Several PGPR strains were selected for their PGP properties in Pantnagar by Rachna Gaur during her thesis in view of subsequent bio-inoculation with AMF trials in rainfed fields (Gaur, 2003). All these PGPR strains could produce the antifungal compound 2,4-diacetylphloroglucinol. Before testing these strains in the fields, it was therefore necessary to determine if they had no deleterious effects on mycorrhizal colonization and spread. Greenhouse pot experiments were undertaken in Pantnagar to test the influence of these PGPR strains on the AMF root colonization (Gaur, 2003; Gaur et al., 2004). An increased percentage of root colonization by AM fungi was observed with several PGPR strain bio-inoculations as compared to non PGPR inoculated controls. These PGPR strains could then be acting as mycorrhiza helper bacteria in the wheat mycorrhizosphere. However, we did not know how these PGPR strains affected directly the AMF hyphal growth or sporulation in the hyphosphere. Our objective was then to design and test an in vitro system to investigate the influence of PGPR on the mycorrhizal spread and development in the hyphosphere with special reference to spore counts, extraradical hyphal biomass and percentage root colonization.

This chapter is a manuscript entitled "*In vitro* compartmental study on the interactions between different rhizospheric bacteria and *Glomus intraradices* in the hyphosphere" that is submitted to Canadian Journal of Botany.

In vitro compartmental study on the interaction between different rhizospheric bacteria and *Glomus intraradices* in the hyphosphere

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4.1 Abstract

The present paper reports an *in vitro* investigation on the effect of different PGPR strains on the AM fungus *Glomus intraradices* in the hyphosphere. The experiment was carried out in a two-compartmental Petri plate system using Ri T-DNA transformed clover roots permitting the separation of the hyphosphere from the mycorrhizosphere. The main objective was to investigate the influence of bacteria on the mycorrhizal spread and development with special reference to spore counts, extraradical hyphal biomass and percent root colonization. Even though the strains tested were all DAPG producers, their effects on the AMF development varied from inhibition to improvement of the hyphal biomass or spore production. Interestingly, there was a positive mutualistic interaction between one bacterial strain, determined as *Pseudomonas synxantha*, and *G. intraradices* that could be explained by the bacterial catabolism of fungal proteins providing a carbon source for the bacteria and a recycled N source for the fungus. *P. synxantha* possessed the strongest proteolytic activity out of all the other tested strains. This finding suggests that the ability to secrete proteolytic enzymes is an important trait related to MHB capabilities.

4.2 Introduction

Arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) have an important role in the improvement of soil fertility and plant health (Barea et al. 2002a). Understanding the interactions between these beneficial microorganisms would improve the management of sustainable agronomic practices.

The production of spores is probably the most important fitness determinant of AMF, at least in disturbed habitats, but vegetative hyphal growth can also be an important way of propagation (Olsson et al. 2002). The stimulation of hyphal growth and spore production could enhance AMF fitness by increasing the chance of contact between fungal hyphae and the plant roots and increase access to potential new resources. Synergistic associations between AMF and PGPR strains would then further improve plant health and soil fertility. Such associations could take place either in the mycorrhizosphere or in the hyphosphere. The mycorrhizosphere is defined as the zone under the joint influence of the root and fungal hyphae (Linderman 1992). The hyphosphere is defined as the zone under the influence of AM hyphae only (Andrade et al. 1998a; Gryndler 2000).

Bacteria beneficial to AMF have been defined as mycorrhiza helper bacteria (MHB) in the review by Garbaye (1994). He reported that mycorrhizal root colonization could be increased by the bacterial production of cell wall softening enzymes or plant hormones. Hyphal growth could be stimulated by the bacterial production of organic acids, vitamins, amino acids or CO_2. Finally, spore germination could be enhanced by the bacterial production of CO_2 (Bécard and Piché 1989).
In a previous study, in the framework of the Indo-Swiss collaboration in biotechnology (ISCB, SA-7), Gaur et al. (2004) have studied the effect of different 2,4-diacetyphloroglucinol (DAPG) producing *Pseudomonas* on mycorrhizal growth in wheat pot cultures. These strains were selected for their promising plant growth promoting properties and could be used as bio-inoculants in marginal rain-fed wheat fields. Despite the fact that DAPG is involved in the disease suppression of many plant fungal pathogens (Keel et al. 1992; Weller et al. 2002) the inoculated strains had no adverse effect on mycorrhizal growth as reported in other studies as well (Barea et al. 1998; Edwards et al. 1998). Moreover, in Gaur's study, some of the bacterial strains tested were stimulating AMF colonization indicating that these rhizobacteria could be functioning as MHB in the mycorrhizosphere. However, besides the AMF root colonization percentage, it remains difficult to detect a direct effect of bacteria on the AMF hyphal growth or spore production not only in the mycorrhizosphere but also in the hyphosphere. Indeed, the complexity of soil matrix and the diversity of microorganisms within it further complicates the monitoring of a high number of different bacterial strains or AM fungus without using marker techniques such as FISH (Amann et al. 1995), green fluorescent protein (Normander et al. 1999), or fluorescence-labelled antibodies (Lübeck et al. 2000). The use of *in vitro* ROC systems can reduce the complexity found in the environment and allow maintaining controlled conditions. Such systems have already allowed to assess a wide range of bacteria and fungi that enhance germination of AM fungal spores and stimulate hyphal growth in the mycorrhizosphere (reviewed in Fortin et al. 2002).

The present paper reports an *in vitro* investigation on the effect of different PGPR strains on the AM fungus *Glomus intraradices* in the hyphosphere. The experiment was carried out in a two-compartmental Petri plate system using Ri T-DNA transformed clover roots permitting the separation of the hyphosphere from the mycorrhizosphere. This separation allowed to apprehend the interactions between the bacteria and AM fungi in the hyphosphere without any other interference into play such as the presence of roots or other soil organisms. The main objective in the present study was to investigate the influence of bacteria on the mycorrhizal spread and development with special reference to spore counts, extraradical hyphal biomass and percent root colonization. In order to test the reproducibility of the experiment, it was conducted at two different laboratories that are project partners under the Indo-Swiss Collaboration in Biotechnology (ISCB, SA6 and SA7 projects), the Laboratory of Microbiology at the Neuchâtel University, Neuchâtel, Switzerland and the Centre for Mycorrhizal Research at the TERI, New Delhi, India. The experimental conditions were set as closest as possible between the two labs and the protocols followed were the same. The combined data obtained in the two laboratories is presented in this paper.

4.3 Materials and Methods

Bacterial strains

Five strains were used as bacterial inocula to study synergistic or antagonist effects on the mycorrhizal growth in the hyphosphere. They were all DAPG producing rhizobacteria isolated from three farmer's fields in the Budaun district near the Ujhani town located in Uttar Pradesh, India (Gaur et al. 2004). The fields had a history of at least 20 years of continuous rice-wheat rotation and the strains were isolated from the wheat rhizosphere. The strains were characterised by amplifying and sequencing a 570 bp fragment of the 16S rDNA fragment according to Tarnawski et al. (2003). The percentage homology with existing 16S rDNA sequences in the *Genbank* database was determined using the BLASTt software (Altschul et al. 1997: http://www.ncbi.nlm.nih.gov).

Preparation of starter cultures

Ri T-DNA transformed roots (transformed at the Centre for Mycorrhizal Research, TERI, India) of clover (*Trifolium subterraneum)* were maintained in the form of actively growing root tips on M media (Bécard and Fortin 1988). The M medium composed of the following chemicals (mg/l): $MgSO_4 \cdot 7H_2O$ (731.0); $Ca(NO_3)_2 \cdot 4H_2O$ (288.0); KNO_3 (80.0); KCl (65.0); $MnCl_2 \cdot 4H_2O$ (6.0); KH_2PO_4 (4.8); $ZnSO_4 \cdot 7H_2O$ (2.65); H_3BO_3 (1.50); KI (0.75); $CuSO_4 \cdot 5H_20$ (0.13); $Na_2MoO_4 \cdot 2H_2O$ (0.0024); NaFeEDTA (8.0); Glycine (3.0); Thiamine·HCl (0.1);

Pyrodixine·HCl (0.1); Nicotinic acid (0.5); Myo-Inositol (50.0); Sucrose (15000) was used. It was gelled using Phytagel (Sigma) at different concentrations depending on its utilisation (0.3% for maintenance, 2% for the starter cultures and 4% for the experiment). Finally, the pH of the medium was adjusted to 5.4 before autoclaving it at 120°C for 20 minutes. After an incubation period of six weeks, at 28 ±1 °C in the dark, the dual culture (roots colonized with the AM fungus *Glomus intraradices)* was sub-cultured in Petri plates containing M media gelled with 2% phytagel. This step was performed to set the symbiosis into a highly active state. These sub-cultures were incubated another 6 weeks and were used as the starter cultures to initiate the experiment. An AMF-free root control of un-colonized transformed clover roots was also maintained.

Mycorrhizal inoculations in two-compartmental Petri dishes

Two-compartmental split Petri dishes, firstly developed by St-Arnaud et al. (1996), were used in this study to delimit a hyphosphere totally free of roots. This system (shown in fig.1) enabled to define the radical compartment or mycorrhizosphere (containing the transformed roots and AMF) and the extraradical compartment or hyphosphere (containing only AMF). Each compartment received approximately 30 ml of minimal M medium gelled with 4% phytagel taking care that both the compartments get equal quantities of media. A small stub of medium of 1.5x1.5 cm surface, containing transformed clover roots and *G. intraradices,* was cut using a sterile scalpel under sterile conditions from the starter cultures. The stub was inoculated in the radical compartment after removing a same sized fresh medium stub. The double-compartment Petri plates were then covered and sealed using parafilm and allowed to grow further.

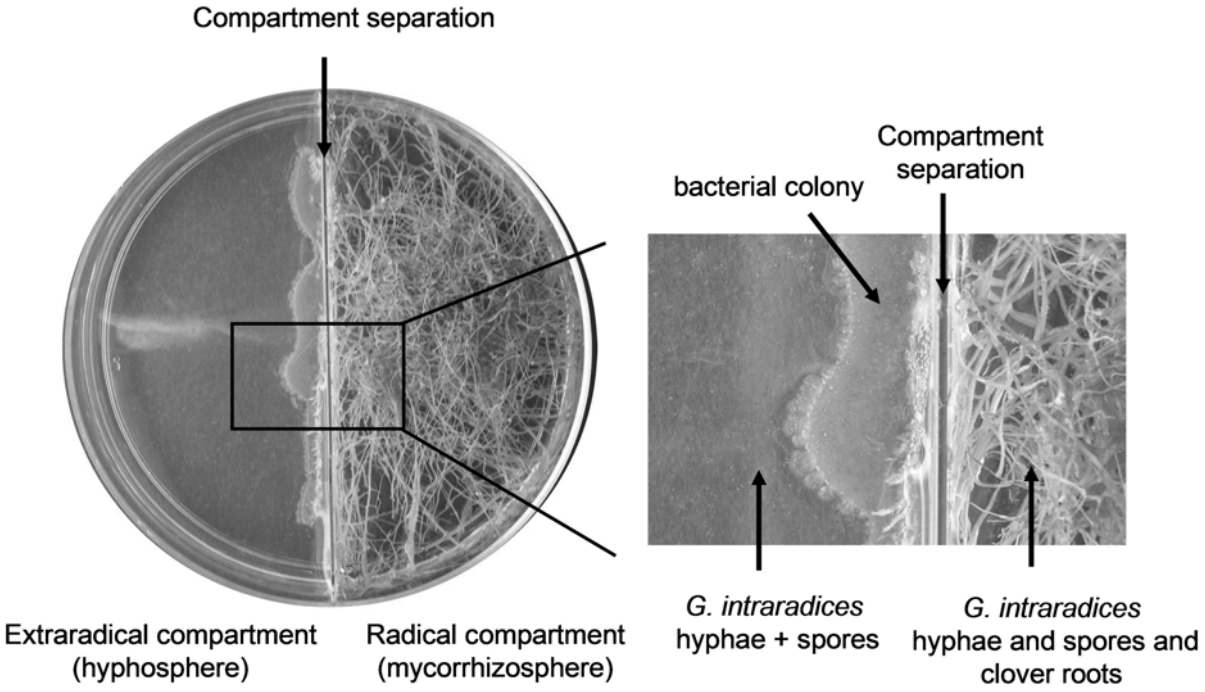

Fig.1. Double-compartment root organ culture system allowing separation of the radical (or mycorrhizosphere) and extraradical (or hyphosphere) compartments. The radical compartment contained Ri T-DNA transformed clover roots infected with *Glomus intraradices*. The extraradical compartment comprised hyphae and spores of *G. intraradices* and the tested bacterial inoculum. The culture had been incubated for three months and is ready for sampling.

Bacterial inoculation

After about one month incubation, the mycelium was able to cross the partition of the compartmental plate. At this stage, a bacterial suspension was added in the extraradical compartment. The bacterial cultures were grown in nutrient broth overnight under agitation at 25°C. They were then centrifuged at 10000 rpm for 1 minute and the pellet was re-suspended in 0.8% saline solution. The suspension was then diluted in the saline solution until reaching a bacterial concentration of 1.5×10^7 cells per ml. From this dilution, 100 µl were added in the extraradical compartment parallel to the partition of the plate as soon as the mycorrhizal hyphae crossed this partition from the radical to extraradical compartment. The purpose of the delayed inoculation was to inoculate fresh active bacteria when the mycelia were crossing the partition of the plate, so that they encounter the bacteria at their most active stage. The culture was further incubated 12 weeks to allow the mycorrhizal fungi to complete its total life span. After one-month incubation, the AMF-free root controls were inoculated the same way as explained above. Following the bacterial inoculation the AMF-free root controls were incubated another 12 weeks. Each double-compartment system with a specific bacterial treatment was replicated three times. As bacteria-free controls, three AMF-inoculated root organ cultures were performed without bacteria.

Sampling procedure

After twelve weeks of incubation, both compartments of the Petri-plates were recovered separately. After the incubation, the gelled M medium was deinonized with an equal volume of 10 mM sodium citrate buffer, pH 6 (Doner and Bécard 1991) achieved by continuous shaking at 150 rpm at 40 °C. After 2 hours incubation in the deionizing solution, the suspensions from the radical and extraradical compartment were recovered from the deionized media by sieving with a 300 BSS mesh. The suspensions were then thoroughly washed with water to remove the adhering buffer solution. In the suspension from the radical compartment (mycorrhizosphere), representative samples of roots were stained using 10% KOH and Trypan blue for the quantification of the percentage of mycorrhizal colonization using the grid-line intersect method (Brundrett et al. 1994). The extraradical suspension was centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the remaining pellet composed of the fungal hyphae and spores was first chopped into small pieces and then re-suspended in a 5 ml of deionised water. Representative 0.5 ml aliquots were drawn and analysed for the spore counts and extramatrical mycelial length with Tennant' s formula (Tennant 1975) using a 1 mm sized grid. This step was repeated five times and the mean of these was considered as the final reading. The value obtained was extrapolated with the total volume of water (5 ml) for the calculation of total spore count and extramatrical mycelial biomass.

The bacterial counts were performed at the step where the extraradical compartment was deionized in citrate buffer. Under laminar flow sterile conditions, 1 ml of the suspension was pipetted into 9ml of 0.8% sterile saline solution and ten-fold serial diluted in sterile saline solution. The last five dilutions corresponding to dilutions 10^{-4} to 10^{-8} were plated on Angle's medium (Tarnawski et al. 2003) and incubated for 48hr at 25°C.

pH measurement

Several double-compartmented systems (with AMF and AMF-free roots) were set-up and inoculated with the R81 strain the same way as previously described. A solution of 1% bromocresol blue was poured on the different compartments after three months incubation. The Petri dishes were then photographed and their image was compared to bromocresol blue standardized solutions (their colour spectra corresponded to a pH value).

SEM analysis

Samples were fixed with OsO_4 1% and air-dried. After coating with gold, the samples were examined with a Phillips XL 30 Scanning electron microscope (SEM) with an acceleration of 10kV. Crystal elemental composition was determined using an EDS (energy dispersive spectroscopy) microprobe.

Statistical analysis

Before statistical analysis the bacterial counts were ln (x+1) transformed. The data were subjected to analysis of variance (ANOVA) and the means were compared with the least significant difference (LSD) test using the S-Plus software vers. 6.1(Insightful Corp, USA).

4.4 Results

The bacterial inocula were either composed of individual strains or consortiums. Two experiments were carried out in parallel. In experiment A, the bacterial treatments were composed of 5 individual strains (R14, R62, R81, R117, R709) and one consortium (mix I composed of R62 + R81 + R117). In experiment B, the bacterial treatments were composed of 3 individual strains (R62, R81, R709) and one consortium (mix II composed of R62 + R81 + R709).

Strain determination

Two strains were affiliated to β-proteobacteria: R14 (*Comamonas acidovorans*, 99% homology) and R117 (*Acidovorax facilis*, 99%). The three other strains were related to the genus *Pseudomonas*: R62 (*P. jessenii*, 99%), R81 (*P. synxantha*, 99%) and R709 (*P. corrugata*, 99%).

Mycorrhizal data

Discrepancies between the spore counts and hyphal biomass of the controls between experiment A and B existed despite having kept the same protocol probably due to differences in incubation conditions. Therefore, in order to limit the location effect and to concentrate on the effect of the different bacterial treatments, the values of the treatments were calculated as the percentage increase (or decrease) with regard to the bacteria-free controls.

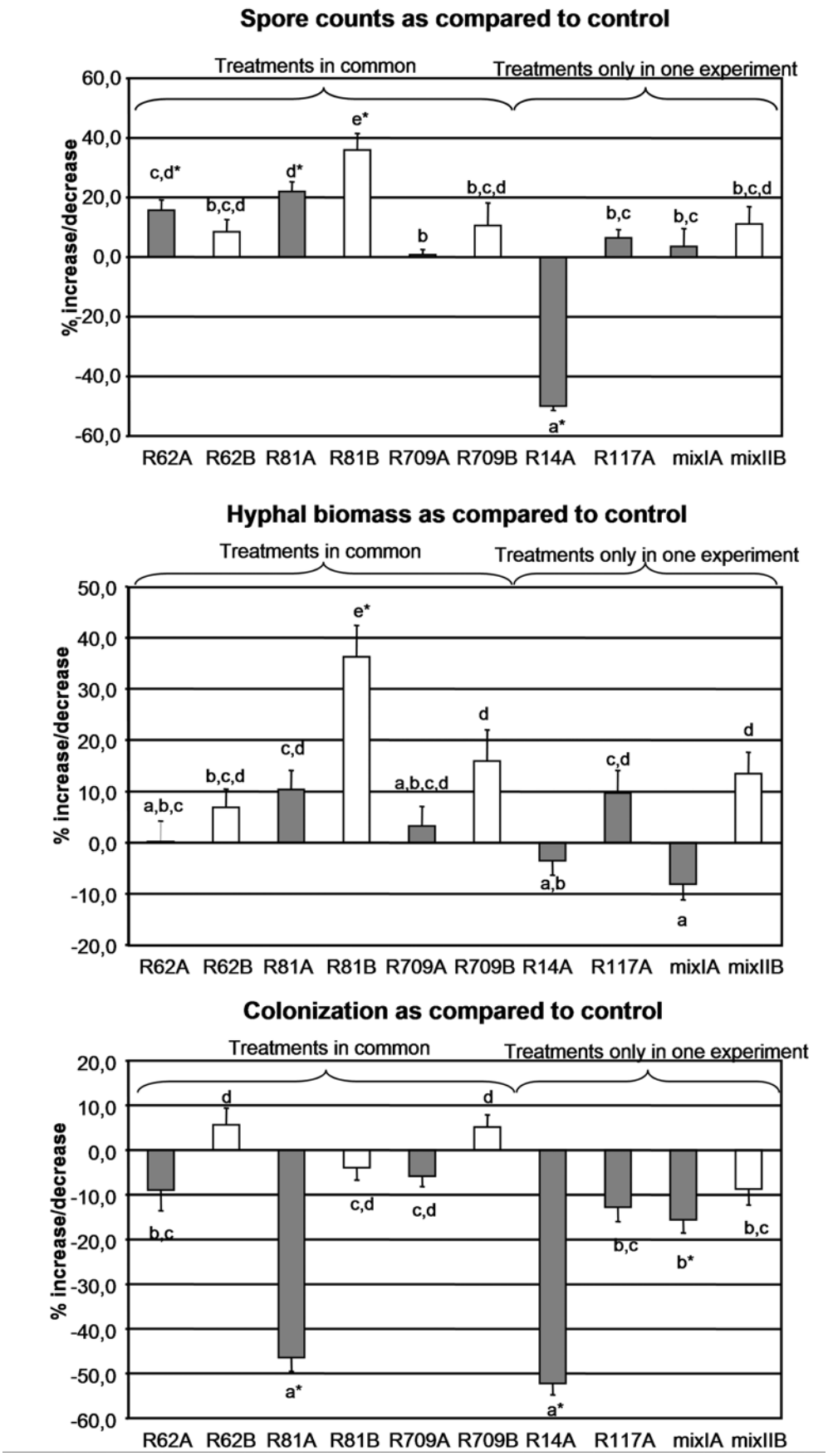

Fig.2. Percentage increase/decrease in spore counts, hyphal biomass and root colonization of different bacterial treatments as compared to their respective bacteria-free controls in the extraradical compartment. Bacterial treatments were inoculated either in both experiments A and B (R62, R81, 709), in experiment A only (R14, R117, mix I) or in experiment B only (mix II). White columns indicate bacterial treatments analysed in experiment A. Grey columns indicate bacterial treatments analysed in experiment B. Values are means of three replicates with standard error bars. Non-significantly different samples ($p<0,05$) have the same letter. Samples significantly different ($p<0,05$) than the control are marked with an asterix.

Similar trends were noticed in the spore counts and hyphal biomass between the bacterial treatments R62, R81, R709, common to experiments A and B (fig.2). The best increase in spore counts and hyphal biomass as compared to the bacterial-free controls was obtained with *P. synxantha* R81. The beneficial effect of this strain was the most significant in experiment B with an increase of over 30% in spore counts and hyphal biomass. However, when this strain was associated with other bacteria, the beneficial effect could not be repeated. Contrarily to all the other strains tested, *C. acidovorans* R14 had a negative effect on the fungal growth. The spore counts was 50% less than the control and the hyphal biomass 3.5% less. The percentage root colonization of the fungus decreased in the presence of most of the bacterial strains tested. This decrease is the most significant with the strains *C. acidovorans* R14 and *P. synxantha* R81 in experiment A. The extent of the decrease in colonization with the *P. synxantha* R81 could not be confirmed in experiment B.

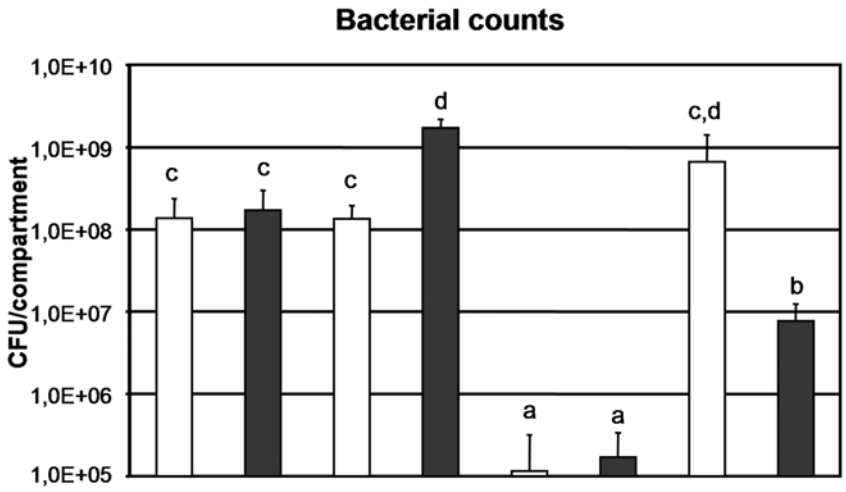

Fig.3. Bacterial colony forming units per extraradical compartment in experiment B. White columns indicate bacterial counts in AMF-free compartments. Black columns indicate bacterial counts in the presence of AMF. Values are shown as means of 3 replicates with standard error bars. Non-significantly different samples ($p<0,05$) have the same letter.

Bacterial counts

The bacterial colony forming units (CFUs) in the extraradical compartment was assessed in experiment B with AMF-free root controls and AMF roots (fig.3). The CFUs of *P. jessenii* R62 and *P. corrugata* R709 were similar whatever *G. Intraradices* was present or absent. However, significantly less CFUs were counted in the mix treatment and significantly more in the *P. synxantha* R81 .

pH measurement

To observe variations of the pH in our two-compartmental system, several cultures of transformed clover roots (with and without *G. intraradices* infection) were set the same way as the previous experiments. The bacterial treatment used was the *P. synxantha* R81 strain alone. To measure the pH, a solution of 1% bromocresol blue was poured on the different compartments after three months incubation. In the radical compartment, the pH was between 5.0 and 5.5 similar to the pH value of the un-inoculated M medium. However in the extraradical compartment the pH increased up to 7 and near the bacteria colonies to more than 8.0. Moreover, precipitates were found near the bacterial colonies in the medium gel. The precipitates were recovered and analysed with SEM microscopy and energy dispersive spectroscopy. It revealed that they were mostly related to calcium carbonate crystals and to a lesser extent to calcium phosphates or magnesia calcites (Braissant, personnal communication). The formation of these crystals (image 1) could be due to the increase in pH localised near the bacterial colonies thus precipitating the carbonate calcium compounds. Contrarily to the extraradical compartment containing hyphae, the pH in the extraradical compartment of the AMF-free controls did not increase around the bacterial colonies and remained between 5.5 and 6.

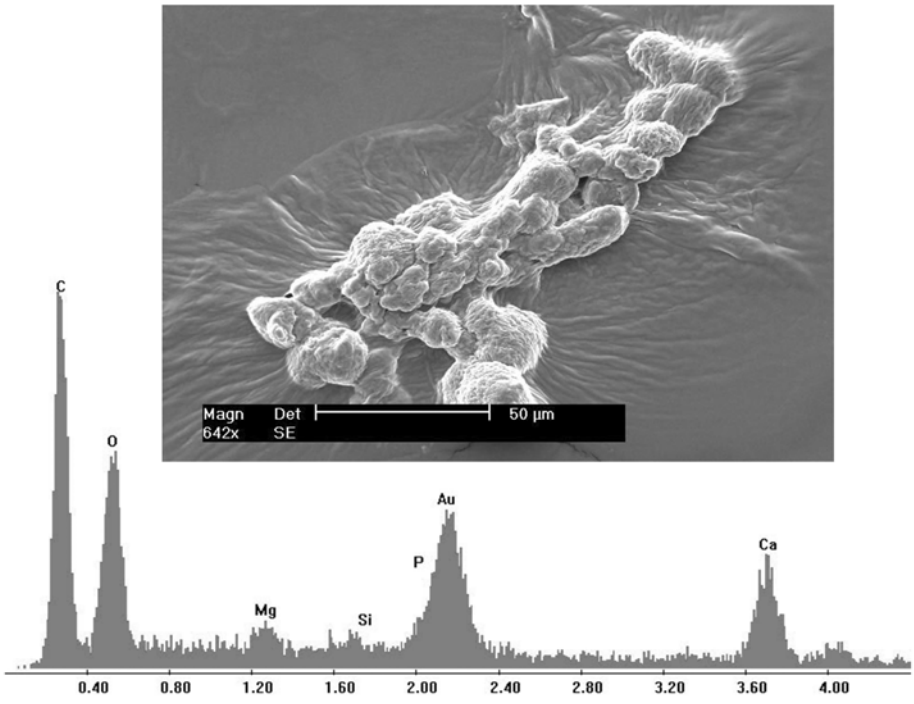

Image 1. Crystal precipitate found near bacterial colonies visualised with scanning electron microscoscopy. EDS graph of the crystal composition is shown below.

Stereomicroscope images of the extraradical compartment

The cultures in experiment B were observed before the sampling under the stereosmicroscope (Olympus) and photographed. In the extraradical compartment, the amount of hyphae and spores was too high to determine quantitatively the number of different hyphal structures. Hence, only a qualitative analysis was performed. In the R81 and R62 treatments, the hyphae formed more branches and arbuscular-like structures (black arrow in image 2) near the bacterial colonies as compared to R709 and the consortium. Moreover, protruding colonies (white arrow in image 2) were formed on the exterior of the main colonies of R62 and R81 indicating a re-growth.

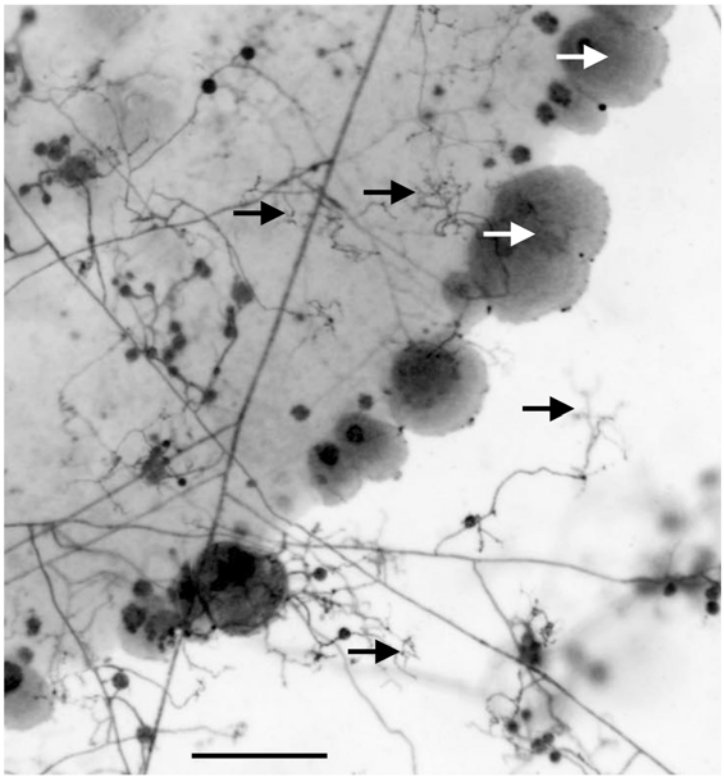

Image 2. Stereomicroscope image of the distal compartment of bacterial treatment R62. Black arrow = formation of arbscular-like structures; white arrow = protruding colony. Measure bar = 0.5 mm.

4.5 Discussion

Experimental design

Compartmented Petri dishes first described by St-Arnaud et al (1996) have been used by several authors successfully to study multiple features of AMF extraradical hyphae. They include studies related to the architecture of the hyphae (Bago et al. 1998), effects on nitrogen uptake and pH modifications (Bago et al. 1996), on phosphorus solubilization and pH modifications caused by the interaction of AM hyphae with soil bacteria (Villegas et al.,

2001), and of soluble substances released by the AM hyphae on different microorganisms (Filion et al. 1999). In the present study, the compartmented system permitted to test the direct effect of different bacterial strains on the growth of extraradical hyphae and on the spore production of *G. intraradices* cultured with Ri-TDNA transformed clover roots. The combined data of experiments run in parallel in two different laboratories were presented here. Differences were observed between the two studies in the fungal colonization percentage. Indeed, the root colonization percentage of the AMF was less important in experiment A as compared to experiment B. These discrepancies might be due to different incubation temperatures or room humidity between the two labs affecting the fungal colonization level. In addition, as the grid-line intersect method used to assess the percentage colonization is based on an observation, visual appreciations might not be similar from one observator to another. Contrarily to the colonization percentage, the hyphal biomass and spore counts showed similar trends between the two experiments. This observation could suggest that the colonization percentage may not necessarily affect the other AM fungi parameters. Indeed, the density of spores is not always correlated to the root colonization level (Smith and Read 1997). The greatest beneficial effect on spore counts and hyphal biomass was observed in both laboratories with the strain *P. synxantha* indicating the reliability of the method used to analyze a direct effect of bacteria on AMF hyphal growth and spore production in the hyphosphere.

Influence of the bacteria on hyphal biomass and spore counts of G. intraradices

Even though the strains tested were all DAPG producers, their effects on the AMF development varied from inhibition to improvement of the hyphal biomass or spore production. Only 10 µM of DAPG can negatively affect mycelial development (Barea et al. 1998). It is, therefore probable that the strains may not be producing DAPG in the M medium Petri plates in sufficient quantities to inhibit fungal growth.

Bacterial treatment with the strain *P. synxantha* R81 had the most efficient effect on AMF growth in the hyphosphere. However, when it was associated with other strains in two different bacterial consortium treatments, no more mycorrhiza helper effect was observed. The presence of other strains might have limited the access of this strain to the media nutrients or might have inhibited its growth by the production of antibiotics. This exclusion in a Petri dish does not necessarily imply an exclusion in the soil or the rhizosphere. Indeed, these environments are much more complex and dynamic than a Petri dish condition, allowing a greater number of niches to be occupied by the bacteria.

The increase in hyphal biomass observed in some treatments could be caused by the bacterial production of volatile or diffusible substances such as CO_2, organic acids, vitamins, and amino acids (Mugnier and Mosse 1987; Tylka et al. 1991, Garbaye 1994). The higher

number of spore counts of the treatments as compared to the control could be reflected by a higher hyphal biomass allowing a greater allocation of hyphal branches for the spore formation. However, in several treatments a higher hyphal biomass was not necessarily correlated with a higher number of spores. In those cases, the increase in sporulation might have resulted in a modification of the nutrient availability affecting the fungal development. Indeed, modifications in the levels of phosphorus and nitrogen have been reported to affect the spore density or germination rate of several AMF species (Douds and Schenck 1990; Bressan 2001). Conversely, the utilisation of sucrose by the bacteria did not imply a competition for this C source as AMF do not uptake carbohydrates via the external mycelium (Pfeffer et al. 1999).

The pH reached 7 in the extraradical compartment when it was covered with AMF hyphae and spores. Moreover, the presence of the fungal hyphae is necessary for the pH to increase (the pH measure in the AMF-free extraradical compartment control near the bacterial colonies remained between 5,5 and 6). Bago et al (1996) report that the modification of the pH in the extraradical compartment could result from the nitrate uptake by the extraradical hyphae coupled to a H^+-symport mechanism. However, pH increase was stronger in the neighbourhood of bacterial colonies. This suggests that catabolism of fungal secretions may be responsible for this alkalinization. Contrary to neutral sugars (such as sucrose), oxidation of organic acids or proteins / aminoacids will induce a pH increase. Glomales are not known to secrete organic acids; however, they are capable of secreting high amounts of proteins in the environment, such as glomalin (Wright and Upadahya 1998).

Admitting the approximative global formula C_4H_9NO for a protein, the following reactions would occur:

Protein oxidation: $2\ C_4H_9NO + 10\ O_2 \longrightarrow 8\ CO_2 + 2\ NH_3 + 3\ H_2O$

Acid-base reaction between ammonia and CO_2 : $CO_2 + H_2O + 2\ NH_3 \longrightarrow CO_3^{2-} + 2\ NH_4^+$

Calcite precipitation: $Ca^{2+} + CO_3^{2-} \longrightarrow \underline{CaCO_3}$

Global reaction:

$2\ C_4H_9NO + 10\ O_2 + Ca^{2+} \longrightarrow 7\ CO_2 + 2\ H_2O + 2\ NH_4^+ + \underline{CaCO_3}$

This explains the formation of calcium carbonate precipitates near the bacterial colonies.

Moreover, the pH measured around the precipitates was around 8, which is close to the equilibrium constant of calcium carbonate (8.4). The alkalinisation process should then not exceed pH 8.4 because the calcium carbonate would then buffer the medium (E. Verrecchia, personal communication).

The protein catabolism could be beneficial for the fungus, which recovers part of the nitrogen lost through protein secretion. Moreover, for the fungus, the assimilation of ammonium is energetically more favourable than nitrate (Hawkins et al. 2000). In addition, fungal self-inhibitory proteins or peptides (Leite et al. 1992) could be removed by the bacterial catabolism. The benefit for the bacteria appears more obvious: the amino acids would serve as carbon substrates, provided the bacterium secretes proteolytic enzymes. Indeed, strain *C. acidovorans* R14, which inhibits fungal proliferation, is not proteolytic, whereas *P. synxantha* R81 produces the highest amount of proteases among the tested strains (Gaur et al. 2004). Moreover, counts of R81 were significantly higher in the extraradical compartment when AMF was present. This suggests that the ability to secrete proteolytic enzymes is an important trait related to MHB capabilities.

Increased absorbing surface

The presence of *P. jessenii* R62 and *P. synxantha* R81 induced a higher level of branches and ramifications on the fungal extraradical hyphae. Some arbuscular-like structures (ALS) as defined by Bago et al. (1998) were visible also near the bacterial colonies. This finding is in accordance with the work of Hildebrandt et al. (2002) who discovered that on M modified medium without root hosts, the presence of *Paenibacillus validus* colonies induced an increase in branching and the formation of densely packed coils of the hyphae of *G. intraradices*. In that study, the formation of the coils and subsequently the spores did not require intimate contact of the bacteria with any of the fungal structures. This observation could indicate the possibility of exchanges between bacteria and the AMF without the influence of plant roots. More ALS could then mean more zones of exchange between the AMF and the bacteria resulting in a mutual benefit for the two organisms. More ALS could also mean a higher transfer of nutrients to the spores (Bago et al. 1998). In support to this hypothesis, *P. jessenii* R62 and *P. synxantha* R81 treatments induced higher spore counts in the extraradical compartment and showed an increased number of ALS as compared to other treatments.

Conclusion and perspectives

The use of an in vitro two-compartmented system allowed to assess successfully a direct mycorrhiza helper effect of different DAPG-producing strains on the hyphal growth and sporulation of *Glomus intraradices* in the hyphosphere. Similar trends were observed in the hyphal length and sporulation in experiments performed parallely in the two laboratories. Interestingly, there was a positive mutualistic interaction between *P. synxantha* R81 and *G. intraradices* that could be explained by the bacterial catabolism of fungal proteins providing a carbon source for the bacteria and a recycled N source for the fungus. Indeed *P. synxantha* R81 possessed the strongest proteolytic activity out of all the other tested strains. This mycorrhiza helper bacterium is now being tested in wheat fields as a bio-inoculant along with different AMF strains to study its plant growth promoting and mycorrhiza helper properties in field conditions. It would also be interesting to evaluate which molecules are secreted by the bacterium and the fungus when they are associated together to evaluate the mechanisms of these beneficial interactions. For example, Filion et al. (1999) have utilized such a system to evaluate the impact of soluble substances released by the extraradical mycelium on different microorganisms. This system could also be exploited for a large-scale primary screening of many PGPR strains in order to detect mycorrhiza helper capacities. It is simple to put in place and to maintain. Moreover, the growth conditions and experimental parameters can be better controlled than in soil microcosms. The best bacterial formulas could then be exploited as bio-inoculants in combination with AMF. A bio-inoculation using mycorrhiza helper bacteria could ensure faster hyphal growth or root colonization that would be beneficial in terms of soil exploration and nutrient uptake making the mycorrhizal plant more competitive. In addition, higher sporulation levels could support greater potential infection rates and increase the AMF presence in the soil in the long term ensuring the sustainability of the agricultural practice.

4.6 Acknowledgments

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5 Root colonization ability of selected PGPR strains marked with a green fluorescent protein

Several PGPR strains showed no deleterious effect on AMF development and some even increased mycorrhizal colonization or hyphal growth. These strains were then selected as bio-inoculants for subsequent field trials in our study site at Budaun. However, a successful introduction of these PGPR strains required evidence of establishment of the inoculants in the rhizosphere *in situ*. Our objective was then firstly to find an appropriate technique enabling to distinguish our PGPR strains from the indigenous bacterial populations and secondly, to test their root colonization capacity in greenhouse pot experiments.

5.1 Introduction

A successful introduction of effective PGPR strains in Indian fields requires evidence of establishment of the inoculants *in situ*. A successful root colonisation implies that the bacterial strain possesses root competence traits such as chemotaxis towards root exudates, flagella, compounds mediating attachment (adhesins, fimbriaie, pili, cell surface proteins and polysaccharides) and a capacity to metabolise root exudate compounds (Chin-A-Woeng and Lugtenberg, 2004). In order to monitor the root colonisation capacity of PGPR strains selected in Pantnagar that will be used as field bio-inoculants, it is necessary to distinguish them from the indigenous bacterial populations. Single cell images of root colonization is now possible as a result of the development of staining and microscopic techniques such as fluorescent antibody labelling, fluorescent in situ hybridization and marker gene technology (Sørensen et al., 2001). Molecular marker genes, conferring specific phenotypes, are now extensively used to monitor microorganisms in their environment (Errampalli et al., 1999). The tagging with the marker gene *gfp* coding for the green fluorescent protein (GFP) offers a better advantage over other reporter genes (e.g. *gus*A, *lac*Z, *lux*A) as GFP expression does not require specific substrates (except oxygen for the chromophore formation) nor complex media nor is subject to high background in most plants and bacteria (Chin-A-Woeng and Lugtenberg, 2004). Moreover, GFP is stable in the presence of many denaturants and proteases, persists at elevated T°C or at a pH range between 6 and 12 (Errampalli et al., 1999). The GFP is a 27 kDa polypeptide, which converts the blue chemiluminescence of the Ca^{2+} sensitive photoprotein, aqueorin, into green light (Chalfie, 1994). Wild type gfp absorbs blue light at 395 nm and emits green light at 510 nm (Ward et al., 1980).

Five rhizobacterial strains with established plant growth promoting properties were selected to be tagged with the GFP (strain description in table1).

Table 1: Characterization of the PGPR strains for the root competence study based on Shani (2002), Gaur (2003) and Gaur et al. (2004), chapter 4. Abbreviations: Fields in Budaun district Uttar Pradesh state (see chapter 6): LL = low input low yield; LM = low input moderate yield; HH = high input high yield; Wheat root fraction: RS = root-adhering rhizospheric soil; RE = rhizoplane/endorhizosphere. Plant growth properties (PGP): P = phosphate solubilization, IAA = Indole-3 acetic acid production, ACC = 1-aminocyclopropane-1-carboxylate deaminase production, Sid = Siderophore production, DAPG = diacetyl-phloroglucinol production.

	Origin		PGP properties					16S rDNA sequence affiliation (chapter 4)
Strain	Field	fraction	P	IAA	ACC	Sid	DAPG	(% homology)
R62	LM	RE	+	+		+	+	*Pseudomonas jessenii* (99%)
R81	HH	RE	+	+	+	+	+	*Pseudomonas synxantha* (99%)
R709	LL	RE	+			+	+	*Pseudomonas corrugata* (99%)
R14	LM	RS				+	+	*Comamonas acidovorans* (99%)
R117	LM	RE			+		+	*Acidovorax facilis* (99%)

The strains were marked chromosomally with the *gfp* gene to maximize genetic stability as well as to reduce the risk of gene transfer to indigenous microorganisms. Before the GFP marking, the natural antibtiotic resistance of the selected PGPR strains had to be assessed (table 2).

Table 2: Natural antibiotic resistance tests of selected PGPR strains. "+" indicates growth on LB medium supplemented with antibiotics.

Luria Bertani agar	R62	R81	R709	R14	R117	Control (*E. coli*)
Control (no antibiotics)	+	+	+	+	+	+
+ Ampicilline (150 μg/ml)	+	+	+	+	+	
+ Gentamycine (30 μg/ml)				+		
+ Chloramphenicol (100 μg/ml)	+	+	+			
+ Kanamycine (200 μg/ml)	+	+	+			
+ Tetracycline (150 μg/ml)						

The pBK-miniTn7-*gfp*2 vector shown below designed by Birgit Koch in Denmark (Koch et al., 2001) was chosen because it contained two antibiotic resistance genes (gentamycine and chloramphenicol) that the strains to be transformed were sensitive to. Indeed, to distinguish transformed from wild type strains an antibiotic resistance gene has to be inserted. This vector is known to insert into a specific neutral intergenic region in tested fluorescent *Pseudomonas* strains (*att*Tn7 site, 22bp downstream of *glmS* gene). There should then be no loss of function by our PGPR strains. The sequence was inserted with the help of two plasmids: a delivery plasmid containing resistance to gentamycine and chloramphenicol and *gfp* gene, and a helper plasmid coding for the transposase genes.

pBK-miniTN7-gfp2 vector

The selected PGPR strains were transformed by electroporation in the laboratory of Prof. Dieter Haas under the guidance of Eric Baehler at the Institut de Microbiologie Fondamentale in Lausanne.

Once the PGPR strains were marked, different tests were carried out to ensure that the GFP strains kept a constant and effective GFP expression as well as similar PGP and growth properties to the wild type strains. Two GFP strains were then selected for a wheat root colonization assay in a greenhouse. The goal of this experiment was to test the survival of introduced bio-inoculants alone on in consortium in the rhizosphere during the wheat growth in non-sterilised soil conditions. As we did not sterilise the soil, the introduced PGPR strains will have to compete with the autochtonous rhizobacteria to colonize the root. In order to localize where and how the PGPR populations colonized the root, confocal laser scanning microscopy observations were carried out.

5.2 Material and Methods

Antibiotic-resistance tests

PGPR strains were grown overnight in Luria-Bertani broth (for 1 liter: peptone 10 g; yeast extract 5 g; NaCl 5g; H_2O ad 1L) and washed with 0,9% saline solution. They were then streaked on LB agar containing the antibiotics (types and concentrations shown in table 2) and on a LB agar control without antibiotics. They were incubated for one week at room T°C.

Preparation of the competent cells and electroporation

This procedure was realised according to Højberg et al. (1999). Cells were grown in LB broth at 30°C. Two millilitres of the overnight culture were transferred in 200 ml of fresh LB in Erlenmyer flasks. The flasks were incubated in a rotary shaker at 35°C (to inactivate the restriction system) until the culture reached an optical density of 0,5 to 1 at 600 nm. The cells were then centrifuged at 4°C and washed twice in 10 ml of an ice-cold solution of 15% (wt/vol) glycerol, and 1mM MOPS (3-morpholinopropanesulfonic acid). The cells were resuspended in 200 µl of the glycerol-MOPS solution, stored on ice and used immediately. Competent cells (40 µl suspension) were mixed in an Eppendorf tube with 0,5 µl of pBK-

miniTn7-*gfp*2 plasmid (conc. 0,1 μg/μl) in TE buffer (10 mM Tris, pH 8,0, 1mM EDTA) and 0,5 μl of pUX-BF13 helper plasmid (conc. 0,1 μg/μl) in TE buffer. The helper plasmid pUX-BF13 carried the genes encoding the transposition proteins necessary for insertion of the Tn7 cassette into the genomic target site. The mixture was transferred to an ice-cold electroporation cuvette and treated in a Bio-Rad electroporator (25 µF, 200 Ω, 5 ms, 2,5 kV/cm). Immediately thereafter, 1 ml of SOC medium (2% [wt/vol] Bacto tryptone, 0,5% [wt/vol] yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2 mM KCl, 20 mM glucose) was added to the cuvette. The cell suspension was transferred to an Eppendorf tube and incubated at 35°C for 3-4 h, followed by spread plating of the entire cultures on LB agar selective plates supplemented with 30 μg/ml of gentamycine and 100 μg/ml of chloramphenicol. Bacterial suspensions spreaded on antibiotics free LB and non electroporated bacterial suspensions were considered as controls. The plates were incubated at 35°C for the first 24 hours and then at 25°C for another 48 hours. GFP expression was evaluated with a Leica DM R epifluorescence microscope equipped with a L5 filter (excitation range in blue, 480 nm).

Growth tests

Wild type and gfp strains were grown overnight in nutrient broth (Biolife). The bacterial culture was then transferred to 50 ml nutrient broth in 250 ml Erlenmeyer flasks to obtain an initial optical density of 0,2 at 436 nm. The Erlenmeyer flasks were incubated in a water bath at 25°C under agitation. The OD was measured at 436 nm every 20 minutes for 400 minutes in order to determine the growth curve of the bacteria. The doubling time was calculated as $\log_{10}2$/(slope exponential phase).

Root colonization experimental set-up

8,5x12 cm diameter pots were autoclaved and filled with sieved organic soil (3 years organic managed wheat field for 3 years after ploughing a calcareous grassland at the Botanical Garden of Neuchâtel (refer to chapter 2.1). Bacterial strains were cultured overnight in 200 ml nutrient broth with constant shaking at root temperature. The cells were washed twice in sterile sodium phosphate buffer (pH 7,0), centrifugation et 7000 rpm for 5 minutes. The bacterial concentration was adjusted to 10^{10} cells per ml. A consortium was composed of an equal volume of each of the three different bacterial suspensions. Pre-germinated UP 2338 wheat seeds (48h in moist filter paper) with roots of equivalent length were added in the bacterial suspension and incubated another 30 minutes under slow agitation. The seedlings where then recovered, excess liquid was removed and they were placed in sterile empty

Petri dishes for subsequent sowing. Three replicates of 5 seedlings per bacterial treatment were crushed, serial-diluted and counts on LB medium containing 30 μg/ml gentamycine and 100 μg/ml chloramphenicol were undertaken to determine the number of GFP-CFU per seedling.

Three seedling bacterial treatments were applied: two with a GFP strain (R62gfp or R81gfp) and one with a consortium of one GFP strain and two non transformed strains (R81gfp + R62wt + R709wt). An unbacterized seedling was considered as treatment control.

Image 1. Root colonization experimental set-up in the greenhouse

Two seedlings were sown per pot. Each treatment was replicated 3 times and placed randomly on a table in a temperate greenhouse in the Botanical Garden of Neuchâtel (image 1) in April 2003. Plants were watered using a Blumat ceramic watering system (Weninger GmbH, Telfs, Austria) linked to 2 L polypropylene flasks (Merck Eurolab SA, France) containing sterile tap water. After 15 days of incubation, 2 L of an autoclaved nutrient solution ($Ca(NO_3)_2 \cdot 4H_2O$ 1mM; KH_2PO_4 0,1mM; K_2SO_4 0,75mM; $Mg(SO_4) \cdot 7H_2O$ 0,65mM; $FeCl_2 \cdot 4H_2O$

0,1mM) replaced the tap water in order to prevent nutrient deficiency. No artificial sunlight was used but the temperature was controlled to remain more than 20°C during the night.

Root colonization sampling and analysis

The sampling was performed at 4 wheat growth stages: 2-leaf (7 days growth), tillering (26d), flowering (47d) and maturity (82d). Three pots per treatment were randomly selected for each growth stage. The two plants with their root system were delicately removed from their pot and shaken to remove non adhering soil corresponding to the non rhizospheric soil (NRS). The roots were then separated from the shoots with sterile scissors. The aerial part of the plants was dried (105°C for 24h) for the plant biomass measure. The root fraction with the residual seed of the first plant was transferred in a sterile Petri dish for subsequent confocal laser scanning microscopy (CLSM) analysis. Bacterial counts were realised on the root fraction of the second plant for which the residual seed was removed. At maturity, the grains were removed from the spikes, weighed and counted.

To separate the adhering rhizospheric soil (RS) from the rhizoplane/endorrhizosphere (RE), the roots with their adhering soil were immersed into a 0,9% NaCl solution and stirred. The washed roots were removed and the remaining suspension constituted the RS suspension. The roots were rinsed with 0,9% NaCl solution and dried on a sterile Whatman paper (Merck AG) to remove the excess rinsing solution. About 0,5 g of roots was crushed sterily in 10 ml of the 0,9% NaCl solution using a mortar and pestle, constituting the RE suspension. The NRS suspension was prepared by adding 20 g of NRS in a glass bottle containing 100 ml of sterilised 0,9% NaCl solution. The bottle was then closed and shaken vigorously during 30 seconds to resuspend the soil particles. NRS, RS and RE suspensions were then serially diluted (1:10) in sterile 0,9% NaCl solution and 0,1 ml of the appropriate dilution was spread on nutrient agar medium supplemented with 30 μg/ml gentamycine, 50 μg/ml chloramphenicol and 150 μg/ml ampicilline. Each sample was diluted and counted twice. The bacterial colonies that fluoresced under the UV lamp were counted as the GFP positive colonies. For each replicate, several fluorescent colonies were observed under the epifluorescence microscope to confirm the presence of GFP.

For the CLSM observations, the GFP strains were monitored using a confocal laser scanning microscope (Zeiss LSM 5 Pascal). The Argon laser excitation wavelength was 488 nm; GFP emission was detected with the Filter set for FITC (505-530 nm). For each treatment, the roots were first observed with their root-adhering soil. They were then washed to remove the RS fraction and observed once again.

Statistical analysis

Before statistical analysis, the bacterial counts were ln(x+1) transformed. The data were subjected to analysis of variance (ANOVA) and the means were compared with the least significant difference (LSD) test using the S-Plus software vers. 6.1 (Insightful Corp, USA).

5.3 Results and discussion

The R14 and R117 strains were not successfully transformed. This failure could be explained by the fact that R14 and R117 are not *Pseudomonas* spp. (R14: *Comamonas acidovorans* and R117: *Acidovorax facilis*, cf. Chapter 4) whereas the transformation protocols and GFP plasmid vector were designed on a fluorescent *Pseudomonas* strain (Højberg et al., 1999; Koch et al., 2001). However the three *Pseudomonas* spp., R62, R81 and R709 were successfully transformed (visible by green colonies that fluoresce under the UV lamp).

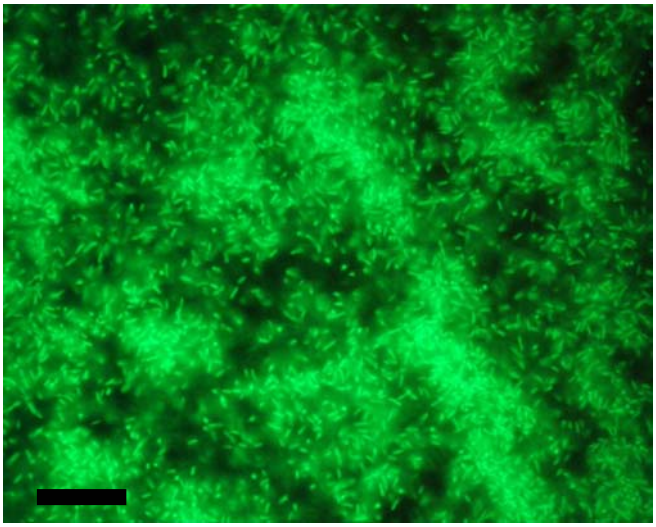

Image 2. Epifluorescence microscopy image (excitation wavelength 480 nm) of GFP-tagged R81 strains after 24 hours growth on nutrient agar. Bar = 20 μm

Five GFP strains of R81 and R709 and one GFP strain of R62 were isolated and further analysed. They were firstly observed under epifluorescence microscope to ensure that these strains fluoresce when excited with the blue wavelength (image2). Growth rate tests were carried out in NB medium to ensure that the growth rate wouldn't be reduced by energy losses caused by the expression of GFP. The figure 1 shows the exponential phase and doubling time of wild type and their corresponding GFP-transformed strains. The growth rate

was slightly reduced in the GFP strains of R81 and R62 as compared to the wild type indicating that the GFP expression could induce a metabolic cost.

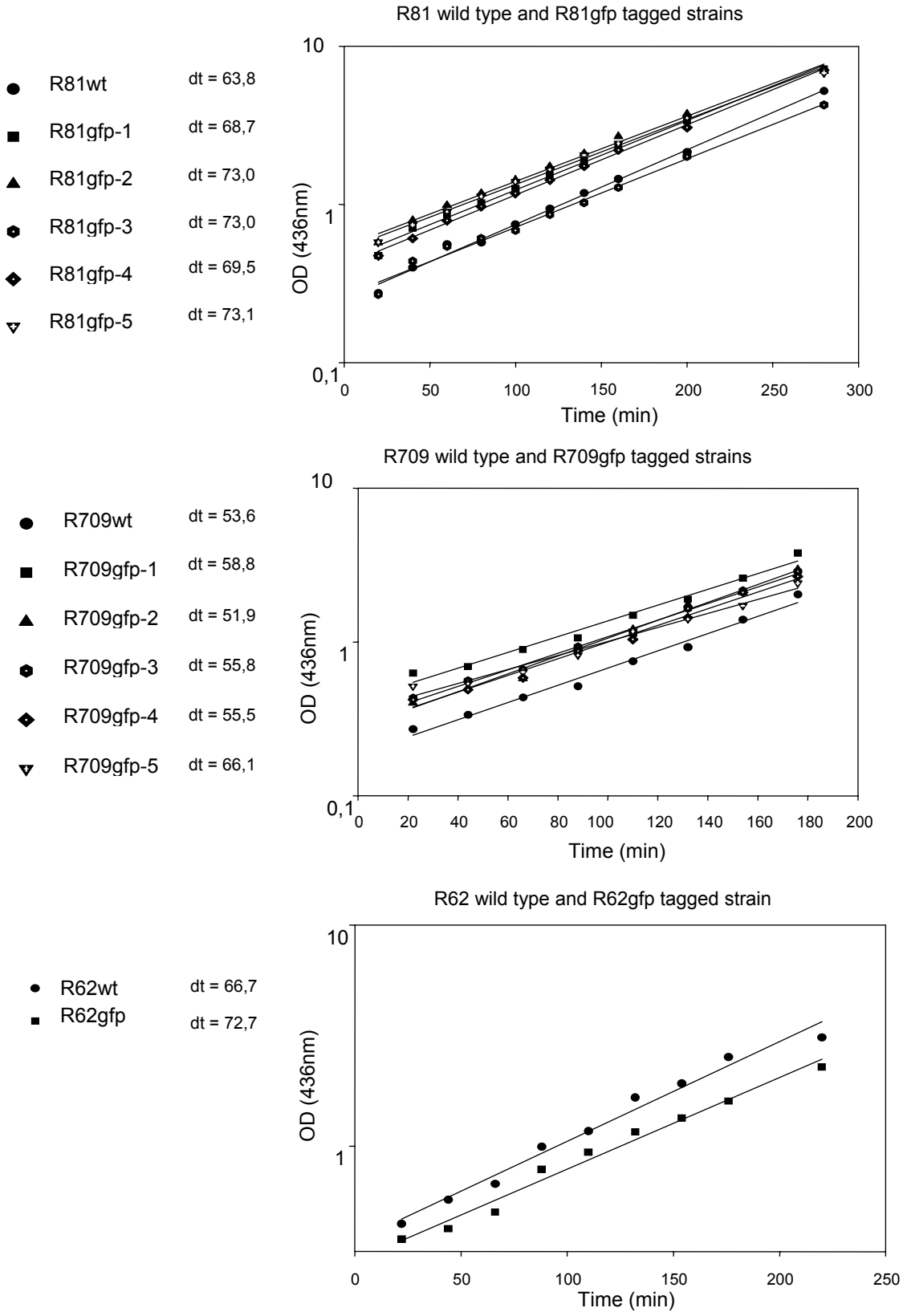

Fig.1. Semi-logarithmic representation of the exponential phase of wild type and their corresponding GFP-tagged strains cultured in nutrient broth. Optical density measures (436 nm) were undertaken every 20 minutes. Doubling time (td) is calculated as log2/slope of the expontial phase and expressed in minutes.

The GFP strains had similar 16SrDNA sequences and PGP properties than the wild type strains (data not shown). The GFP strains were re-inoculated for 25 generations in NB medium diluted 10 times to check the genetic stability of the insertion and to test if they still expressed the GFP with the same intensity as the first generation.

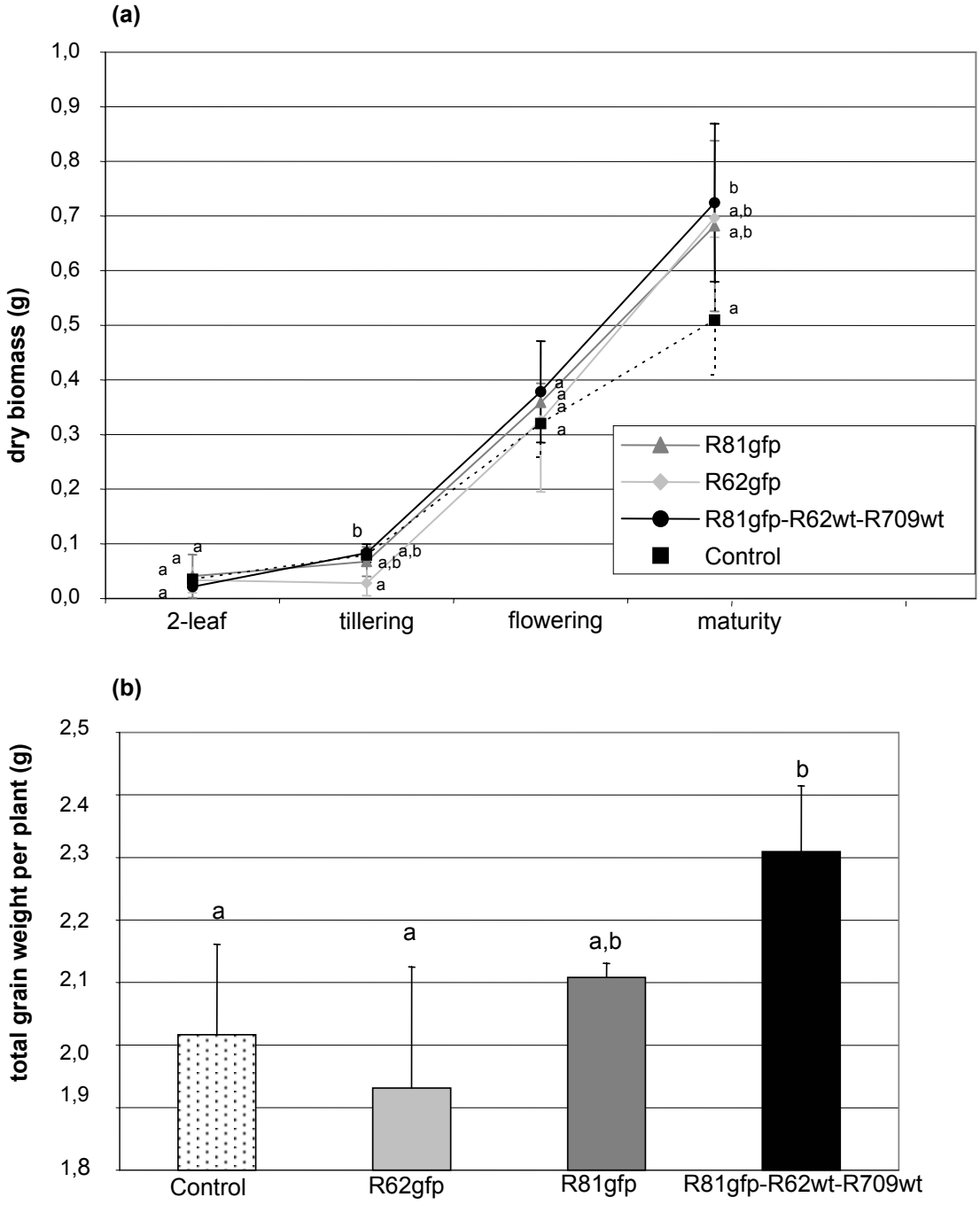

Fig.2. (a) Dry biomass (aerial part) of the PGPR-free control and the PGPR bio-inoculated plants. Values are means ± standard deviation of 3 treatment replicates. Identical letters indicate non-significantly different means between treatments at a particular growth stage according to LSD test ($p < 0,05$, $n=3$). (b)Total grain weight of the PGPR-free control and the PGPR bio-inoculated plants at the maturity stage. Values are means ± standard deviation of 3 treatment replicates. Identical letters indicate non-significantly different means between treatments according to LSD test ($p < 0,05$, $n=3$).

There was no loss of GFP expression nor intensity between the first and the 25th generation of all the gfp strains tested. This persistence of GFP expression is fundamental for root colonization assays as a loss of GFP expression could induce artefacts in the monitoring. As the chromosomal insertion of the *gfp* gene remained stable, the risk of loosing its expression due to an absence of pressure of selection (no antibiotics were added in the growth medium) was therefore considered as minimal.

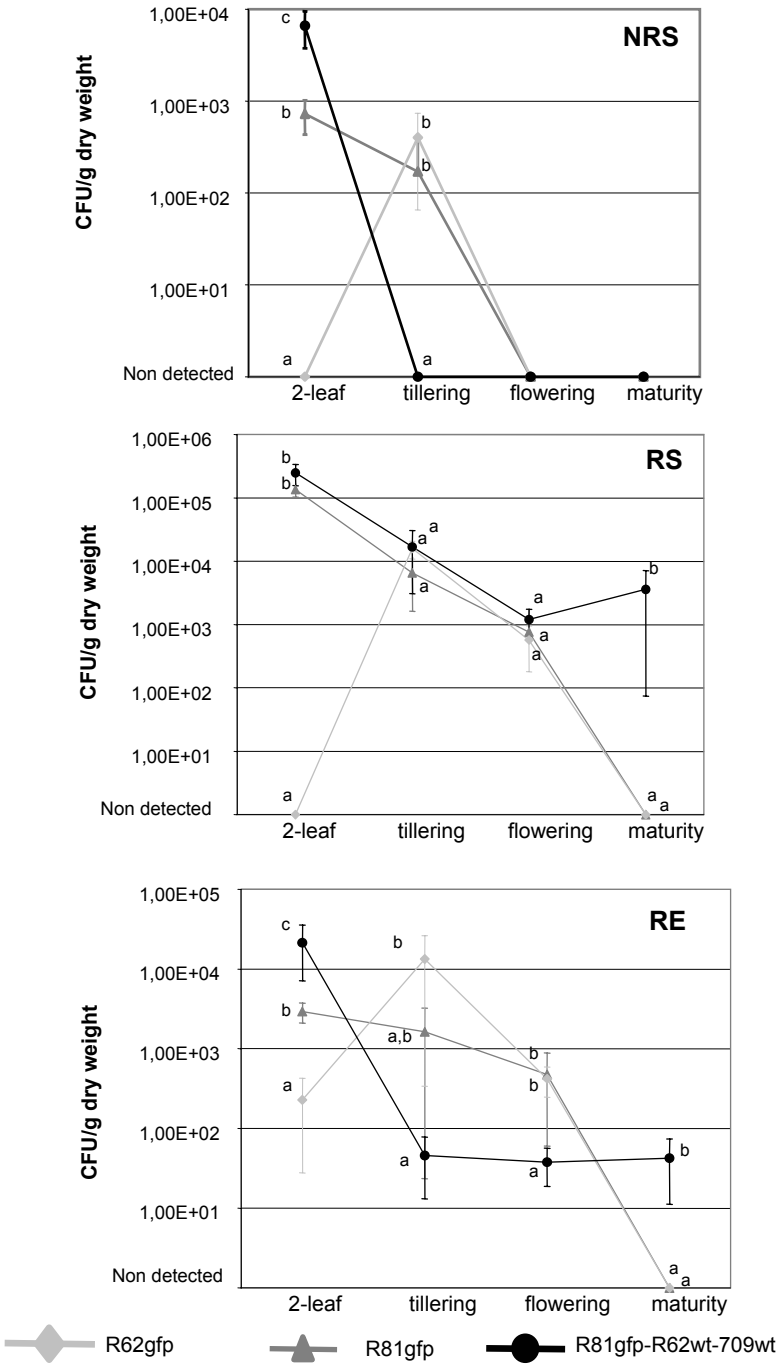

Fig.3. GFP-tagged strain counts on nutrient agar medium supplemented with 30 μg/ml gentamycine, 50 μg/ml chloramphenicol and 150 μg/ml ampicilline. NRS = non rhizospheric soil; RS = adhering rhizospheric soil; RE = rhizoplane/endorrhizosphere. Values are means ± standard deviation of 3 treatment replicates. Identical letters indicate non-significantly different means between treatments at a particular growth stage according to LSD test (p< 0,05, n=3).

For root colonization assays in greenhouse pot experiments, R62gfp alone and R81gfp alone or in a consortium with R62wt and R709wt were bio-inoculated as seed treatment on the wheat variety UP2338 which is sown in our test fields of Budaun, Uttar Pradesh. Bacterial counts were about 10^6 GFP-CFU per seedling before sowing. The PGP effect of the GFP-tagged strains confirmed the previous pot experiments undertaken with the wild type strains (Gaur, 2003). Indeed, at maturity stage of wheat growth, the PGP effect of the treatments was measured by an increase in the plant dry biomass in all the PGPR treatments as compared to the PGPR-free control (fig. 2a). This result implied that the marking of these PGPR strains with GFP did not alter their plant growth promoting properties. Bacilio et al. (2004) and Rothballer et al. (2003) also observed that the GFP marking did not affect the plant growth promoting capacities of PGPR strains. The use of our GFP-tagged strains is then very promising for future studies not only concerning PGPR-plant interactions but also interactions with other soil or rhizosphere microorganisms such as protozoans or AMF. Finally, the total grain weight per plant was significantly higher in the consortium than in the control (fig. 2b). However, it was not possible to tell if the positive effect on grain yield was due to R81gfp, R62wt, R709wt or a combination of the three strains.

The GFP counts on antibiotic medium showed that the GFP-tagged PGPR strains survived better in the rhizosphere than in the non-rhizospheric soil (fig. 3). This was expected as these PGPR strains were isolated from the wheat rhizosphere (Gaur et al., 2004) and therefore are more adapted to this environment providing energy-rich ready available substrates than to the limited resources found in the non rhizospheric soil. This finding implied that the method of inoculation of the PGPR strains should favour their contact with the roots. Seed coating would then be preferred as the method of bio-inoculation than other practices such as direct PGPR soil amendment or mixing the PGPR strains with a vermicompost (Mazzola et al., 1992; Raj et al., 2003; Jeyabal and Kuppuswamy, 2001). The bacterial counts in the rhizospheric soil were in general higher than for the rhizoplane/endorrhizosphere and might result from a high number of loosely attached PGPR cells on the root surface which detach when the roots are immerged in the 0,9% saline solution to recover the rhizospheric soil.
The GFP-tagged PGPR counts were quite low in the rhizosphere as compared to the *Pseudomonas* counts in the UP2338 wheat rhizosphere by Gaur (2003) that reached 10^8 CFU/g at the flowering stage. However, the biomass of the treated plants was still higher than that of the control plants. This result indicated either that a small number of PGPR could produce a sufficient amount of PGP compounds (such as root hormones) or that their high concentration on the seedling surface at the time of sowing might have affected the surrounding microbial community, stimulating the activity or growth of plant beneficial microorganisms (see chapter 6). R62wt and R81wt can for instance interact beneficially with

arbuscular mycorrhizal fungi (chapter 4) whose presence could improve the nutritional level of the plant. In addition, even if the GFP-PGPR strains were not recovered on the plating medium they could still be present in the rhizosphere in a viable but not cultivable state. Indeed, cells in the rhizosphere are subjected to moderate to a severe starvation resulting in a loss of activity and only a few cells of the original inoculum appear to remain active over long periods of time (Marschner and Crowley, 1997; Sørensen et al., 2001).

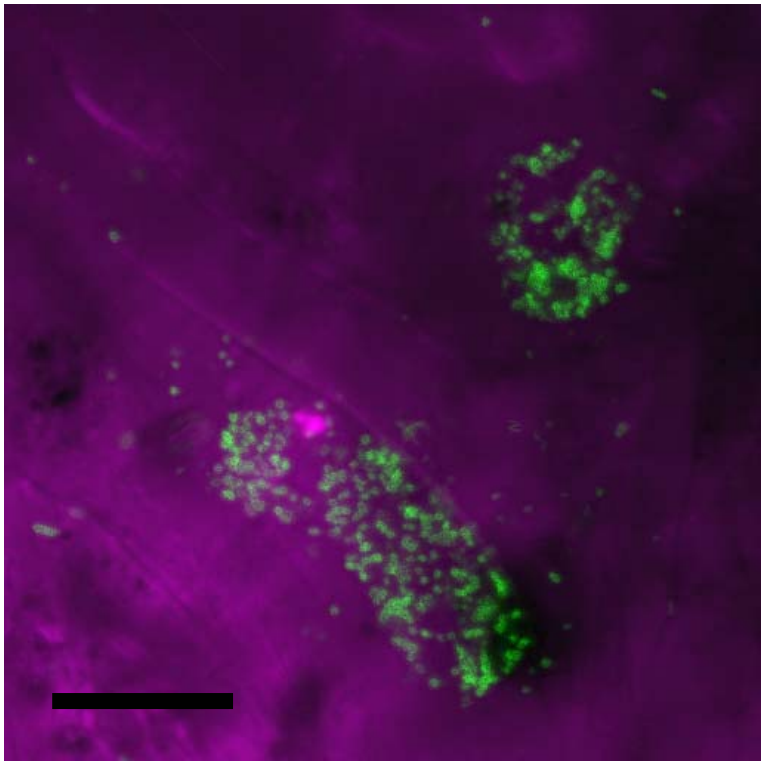

Image 3. Localization of R81gfp on the upper part of the wheat root at the two-leaf stage as visualized by CLSM. R81gfp cells are mainly found in micro-colonies near the crevices of epithelial cells. Bar=20 μm

R62gfp was not detected at the two-leaf stage but was detected at tillering. This result implied that R62gfp had colonized the root at a later stage than R81gfp. This sudden increase in R62gfp numbers at the tillering stage might have been triggered by the appearance or a higher level of a particular exudation product metabolised by R62gfp. A later rhizosphere colonization of R62gfp might explain that the plant biomass for this treatment was less than that for the control and the R81gfp treatments at the tillering stage. However, after tillering, the plant biomass of the R62gfp treatment increased strongly until reaching similar values to the R81gfp treatments. Jacoud et al. (1998) suggested that an optimal bacterial density appears soon after inoculation and is more important in growth promotion than the development of large bacterial populations during the whole growth period. Our

results could then indicate that the optimum bacterial density of R62gfp on the root for the plant growth promotion appears later in the growth period than that for R81gfp.

There was a continuous decrease of the R81gfp strain when bio-inoculated alone in the RS and RE throughout the wheat growth period, even reaching an undetectable level at maturity (fig.3). However, when R81gfp was associated with R62wt and R709wt in the treatment it remained detectable in the RS and RE even at maturity. In addition, in the RE at tillering and flowering, R81gfp counts were smaller in consortia than R81gfp alone or R62gfp counts. This result suggested that there was a competition for root exudation sites between R81gfp, R62wt and R709wt. Resulting from this competition, R81gfp might have adapted to more zones on the root than if it were alone. Indeed, R81gfp was detected near the root elongation zone (image 4) only when it was inoculated in consortium. When inoculated alone, it was found mainly on the upper part of the root (image 3). Actually, most studies based on microscopic observations of introduced rhizobacteria detect them on the upper part of the root indicating that most bacteria remain close to the inoculation site after sowing (Chin-A-Woeng et al., 2004; Unge and Jansson, 2001; Hansen et al., 1997) Occasionally, single cells would detach from older parts of the root and travel along the root tip to establish new colonies (Bloemberg et al., 2000).

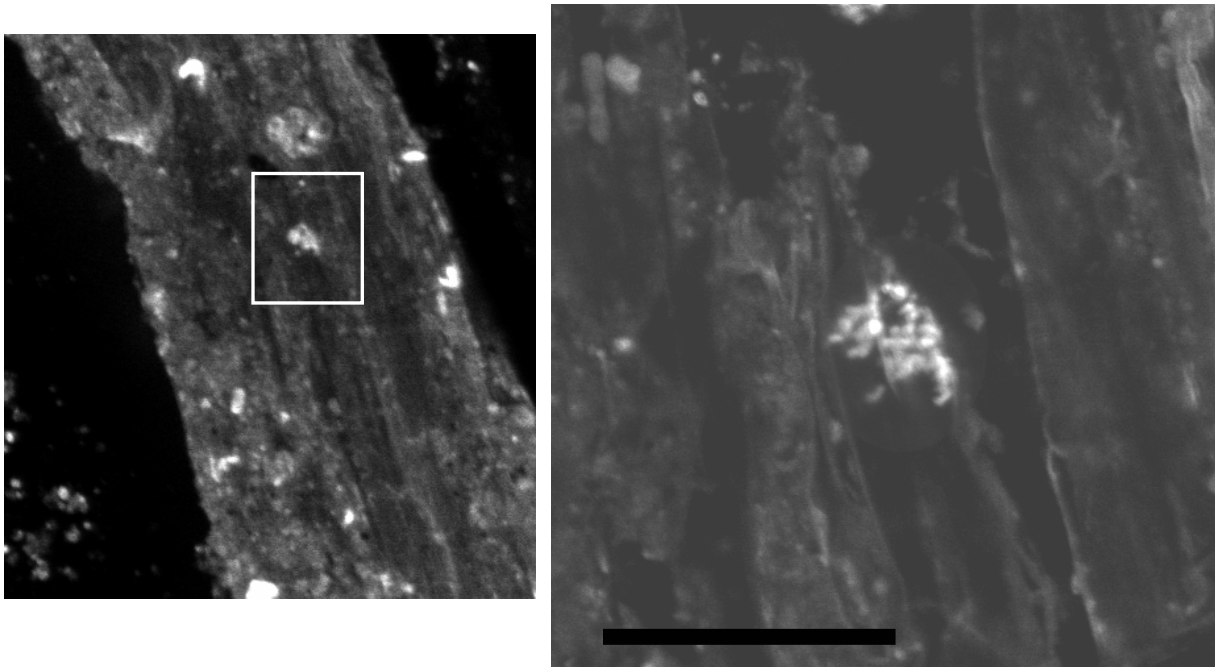

Image 4. Localization of R81gfp near the wheat root elongation zone at the two-leaf stage as visualized by CLSM. R81gfp cells have formed micro-colonies near the crevices of epithelial cells. Bar=20 μm

Pseudomonas cells are often found in microcolonies or in strings in the crevices between the root epithelial cells (Normander et al., 1999; Bloemberg et al., 2000; Unge and Jansson,

2001; Lübeck et al., 2000). In this study, it was difficult to detect GFP strains with the CLSM after the two–leaf stage as the number of bacterial cells was too low (only individual cells were found) and there was a higher autofluorescence from the root cells at later growth stages. This might explain why most of the root colonization studies using CLSM are carried out on young plants. At the two-leaf stage, R62gfp was observed only on the seed coat (image 5). The seed coat with its cracks and leakage of cytoplasmic solutes provides a nutrient rich zone for bacterial proliferation (Unge and Jansson, 2001). R81gfp was detected as single cells or microcolonies on the root surface (images 3 and 4). This bacterial development on the root surface was probably triggered by the root exudates leakaging out between the epithelial cells providing a favourable micro-habitat (Sørensen et al., 2001).

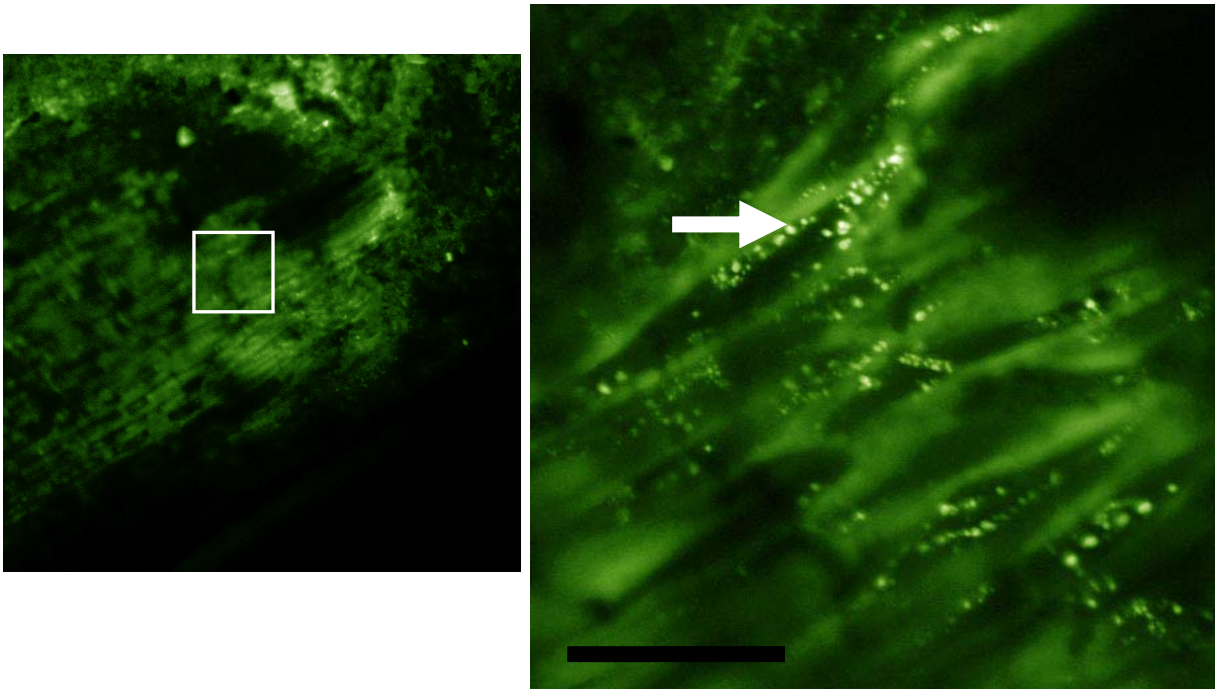

Image 5. Localization of R62gfp on the seed coat of wheat at the two-leaf stage as visualized by CLSM. R62gfp cells are mainly found near the crevices of pericarp cells (white arrow). Bar=20 μm

ts, that the optimum bacterial density of R62gfp on the root appeared later in the growth period than R81gfp and the fact that R81gfp remained detectable in the presence of other PGPR strains could imply that these strains would not out-compete one-another for exudation sites and could act synergistically to improve plant growth. A bio-inoculation formula combining these PGPR strains would then be efficient in term of plant growth promotion. The R62 and R81 consortium is now being tested extensively in Indian wheat fields at different location sites and with different wheat varieties. The first results of these field assays is presented in chapter 6.

6 Studying the dynamics of the wheat rhizobacterial community

The wheat rhizobacterial community structure was assessed in the Budaun rainfed fields at two different wheat cropping seasons.

The first study was performed during the rabi season that lasted from November 2001 till March 2002. It consisted of analysing the rhizobacterial community dynamics during the wheat growth period in three different fields with different fertilizer inputs and yields. Our objective was to assess which factors, between the field conditions or plant age influenced the most the bacterial community structure.

The second experiment, performed during the season after (rabi season from November 2002 till March 2003), aimed at analysing the effect of PGPR/AMF bio-inoculations on the rhizobacterial community and wheat growth. Moreover, this field study enabled to carry out the first test in field conditions of selected PGPR strains and AMF bio-inoculations and to confirm the positive interactions between the PGPR strains and the AMF reported in our previous experiments.

This chapter is a manuscript entitled “Plant growth stage, field condition and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rainfed wheat fields” that will shortly be submitted to Soil Biology and Biochemistry.

Plant growth stage, field condition and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rainfed wheat fields.

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Keywords: wheat; rhizosphere, growth stage, PGPR, AMF, DGGE

6.1 Abstract

The goal of this study was to assess the changes in the wheat bacterial rhizospheric community with respect to field conditions, plant age and PGPR/AMF bio-inoculation. The bacterial community structure was determined with PCR-denaturing gradient gel electrophoresis. Rainfed wheat fields with different fertilizer levels and yields were investigated in the Budaun district of Uttar Pradesh. The bacterial community of the root-adhering rhizospheric soil and the rhizoplane/endorhizosphere were analysed. The bacterial community of the root-adhering rhizospheric soil was more influenced by the field conditions such as an increase in fertilizer input than the bacterial community of the rhizoplane/endorrhizosphere. Consequently, in order to prevent discrepancies in the plant responses due to different field conditions, we propose that a PGPR consortium should contain one or several strains that were isolated from the rhizoplane/endorrhizosphere. The bacterial community structure was also dependent on the plant's growth stage. After the flowering stage, the bacterial community structure diverged from earlier wheat growth stages probably resulting from a drop in the amount of rhizodeposits. The type of PGPR consortium affected more the bacterial community structure than the mycorrhizal colonization possibly resulting from a PGPR-induced quorum sensing response and a faster root colonization process of the bacteria.

6.2 Introduction

This study is integrated in a project of the Indo-Swiss collaboration in biotechnology (ISCB) whose main goals are to develop new biotechnologies such as the use of bio-inoculants for improving plant growth and soil health in marginal rainfed regions of India. In 1995, the areas planted with rice and wheat crops in India were 43 and 25 million ha, respectively. Nearly 25% of the area under rice and 40% of the area under wheat are currently cropped in rice-wheat rotations (Abrol, 1999). In this system rice is grown in the kharif (rainy) season and followed by wheat in the rabi (winter) season. The Indian “Green Revolution”, that took place in the 1960s, has increased dramatically crop yield by introducing high yielding varieties using large amounts of mineral fertilizers or pesticides. Demand for rice and wheat will grow at 2.5% per year over the next 20 years (Hobbs and Gupta, 2001). However in some regions, the gains in food grain production have stagnated or even declined in recent years for both rice and wheat crops (Dawe and Dobermann,1999). Causes of decline may include changes in biochemical and physical composition of the soil organic matter, a depletion and diminution in bio-availability of soil nutrients, a scarcity of surface water and groundwater as well as poor water quality (salinity), and the buildup of pests (Abrol, 1999; Ladha et al., 2000; Timsina and Connor, 2001). Such areas will require an integrated management including the use of biotechnology, improving not only the crop, but also the interaction of roots with its soil microbial partners such as the bacterial community and arbuscular mycorrhizal fungi (AMF). Microbial communities in soil or in the rhizosphere contribute to plant growth by recycling nutrients and making them available (Lynch, 1990), increasing the root health by competition with root pathogens (Weller et al., 2002) or enhancing nutrient uptake (Smith and read, 1997). Wheat transfers about 30% of carbon assimilates into the soil through the process of rhizodeposition and part of this below-ground translocated C is incorporated in rhizosphere microorganisms (Kuzyakov and Domanski, 2000). Therefore, the rhizobacterial abundance and tunrnover increases in the rhizosphere as compared to the bulk soil as they metabolize root exudates. Within the rhizosphere microbial populations are a group of plant beneficial bacteria designed as plant growth promoting rhizobacteria (PGPR). PGPR are beneficial to the plant via nutrient acquisition (Rodriguez and Fraga, 1999; Dobbelaere et al., 2003; Ladha et al., 2003), biocontrol (Walsh et al., 2001; Chin-A-Woeng et al., 2003), plant hormone-like production, lowering of plant ethylene level (Glick, 1995; Steenhoudt and Vanderleyden, 2000) and induction of systemic resistance (van Loon et al., 1998). Another beneficial microbial partner of the plant are the symbiotic arbuscular mycorrhizal fungi (AMF). Beneficial effects of AMF in agriculture comprise an improved plant nutrition (mainly via phosphorus acquisition) and hydric stress resistance (Smith and Read, 1997), biological control against pathogens (Azcón-Aguilar and Barea, 1996), and better soil structuration

(Miller and Jastrow 1990). The rhizosphere microbial communities can be affected by a wide range of factors including plant type (Grayston et al., 1998; Germida and Siciliano, 2001), plant age (Marschner et al., 2004), distance from the soil to the root (Marilley and Aragno, 1999), soil characteristics (Latour et al., 1996; Buyer et al., 1999), agronomic practices (Lupwayi et al, 1998; Alvey et al:, 2003; Kennedy et al., 2004) and mycorrhizal infection (Marschner et al., 2001). These changes in the rhizosphere community might affect plant growth negatively (e.g. caused by root pathogens development) or positively (increase in the proportion of PGPR populations). It is therefore necessary to study the microbial community dynamics in the fields before applying a modification in the agricultural practice especially when using bio-inoculants to improve soil health or crop yield. The goal of this study was to assess the changes in the wheat bacterial rhizospheric community with respect to field conditions, plant age and PGPR/AMF bio-inoculation. The bacterial community structure was determined with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). This molecular fingerprinting technique allows to detect the most abundant bacterial populations, culturable or not, in the rhizosphere. The studied site was composed of wheat fields in the Budaun district of Uttar Pradesh where rice and wheat rotation crop practices have been carried out for over 20 years. The area is without modern irrigation facilities and is therefore considered as rainfed.

6.3 Material and Methods

Experimental sites

Two experiments were carried out in a study site located in Bhavnipur village (Budaun district latitude 28.02 N, longitude. 79.10 E°, alt 600m) in the Uttar Pradesh (UP) state. The first experiment consisted of analysing the rhizobacterial community dynamics during the wheat growth period in three different fields. It was performed during the rabi season that lasted from November 2001 till March 2002. The second experiment, performed the season after (rabi season from November 2002 till March 2003), aimed at analysing the effect of PGPR/AMF bio-inoculations on the rhizobacterial community and wheat growth. The study site is rainfed; characterized by twenty years of rice-wheat rotation and by a basic irrigation system. For wheat cropping, standard agronomic practices were followed, such as regular sowing, irrigation and weeding. Three fields of 4000 m^2, separated by less than 2 km, were selected on this site, differing mainly for several years by their fertilizer level and wheat grain yields (field characteristics are presented in table 1). They were classified as low input low yield (noted LL), low input moderate yield (LM), and high input high yield (HH). The fields' soils have sandy loam soil textures and belongs to the following USDA classification: Entisol (order), Psamment (Sub order), Doarse (Family), Hyperthermic (Regime). The same wheat

cultivar UP 2338 (provided by the GP Pant University of Agriculture and Technology, Pantnagar, Uttaranchal State) was grown for more than five years on the three fields in rotation with rice. Wheat was sown in the second half of November.

Table 1. Fertilizer inputs and soil physico-chemical characteristics of the studied fields LL, LM and HH before sowing in 2001. DAP= Diammonium phosphate; AV. P = available phosphorus; Av. N = Available nitrogen; Exch. K = Exchangeable potassium, OM = organic matter, Cu = copper; Mn = Manganese; Zn = Zinc; Fe = Iron

Field	Fertilizer input		pH	Total P	Av. P	Total N	Av. N	Exch. K	OM	Cu	Mn	Zn	Fe
	Urea (kg ha^{-1})	DAP (kg ha^{-1})		(kg ha^{-1})	(kg ha^{-1})	(kg ha^{-1})	(kg ha^{-1})	(kg ha^{-1})	(%)	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
LL	50	0	7.2	919.0	11.4	1198.2	95.8	136.1	1.06	1.65	18.54	0.62	22.1
LM	50	0	7.4	1040.7	15.4	1219.1	99.4	190.6	1.11	3.02	24.12	1.62	20.7
HH	60	20	7.2	1672.7	28.5	1581.4	100.8	170.2	1.42	1.36	19.25	0.59	38.3

Bio-inoculant characterization and seed treatment

The bacteria used in the PGPR consortia were *Pseudomonas spp*. strains selected as follows: First, 3000 strains were isolated from the rhizosphere of the UP 2338 wheat variety in LL, LM and HH fields. From this pool, a selection of 20 strains with the most promising PGP properties were then tested in different consortia for plant growth promoting effects in greenhouse experiments in pots containing soil from LL, LM and HH sowed with UP238 wheat (Gaur, 2003). The consortia which caused the best plant growth were selected for the field trials presented here. The bacterial strains were maintained in Kings' B agar slants (Difco Laboratories, Sparks, USA) and stored under glycerol. The plant growth promoting properties of the PGPR strains used in this study are summarized in table 2.

The mycorrhizal inoculum was composed of an indigenous AMF consortium isolated from the LL field and subcultured by our partners at the Tata Energy Research Institute (TERI) in New Delhi. The mycorrhizal subculturing was performed in trap cultures according to Oehl et al. (2003). The host plants were *Allium cepa*, *Tagetus* sp, *Daucus carotus*, *Medicago sativa*, and *Trifolium alexandrianum* for a first growth cycle and *Gossypium* sp, *Vetiveria zizanioides*, *Sorghum* sp and *Tagetus* sp for the 2^{nd} and third cycle. The substrate was Terragreen: American Aluminium Ixide, Oil Dry US Special, Type R and soil sediment in the ratio of 1:1.

Wheat seeds were coated according to Sharma et al (2003). Briefly, overnight grown PGPR cultures were washed and re-suspended in 10mM phosphate buffer saline (PBS). Optical density was adjusted to 0,6 (~10^8 cfu ml^{-1}). PGPR consortia were prepared by adding equal volume (v/v) of bacterial suspension. The AMF inoculum along with carboxymethyl cellulose (final concentration 0.1%) was added to allow the bacteria and fungal propagules to stick properly to the seeds. Seeds (ratio of 1g ml^{-1}) were added in the suspension, mixed properly and air dried. The number of CFU per seed counted on King'sB agar was approximately 10^6-10^7.

Table 2. Characterization of the *Pseudomonas* spp. bacterial bio-inoculants used as treatments in the second experiment based on Gaur (2003) and Gaur et al (2004). Abbreviations: RE = rhizoplane/endorrhizosphere; RS = rhizospheric soil; "+" indicates that the strains possess the following plant growth properties: P = phosphate solubilization, IAA = Indole-3 acetic acid production, ACC = 1-aminocyclopropane-1-carboxylate deaminase production, Sid = Siderophore production, DAPG = diacetyl-phloroglucinol production.

	Origin		PGP properties				
Strain	Field	fraction	P	IAA	ACC	Sid	DAPG
R62	LM	RE	+	+		+	+
R81	HH	RE	+	+	+	+	+
R103	LL	RS	+	+	+	+	
R110	LL	RS		+	+		

Six different seed treatments were examined:

- Control — Control containing no AMF or PGPR strain
- M — Mycorrhiza without PGPR consortia
- R62/R81 — R62 and R81 PGPR consortium alone
- R62/R81/M — R62 and R81 PGPR consortium with AMF
- R103/R110 — R103 and R110 PGPR consortium alone
- R103/R110/M — R103 and R110 PGPR consortium with AMF

The seeds were sown in mid-November 2002 in the LL field that was separated in 18 plots of 4 x 4 m. Each type of treatment was applied in three random plots of 16 m^2.

Sampling procedure for bacterial community analysis

In the first experiment (season 2001-2002), samples for bacterial community analysis were taken at different days of wheat growth: 25 days (corresponding to the crown root initiation growth stage), 45 (tillering), 90 (flowering), 120 (maturity). In the second experiment (season 2002-2003), the samples for the bacterial community analysis were taken at 90 days growth corresponding to the flowering stage. Three to four wheat plants with roots and soil cores were sampled sterily using a soil corer at a depth of 0 to 30 cm at 8 random spots in each field or plot. The coarse stones and stubs were removed. The bulk soil was separated from the adhering soil by shaking the plants. The root systems with adhering soil from the 8 spots were separated from the aerial part of the plant using sterile scissors and pooled. The resulting composite samples were stored at –80°C for subsequent molecular analysis.

Plant data

At harvest in rabi season 2001-2002, all the plants from three 1 m^2 surfaces per field were harvested together, grain was removed from straw, then the straw weight and grain weight were recorded. In rabi season 2002-2003, plant height and grain weight were measured from

the plants in 1 m^2 surfaces in each of the 16 m^2 plots. For nutrient analysis the following procedure was applied: One g seed was digested in a diacid mixture of HNO_3 and $HClO_4$ (9:4). The resulting ash was analysed for nutrient content. The potassium and the micronutrients Zn and Fe were determined by flame photometer. The concentration of organic C was determined by the Walkley and Black method (Walkley and Black, 1934). Total nitrogen was determined by the Kjeldhal's method (in Allen et al., 1974) and converted into protein content by multiplying with the constant factor 5.73. Finally phosphorus content was determined with molybdenum-ascorbic acid colorimetry method (Hansen, 1950).

DNA extraction from RS and RE samples

A fraction of the root systems with adhering soil was immersed into sterile 0.1 M sodium phosphate buffer (pH=7) and stirred to separate the root-adhering rhizospheric soil (RS) from the rhizoplane-endorhizosphere (RE) fractions. The washed roots (RE) were removed. The remaining suspension considered as the RS was weighed. The roots were rinsed with sterile deionised water and dried on sterile Whatman paper (Merck AG). One g of fresh weight RE was crushed sterily in 10 ml phosphate buffer in a mortar with a pestle, constituting the RE suspension. The root dry weight was determined. One ml of the RE suspension was tenfold diluted in sterile sodium phosphate buffer (0.1 M, pH7).

DNA was extracted from the RS and RE fractions by the bead-beater technique (FastPrep FP120, SAVANT, BIO101, Carlsbad, USA) using a FastDNA spin kit for soil DNA extraction (BIO101) according to the manufacturer's protocol. The extracted DNA was further purified with a GENECLEAN® II kit (BIO101) and stored at –20°C in TE buffer.

DGGE analysis

For the first season (2001-2002), two DNA extractions and DGGE analysis were carried out per field condition and wheat growth stage. For the second season (2002-2003), one DNA extraction and DGGE analysis was carried per plot (three plots per seed treatment). A double step PCR was used to amplify the V3 region, a fragment of about 200 bp of the bacterial 16S rDNA, according to Weisskopf et al (2004). A composite mix of different bacterial 16S rDNA fragments was added on each side of the DGGE gel as a reference DGGE pattern: *Pseudomonas fluorescens* ATCC 27663, *Acidovorax facilis* DSM 550, *Bacillus subtilis* ATCC 14893, *Sinorhizobium meliloti* DSM 1981 and *Aquaspirillum dispar* ATCC 27650. DGGE was performed using a 8% (w/v) acryl-bisacrylamide gel (37.5:1, Qbiogene, Illkirch, France) with 30-60% linear urea/formamide (Fluka, Buchs, Switzerland, Qbiogene) denaturing gradient (100% denaturant corresponds to 40% formamide + 7 M urea). 500 ng of the PCR product were electrophorated in 1x TAE buffer (Qbiogene, France) at 60°C with a constant voltage of

150 V during 5.5 hours using the BioRad D-Code Electrophoresis System (Bio-Rad Inc. California, USA). The gels were stained in the dark for 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden, The Netherlands) in 1x TAE solution. The gels were photographed with the Multi-Analyst package (Bio-Rad Inc., California, USA). The DGGE fingerprints were normalised according to the reference patterns and were compared using the GelCompar software (Applied Maths, Kortrijk, Belgium). An example of DGGE profile obtained is shown in figure 1. DGGE banding patterns were then converted into a numerical matrix used in the statistical analysis. For each sample analysed, two DGGE gels were run and the mean value of these two profiles were considered as the final fingerprint values. Each band was considered as corresponding to a single bacterial population and the band intensity was representative of the relative abundance of the population (Fromin et al, 2002). The bands whose average relative contribution was below 1% were discarded for the analysis. Richness of the DGGE profiles was considered as the number of detectable bands and Shannon diversity index was calculated as $-\Sigma$ pi $\log_2$(pi) where pi represents the relative abundance of a given population in the profile.

Statistical analysis

The plant data were subjected to analysis of variance (ANOVA) and the means were compared with the Tukey test using the S-Plus software vers. 6.1 (Insightful Corp, USA). The means of the DGGE profile richness and diversity were compared with the Student's t tests. Ordination methods were applied on the basis of numerical data matrices converted using the program Progiciel R (Legendre and Vaudor, 1991) and calculated with the Canoco 4.0 software (Canoco 4.0, Microcomputer Power, Ithaca, USA). The DGGE profile matrix was composed of rows of objects representing the samples and columns of species representing the DGGE band position along the vertical gel gradient. The relative abundance of a species in a sample corresponded to the DGGE band's relative intensity with regards to the sum of all band intensities in a pattern. In order to quantify and test effects of various sets of explanatory variables on the DGGE profile variation, Canonical Correspondence Analysis (CCA) was applied. In order to quantify and test effects of various sets of explanatory variables on the plant feature variation, the plant data were first standardized and then submitted to Redundancy Analysis (RDA). Variation partitioning analysis (Borcard et al., 1992) enables to display the variability of patterns constrained by the factors of interest. Therefore this analysis was used to display the relative importance of the contributions plant age, field condition, type of PGPR consortium, or presence of AMF on the bacterial community profiles. The significance of the result was tested with the Monte Carlo permutation test. Variation partitioning analysis was performed with Canoco 4.0. Mantel tests

were performed with the Progiciel R to determine the correlation between plant features and the DGGE numerical data matrices.

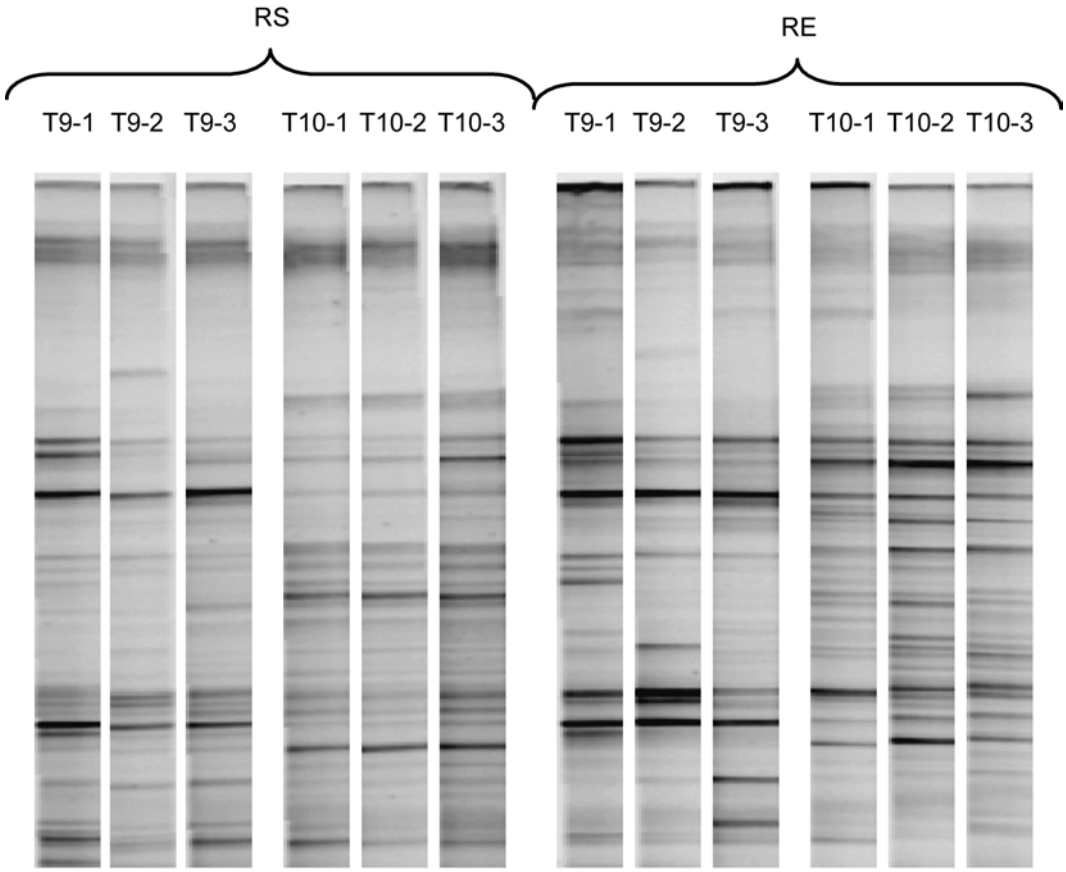

Fig.1. Example of DGGE profiles of the rhizospheric soil (RS) and the rhizoplane/endorhizosphere (RE) bacterial communities of the season 2002-2003 . T9-1, T9-2, T9-3 = plot replicates of treatments containing AMF alone. T10-1, T10-2, T10-3 = plot replicates of control without treatment.

6.4 Results

Wheat rhizosphere bacterial community assessment in the fields LL, LM and HH during the rabi season 2001-2002

The straw and grain yields differed between the three fields (straw and grain yield respectively in t ha^{-1}): LL (1.06 and 1.55), LM (1.54 and 2.19), HH (2.01 and 2.88). The wheat rhizobacterial community was very dynamic during the growth season as strong shifts in the DGGE patterns were observed between the different wheat growth stages. In order to determine the most discriminant factors influencing the bacterial communities, the DGGE profiles of the RS and RE fractions were analysed by canonical correspondence analysis (CCA) as shown in figure 2.

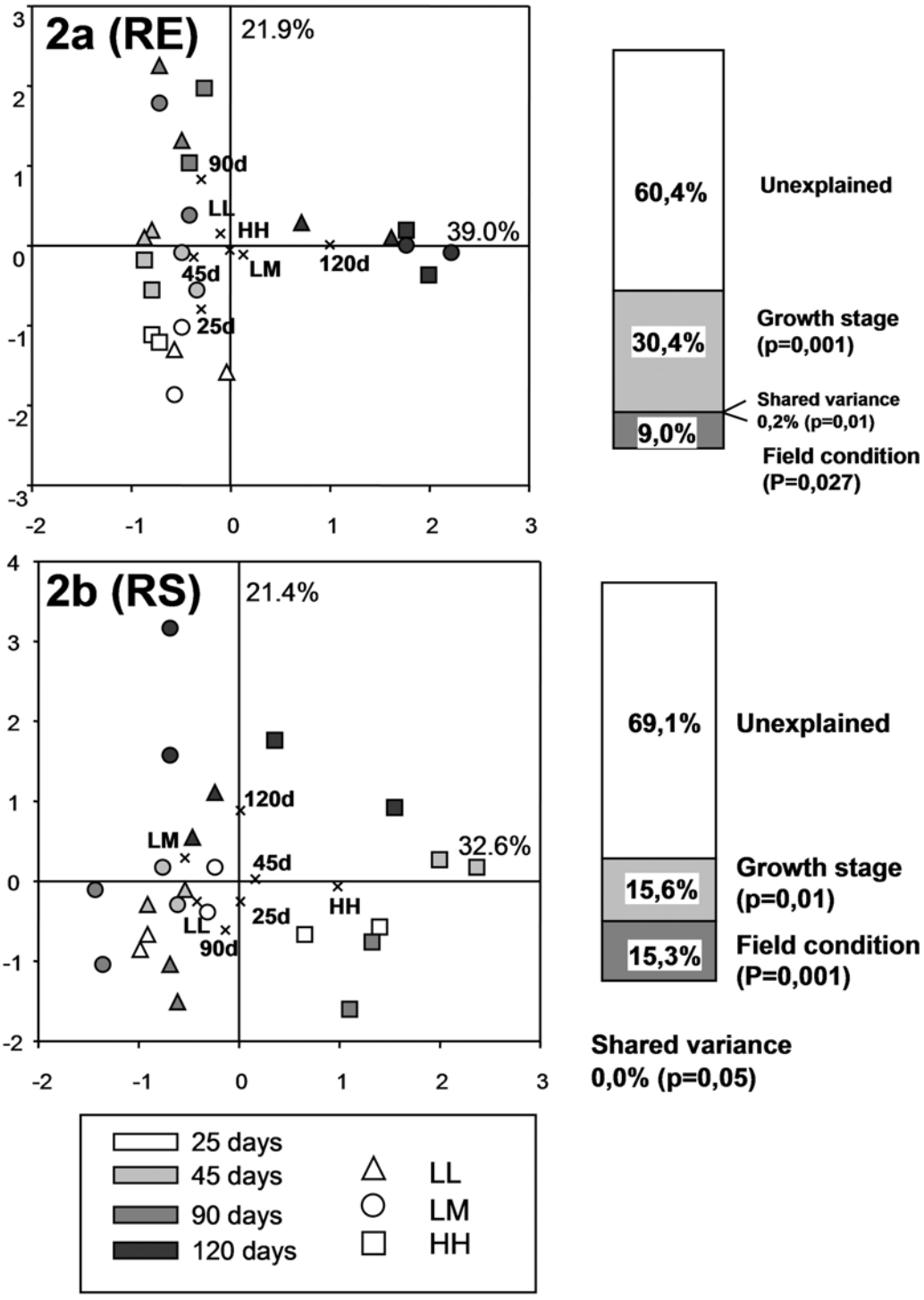

Fig.2. Canonical correspondence analysis (CCA) of DGGE bacterial community profiles for different wheat growth stages and fields at season 2001-2002. Time 25, 45, 90, 120 days and fields LL, LM and HH are used as qualitative explanatory variables (centroids). Values on the axes indicate % of total variation explained by the axes. The variance decomposition of the CCA of the bacterial community profiles is represented as a bar diagram. (a) Analysis of the community profiles of the rhizplane/endorhizosphere: sum of all canonical eigenvalues, 1.127; total inertia, 2.846; Monte Carlo overall 999 permutation test, p = 0.001. (b) Analysis of the community profiles of the rhizospheric soil: sum of all canonical eigenvalues, 0.881; total inertia, 2.850; Monte Carlo overall 999 permutation test, p = 0.001.

Objects (DGGE profiles represented as squares, triangles and circles) close together are likely to have similar bacterial community profiles. Objects close to centroïds points (X in fig. 2) are bacterial community profiles that are likely to contain speciess (DGGE bands) that are found frequently (or more abundantly) in the conditions of the qualitative explanatory variables.

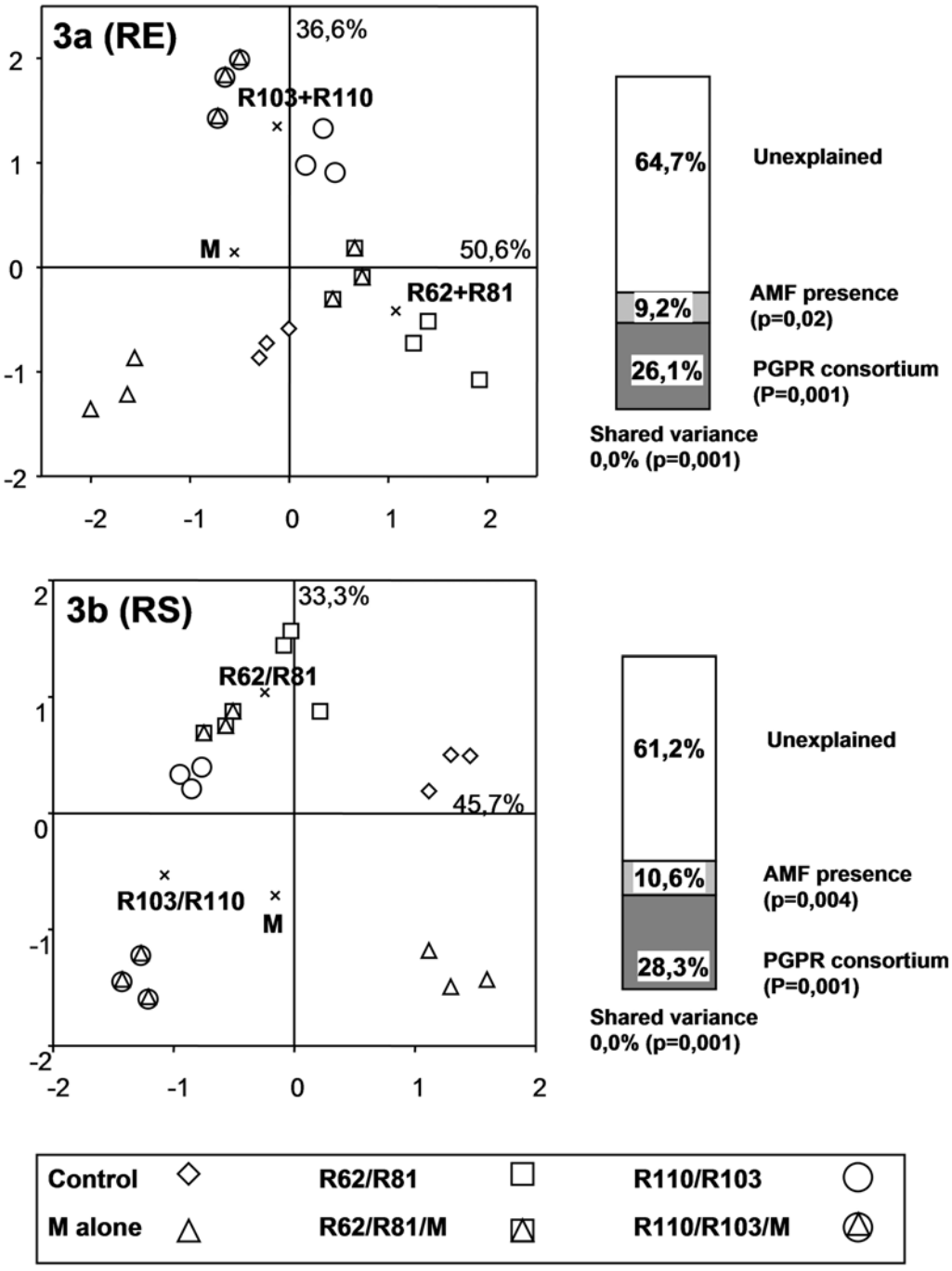

Fig.3. Canonical correspondence analysis (CCA) of DGGE bacterial community profiles for different consortia in LL field at season 2002-2003. The PGPR consortium (R62/R81 or R103/R110) and the presence of arbuscular mycorrhizal fungi (M) are used as qualitative explanatory variables (centroids). Values on the axes indicate % of total variation explained by the axes. The variance decomposition of the CCA of the bacterial community profiles is represented as a bar diagram. (a) Analysis of the community profiles of the rhizplane/endorhizosphere: sum of all canonical eigenvalues, 0.850; total inertia, 2.408; Monte Carlo overall 999 permutation test, p = 0.001. (b) Analysis of the community profiles of the rhizospheric soil: sum of all canonical eigenvalues, 1.001; total inertia, 2.575; Monte Carlo overall 999 permutation test, p = 0.001.

In the CCA of the RE (fig. 2a), the objects grouped by growth stage (25, 45, 90, 120 days) indicating a predominant effect of the wheat growth stage over the field condition on the bacterial community structure. Indeed, in the RE, the growth stage explained 30.4% (p=0.001) of the variance in the DGGE profiles whereas the field condition explained only 9.0% (p=0.027), with a shared variance of 0.2% (p=0.01). However, in the RS, the field condition had a greater influence on the bacterial community structure (fig.2b) than in the RE. In the RS, the variance in the DGGE profiles could be explained by 15.6% (p=0.01) by the wheat growth stage and 15.3 % (p=0.001) by the field condition.

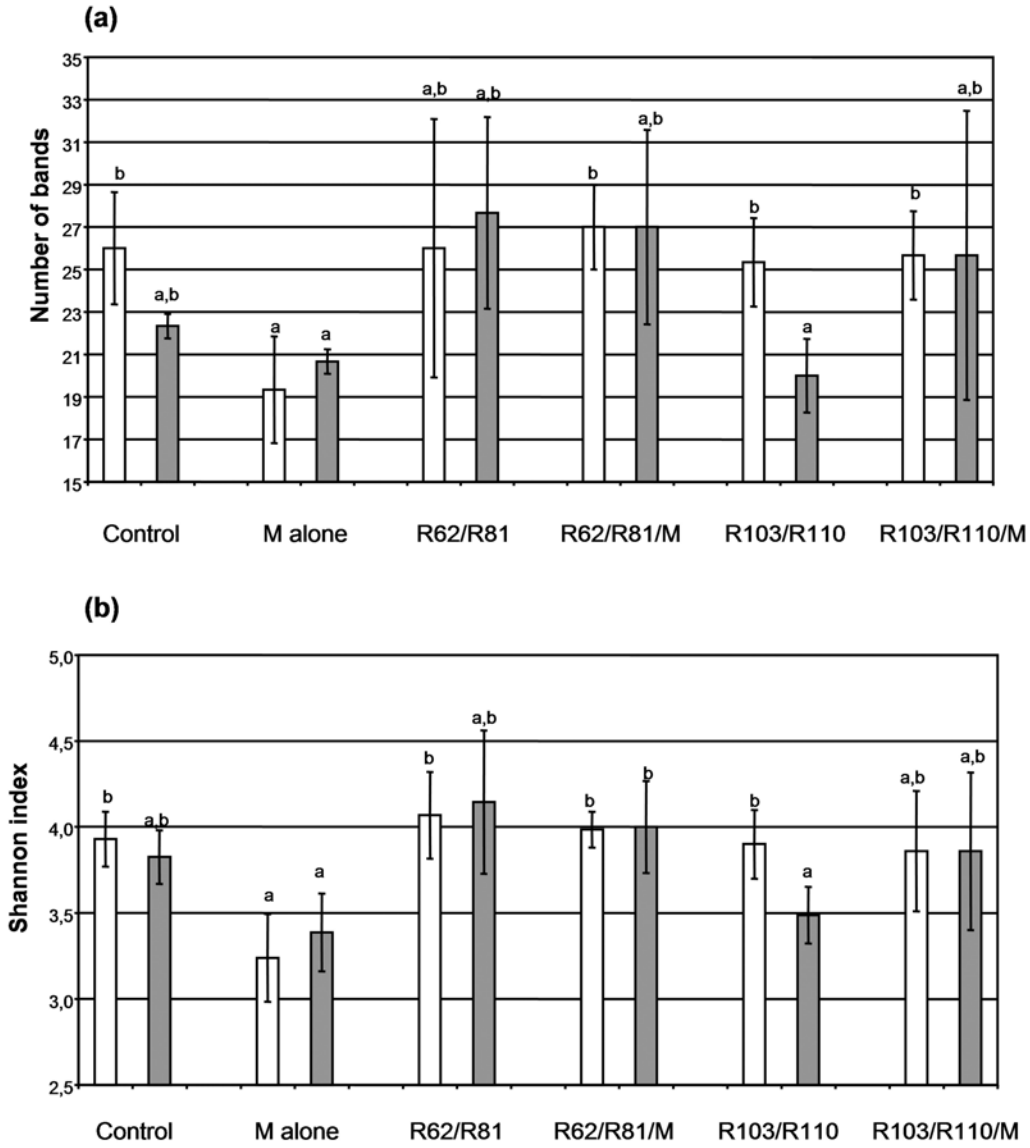

Fig.4. Richness (a) and diversity (b) of bacterial communities according to consortia and root fraction. Richness was determined by counting the number of bands per DGGE profile and the Shannon index was calculated as as $-\Sigma$ pi $\log_2$(pi) where pi is the relative abundance of a given band in the profile. Values are means ± standard deviation of 3 plot replicates. Identical letters indicate non-significantly different counts according to Student's *t*-test ($p< 0.05$, n=3). Column in blank = rhizoplane/endorhizosphere; Column in grey = rhizospheric soil.

Rhizosphere bacterial community assessment of wheat treated with PGPR and AMF in the LL field during the rabi season 2002-2003

LL was selected because for several years, lower wheat yield levels were observed in this field as compared to LM and HH. In addition, the rhizobacterial community response was assessed at the flowering stage when the total mesophilic cultivable bacteria were at their highest level (Gaur, 2003). Changes were observed between the DGGE profiles of the control and the bio-inoculation treatments and there was a high homogeneity between the plot replicates (fig.1, fig.3). In order to determine which of the AMF or PGPR presence affected more the bacterial community structure, the DGGE profiles were analysed by CCA. There was a predominant influence of the PGPR consortium (R62/R81 or R103/R110) as compared to the AMF presence on the bacterial community structure. In the RS, the PGPR consortium explained 28.3% (p=0.001) of the variance in the DGGE profiles, whereas AMF 10.6% (p=0.004), with no shared variance (fig.3). In the RE, this proportion was similar as in the RS: the PGPR consortium explained 26.1% (p=0.001) of the variance and the AMF 9.2% (p=0.02) with no shared variance (fig.3). The treatment AMF alone diminished significantly the bacterial richness and diversity as compared to the control in the RE fraction (fig. 4). In the RS, this trend is also observed.

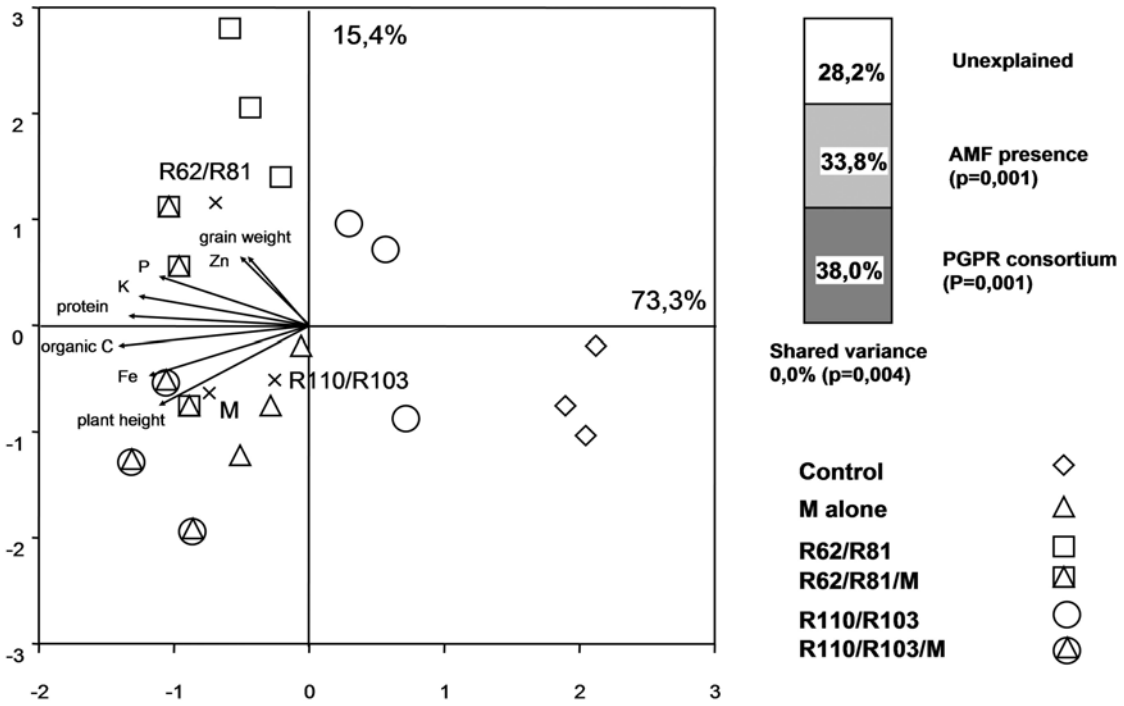

Fig.5. Redundancy analysis (RDA) of plant data for different treatments in LL field at season 2002-2003. The plant data have been standardised before the analysis. Descriptors (arrows) are the plant height, grain weight, organic carbon (organic C), protein content (protein), phosphorus (P), potassium (K), Iron (Fe) and Zinc (Zn). The PGPR consortium (R62/R81 or R103/R110) and the presence of arbuscular mycorrhizal fungi (M) are used as qualitative explanatory variables (centroids). Values on the axes indicate % of total variation explained by the axes. The variance decomposition of the RDA of the plant data is represented as a bar diagram. Sum of all canonical eigenvalues, 0.718; total inertia, 1.000; Monte Carlo overall 999 permutation test, p = 0.001.

The effect of the different treatments on the plant growth was also significant (table 3 and fig.5). The influence of the PGPR and AMF with regards to plant features is equivalent. Indeed, the PGPR consortium explained 38.0% ($p=0.001$) of the variance, the AMF presence 33.8% ($p=0.001$), with no shared variance. The plant height increased in all the treatments as compared to the control especially when PGPR and AMF were co-inoculated but the increase was significant only in R103/R110/M ($p<0.01$). The grain weight was also higher in all the treatments as compared to the control however non significantly. The grain quality of the wheat plants under treatments had considerably improved as compared to the control.

Table 3. Wheat plant features at harvest after the rabi season 2002-2003. Values are means ± standard deviation of 3 plot replicates. Identical letters indicate non-significantly different counts according to the Tukey test ($p< 0.01$, $n=3$).

Treatments	Plant Height (cm)	1000 Grain Weight (kg)	Grain nutrient content					
			Organic C (%)	Protein content (%)	P (mg kg^{-1})	K (mg kg^{-1})	Zn (mg kg^{-1})	Fe (mg kg^{-1})
Control	87.7a	32.7a	23.2a	9.2a	35.4a	48.7a	0.26a,b	0.48a
M alone	94.3a	34.7a	29.0b	11.6b	65.5b,c	58.0c	0.26a,b	1.35d
R62/R81	89.3a	38.0a	29.3b,c	12.4b,c	77.2d	59.5c,d	0.31b	0.84b
R62/R81/M	94.7a	38.0a	30.8c	14.05c	68.8c	60.5d	0.28a,b	1.33d
R103/R110	90.0a	38.7a	28.4b	12.8b,c	60.1b	53.8b	0.23a	0.47a
R103/R110/M	102.3b	34.7a	33.5d	14.4c	67.2c	57.7c	0.28a,b	1.17c

Organic carbon content was significantly higher in the grain of plants treated as compared to the control with maximum values obtained when the PGPR were co-inoculated with the AMF. The same trend occurred in the protein content. The phosphorus concentration was almost double in the grains of the plants treated with the bio-inoculants as compared to the control. Finally, grain iron content more than doubled in treatments where AMF were applied. In order to determine if the DGGE profiles and plant data were correlated, the Mantel correlation test was carried out. In the RS, a significant correlation (r Mantel, 0.28; $p<0.01$) was found between the DGGE profiles and plant data. However this was not the case in the RE ($p = 0.12$). This means that in the RS, a specific bacterial community could be affiliated to specific plant features.

6.5 Discussion

In the first experiment, the wheat rhizobacterial community structure was highly dynamic and strongly influenced by the plant age. Indeed, the growth stage explained 30.4% of the variance in the DGGE profiles in the RE and 15.6% in the RS. Community shifts during the plant growth have probably resulted from a modification in the root exudation pattern of the plants during their growth (Lynch, 1990). The bacterial community structure at 120 days

growth (maturity stage) differed more than the other growth stages. Moreover, a previous study carried out in these fields showed that the bacterial abundance measured by total mesophilic bacterial counts reached a maximum at 90 days and diminished at 120 days in both RS and RE fractions (Gaur, 2003). This result could be explained by a strong modification in the exudation pattern at the maturity stage. Actually, after flowering, most of the carbon assimilated is transported to the grain and therefore at a later stage of wheat growth, the amount of rhizodeposits diminishes (Kuzyakov and Domanski, 2000). In the RS, the impact of plant age was less than in the RE and the field conditions explained the same proportion of variance in the DGGE profiles. In the RS, the bacterial community structure of the HH field was very different than LL and LM. The main difference in agricultural practice between HH and the two other fields was a higher input of fertilization. HH received 60 kg/ha of urea and 20 kg/ha of diammonium phosphate (DAP) as compared to 50 kg/ha of urea and no DAP in LL and LM. This higher level of fertilization had likely affected the rhizobacterial community as reported previously (Liljeroth et al., 1990, Marschner et al., 2004; Sturz et al., 2004). The introduction of no tillage or modifications in the rotation of crops can affect the bacterial community (Lupwayi et al., 1998; Alvey et al., 2003). However, in these studies, either the RS only was analysed or both RS and RE fractions were not separated. Our results indicate that the bacterial community of the RS would be more subjected to changes in the agricultural practice than the one of the RE. It would then be more appropriate to select PGPR strains isolated from the RE or that colonize this fraction more efficiently. These strains would be more dependent on the plant species or growth period and be less influenced by field conditions or changes in agricultural practices. This is particularly important if a PGPR strain or consortium have to be applied in fields with different soil characteristics and management.

Positive effect of the PGPR and AMF bio-inoculants on wheat growth observed in previous greenhouse experiments (Gaur, 2003) was confirmed in the fields. The values of plant height and grain weight were higher in bio-inoculated plants as compared to the control but in general not significantly. However, a significant effect of the bio-inoculation was observed on grain quality. Grain iron content more than doubled in the treatments where AMF were applied as compared to the un-inoculated controls. AMF are known to uptake the nutrients phosphorus, potassium, nitrogen and the micronutrients zinc and copper (Smith and Read, 1997) but reports on Fe uptake by AMF are scarce. An increase in Fe plant concentration in maize and sorghum mycorrhizal plants has nevertheless been reported (Clark and Zeto, 1996; Caris et al., 1998). However contrarily to these authors, are soils were neither alkaline nor Fe depleted (see table 1). Organic carbon and protein contents were significantly higher in the grain of plants treated as compared to the untreated control with maximum values

obtained when the PGPR were associated with the AMF. Moreover, there was a significantly higher level of phosphorus content that doubled in the bio-inoculated plants. The positive responses of wheat to the PGPR consortia and AMF inoculation might be due to the fact that these micro-organisms were adapted to their environment in terms of soil characteristics, plant genotype and climate. Indeed, they had been selected in the wheat rhizosphere from the same plant and area of cropping. This approach might have limited the discrepancies that could have occurred in the plant responses between greenhouse and field trials using PGPR bio-inculations. Indeed, reviewing the applications of PGPR in agronomy, Lucy et al. (2004), stress the inconsistency of results between the laboratory, greenhouse and field studies due to differences in soil type or climatic variability and the better plant response obtained if the PGPR strains were isolated from the native rhizosphere. Even if PGPR strains R62 and R81 produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) which is known for its antifungal properties (Weller et al., 2002), the AMF growth was probably not affected. This confirms the finding of Barea et al (1998) and Gaur et al (2004) who reported that 2,4-DAPG-producing rhizobacteria did not adversely affect AMF growth. When analysing globally the plant data, the type of bacterial consortia explained 38.0% and the AMF treatments 33.8% of the variance meaning that the plant response is mediated approximately equally by the type of bacterial consortia and by the presence of AMF. This finding could imply that there was no competition for exudates between the AMF and PGPR strains. Moreover, when selected appropriately, a bio-inoculation combining AMF and PGPR strains may have complementary roles in the growth promotion of the plant. A synergistic effect between the AMF and PGPR was even observed on the organic carbon and protein contents when they were co-inoculated. This result is in accordance with several studies reporting a positive interaction between AMF and a wide range of PGPR including phosphate solubilizing bacteria (Toro et al., 1997), nodule-forming N_2-fixing Rhizobia or free-living *Azospirillum* spp. (Barea et al, 1996; Biro et al., 2000) and *Pseudomonas* spp. (Vàzquez et al., 2000) on plant growth.

In our study, the different bio-inoculation treatments have strongly modified the bacterial community structure in the RS and RE. When inoculated alone, the AMF have diminished significantly the bacterial richness and diversity as compared to the control in the RE fraction. In the RS the same trend was also observed but not significantly. The response of the rhizobacterial community to AMF infection varies depending on the fungus, soil type, plant species and experimental conditions. Indeed, some studies report a decrease (Christensen and Jakobsen, 1993), or on the contrary an increase in bacterial abundance or diversity (Posta et al., 1994), whereas others don't observe a significant difference (Mansfeld-Giese et al., 2002). A reduction in bacterial diversity might have implications in the long-term use of AMF as bio-inoculants. A combined inoculation of AMF with PGPR could resolve this

problem as the treatments with PGPR/AMF co-inoculations in this study did not affect significantly bacterial richness or diversity as compared to the non-inoculated control.
The bio-inoculation of PGPR in the form of seed coating or in the soil close to the seed has a wide range of effects on the bacterial community. It may cause shifts in the community or in specific populations either in small or high magnitudes (Nacamulli et al., 1997; Pandey et al., 1998; Bakker et al., 2002; Bankhead et al., 2004, Kozdrój et al., 2004). In other cases, it had no significant effect (Mahaffee and Kloepper, 1997; Lottman et al., 2000). Moreover, this effect is probably dependent on the type of application method of the PGPR bio-inoculation (Ciccillo et al., 2002). In our study, not only did the PGPR consortia affected significantly the bacterial community structure but also this latter was more dependent on the type of PGPR consortium (R62/R81 or R103/R110) as compared to the presence of AMF. Indeed, in the RS, the bacterial consortia explained 28.3% and the presence of AMF explained 10.6% of the variance and in the RE, the proportion was similar. The predominant influence of the PGPR strains over AMF probably results from the faster root colonizing ability of the bacteria. It takes several days for the fungus to penetrate into the root's epidermal cells and form infection units (Smith and Read., 1997). For the PGPR strains, the process of root colonization is probably immediate. Indeed, the PGPR strains used in this study are r strategists that increase rapidly in number and activity in response to flushes of readily available energy substrates such as the ones found once the roots begins to provide a sufficient amount of exudates. Moreover, the PGPR strains are coated in high numbers around the seed (10^6-10^7 CFU/seed). This high PGPR density around the seed could induce a state of quorum sensing amongst the bacterial populations. Quorum sensing has been defined as the ability of bacteria to monitor cell density before expressing a phenotype (Whitehead et al., 2001). Low molecular-mass molecules, whose extracellular concentration is related to the population density, are produced. If their concentration surpasses a characteristic threshold, gene expression is induced. Amongst the gene responses caused by quorum sensing is the production of secondary compounds such as antibiotics (Wood and Pierson, 1996). A localised production of antibiotics could therefore diminish the abundance of sensitive populations and increase those of the more resistant ones, explaining the differences in bacterial community structures observed in our studies.

The bacterial community profiles were positively correlated with the plant features in the RS. Also, the bacterial community structure could be associated with a positive plant response. Therefore, the positive effect of the bio-inoculants on plant growth in our study might not only result from a direct PGP effect but also from an indirect modification of the bacterial community. To corroborate this hypothesis, Pandey et al (1998) have reported that improvements in yield and plant growth resulted in part from the stimulation of N_2-fixing

bacteria in the rhizosphere of maize after the bio-inoculation of two PGR strains. A positive change in the bacterial community could result from an increase in the plant-beneficial rhizosphere bacteria *Pseudomonas* spp. This group of bacteria possesses multiple PGP characteristics (Glick et al., 1995; Weller et al., 2002; Lucy et al., 2004), are known to be the most abundant rhizosphere bacteria (Kragelund et al., 1996; Marilley et al., 1999; Tarnawawski et al., 2003) and are often resistant to antibiotics (Goddard et al., 2001; Bensasson et al., 2004). Finally, a modification in the bacterial community caused by a PGPR bio-inoculation could explain the fact that a PGP effect lasts during the plant development even if the PGPR population decreases rapidly (Jacoud et al.,1998).

In conclusion, the bacterial community of the root-adhering rhizospheric soil is more influenced by modifications in its soil environment such as an increase in fertilizer input than the one of the rhizoplane/endorhizosphere fraction. Consequently, in order to prevent discrepancies in the plant responses due to different field conditions, a PGPR consortium should contain one or several strains that were isolated from the RE fraction. As these beneficial rhizobacteria should *a fortiori* colonize best the RE fraction, they would be less subjected to environmental changes than other strains colonizing the RS. The rhizobacterial community structure was also dependent on the plant's growth stage especially in the RE. The PGPR strains or consortia could then be applied not only at sowing but also at stages of plant growth they are best adapted to. The effect of the PGPR consortium on the bacterial community structure was predominant with regards to the one of AMF possibly resulting from a PGPR-induced quorum sensing response and a faster root colonization process. The AMF and the PGPR strains used as bio-inoculants in this study were probably adapted to the agro-climatic conditions. This explains why when used as AMF-PGPR consortia, their effect on the plant development was not only positive but also in some aspects synergistic. Therefore, formulations for the bio-inoculum preparation should take into account the PGPR or AMF strains that are adapted to local conditions. This is why a diverse set of PGPR or AMF consortia are now being tested in fields with different plant genotypes, cropping practices, soil and climate in the continuum of the ISCB project. Moreover, testing of shelf-life and inoculation methods of the bio-inoculants are actually undertaken in order to meet the needs for a large-scale production of PGPR and AMF bio-inoculants.

6.6 Acknowledgements

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7 General discussion

7.1 Interactions between arbuscular mycorrhizal fungi and the wheat rhizobacterial community

7.1.1 AMF do affect the rhizobacterial community structure but in a lesser extent than the plant

Arbuscular mycorrhizal fungi can change the number and species composition of the bacterial community in the mycorrhizosphere (see table 2 in paragraph 1.8.3). In our studies comparing AMF and non-AMF rhizobacterial communities (chapters 2.1 and 2.2), there is also evidence of shifts in the bacterial population composition due to AMF colonization in the mycorrhizosphere. However, the type of rhizospheric fraction (non rhizospheric soil, rhizospheric soil and rhizoplane/endorrhizosphere), the plant age and the plant species (wheat or green gram) had more influence on the bacterial community structure than the presence of AMF. This result is in accordance with the study of Marschner et al. (2001) and Marschner and Baumann (2003) who assessed the bacterial community structure in mycorrhizal and non mycorrhizal maize rhizosphere also with the technique of DGGE analysis. These authors found that the bacterial community structure was affected mainly by the plant age, the root fraction or zone and to a lesser extent by AMF, mostly at later stages of plant growth. In addition, Söderberg et al. (2002) showed with PLFA patterns that the plant species had greater effects on the bacterial community structure of the rhizosphere than AMF colonisation. Finally, Andrade et al. (1997) counted less bacteria in the hyphosheric soil than in the rhizosphere.

This predominant plant effect might result from the smaller amount of organic compounds released by fungal structures as compared to the root (Barea et al., 2002a). Moreover, different exudation patterns exist between plants of different species or within a plant at different ages (Curl and Truelove, 1986; Aulakh et al., 2001). Also, the amount and quality of the rhizodeposition depends not only on the zone of the root (Lynch, 1990) but also on the distance to the root (Jones et al., 2004). Utilisation of AMF for agronomical purposes should therefore not be rejected on a pretext that indigenous microflora could be modified. Indeed, not only, could this modification be favourable for the plant, but also other factors related to the crop (species, cultivar, age, etc…) or the cropping practice (rotation, tillage, etc…) have probably much greater impacts on the microbial community in agroecosystems.

7.1.2 How AMF affect the bacterial community in the mycorrhizosphere and the concept of mycorrhizosphere competence

In order to colonize successfully the rhizosphere environment, rhizobacteria need to possess certain rhizosphere competence traits (see paragraph 1.6.3). We could extend the rhizosphere competence concept to the mycorrhizosphere or hyphosphere. As reported in our studies, particular bacterial populations develop more favourably in the presence of AMF. These bacterial populations could then possess particular characteristics facilitating their survival when AMF are present that could be defined as mycorrhizosphere or hyphosphere competence traits. One of these competences would be the capacity to metabolise a higher variety of root exudation products as exudate composition changes with AMF infection (Mada and Bagyaraj, 1993; Bansal and Mukerjii, 1994). Other competence traits, observed in our study and by other authors are described below.

7.1.2.1 Nutrient competition

One of the main functions of AMF is the translocation of nutrients, mainly inorganic phosphorus and nitrogen, from the soil to the plant (Smith and read, 1997). Phosphorus is an extremely immobile nutrient in the soil. It is adsorbed very strongly to the soil particles surface and quickly form insoluble compounds by reacting with common soil elements such as iron, aluminium and calcium (Ladha et al., 2000). In the microcosm study (chapter 2.1), the most pronounced effect of AMF on the cultivable bacterial composition was the strong increase in the proportion of phosphate solubilizing bacteria (PSB) in AMF related zones. This proportion even reached 25% in the rhizoplane/endorrhizosphere fraction of the mycorrhizosphere at the maturity stage. An increase in PSB populations in the vicinity of AMF in synergy with solubilization of insoluble P has been reported (Andrade et al., 1998a; Villegas and Fortin, 2001). PSB growth could be stimulated by the sparingly available phosphate close to mycorrhizal hyphae. Indeed, the soluble phosphate ions (Pi) are taken up predominantly by the AMF and transported to the plant, thus the level of mineralised or solubilized Pi decreases dramatically in close vicinity of the hyphae. However, PSB can produce organic acids that acidify the surrounding of the bacterial cells. The protons in excess could release Pi from calcium phosphates by proton substitution with calcium. Pi solubilized is subsequently uptaken by the PSB, fulfilling the bacterial P demand and enabling bacterial growth and division. The number of PSB cells increases then more than the other bacteria.

- *Mycorrhizosphere competence trait: Capacity to solubilize phosphorus from inorganic sources (by producing organic acids or siderophores) or organic sources (by producing phosphatases or phytases).*

In addition, in the the chapter 2.1 study, NH_4^+ and NO_3^- diminished in the mycorrhizosphere and the hyphosphere. Consequently, this ion uptake by AMF could change the pH (Bago and Azcón-Aguillar, 1997).

7.1.2.2 AMF affect the pH locally

We observed a pH increase in the extraradical compartment, corresponding to the hyphosphere, of *G. intraradices in vitro* cultures (chapter 4). In the chapter 2.1 study, the pH was higher in the AMF related zones and was significantly correlated with the bacterial community structure. These results confirm the findings of Marschner and Baumann (2003) who suggested a pH-induced modification of the bacterial community structure following AMF colonization in the rhizosphere of split-root maize. In addition, Bethlenfalvay et al. (1997) hypothesised a functional relationship between a higher soil pH value and water stable aggregates stability (i.e.. low soil pH diminishes the bridging of clays and organic materials). An important fact is that a modification in soil pH can modify the availability of the phosphorus. For example, in neutral to alkaline conditions, inorganic phosphate complexes with calcium and in acid soils, where Ca-P would be dissolved, it forms metal complexes (Fe-, Mn-, Al-phosphates (Vance et al., 2003).

- *Mycorrhizosphere competence trait: Capacity to produce both organic acids and siderophores in order to be competitive for P uptake in both acid and alkaline soils*.

7.1.2.3 Bacteria feeding on fungal structures

The study of the bacterial community associated to *Glomus geosporum* and *G. constrictum* spores revealed that the bacteria were probably feeding on fungal structures (chapter 3). Indeed, the outer hyaline wall layer composed in major part of chitin was gradually degraded by spore surface bacteria. In addition, the most dominant bacterial populations found associated to the spores belonged to genera possessing bio-polymer degrading traits such as the production of cellulase, chitinase, protease, etc… For example, 4 out of the 15 bands sequenced including the most dominant one were affiliated to the *Flexibacter* genus which is known for its high bio-polymer degrading capabilities. Interestingly, 4 out of 9 AMF-related dominant bands sequenced in the microcosm experiment (chapter 2.1) were also affiliated to the *Flexibacter* genus. Moreover, a higher number of active bacterial populations were found in mycorrhized plants as compared to none mycorrhized plants (chapter 2.2). These bacteria could not only feed on the fungal spore wall but also on the extraradical hyphae. Indeed, external hyphae represent a large carbon input in the environment and can represent 8 to 25 km hyphae per liter soil (Schreiner et al., 1997).

- *Mycorrhizosphere competence trait: Capacity to feed on the fungal structures by producing bio-polymer degrading enzymes such as chitinases.*

7.1.2.4 Bacteria feeding on fungal exudates

Bacteria could also feed on fungal exudates relased in the mycorrhizosphere or hyphosphere. Filion et al. (1999) reported that a crude extract of *Glomus intraradices* extraradical hyphae increased *Pseudomonas chlororaphis* growth. Bacterial consumption of exudated fungal proteins was hypothesised in chapter 4. A pH increase was found in the neighbourhood of bacterial colonies in extraradical hyphal compartments of *G. intraradices in vitro* cultures. We suggested that the catabolism of fungal secretions may be responsible for this alkalinization. Glomales are capable of secreting high amounts of proteins in the environment, such as glomalin which contributes to the stabilization of soil agreggates (Wright and Upadahya, 1998). Bacteria could therefore feed on the exudated glomaline.

- *Mycorrhizosphere competence trait: Capacity to produce proteases in order to feed on secreted fungal proteins*

7.1.2.5 Bacterial production of exopolysaccharides

The production of bacterial mucilage such as extracellular polysaccharides is necessary for a firm anchoring on the root or hyphal surfaces. For example, Bianciotto et al. (1996) demonstrated that rhizobia and pseudomonads can attach to spores and hyphae of the AM fungus *Gigaspora margarita* germinated under sterile conditions in vitro. First stages of attachment were governed by general physico-chemical parameters such as electrostatic attraction. Secondary attachment was provided by the production of extracellular polysaccharides (Bianciotto et al., 2001).

- *Mycorrhizosphere competence trait: Capacity to attach firmly on AM hyphae or spores by possessing appendages such as fimbriae, and producing large amounts of extracellular polysaccharides.*

7.1.4 Mycorrhiza helper bacteria

PGPR strains would be more effective if not only they possessed mycorrhizosphere competent traits but also could stimulate fungal development. Bacteria that have the faculty to improve mycorrhizal growth or activity have been defined as mycorrhiza helper bacteria (Garbaye, 1994).

Several PGPR strains were selected in Pantnagar by Rachna Gaur during her thesis in view of subsequent bio-inoculation trials in rainfed fields (Gaur, 2003). Before testing these strains

in the fields, it was necessary to determine if they had no deleterious effects on mycorrhizal growth. An increased percentage of root colonization by AM fungi was reported in pot experiments with several PGPR strains bio-inoculations as compared to non PGPR inoculated controls (Gaur, 2003; Gaur et al., 2004). These PGPR strains could then be acting as mycorrhiza helper bacteria in the wheat mycorrhizosphere. However, we did not know if these PGPR strains affected the AMF hyphal growth or sporulation directly in the hyphosphere. Therefore we designed an *in vitro* device consisting of a two-compartmental Petri plate system using Ri T-DNA transformed clover roots permitting the separation of the hyphosphere from the mycorrhizosphere (chapter 4). The effects of the PGPR strains tested on the AMF development varied from inhibition to improvement of the hyphal biomass or spore production. Interestingly, there was a positive mutualistic interaction between *Pseudomonas synxantha* R81 and *Glomus intraradices* that could be explained by the bacterial catabolism of fungal proteins. The protein catabolism could be beneficial for the fungus, enabling it to recover a part of the nitrogen lost through its protein secretion. In addition, fungal self-inhibitory proteins or peptides (Leite et al. 1992) could be degraded by the bacterial catabolism (Barea et al., 2002a). This finding suggests that the ability to secrete proteolytic enzymes is an important trait related to MHB capabilities.

Other bio-polymer-degrading bacteria isolated from AMF fungal structures seem not to affect AMF development negatively. On the contrary, chitinase-producing actinobacteria isolated from spores of *G. macrocarpum* were shown to increase the percentage of mycorrhizal root colonization and the density of hyphae in the onion's mycorrhizosphere (Ames et al., 1989). A limited bacterial degradation of AM hyphae could stimulate fungal activity in particular to accelerate the hyphal turnover in soils which takes place in 5 to 6 days (Staddon et al., 2003; Lussenhop, 1996). Moreover, fungi produce lytic enzymes that weaken the structural microfibrils in their hyphal wall making it unable to withstand the high turgor pressure. The hyphal surface is then enlarged and new microfibrills are generated to extend the old ones resulting in the growth of the hyphae (Smith and Berry, 1978). Bacterial lytic enzymes such as chitinases could then also weaken the hyphal wall thus stimulating fungal growth. Finally, the process of maturation and eventual germination of the AMF spores might benefit from the activity of the surface microorganisms degrading the outer hyaline layer as hypothesised in chapter 3.

Bacteria with known antifungal properties seem not to impair AMF development and are often present in the mycorrhizosphere (Citernesi et al., 1996; Edwards et al., 1998; Vàzquez et al., 2000). For example, Barea et al. (1998) reported that not only DAPG-producing *Pseudomonas* F113 did not inhibit *G. mosseae* functioning but also that this strain could

colonize the *G. mosseae* spores and stimulate mycelial development from the spores. In our studies, the PGPR strains *P. synxantha* R81 and *P. jessenii* R62 had antagonistic effects on *Helminthosporium sativum*, *Fusarium oxysporium* and *Rhizoctonia solani* growth due to the production of the antifungal compound 2,4-diacetylphloroglucinol (Gaur, 2003). Despite possessing these antifungal traits, the AMF formation and functioning was not impeded when the fungus was associated with these two strains. On the contrary, hyphal growth and sporulation of extraradical hyphae *in vitro* and root colonization in pot experiments were even stimulated (Chapter 4; Gaur, 2003). Moreover, in the fields, treatments combining R62/R81 and AMF had synergistic effects on grain quality (Chapter 6). These biocontrol bacterial strains can therefore be used in association with the AMF in plant growth promoting formulas.

7.2 Bacterial community structure in the wheat rhizosphere

7.2.1 A dynamic bacterial community during the cropping period

Bacterial community diversity or structure may be a sensitive indicator of ecosystem functioning and for evaluating disturbed or contaminated systems as it can be quickly affected by changes in the ecosystem processes (Kennedy and Smith, 1995). In order to select and apply PGPR and AMF bio-inoculants, we needed not only to test the effectiveness of the bio-inoculant strains in terms of plant growth but also to get a clearer picture of the functioning of the wheat rhizosphere's indigenous bacterial community. Our studies brought insights on the factors affecting the wheat rhizosphere bacterial community and to which extent (chapters 2.1; 2.2 and 6). A major result showed that the wheat's rhizobacterial community structure was highly dynamic and strongly influenced by the plant's age. These community shifts have probably resulted from a modification in the root exudation pattern of the plants during their growth (Lynch, 1990). A temporal variation in root exudation composition and a preferential utilisation of some products by PGPR strains could also explain why the root was not colonized simultaneously by our two gfp-tagged PGPR strains (chapter 5). The PGPR bio-inoculants could then be applied not only at sowing but also at the stages of plant growth they are best adapted to.

7.2.2 Distance-related effect of the root

The bacterial populations in the root-adhering rhizospheric soil were more affected by differences in field conditions than the ones colonizing the rhizoplane/endorrhizosphere corresponding to the washed roots (chaper 6). These latter were more sensitive to the plant age. The influence of the plant on the rhizosphere microbial communities in our studied

agroecosystem probably depends on the distance separating the bacterial populations from the root: the closer to the root they are, the stronger the influence of the plant is. Similarly, Marilley et al. (1998) and Marilley and Aragno (1999) found that the bacterial diversity in the rhizosphere of *Trifolium repens* and *Lolium perenne* decreased as the root proximity increased from non rhizospheric soil, adhering rhizospheric soil to rhizoplane/endorhizosphere (that is diversity in the nrs> diversity in the rs> diversity in the washed roots). Moreover, Lupwayi et al. (1998) reported that tillage in wheat cropping affected more the microbial diversity of the bulk soil than the one in the rhizosphere. Thus, when the bacterial populations are more distant from the root, they are more sensitive to the field conditions.

It would then be more appropriate to select PGPR strains isolated from the rhizoplane/endorrhizosphere or that colonize this fraction efficiently. These strains would be more dependent on the plant species or growth period but less influenced by field conditions or changes in agricultural practices. Indeed, the internal tissues of plants provide a relatively uniform and protected environment which is above all subjected to variations in plant physiology (Chen et al., 1995; Kozyrovska et al., 1996). Consequently, endophytic bacteria, defined as bacteria that can invade the tissues of healthy plants without causing symptoms of disease, could be used as PGPR (Wilson, 1995). Bacterial endophytes can be obligatory root symbionts such as for the *Rhizobia*-legume symbiosis. Other endophytic bacteria are actually a subset of bacterial populations previously living on the root surface or in the root-adhering soil and belong to genera like *Pseudomonas*, *Bacillus* and *Azospirillum* (Chanway, 1989; van Peer et al., 1990; Germida et al., 1998). Many endophytic strains can promote plant growth through N_2 fixation (Ladha et al., 2000), root elongation by the production of plant hormones (Kozyrovska et al., 1996) and increased resistance to pathogens and parasites (Chen et al., 1995).

7.2.3 Effect of the PGPR bio-inoculation on the indigenous bacterial community

In our field study (chapter 6), not only did the PGPR consortia affected significantly the bacterial community structure but also this latter was more sensitive to the type of PGPR consortium than to the presence of AMF. The predominant influence of the PGPR strains as compared to AMF possibly resulted from the faster root colonizing ability of the bacteria and from an induced quorum sensing effect. Interestingly, in this study, the bacterial community profiles of the rhizospheric soil were positively correlated with the plant features indicating that aspects of the plants could be related to the contribution of particular bacterial populations. Moreover, our root colonization assays (chapter 5) showed that a PGP effect remained even though there was a continuous drop of the PGPR counts in the rhizosphere

which were monitored throughout the wheat growth period. Therefore, the stimulating effect of the bio-inoculants on plant growth in our study might not only result from a direct PGP effect but also from an indirect modification of the bacterial community. In agreement with this hypothesis, Pandey et al (1998) have reported that after the bio-inoculation of two PGR strains, the improvements in yield and plant growth resulted in part from the stimulation of N_2-fixing bacteria in the rhizosphere of maize.

A modification in the bacterial community structure caused by a temporary disturbance could be buffered by the phenomenon of ecosystem resiliency which is driven by the level of diversity and interactions of the system (Elliot and Lynch, 1994; Kennedy, 1999; Lynch, 2002). Moreover, there is probably an over-riding influence of the plant over the presence of microbial inoculants (Lottman et al., 2000; Mahafee and Kloepper, 1997; paragraph 7.2.1).

7.2.4 Functional diversity of bacteria

Functional diversity, in opposition to taxonomic diversity, is critical to ecosystem functioning because of the variety of processes for which bacteria are responsible (Kennedy, 1999; Nannipieri et al., 2003). A loss of bacterial species may not change the functioning of the system as different bacterial species can carry out the same function, a phenomenon defined as the bacterial redundancy (Kennedy, 1999, Nannipieri et al., 2003; Othonen et al., 1997). This means that even though anthropogenic activites might affect the genetic composition of the soil microbial communities, gross microbial processes and their role in maintaining soil quality could remain unaffected (Crecchio et al., 2004). On the other hand, natural or human-induced perturbations may influence the level of soil microbial activities without changing the composition of microbial communities (Giller et al., 1997). Functional diversity in soils includes the magnitude and capacity of soil inhabitants that are involved in key roles such as nutrient cycling, decomposition of various compounds and other transformations (Zak, 1994). A loss of one of these functions could have dramatic consequences to the equilibrium of the ecosystem. For the assessement of functionnal diversity, community level physiological profiles (CLPP) generated with sole-carbon-source-utilization tests from Biolog® can provide physiological data of the soil rhizosphere populations (Garland and Mills, 1991; Konopka et al., 1998; de Fede et al., 2001). To assess the role of a particular function in soil-plant systems, one can study this function at the genetic level.

7.2.5 Functional genes

Functional gene analysis provides informations on the allelic diversity and potential activity of the studied function (Wellington et al., 2003). Some genes coding for a particular function can be related to the taxonomic affiliation of the bacteria. Therefore, a taxonomic diversity

study could be sufficient to understand the functioning of the system. For instance the *nif*H, gene coding for the dinitrogenase reductase, a key enzyme in the nitrogen fixation process, has evolved similarly to the 16S rDNA gene and can be used as an evolution molecular marker (Young, 1992). Since only 0,1 to 10% of bacterial cells in the soil are cultivable in currently used media (Amann et al., 1995; Nannipieri et al., 2003), the direct DNA extraction followed by functional gene analysis could give a more accurate image of the total functional genes diversity. An example of this approach is presented in annex 2. In this study, a two-step DNA amplification procedure was realised in order to increase the *phl*D gene detection level and to assess the allelic diversity of the *ph*D gene in study sites located in Budaun and Ghaziabad. The *phl*D gene is a useful marker of genetic and phenotypic diversity of 2,4-DAPG producing rhizobacteria. The antibiotic 2,4-diacetyphloroglucinol (2,4-DAPG) is a major determinant in the biocontrol of the PGPR associated with crops of agronomic relevance. Our field studies revealed that there was a genetic homogeneity of the *phlD* gene pool within a same field condition and rhizosphere fraction. Moreover, we hypothesized that the low diversity of the *phl*D gene pool might have resulted from the continuous rice-wheat rotation for twenty-five years and the use of the same wheat cultivar in these fields. The *phlD* sequences already available in the databases originate from *Pseudomonas* spp. that were isolated on culture media. However, in our study, a cluster of sequences not yet described was found, indicating that non-cultivable (or not-yet cultured) bacterial strains could play a significant role in biocontrol and should not be neglected. Similarly, Hamelin et al. (2002), studying *nifH* gene diversity in the rhizosphere of *Molinia coerulea*, demonstrated that the most dominant *nifH* cluster included only environmental clones and no sequences related to cultivable diazotrophs.

Phenotypic expression of functional genes by the soil or rhizosphere bacteria can be monitored with the mRNA reverse-transcription PCR analysis. Using this approach, Burgmann et al. (2003) detected that the *nif*H gene expression was positively correlated with the bulk nitrogen fixation activity in soil. Microarrays of DNA are powerful for rapidly characterising the composition and functions of microbial communities: a single array contains 500 to 1000 DNA spots with the possibility of a broad identification capacity (Tiedje et al., 2001). For example, Taroncher–Oldenburg et al. (2003) have developped microarrays to monitor the dynamics of the functions involved in the nitrogen cycle in soils such as nitrogen fixation, denitrification and nitrification. To have a more accurate view of the role of microbial populations in the functioning of ecosystems it is however necessary to study the relation between microbial taxonomic diversity and functional diversity.

7.2.6 Linking taxonomic with functional diversity

Multi-technique approaches comprising both structural and functional diversity analyses can be used to assess the functional and taxonomic diversity of bacterial communities and their inter-relations (Miethling et al., 2003; Larkin, 2003). For example, Crrechio et al. (2004) demonstrated with combined DGGE, CLPP analysis and ATP content analysis of soils, that saline water irrigation induced a genetic and metabolic alteration of soil microbial communities, corresponding to a lower microbial activity.

A major advance in linking functional activity to community structure came with the development of SIP (stable isotope probing). With this technique, a wide range of microbial communities were related with their functional activity such as methylotrophs (Radajewski et al., 2000), methanotrophs (Morris et al., 2002), ammonia oxidisers and phenol degrading bacteria (Manefield et al., 2002). Metagenomics is the culture-independent genomic analysis of microbial communities (Schloss and Handelsman, 2003). With this method, high molecular-weight soil DNA is archived in the form of bacterial artificial chromosomes (BAC) which can be analysed at a phylogenetic and functional level (Rondon et al., 2000). The function-driven analysis identifies clones that express a specific trait such as antibiotic production followed by characterization of the active clones by sequence and biochemical analysis. The sequence-driven analysis, on the other hand screens, metagenomic libraries for clones that contain sequences of interest via hybridization or gene-specific PCR (Schloss and Handelsman, 2003). Sequencing of the flanking DNA of some clones enables to obtain a phylogenetic affiliation of the organisms from which the DNA was isolated (Schloss and Handelsman, 2003).

8 Outlook

8.1 Traits for an effective PGPR bio-inoculation in the wheat mycorrhizosphere

The studies performed in this thesis permitted to define traits that a PGPR strain should possess to increase its efficiency as a bio-inoculant. The next generation of PGPR strains isolated from Indian fields should be selected taking into account these criteria and then tested in wheat microcosms to confirm our suggestions.

For instance, a PGPR consortium should contain at least one endophytic microorganism which is adapted to the crop of interest. This is particularly important if a PGPR strain or consortium have to be applied in fields with different soil characteristics and management. The endophyte would be adapted to the crop and thus be less influenced by environmental factors. In rice-wheat rotation systems for instance, endophytic diazotrophs have been introduced in rice plants to increase plant nitrogen levels (Ladha and Reddy, 2003). The endophyte does not necessarily have to be a prokaryote but could also be an eukaryote as for example the biocontrol fungus *Trichoderma* or the symbiotic AMF.

Another important task of the PGPR strain is to interact positively with the indigenous or co-inoculated AMF. PGPR that possess mycorrhizosphere competence traits should therefore be preferentially selected. These traits comprise the production of biopolymer degrading enzymes (proteases, chitinases), phosphate solubilising capacities (production of siderophores, organic acid, phosphatases, or phytases) as well as hyphal attachment capacities (production of exopolysaccharides). In addition, the consortium should contain at least one mycorrhiza helper bacteria that would ensure the stimulation of indigenous or co–inoculated AMF. For example, we suggested that the bacterial production of proteases would be an important mycorrhiza helper trait. To discover new traits involved in the mycorrhiza helper effect or to assess the outcome of mycorrhiza helper bacteria on the fungal metabolism, 2D gel proteomics could be used (Anderson and Anderson, 1998). In particular, the analysis of the proteins secreted by the bacteria when they are in the presence of the fungus by bidimensional eletrophoresis could be worthy of interest. In addition, to improve our understanding on the mechanisms implied in the mycorrhiza helper effect we could use the *in vitro* cultures designed in this thesis. For instance, mycorrhizal roots could be grown on gelosed media containing nutrients isotopically marked with ^{15}N, ^{32}P or ^{13}C to determine trophic interactions between the fungus and the tested bacteria.

8.2 How to monitor a consortium of different PGPR strains

The PGPR survival in the rhizosphere is a critical issue regarding commercialization of bio-products. Indeed, if they are not able to compete with the already acclimated indigenous microflora and to survive in the rhizosphere at the early stage of plant growth, there might not be any beneficial effect for the plant. To be able to monitor our PGPR strains in the wheat rhizosphere, we tagged them with a green fluorescent protein (gfp) and performed greenhouse experiments (chapter 5). We stated that *P. jessenni* R62gfp and *P. synxantha* R81gfp would not out-compete one-another for exudation sites, assuring that the plant growth promoting effect would not be hampered. To confirm this statement, a bio-inoculation formula combining these PGPR strains was effective in terms of plant growth promotion in our tested fields (chapter 6).

When multiple PGPR strains are in a consortium, it becomes difficult to monitor the precise localisation of individual bacterial cells. To differentiate these PGPR strains from one-another, each strain can be transformed with a different coloured fluorescent protein such as green, yellow, blue and red fluorescent proteins (Bloemberg et al., 2000). Moreover, to monitor the expression of crucial proteins in consortia such as the production of specific antibiotics, reporter gene systems with the gene coding for the protein of interest can be used (Chin-A-Woeng et al., 2001). Such techniques could then be used to follow-up the gene activity during mass production, formulation, storage and application of our bio-inoculants. The monitoring of PGPR strains or gene expression would also provide valuable data for registration purposes (Gerhadson, 2002).

8.3 Importance of early PGPR colonization

In paragraph 7.2.3, we hypothesised that the positive effect of the bio-inoculants on plant growth in our study might have resulted from an indirect modification of the bacterial community through the stimulation of beneficial populations. This finding means that benefical microorganisms would have to be introduced early in the cropping period in order to affect sufficiently the indigenous microbial community. Indeed, in mature communities, positive interactions among autochtonous populations are usually better developed, allowing a higher stability than in newly established communities and consequently, PGPR establishment in mature colonies is more difficult (Sturz and Nowak, 2000).

Seed bacterization would then be the optimal method of inoculation for an immediate root colonization by the PGPR strains. In addition, seed bacterization by biocontrol strains would provide a barrier against infection by soil-borne phytopathogens. New coated seeds would have to be prepared at each cropping season as our PGPR strain numbers declined rapidly in the non rhizospheric soil (chapter 5).

8.4 Increasing the effectiveness of bio-inoculations: the necessity to select bio-inoculants with different ecological properties, of adaptations to local field conditions and to use them in complement of sustainable agronomical practices

The positive responses of wheat to our PGPR/AMF consortia inoculation might be due to the fact that these micro-organisms were adapted to their environment in terms of soil characteristics, plant genotype and climate (chapter 6). Indeed, they had been selected in the wheat rhizosphere from the same plant cultivar and area of cropping. Therefore, a more thorough field research with different crops or field conditions are necessary to confirm the potential of our PGPR formulas. This research is actually been carried out in the continuity of the project by our Indian partners in Pantnagar.

In general, the use of PGPR to increase crop yield has been limited up till now due to the variability and inconsistency of the results between laboratory, greenhouse and field studies (Lucy et al., 2004). To remediate this problem, the application of bio-inoculants would probably be more effective if the consortium contains micro-organisms with different functional or ecological properties and who are adapted not only to their host plant but also to the local agroclimatic or soil conditions. Preparing a consortium containing micro-organisms with complementary properties like for instance phosphate-solubilizing bacteria and AMF should increase the effectiveness of these bio-inoculants. Moreover, instead of focusing on a few PGPR strains with the highest PGP potential or genetically modifying a bacterium to make it a “superbug”, it would be more appropriate to generate a vast pool of bio-inoculants with different ecological properties. The PGPR formulas would then contain a selection of bio-inoculants from this pool susceptible to interact together positively and to be adapted to the local agronomic specificities. A vast PGPR bank could then be developed by creating networks between agronomic and research institutes throughout India and other parts of the

world. Once an effective PGPR formula is chosen, the mass production and distribution of the product would be left to biotechnology companies.

The use of promising PGPR and associated AMF requires also soil managment in a way that ensures acclimation of these organisms in the long term. Therefore, the development of bio-inoculants should be considered as part of a global approach towards sustainable development of soil fertility which includes other agricultural practices and which avoid soil intensive overexploitation. Sustainable agronomic practices for instance include the use of reduced tillage, inputs of organic materials and nutrient cycling strategies based on crop rotations (Pankhurst et al., 1996). Efforts are also needed in the field of Integrated Pest Management to face the progressive build-up of weed, pathogen and pest populations inherent to the cropping system. Suggestions of a combination of sustainable agronomic practices and of the use of bio-inoculants are presented below.

8.5 Examples of AMF and PGPR combined bio-inoculations in low-input fields

8.5.1 Endophytic bacteria and AMF

Interactions of AMF with endophytic bacteria such as the N_2-fixing symbiotic bacteria greatly benefit the biological N inputs to the soil-plant systems (Barea et al., 2002a). AMF can improve nodulation and N_2 fixation in bacteria-AMF-legume tripartite symbiotic relationships probably because the mycorrhiza supplies the plant and the rhizobacteria with P (Johansson et al., 2004). In turn, diazotrophic bacteria can provide not only the plant but also the fungus with fixed N_2 (Barea et al., 1992, 2002). An example of bio-inoculation of AMF with endophytic N_2-fixing *Rhizobium* is provided by the study of Requena et al. (2001). These authors reported that the planting of the legume *Anthyllis cytisoides,* co-inoculated with *Rhizobium* spp and AMF inoculum based on indigenous taxa, induced an amelioration of the physico-chemical properties of a destertified semi-arid ecosystem.

8.5.2 Rock phosphate fertilization and PSB/AMF bio-inoculations

Phosphorus assumes particular importance in rice-wheat cropping systems. The phenomenon of wetting and drying, resulting in soils being exposed to alternations of anoxic and oxic conditions and subsequent draining of the rice fields, increases P absorption and immobilization during the wheat phase of the rotation (Ladha et al., 2000). An increased use of inorganic fertilizers to compensate the phosphorus depletion would further diminish the

soil fertility (Ladha et al., 2000). In India, there are enormous deposits of rock phosphate (phosphate-bearing minerals such as fluorapatite), which have a potentially high phosphate content (Sahu, 2000). Rock phosphate is a cheaper and less soil-degrading phosphate source than conventional water soluble P fertilizers such as superphosphates (FAO, 2004). AMF and PSB could act in synergy to improve phosphorus uptake from the soil, the PGPR solubilizing rock phosphate and the AMF taking up and transporting this solubilized phosphate to the plant. Synergistic improvement in growth of wheat and onion due to AMF and PSB co-inoculations and rock phosphate as P amendment have been reported (Toro et al., 1997; Singh and Kapoor, 1999). Moreover, as shown in chapter 2.1, the AMF presence might increase the indigenous PSB populations' densities thus accelerating the P solubilization process. These findings have major implications for the use of phosphate-solubilizing PGPR and AMF as bio-inoculants in low input agricultural sites where phosphorus availability can become the limiting factor for plant growth. The use of these AMF and PSB as biofertilizers would provide a cheap and environmentally friendly alternative to inorganic chemical fertilizers.

8.5.3 Prevention of pest re-emergence in no or reduced tillage systems

Tillage causes a physical disruption of fungal mycelia and may change physico-chemical properties of the soil with consequences for long-term soil health (Johansson et al., 2004). Reduced tillage enables to conserve soil water and diminish erosion or soil compaction caused by conventional intensive management (Timsinna and Connor, 2001). Moreover, in rice-wheat systems, reduced tillage diminishes the delay in wheat sowing after rice. Actually, delays in sowing wheat can reduce yields by as much as 1.5% per day (Hobbs et al., 2000). Therefore, reduced and zero tillage practices for the establishment of wheat after rice crop are being increasingly used in South Asia. The acreage of zero tillage crops has risen from a few hectares in 1996 to more than 100000 ha in 2000 in northwest India and Pakistan (Hobbs et al., 2000). One of the major impediments to the no tillage practices could be the re-emergence of pests and pathogens. Indeed, reduced tillage concentrates plant debris and consequently microbial biomass in the topsoils thus promoting proliferation of pathogens such as *Helminthosporium* spp., agent of the leaf blight disease (Sturz et al., 1997).

The introduction of the raised bed technique in rice-wheat systems has enabled to increase wheat yield significantly by optimising the water use and by keeping a permanent oxic zone during the rice flooding, thus increasing the survival of beneficial soil microorganisms such as the AMF (Hobbs et al., 2000; Alok Adholya, personnal communication). Nonetheless, the raised beds system, by combining reduced-tillage and a permanent oxic zone, could in turn favour the re-emergence of pests and diseases kept at low levels during the rice season.

The use of chitinase or antibiotic-producing PGPR strains in association with AMF could to prevent the development of pests in no/reduced tillage and raised beds systems. Not only the antibiotic strains used in our study did not adversely affect mycorrhizal growth but AMF can also offer plant protection against pathogens. AMF can limit pathogen development by improving plant nutrition, competing for photosynthates or infection sites, by local elicitation of plant defence mechanisms (Azcon-Aguilar and Barea, 1996) or by stimulating saprotrophs and PGPR (Kapoor and Mukerjii, 1998; chapters 2.1; 3; 4). A limited pest development by the use of these bioinoculants would limit the costs and ecological risks due to an extensive pesticide use.

8.5.4 Increase of the soil organic matter mineralization by bio-inoculants

Soil quality or productivity is closely linked to soil organic matter (SOM) status. Maintaining or increasing soil organic matter permits the recycling of plant nutrients and improves the soil physical structure thus minimizing the need for inorganic fertilisers (Brady, 1990; Gobat et al., 2004). The SOM can be increased by the addition of organic amendments such as green manure, farmyard manure, crop residues or compost (Caravaca et al., 2002; Mäder et al., 2002; Yadvinder-Singh et al., 2004; Celik et al., 2004). Quality composts for instance influence plant development by improving soil structure and supplying an elevated soil humus content as well as macro and micronutrients (Gobat et al., 2004). AMF associated with bacteria and other fungi could play an important role in nutrient mobilisation from crop residues. Hodge et al. (2001) demonstrated that the presence of *Glomus hoi* enhanced decomposition of plant litter in soil and resulted in increased N capture from the litter. Hyphal growth of the fungal symbiont increased also in the presence of organic material. The mechanism of a direct AMF decomposition of organic matter remains yet unclear although evidence of AMF phosphatase activity has been reported (Joner and Johansen, 2000). Nonetheless, an increase in bacterial activities due to organic compounds degradation could also explain this increase in fungal growth (Joner and Jakobsen, 1995; Ravnskov et al., 1999). The presence of AMF extradical hyphae might stimulate the saprophytic microflora by providing a surface for bacterial attachment as well as a carbon source. This AMF-induced increase in microbial activity could then in turn increase SOM mineralization. Morever, saprophytic microorganisms would be transported to new microhabitats containing not yet degraded organic matter. Mycorrhizosphere competent biopolymer-degrading strains such as *Flexibacter* (paragraph 7.1.2.4) could be inoculated in association with AMF in systems for which organic matter degradation processes are essential. Actinobacteria could also be used as bacterial co-inoculants to degrade organic matter as they are found in association with AMF and are considered as major saprophytic microorganisms (Ames et al., 1989; Carpenter-Boggs et al., 1995). Bacterial production of lytic enzymes might in turn increase

the turnover or growth of AM hyphae. Consequently, hyphae could reach faster other microhabitats of the soil where nutrients have not yet been uptaken. Moreover, in order to release N and P incorporated in the bacterial biomass, protozoans could be integrated in the bio-inoculation. To study the interactions between AMF, saprophytic bacteria and protozoans in terms of organic matter degradation, the Stable Isotope Probing Technique could be used (Radajewski et al., 2000). SIP should permit to assess which populations are stimulated by a ^{13}C-labelled organic substrate by separating the heavier DNA by ultracentrifugation. The labelled DNA can then be analysed for functional or taxonomic marker genes representative of the AMF, bacteria and protozoans .

In conclusion, taking into account what was previously described, the research on interactions between the microorganisms in agroecosystems is a fascinating domain and offers a wide range of perspectives for future studies.

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Annex 1

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Minireview

Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns

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Summary

Technical developments in molecular biology have found extensive applications in the field of microbial ecology. Among these techniques, fingerprinting methods such as denaturing gel electrophoresis (DGE, including the three options: DGGE, TGGE and TTGE) has been applied to environmental samples over this last decade. Microbial ecologists took advantage of this technique, originally developed for the detection of single mutations, for the analysis of whole bacterial communities. However, until recently, the results of these high quality fingerprinting patterns were restricted to a visual interpretation, neglecting the analytical potential of the method in terms of statistical significance and ecological interpretation. A brief recall is presented here about the principles and limitations of DGE fingerprinting analysis, with an emphasis on the need of standardization of the whole analytical process. The main content focuses on statistical strategies for analysing the gel patterns, from single band examination to the analysis of whole fingerprinting profiles. Applying statistical method make the DGE fingerprinting technique a promising tool. Numerous samples can be analysed simultaneously, permitting the monitoring of microbial communities or simply bacterial groups for which occurrence and relative frequency are affected by any environmental parameter. As previously applied in the fields of plant and animal ecology, the use of statistics provides a significant advantage for the non-ambiguous interpretation of the spatial and temporal functioning of microbial communities.

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Fingerprinting techniques applied to microbial communities

Molecular approaches in microbial ecology

A major challenge in the field of microbial ecology is to assess the diversity of the microbial cells present in a defined habitat. Assessing the diversity of microbial communities (in terms of richness and structure) is a way to address how they evolve in their environment. In a more general way, it is a possible means to address the question of the modulation of microbial communities by environmental factors. Phylogenetically meaningful macromolecules, particularly 16S rDNA directly amplified from environmental DNA, are now widely used for such purposes (Ranjard *et al.,* 2000a; O'Donnell *et al.,* 2001; Schäfer and Muyzer, 2001).

However, information collected by these molecular tools quickly revealed the unsuspected complexity of whole bacterial communities (Ward *et al.*, 1990). They were shown in turn to be limited in a practical way (O'Donnell *et al.,* 2001). The amount of time and resources needed for the now classical 'cloning-sequencing' technique (which potentially supply an exhaustive description of microbial communities), coupled with the impracticability of complete counts of organisms at present (Dunbar *et al.,* 2002), led to the development of alternative solutions. An original way was to separate polymerase chain reaction (PCR)-amplified fragment pools produced from whole microbial communities by electrophoresis techniques (Table 1). These fingerprinting methods are now widely adopted in the field of bacterial ecology and permit the simultaneous analysis of numerous samples (Ferrari and Hollibaugh, 1999).

DGE fingerprinting of microbial communities

Muyzer *et al.* (1993) first applied denaturing gel electrophoresis (DGE) techniques for the analysis of whole bacterial communities. Denaturing gel electrophoresis allows

Table 1. Fingerprinting methods used for the characterization of microbial communities, with recent publications in the related field.

Amplified ribosomal DNA restriction analysis (ARDRA)	Smit *et al.* (1997); Tiedje *et al.* (1999)
Denaturing gel electrophoresis (DGE)	Muyzer and Smalla (1998)
Ribosomal intergenic spacer analysis (RISA)	Fisher and Triplett (1999); Ranjard *et al.* (2000b)
Single strand conformation polymorphism (SSCP)	Schwieger and Tebbe (1998); Dabert *et al.* (2001)
Terminal restriction fragment length polymorphism (T-RFLP)	Moeseneder *et al.*, (1999); Dollhopf *et al.* (2001)

the separation of small polymerase chain reaction products, commonly up to 400 bp. The separation of the DNA fragments is achieved as a function of their different G + C content and distribution. Consequently, the fingerprinting pattern is built according to the melting behaviour of the sequences along a linear denaturing gradient (Myers *et al.*, 1985). Such a gradient is obtained using either denaturing chemicals for denaturing gradient gel electrophoresis (DGGE) or heat for temperature gradient gel electrophoresis (TGGE) and temporal temperature gradient electrophoresis (TTGE).

The DGE techniques were applied using 16S rDNA fragments to the analysis of bacterial communities in numerous habitats such as soil and rhizosphere (Bruns *et al.*, 1999; Yang and Crowley, 2000; Duineveld *et al.*, 2001; Ibekwe *et al.*, 2001; McCaig *et al.*, 2001; Smalla *et al.*, 2001) and aquatic environments (Murray *et al.*, 1996; 1998; Ferrari and Hollibaugh, 1999; Moeseneder *et al.*, 1999; van der Gucht *et al.*, 2001; Schäfer and Muyzer, 2001; Schäfer *et al.*, 2001). Interestingly, an increasing number of studies based on DGE are carried out on archaeal (Murray *et al.*, 1998; Rölling *et al.*, 2001) or eukaryal communities (van Hannen *et al.*, 1999; van Elsas *et al.*, 2000; Diez *et al.*, 2001; Mohlenhoff *et al.*, 2001).

The sensitivity of DGE analysis can be refined with the targeting of precise (and even non-dominant) taxonomic groups, by using specific PCR primers (Heuer *et al.*, 1997; Nübel *et al.*, 1997; Heilig *et al.*, 2002) or by identifying community members by hybridization of blotted DGE gels with group-specific oligonucleotide probes (Heuer *et al.*, 1999). Other developments were based on the use of 16S rRNA as a target (Felske and Akkermans, 1998; Kowalchuk *et al.*, 1999; Duineveld *et al.*, 2001; Schäfer *et al.*, 2001) to highlight metabolically active populations only. Functional genes (Watanabe *et al.*, 1998; Bruns *et al.*, 1999; Lovell *et al.*, 2000; Fjellbirkeland *et al.*, 2001) or even their transcripts (Wawer *et al.*, 1997) were also analysed, which heralds very interesting prospects in clarifying the functioning of microbial communities.

Guidelines for the interpretation of DGE fingerprinting patterns

Some features of the fingerprinting techniques have to be considered before applying statistics for the analysis of DGE profiles.

In DGE analysis, the generated banding pattern is considered as an 'image' of the whole bacterial community. An individual discrete band refers to an unique 'sequence type' or phylotype (Muyzer *et al.*, 1995; van Hannen *et al.*, 1999), which is treated in turn as a discrete bacterial population. The term population classically refers to a group of bacterial cells present in a specified habitat and belonging to the same species. We are expecting that PCR fragments generated from a single population to display identical electrophoretic mobility in the analysis. This was confirmed by Kowalchuk *et al.* (1997) who showed that co-migrating bands generally corresponded to identical sequences. However, it was shown that rDNA fragments of closely related bacteria are not necessarily resolved (Buchholz-Cleven *et al.*, 1997) or may produce separated bands (Jackson *et al.*, 2001). Moreover, non-related sequences might co-migrate at an identical position (Vallaeys *et al.*, 1997), especially when treating complex community patterns (Kowalchuk *et al.*, 1997; Ben Omar and Ampe, 2000). In this case, the question of the resolution of the gel needs to be addressed. Crowding of the gel has been discussed already and algorithms to assess it were proposed by Nübel *et al.* (1999a). Degenerated primers should be avoided also as one single bacterial strain, or even a single clone, may generate a multiple band pattern (Kowalchuk *et al.*, 1997; Piceno *et al.*, 1999). Some authors have also detected artificial bands when analysing complex DNA templates, probably induced by heteroduplex molecules (Ferris and Ward, 1997). Consequently, care should be taken in assigning a single band to a single bacterial population.

Another assumption for DGE fingerprinting interpretation is that the band intensity is directly related to the density of corresponding bacterial phylotypes within the sample. Results obtained by Murray *et al.* (1996) suggested a relationship between band intensity and relative abundance of the corresponding phylotype in the template DNA mixture. Such an assumption implies that no bias was obtained during the whole extraction–amplification procedure of the bacterial genomes (Muyzer *et al.*, 1993; Wang and Wang, 1997; Garcia-Pichel *et al.*, 2001). The DGE analysis should probably be restricted to samples treated using identical methods, in which DNA extraction and amplification biases are supposed to occur homogeneously. Moreover, it is commonly accepted that the main populations only (those representing more than 0.1–1% of the target organisms in terms of relative proportion) are

displayed in the profiles (Muyzer *et al.*, 1993; Murray *et al.*, 1996). As a result, all populations present within a habitat do not appear on DGE banding patterns. When assessing the above considerations, the image of the communities which is provided by DGE fingerprinting patterns probably relates more to its structure, i.e. to the relative abundance of the main bacterial populations, than to its total richness (Muyzer and Smalla, 1998). These features and restrictions are nevertheless common to all PCR-based approaches (Lee *et al.*, 1996; Fisher and Triplett, 1999; Schäfer and Muyzer, 2001).

The last consideration about this analytical technique is about the reproducibility of the DGE analysis. Reproducibility of sample analysis depends on the upstream analytical steps (from the sampling to the DNA extraction and amplification procedures) as well as the care brought to the DGE gels themselves. A thorough standardization at each level of the experiments results in very high reproducibility. The use of reference patterns, the loading of precise amounts of PCR-amplified fragments and the precision of gel staining are required. As a consequence, identical samples loaded on a single gel display identical patterns (Simpson *et al.*, 1999; Schäfer *et al.*, 2001; Yang *et al.*, 2001) and patterns from different gels can be compared with a high degree of confidence. The analysis of large numbers of samples can be exploited for the characterization of the intrinsic variability of the bacterial community structures. This large amount of data can be analysed in turn with statistical tools, which provide a significant advantage for the non-ambiguous interpretation of the observed variability (Morris *et al.*, 2002).

Analysis and comparison of DGE community profiles

Denaturing gel electrophoresis techniques have been extensively used to monitor bacterial communities in space and time (Ferris and Ward, 1997; Murray *et al.*, 1998; Nübel *et al.*, 1999b; van der Gucht *et al.*, 2001) or to evaluate the impact of environmental disturbances (Ibekwe *et al.*, 2001; Müller *et al.*, 2001). The variations between DGE profiles were classically described visually on a single DGE gel by the disappearance, the appearance or the changes in the intensity of selected bands. However, an increasing number of studies propose statistical investigations of DGE banding patterns, which undoubtedly lead to refined results. These advanced analyses are based on a computer-assisted characterization of the banding patterns and the subsequent treatment of the data using a statistical approach.

An example of computer-assisted guideline for the analysis of fingerprinting profiles was proposed by Rademaker *et al.* (1999) using the GelCompar software package (Applied Maths, Kortrijk, Belgium). Briefly, banding patterns were first standardized with a reference pattern included in all gels. Each band was described by its position (Y, in pixel on the image file) and its relative intensity in the profile (P_i), which could be calculated by the relative surface of the peak in the profile ($P_i = n_i/N$, where n_i is the surface of the peak i, and N is the sum of the surfaces for all the peaks within the profile). Using these data various statistical methods can be applied, based either on single band or on whole DGE profile analysis.

Analysis of DGE profiles based on single bands

One way to analyse DGE fingerprinting patterns is to observe the possible changes in the presence/absence or in the variation of intensity of a single band (Murray *et al.*, 1996). Putative indicator bands highlighted in this way can be excised from the gels and their sequences analysed using a cloning–sequencing procedure (Kowalchuk *et al.*, 1997; Watanabe *et al.*, 1998; Ibekwe *et al.*, 2001).

The variation in band presence or intensity can be exploited in two different ways. First of all, the relevance of indicator bands can be evaluated by testing their occurrence in relation with various biological and physicochemical parameters (Widmer *et al.*, 2001) as well as with the presence or absence of other bands in the patterns. In the example shown in Table 2 16S rDNA TTGE banding patterns of 30 raw milk samples were analysed in this way. The occurrence of each TTGE band was tested against qualitative descriptors using a Fisher's exact test and bands found at the positions $Y = 230$ and $Y = 300$ were positively correlated to the cleaning frequency of the milking device and to the hygienic status of the cow tits respectively.

Second, single band analysis can also be used for computing a regression between band intensity (dependent quantitative variable) and an environmental descriptor (independent quantitative variable). In the example

Table 2. Significant correlation ($P < 0.05$, Fisher's exact test) between the presence of a selected band within a gel pattern and a qualitative descriptor. The bands were identified using a cloning-sequencing procedure (P. Rossi, unpublished data).

	Descriptors		
Position of the band (in pixels on Y axis)	Frequency of cleaning of the milking device	Hygienic status of the cow tits before milking	Identification of 16S rDNA fragment (% identity)
Y = 230	$P = 0.0001$	No correlation	*Bacillus* sp. (>95%)
Y = 300	No correlation	$P = 0.004$	*Pseudomonas* sp. (> 95%)

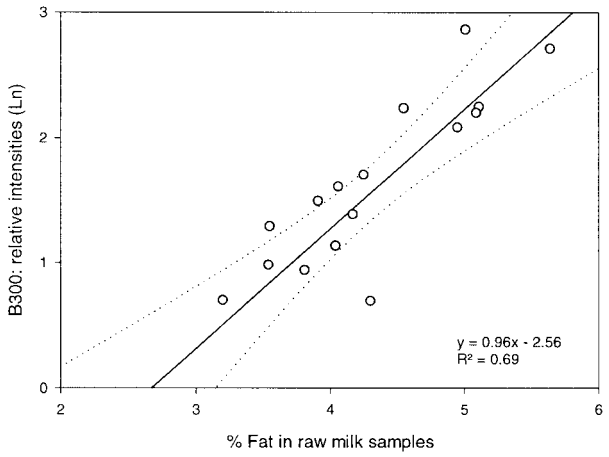

Fig. 1. Regression analysis between the relative intensity (ln) of the band at the $Y = 300$ position and the percentage of fat found in the corresponding raw milk samples (P. Rossi, unpublished data).

given above, the TTGE patterns were analysed by plotting the relative intensities (P_i) of each band versus various physical parameters measured from the same samples. A positive correlation ($R^2 = 0.69$) was found between the relative intensity of the band $Y = 300$ (identified as *Pseudomonas* sp.; Table 2) and the per cent of fat measured in the raw milk (Fig. 1).

Whole profile analysis

The second approach for a comparative analysis of DGE patterns is based on the whole set of bands present within the profiles. The total number of bands (called sometimes 'band richness') in each sample pattern is related to the number of dominant phylotypes, and can be used for comparison purposes (Müller *et al.*, 2001; van der Gucht *et al.*, 2001). Comparison of profiles can be refined by taking into account the relative intensity of each band (P_i). Thus, diversity indices, such as Shannon-Weaver and Evenness indices (Nübel *et al.*, 1999a; Simpson *et al.*, 1999; Kocherginskaya *et al.*, 2001; McCaig *et al.*, 2001; Ogino *et al.*, 2001), can be calculated to describe possible changes in the dominance among phylotypes. An interesting feature is to combine these indices with other sets of environmental data. For instance, Nübel *et al.* (1999a) found a positive linear correlations between Shannon-Weaver indices calculated from both DGE patterns and carotenoid types in oxygenic-phototrophic microbial communities.

Computation of similarity matrix

When considering the presence/absence of the bands, similarities between banding patterns, taken in pairs, can be expressed as a percentage value of a similarity coefficient such as Jaccard (Diez *et al.*, 2001) or Dice (van der Gucht *et al.*, 2001) coefficient, or a distance coefficient such as Euclidean measure (McSpadden Gardener and Lilley, 1997). Other coefficients, such as the Steinhaus coefficient (Fig. 2) or the product moment, also named Pearson correlation coefficient (Rölling *et al.*, 2001; Smalla *et al.*, 2001), allow to take into consideration the relative intensity (P_i) of each band (Legendre and Legendre, 1998; Rademaker *et al.*, 1999). As noticed by Murray *et al.* (1998), the use of these similarity coefficients for the calculation of pair-wise levels of similarity between patterns does not require a one-to-one correspondence between the number of bands and the number of

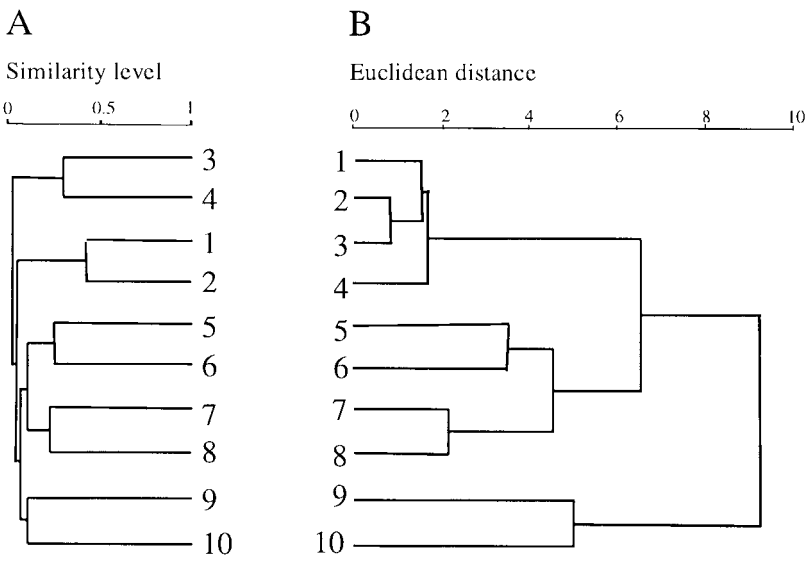

Fig. 2. UPGMA clustering of 10 samples taken along a vertical gradient form the small eutrophic Lake Loclat (Neuchâtel, Switzerland). Samples are ranked by depth: 1, corresponds to the surface; 10, to the bottom of the lake (8.7 m).
A. Clustering according to DGGE data (using Steinhaus coefficient).
B. Clustering according to 23 physical and chemical variables using Euclidean distance (Forestier *et al.*, 2002). Linkage levels were computed using the R package (Casgrain and Legendre, 2001).

sequence types. Similarity or distance matrices can be displayed graphically as a dendrogram, but also give way to clustering and ordination methods.

Clustering techniques

Clustering techniques, such as the unweighted pair group method using arithmetic averages (UPGMA), are applied to the DGE profiling with the aim of identifying the samples which generate similar patterns (Ibekwe *et al.,* 2001; Yang *et al.,* 2001; Boon *et al.,* 2002). One advantage of this presentation is that the coherence of the fingerprinting patterns can be assessed rapidly.

In the example given above (Forestier *et al.,* 2002), 10 samples were taken from a holomictic eutrophic lake along a vertical gradient and were analysed for major ions, organic content, physical parameters and DGGE analysis of 16SrDNA fragment genes. Computation of the DGGE and environmental parameters matrices was carried out using the Euclidean distance and Steinhaus coefficient, respectively, and UPGMA was selected as a clustering method for the presentation of the results. The resulting dendrograms (Fig. 2) showed that the samples were clustered according to the depth of their sampling, in agreement with measured physical and chemical parameters.

Ordination methods

Another way of analysing DGE profiles is to bring out major tendencies of the variance of the samples for the whole set of descriptors using multivariate ordination methods. Legendre and Legendre (1998) provide a excellent review of these methods which are commonly used in the field of ecology. These methods are used for the integration of complex sets of data (i.e. bands in the DGE patterns) into new mathematical variables which can be projected into a few-dimension perspective (reduced space). The major advantage of these methods is to display the whole set of samples on a simple scheme, and to highlight the possible descriptors which are governing their dispersion (ter Braak *et al.*, 1995). Of course, true correlation can only be deduced when sufficient amounts of data are provided: the results of proposed statistical analysis should be considered with care, as coincidence or convergence mechanisms cannot be excluded.

Common ordination methods include non-metric multidimensional scaling (NMDS), principal component analysis (PCA), correspondence analysis (CA), canonical variate analysis (CVA) and canonical correspondence analysis (CCA). Several complementary statistical procedures can be applied to analyse DGE data (Yang *et al.,* 2001). Details on the specific underlying theory of each of these methods can be found elsewhere (McSpadden Gardener and Lilley, 1997; Legendre and Legendre, 1998).

Non-metric multidimensional scaling is an ordination method which reduces complex DGE patterns to a point in a two-dimensional space. By connecting the consecutive points, the relative changes in the bacterial community can be visualized. van Hannen *et al.* (1999) proposed to calculate Nei-Li distances from the binary data resulting from DGE profile analysis and to represent these distances using this ordination method. The authors showed that bacterial communities that developed on two distinct detritus substrates differed significantly: the distances calculated between communities from different substrates were greater ($P < 0.05$) than the distances calculated between the replicates for a given substrate. Non-metric multidimensional scaling was used elsewhere for the interpretation of DGE data (Diez *et al.,* 2001; Schäfer *et al.,* 2001). The advantage of NMDS is to represents the objects in two or three dimensions, with dissimilar objects far apart and similar objects close to one another in the ordination space.

Principal component analysis generates new variables, called principal components (linear components of the original variables), which explain the highest dispersion of the samples. This method was often used for the interpretation of DGE community fingerprinting analysis (van der Gucht *et al.,* 2001; Müller *et al.,* 2001; Ogino *et al.,* 2001; Yang *et al.,* 2001). As an example, Müller *et al.* (2001) used PCA to compare 16S rDNA DGGE profiles for bacterial communities present in mercury-contaminated soils. Their investigations showed that the DGGE approach generated more distinctive results than colony morphotyping and substrate utilization. van der Gucht *et al.* (2001) showed that the composition of bacterioplanktonic communities differed between two lakes and during seasons using a PCA applied to presence/absence of bands within 16S rDNA DGGE patterns. Using Spearman's rank correlation, the observed seasonal variations were found to be positively correlated with environmental variables such as temperature, nitrate concentration or microbial biomass. However, PCA is probably not the most suitable statistical approach for analysing DGE patterns, as its underlying model assumes that biological populations have a linear response curve along the axes of ecological variation. Niche theory tells us that populations have ecological preferences. An unimodal (i.e. bell-shaped) response distribution of the different bacterial populations present in a community is probably closer to reality, with more individuals near some optimal environmental values.

Correspondence analysis may be applied to any data table that is dimensionally homogenous. ter Braak (1985) showed that the underlying model was adapted to presence/absence or abundance data tables and consequently, that the analysis was well suited for populations

with unimodal distribution along environmental gradients. Using this statistical analysis, Jourdain-Miserez *et al.* (2001) analysed 16S rDNA gene fragments issued from milk samples on TTGE gels. The results clearly showed different community structures between organic and conventional farming practices (Fig. 3). Correspondence analysis was also used elsewhere for similar approaches (Ibekwe *et al.*, 2001; Yang *et al.*, 2001).

Interpretation of DGE patterns with environmental variables

From our point of view, the greatest opportunity of the statistical analysis of DGE patterns is offered when the community profiles are combined in a joint analysis with environmental data sets. The relevant question here is to know whether the variations observed between different banding patterns could be associated with the variations of measured environmental variables.

McCaig *et al.* (2001) applied multivariate analysis to reduce the original data for grassland DGGE community patterns into six principal components. They showed clear differences between improved and non-improved grassland communities using CVA. This method requires an *a priori* definition of groups and finds linear combinations of variables that maximize the ratio between-group variation to within-group variation.

The 'community matrix' obtained from DGE profiles can be tested also against a second matrix obtained from environmental data sets measured on the same samples. Canonical correspondence analysis is a powerful canonical ordination technique (multivariate direct gradient analysis) allowing the explanation of the structure of a 'species' data table by quantitative environmental descriptors and assuming unimodal distributions of species (ter Braak, 1986). Using this technique, Yang and Crowley (2000) compared bacterial rhizospheric communities associated to barley plants under iron-limiting and iron-sufficient growth conditions. As a result, they showed that about 40% of the variation between microbial communities could be attributed to plant iron nutritional status. Figure 4 presents a CCA of a DGGE analysis carried out on samples taken from the water column of Lake Loclat (Forestier *et al.*, 2002). In this case, five environmental variables were selected according to their high probability of correlation with the samples ($P < 0.05$) using Mantel tests (Mantel, 1967). This test is based on the linear correlation between two distance or similarity matrices obtained from independent data. As shown in the Fig. 4, the redox and the dissolved organic carbon were the variables which influenced mostly the dispersion of the samples. The first five samples taken from the aerobic zone (samples 1–5) are closely related, defining a homogenous bacterial structure. The samples taken from the anaerobic section of the water column (points 6–10) were displayed according to depth indicating a continuum of different bacterial communities.

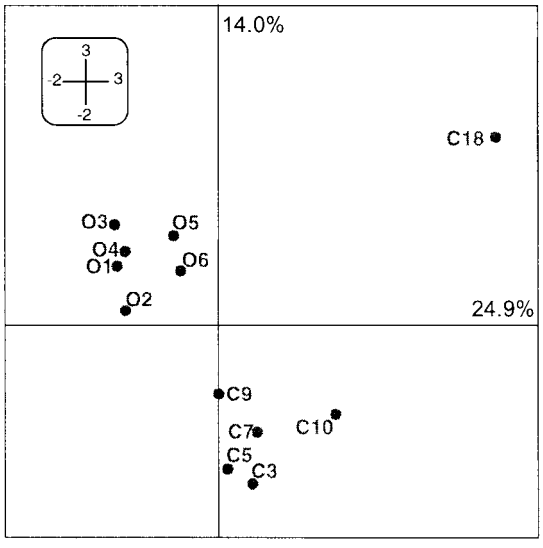

Fig. 3. Correspondence analysis of TTGE community profiles from milk samples from 12 farms (Switzerland). TTGE data of five samples taken on five consecutive days were pooled before being analysed (sum of unconstrained eigen values: 1.74). C, conventional; O, organic farming practices. Numbers refer to the different farms (Jourdain-Miserez *et al.*, 2001).

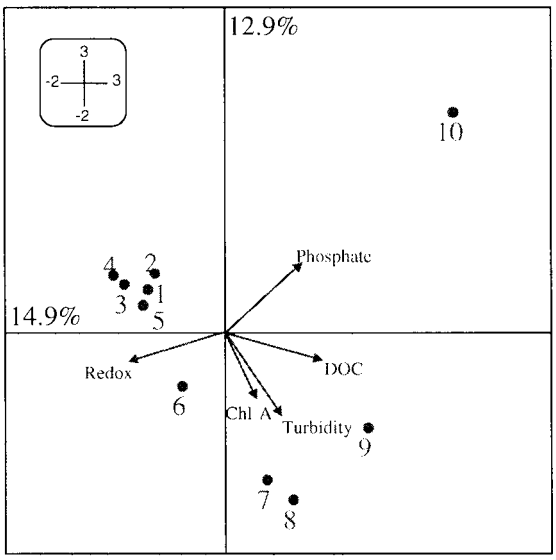

Fig. 4. Canonical correspondence analysis of microbial community patterns generated by 16S rDNA DGGE analysis for 10 water samples (Lake Loclat) ranked by depth (1 for surface to 10 at 8.7 m depth). The total inertia of the matrix was 4.45 and the selected variables explained 59% of the variance of the DGGE data set (sum of canonical eigenvalues: 2.62) (Forestier *et al.*, 2002).

Conclusions

Denaturing gel electrophoresis fingerprinting techniques are very effective methods for the characterization of bacterial community structures. These techniques are convenient for the simultaneous analysis of numerous samples. They are consequently well suited for the monitoring of whole communities, focusing on phylotypes for which the occurrence and/or the relative frequency are affected by any environmental change. As shown above, emphasis should be brought to the standardization of the whole analytical procedure as a means for increasing the reliability of the method and the reproducibility of the patterns. For instance computer-assisted analysis of the profiles should be generalized, escaping the merely qualitative reading of the fingerprinting patterns.

The exploratory aspect of the statistical techniques applied to DGE patterns that we present here is the consequence of statistical developments brought to the field of plant and animal ecology. It is now possible to approach causality in microbial ecology with statistical methods using experimental designs which were impossible to conceive a few years ago, principally because of the time and resources needed for the analysis of high number of samples. Examples provided above showed that it is possible to apply statistical tools to DGE data sets efficiently. The first result is in the validation of the interpretation of the patterns, such as shifts in the microbial community structure or the identification of key-populations which may be affected by changing conditions. Moreover, whole pattern data generated by the DGE analysis can be directly tested for correlation analysis against any single or combination of environmental sets of variables.

However, care should be taken in the choice of the statistical analytical procedure. As shown above, the underlying theoretical model should be carefully assessed before any attempt of application. Some analysis used up to now were probably not well suited to this type of data sets. On the contrary, CA is particularly well suited for abundance data sets, and PCA (normalized using correlation) is perfectly adapted for the analysis of environmental data sets (standardized descriptors).

The complementation of DGE analysis with a statistical approach leads to the definition of new hypotheses and to new prospects in terms of spatial or temporal functioning of microbial systems. Statistical methods reveal putative correlations between different sets of variables. They do not permit, however, conclusions to be drawn regarding the causality of these correlations. Therefore, statistical analyses should not be considered alone, but in a dialectic relationship with an ecological hypothesis. Automated pattern recognition and mechanistic dynamic modeling (combined with field and laboratory experiments) will probably very soon be the future steps in this field. In this sense, it will be conceivable to describe more precisely the relations between the observed diversity of the organisms and their ecological niches, leading to the development of the promising concept of 'bacterial sociology'.

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Annex 2

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Diversity of *phl*D alleles in the rhizosphere of wheat cropped under annual rice-wheat rotation in fields of the Indo-Gangetic plains: influence of cultural conditions

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Keywords
2,4-DAPG; *phl*D; wheat rhizosphere; rice-wheat

Abstract

The antibiotic 2,4-diacetyphloroglucinol (2,4-DAPG) is a major determinant in the biocontrol of the plant growth promoting rhizobacteria associated with crops of agronomic relevance. The *phl*D gene is a useful marker of genetic and phenotypic diversity of 2,4-DAPG producing rhizobacteria. A two-steps amplification procedure was developed in order to assess directly the presence of *phl*D in environmental DNA avoiding the tedious procedure of *phl*D positive strain screening and isolation. We hypothesise that the continuous rice-wheat rotation for twenty-five years and the use of the same wheat cultivar in the tested fields would lead to an enrichment of particular *phl*D genotypes, following a process similar to the one occurring in monoculture suppressive soils.

Introduction

The high input agricultural management initiated by the Green Revolution in India tends to be replaced by ecologically sustainable systems that include the use of reduced tillage, input of organic materials, and nutrient cycling strategies based on crop rotations. The limited incidence of soil-borne pathogens in the rice-wheat (R-W) systems, is probably due to the repeated transitions from anaerobic (rice season) to aerobic conditions (wheat season). However, survival of pathogens during the rice phase [1] as well as their proliferation on crop residues [2] may reduce the yield up to 20% if their propagation occurs before leaf emergence [3,4].

In the R-W cropping fields of the Indo-Gangetic plains region, adoption of the raised beds system allows the upper part of the bed to remain in oxic conditions even during the rice season. The use of this technique optimises water use efficiency, improves weed management and opens up investment opportunities by diminishing the production costs [1,5]. Nonetheless, raised beds system, by combining reduced-tillage and a permanent oxic zone, could favour re-emergence of pests and diseases kept at low levels during the rice season.

The biological approaches that are currently being developed to control a variety of phytopathogenic agents include the use of beneficial free-living bacteria, usually referred to as biocontrol agents [6]. Bacterial biocontrol agents are involved in disease management by different mechanisms that include niche exclusion through microbial competition, stimulation of plant defence or production of antibiotics [7,8,9].

Phloroglucinol antibiotics are bacterial or plant phenolic metabolites with antifungal, antibacterial, anthelminthic, and phytotoxic properties [10]. The broad spectrum antibiotic

2,4-diacetyphloroglucinol (2,4-DAPG) is a major determinant in the biocontrol activity of plant growth promoting rhizobacteria (PGPR) [11]. Moreover, the 2,4-DAPG producing fluorescent pseudomonads of worldwide origin and from various crops have been shown to share the same biosynthetic locus [12, 13]. The *phl*D gene is located in the *phl*ACBD operon coding for the production of 2,4-DAPG [14]. In addition, the *phl*D gene was shown to be a useful marker of genetic and phenotypic diversity of 2,4-DAPG producing rhizobacteria [15,16,17]. This diversity has been studied in relation to biological control, root colonisation, and soil suppressiveness [18]. Studies carried out on pseudomonad populations of world-wide origin suggest that strains with different *phl*D alleles may coexist in the rhizosphere of the same dicot at a particular geographic location [17,19]. Moreover, Mavrodi *et al*. [15] reported that in soils subjected to wheat monoculture up to four different *phl*D alleles could be found and that a single *phl*D-based genotypic group dominated in the rhizosphere at each location studied. Recently, Ramette *et al*. [19] suggested that the diversity of *phl*D alleles recovered from fluorescent pseudomonad strains is not predictive enough of the disease-suppressive or conducive status of soils. However, to date, diversity studies of 2,4-DAPG producers are mostly based on screening approaches targeting *Pseudomonas*-genus isolates. Several diversity studies of functional bacterial groups showed that only a small fraction of the diversity is revealed by cultivable strains. For example, Hamelin et al [20] demonstrated that the most dominant *nif*H cluster included only environmental clones and no sequences related to cultivable diazotrophs, in a study on *nif*H gene diversity in the rhizosphere of *Molinia coerulea*. We can therefore expect that some key 2,4-DAPG-producing organisms may be ignored by culture-based experiments. Little attention has been given on *phl*D gene diversity in relation to rice-wheat systems and to sustainable farming options using the beneficial activities of soil microorganisms for the enhancement of soil quality.

In order to avoid a cultivation bias, the purpose of this study was to assess the allelic diversity of the *phl*D gene from DNA extracted directly from the root environment in fields under annual rice-wheat rotation. The diversity of the *phl*D sequence pools was compared in root and root-adhering soil with respect to, (i) raised beds versus traditional plain fields, (ii) plain fields with the same practice but with different grain yields.

Material and methods

Experimental sites

Trials were carried out in two experimental sites located in Uttar Pradesh (UP) state, India. The first one is located in Bhavnipur village (Badaun District latitude 28,02 N, longitude 79,10 E) and the second, near Ghaziabad (Ghaziabad District latitude 28,40 N, longitude 77,28 E). Bhavnipur site is characterized by twenty years of rice-wheat rotation and by a basic irrigation system devoid of canals. For wheat cropping, standard agronomic practices were followed, such as regular sowing, irrigation and weeding. Two fields of 4000 m^2 were selected on this site, differing mainly by their wheat grain yields (table 1): low input low yield (noted LL) and low input high yield (LH), separated by less than 2 km. The sandy loam soil texture displayed homogenous characteristics. The same wheat cultivar UP 2338 (provided by the GB Pant University of Agriculture and Technology, Pantnagar, Uttaranchal, India) was grown for more than five years on both fields in rotation with rice. The traditional agricultural practice (plain field) was applied in this site. Wheat was sown in the second half of November. The same dose of urea was applied to both fields at their preparation stage. Neither chemical fertiliser nor pesticide was added.

Table 1. General characteristics of the studied fields. Fields LL (low input, low yield) and LH (low input, high yield) are located in Bhavnipur. The two agricultural practices of Ghaziabad (GH) fields are designated RB (raised beds) and PF (plain fields). The input of the different fertilisers used in the fields are reviewed (urea, DAP: diammonium phosphate, MOP: mureate potash and Zn: zinc). NM = not measured

			Field			
Season		Fertiliser	LL	LH	GHRB	GHPF
2001-2002	Fertiliser input (kg/ha)	Urea	125	125	120-150	120-150
		DAP	-	-	40-60	40-60
		MOP	-	-	40-60	40-60
		Zn	-	-	25	25
	Grain Yield (Quintals/ha)		23.5	34	NM	NM

In Ghaziabad, two fields characterized by twenty years of rice-wheat rotation were selected. They differed only in the agricultural practices, one being a conventionally tilled plain field (pf) and the other a three-year-old practiced raised bed field (rb). Raised beds were built using a tractor-drawn machine and seed sowing was carried out by a tractor using seed drill. Standard agronomic practices (regular hoeing, irrigation and weeding) were followed. The crop was irrigated six times, covering all the critical stages of growth. Wheat cultivar UP 2338 was also grown on this site. Phosphorus was applied in the form of diammonium phosphate (DAP) and potash in the form of potash mureate (MOP), mixed thoroughly with the soil

before sowing. The first nitrogen dose was provided at the time of planting in the form of DAP and the second one after one month in the form of urea. Zinc was applied just after rice transplantation. For more details, see table 1.

Sampling procedure

At Bhavanipur sites, sampling was performed at the tillering stage (45 d) of wheat growth, and in Ghaziabad sites at the late tillering stage (60 d). Three to four wheat plants with roots and soil cores were sampled under semi-sterile conditions using a soil corer at a depth of 0 to 30 cm, at 8 random spots in each field. The coarse stones and stubs were removed. The bulk soil was separated from the adhering soil by shaking the plants. For each field, the root systems with adhering soil from the 8 spots were separated from the aerial part of the plant using sterile scissors and mixed. The resulting composite samples were stored at –80°C for subsequent molecular analysis.

DNA extraction from RS and RE samples

A fraction of the root systems with adhering soil was put into sterile 0.1 M sodium phosphate buffer (pH, 7.0) and stirred to separate the rhizosphere soil (RS) from the rhizoplane-endorhizosphere (RE) fraction. The washed roots (RE) were removed. The remaining suspension considered as the RS, was weighed. Roots were rinsed with sterile deionised water and dried on sterile Whatman paper (Merck AG). One g fresh weight RE was crushed under sterile conditions in 10 ml phosphate buffer in a mortar with a pestle, constituting the RE suspension. Root dry weight was determined. One ml of RE suspension was ten-fold diluted in sterile phosphate buffer (0.1 M sodium phosphate buffer, pH 7.0). DNA was extracted from RS and RE fractions by a bead-beater technique (Fast Prep FP120, SAVANT, BIO101, Carlsbad, USA) using a FastDNA Spin Kit (BIO101) according to the manufacturer's protocol. The extracted DNA was further purified with a GENECLEAN® II kit (BIO101) and stored at –20°C in Tris-EDTA (pH 8,0) buffer.

Design of new phlD primers

Eleven *phl*D DNA sequences from Genbank ([21] were aligned using the GeneBase software (Applied Maths, Kortrijk, Belgium) in order to find and select suitable loci. These sequences were those of strains CHA0 (accession numbers: AJ278806 and AF214456), CM1'A2 (AJ278808), F113 (AJ278801), M1-96 (AF207692), Pf5 (AF214457), PILH1 (AJ278810), PITR2 (AJ278809), Q2-87 (U41818), Q65c-80 (AJ278807) and Q8r1-96 (AF207693). Two low-polymorphic loci were defined (positions 310-326 and 520-536, table 2). A first forward primer, phl310chf, was designed on the basis of a *phl*D target sequence entirely conserved

among CHA0 and Pf5 available sequences. A second forward primer, phl310f, was designed on the basis of the same locus from the remaining strain sequences and displays one degenerated position in order to include Q2-87 related sequences. The two bases-degenerate reverse primer phl536r was designed to target the 520-536 locus within the *phl*D gene sequence (table 2). The predicted size of the amplicon was 227 bp.

Table 2. List of primers used in the present study. The relative nucleotide positions were set according to the position of primer phl2b developed by Raaijmakers et al. [13] on the *phlD* gene of the strain Q2-87 (Genebank U41818). The annealing temperatures were defined experimentally.

Designation	5'-3' Sequences	Size	Positions	T_m (°C)	Reference
phl2a	GAGGACGTCGAAGACCACCA	20bp	728-747	59.5	[13]
phl2b	ACCGCAGCATCGTGTATGAG	21bp	1-20	57.5	[13]
phl310f	CTCTGCTATCAACCMCA	17bp	310-326	51.0	This study
phl310chf	CTGTGCTACCAGCCGGA	17bp	310-326	53.0	This study
phl536r	TTRATGGAGTTCATSAC	17bp	520-536	47.0	This study

PCR amplification of phlD gene fragments

Primer testing and optimisation of PCR conditions were performed on *phl*D positive reference strains CHA0 [22], PGNL1 [12], PGNR1 [12], PITR2 [12], PILH1 [12], TM1'A4 [12] and TM1A3 [23], and on *Escherichia coli* (Neuchâtel collection of microorganisms Neu 1006), *Pseudomonas aeruginosa* (ATCC 10145), *Alcaligenes faecalis* (Neu 1033), *Aquaspirillum autotrophicum* (ATCC 29984) and *Micrococcus luteus* (Neu 1013) as *phl*D negative reference strains. Bacterial cells were grown overnight on nutrient agar (Biolife) at 28°C and DNA was extracted using the *Wizard® Genomic DNA Purification kit* (Promega, Madison, WI, USA).

*phl*D fragments from the RS and RE crude DNA were obtained using a two-step amplification procedure. The first amplification was carried out using phl2a and phl2b primers (table 2). The amplification reaction mix was composed of 1X Taq DNA polymerase reaction buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Gibco, Cheshire, England, UK), 0.25 μM of each primer (Microsynth, Balgach, Switzerland) and 0.05 U/μl Taq DNA polymerase in buffer B (Promega). A final concentration of 0.1-1 ng/μl of crude extracted DNA was used as a template for a 20 μl reaction. Amplification was carried out in a PTC-200 Thermocycler (MJ Research Inc., Reno, USA), with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles at 94°C for 40 s, 59°C for 30 s, and 74°C for 45 s, and completed by a final elongation step at 74°C for 10 minutes.

The products of the first amplification were diluted tenfold in sterile deionised water before being used for the nested amplification reaction. Primers phl310f and phl310chf were mixed (50:50) prior to use in the PCR mix, which was composed of 1X Taq DNA polymerase reaction buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Gibco), 0.25 μM of the mix containing phl310f and phl310chf (Microsynth), 0.25 μM of primer phl536r

(Microsynth) and 0.05 U/μl Taq DNA polymerase in buffer B (Promega). The nested amplification program consisted of an initial denaturation at 95°C for 4½ min, followed by 34 cycles at 94°C for 30 s, 52°C for 1 min and at 74°C for 30 s, and completed by a final elongation step at 74°C for 10 min. The size of the amplicons obtained was checked after electrophoresis on a 1.3% Standard Agarose gel (Eurobio, Les Ullis, France) using a "Low DNA Mass Ladder" (Gibco BRL) as a size ladder.

Cloning and sequencing of the phlD fragments

PCR products were purified employing a *NUCLEOTRAP-CR kit* (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The ligation was carried out in pGEM®-T Vector System (Promega), following manufacturer's protocol. Transformation was performed by electroporation using the *Bio-rad Gene Pulser XCell and PC module* into *E. coli* XLI-Blue. The transformed colonies were plated onto Luria–Bertani (LB) agar containing ampicillin (150 μg/ml), X-Gal (0.1 mM) and IPTG (0.2 mM) [24]. Plasmids were recovered from white colonies using a NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer's protocol. The presence of the insert was checked by PCR using the set of primers T7 and SP6 [24]. The inserts were sequenced by Synergen (Schlieren, Switzerland).

Similarity analysis of phl*D sequences from clones and isolates*

*phl*D sequences were aligned using the ClustalX software [25] and trees were constructed using the neighbour-joining method [26] on 227 bp sequences with the NJplot software (ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/njplot) [27]. The topology of the distance tree was tested by resampling data with 100 bootstraps [28] to provide confidence estimates for tree topologies.

Results

PCR validation

The two-step PCR assay was validated on 2,4-DAPG producing strains and was applied to RS and RE DNA extracts from both field sites. Trials attempting an amplification of the gene from environmental DNA in a single step, using the primers phl2a and phl2b did not provide positive signals (data not shown). Direct amplification of *phl*D sequences from environmental DNA using phl310chf/phl310f and phl536r did not generate sufficient amplification product. Consequently, a two-step amplification procedure was applied for assessing the diversity of *phl*D gene pools. A DNA fragment about 227 bp in size was generated after nested PCR for the positive reference strains as well as for all the environmental samples, as predicted from known *phl*D sequences [14]. No PCR product was obtained from *phl*D$^-$ reference strains.

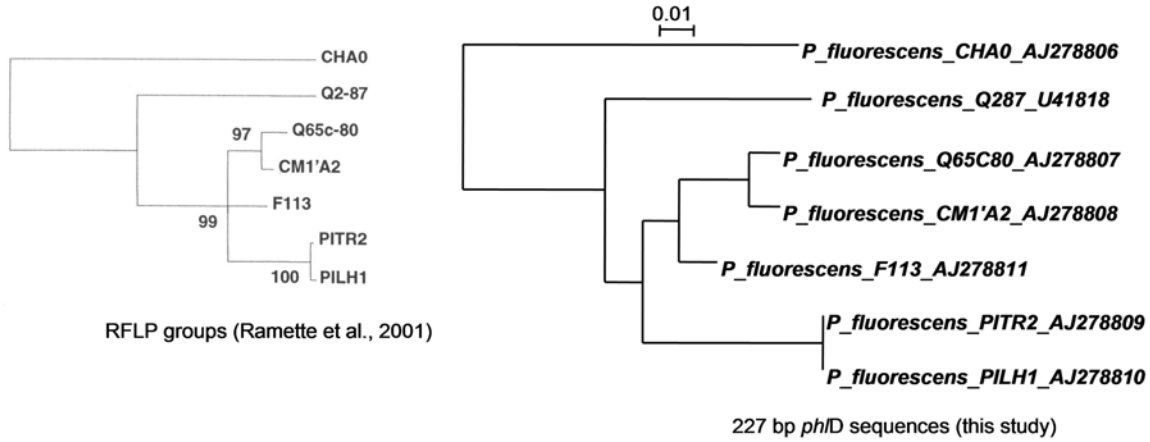

Fig.1. Phylogenetic relationship on the basis of 227bp *phl*D nucleotides between representative Phl+ biocontrol isolates of the seven *phl*D PCR–RFLP groups defined by Ramette *et al*. [16]. The tree was constructed by Neighbour-Joining with Njplot. Scale bar indicates the number of substitution per site.

Diversity of phl*D sequences from Ghaziabad and Bhavanipur fields*

Amplicons obtained from environmental DNA were cloned and sequenced. All the products were related to *phl*D sequences (sequence identity > 93%). The phylogenetic relationship of the cloned sequences was constructed according to previously defined clusters by Ramette *et* al. [16] based on sequence polymorphism analysis (fig.1). Figures 2 and 3 represent the phylogenetic position of the partial *phlD* sequences compared to *phl*D sequences for known DAPG producers, for Ghaziabad and Bhavanipur sites, respectively. Sequences with a high level of similarity were grouped into clusters (A, B, C).

For Ghaziabad environmental sequences, two main clusters were identified on the basis of the reference sequences and were defined as cluster A and cluster B (fig.2). Cluster A

included the *phlD* sequence of the *Pseudomonas* sp. biocontrol strain *M196* (AF207692). This cluster also included three of the seven clones of the root fraction of wheat plant cropped under the raised beds agricultural practice. Cluster B included sequences retrieved from RS and RE from both plain field and raised bed practices, and were not related to any reference sequences.

The environmental *phlD* sequences from Bhavanipur field samples grouped into three different clusters (A, B and C), according to the field or the origin of the root fraction (fig.3) *i.e.*, Cluster A and B (as for the sequences from Ghaziabad fields) and cluster C, which included CHA0 *(*AJ278806) reference strain. Cluster A contained exclusively sequences of the LH field (RS and RE fractions) whereas Cluster B contained sequences from the RE fraction for both LL and LH fields; cluster C contained sequences from the RS fraction of LL.

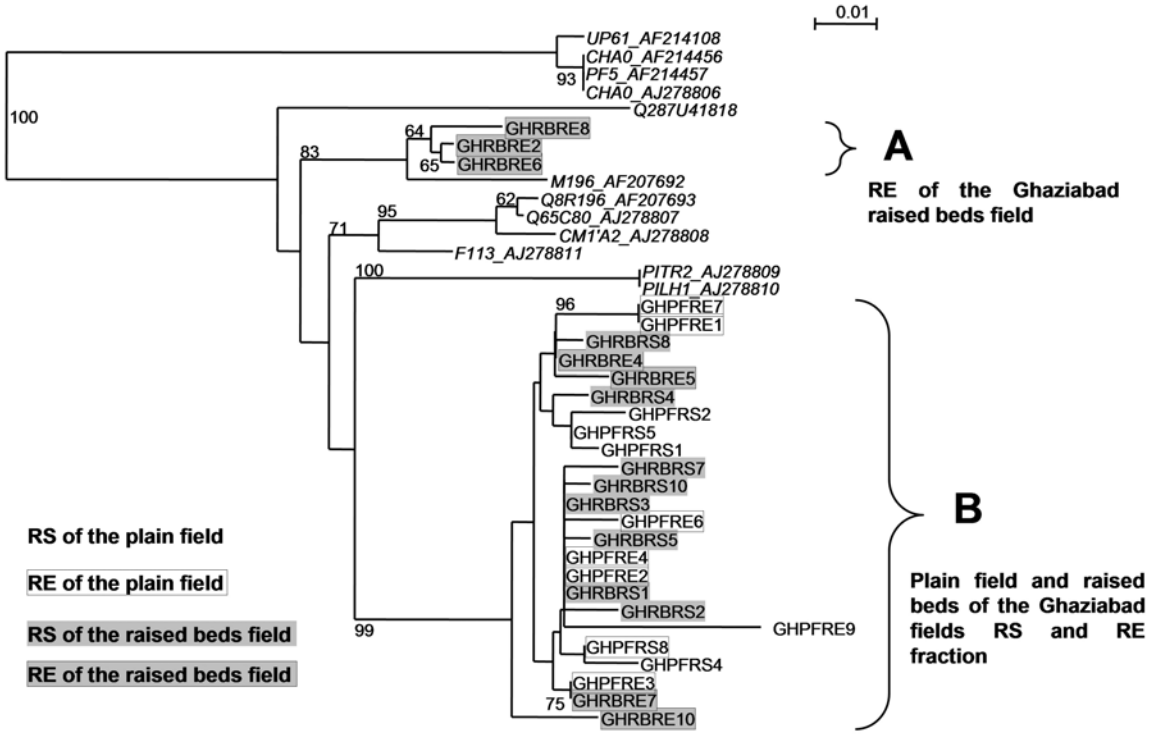

Fig.2. Phylogeny to *phl*D nucleotide sequences using partial *phl*D gene sequences from the GenBank database (bold and italic) and sequences obtained from the cloning of the 227bp amplicons from DNA extracted from the soil and root fractions of the wheat rhizosphere, on the basis of Ghaziabad raised beds and plain fields samples. The tree was constructed by Neighbour-Joining method [26] with Njplot [27]. The robustness of the inferred tree was assessed by 100 bootstrap replicates. Nodal support is indicated when higher than 50%. Scale bar indicate the number of substitution per site.

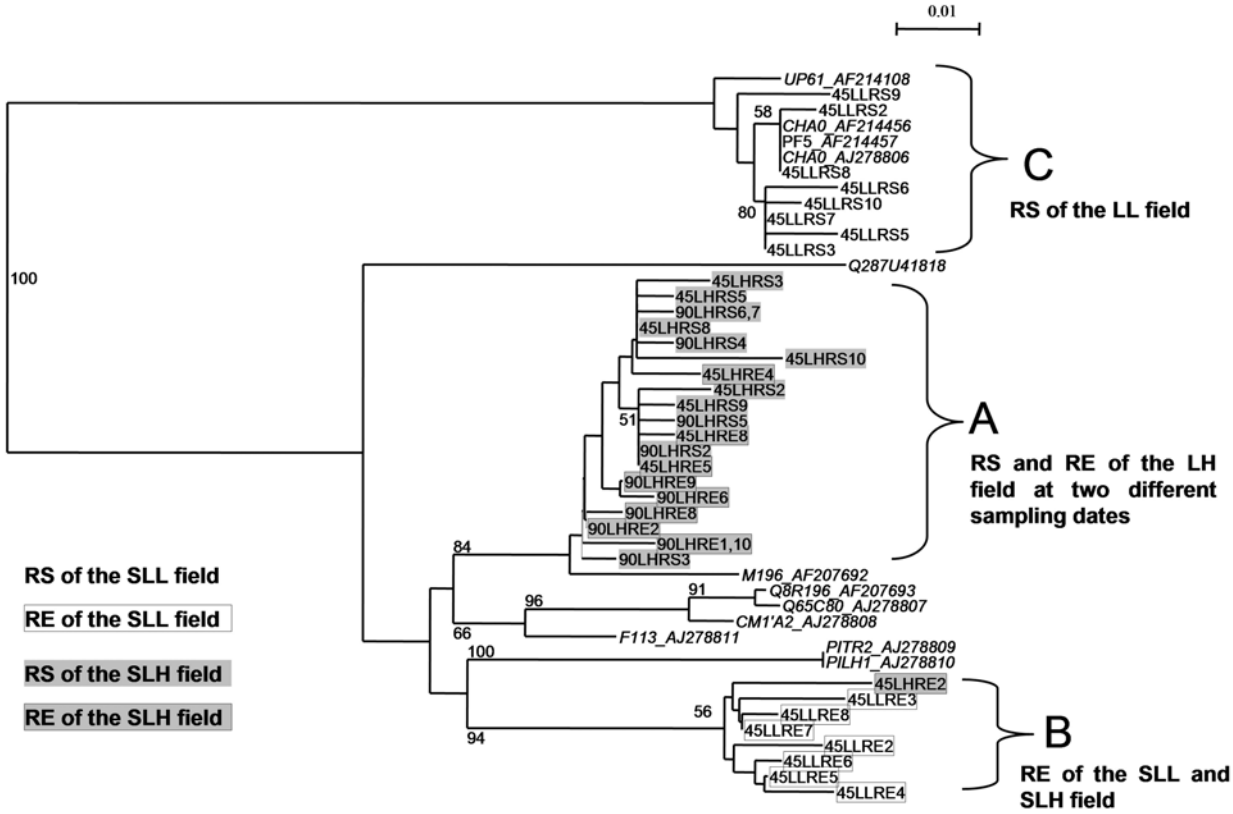

Fig.3. Phylogeny to *phl*D nucleotide sequences using partial *phl*D gene sequences from the GenBank database (bold and italic) and sequences obtained from the cloning of the 227bp amplicons from DNA extracted from the soil and root fractions of the wheat rhizosphere, on the basis of Bhavnipur samples. The tree was constructed by Neighbour-Joining method [26] with Njplot [27]. The robustness of the inferred tree was assessed by 100 bootstrap replicates. Nodal support is indicated when higher than 50%. Scale bar indicate the number of substitution per site.

Discussion

Direct amplification of the phl*D gene from environmental samples*

The nested-PCR procedure developed in the present study was used for specific amplification of *phl*D gene fragments directly from environmental DNA extracts, circumventing any isolation step. Until now, studies on *phl*D–harbouring bacteria have all focused on the cultivable fluorescent pseudomonad group [18]. Part of the diversity might therefore have been missed, as it has not yet been demonstrated that others, non-*Pseudomonas* or other non-culturable bacteria (using standard culture media and conditions) do not produce DAPG. Using S1 medium, known to be selective for the recovery of

fluorescent pseudomonads [29], Picard *et al*. [30] isolated *phl*D$^+$ strains not only related to the fluorescent pseudomonads group but also to the family *Enterobacteriaceae*.

The new primer set (phl310f/phl310chf and phl 536r) was designed so as to consider all the available *phl*D sequences. In addition, the sequence comparison of the 227bp *phl*D fragment allowed to distinguish *phl*D alleles that could be resolved by PCR-RFLP using the whole 745 bp *phl*D sequence [17]. The two-steps amplification procedure was designed to increase the *phl*D fragment yield when directly amplified from environmental DNA samples. The fact that primers phl2a and phl2b have been designed on the basis of the single strain Q2-87 could explain the low yield at the initial PCR step. However, use of newly defined primers enabled assessment of the presence and diversity of *phl*D in environmental DNA. In Ghaziabad and Bhavanipur fields, *phl*D molecular tools applied from environmental DNA allowed to recover new sequences with no close relative among known *phl*D sequences (cluster B). The low allelic diversity observed could be inherent to the study sites. Indeed, in a previous experiment amplifying the *phl*D gene using these new primers allowed the retrieval of five main *phl*D clusters from the rhizosphere of tobacco planted in vineyard soils (F. Poly, unpublished). The impact of DAPG producing non-cultivable (or not yet cultured) bacteria in biocontrol should not be underestimated. Indeed, these microorganisms may be numerically dominant [31]. Moreover, their genotypic background could imply critical phenotypic traits in terms of biocontrol, *i.e.* a specific genotype could be related to an increased or more diverse antibiotic production [12,32,33].

Influence of agricultural management on the diversity of phlD gene fragments

A majority of sequences retrieved from the LH field were found in a single cluster (A) whereas sequences from the LL field were split in two clusters (B and C). Furthermore, *phl*D sequences from DAPG-producing *Pseudomonas* spp. strains isolated from the same fields during the previous year [34] grouped into the same dominant clusters (data not shown). These results suggest that there was a predominance of one or two *phl*D alleles in a single field. This is in conformity with the conclusions of Mavrodi *et al*. [15] and Picard and Bosco [35], who hypothesised that a single *phl*D genotype predominated in monocotyledon crops. Picard *et al*. [30] observed a spatial selection gradient for DAPG producers, which most probably resulted from the selective influence of root exudates. Similarly, a particular allele was selected with respect to vicinity of the root in case of LL field. For Ghaziabad, the discrepancy between the *phl*D sequences related to plain field and raised bed practice could have been triggered by the recent introduction of the latter. This result is in accordance with several other studies suggesting that modifications in the agronomic practices influence the

bacterial community structure at the rhizosphere scale [36,37]. However, discrepancy was also noticed between the two Bhavanipur fields wherein a same practice was carried out. Probably some variation in the field environmental factors might play a critical role in the differentiation of the *phl*D populations. Indeed, Cu and Zn levels were two times higher in the LH fields as compared to LL (data not shown). Nevertheless more sequences would be necessary to confirm this hypothesis.

Mavrodi et al. [15] demonstrated that a low diversity is evidenced in different take-all decline fields that were subjected to wheat monoculture. Their data suggests that monoculture conditions maintained for several decades would promote an enrichment of certain genotypes of DAPG-producing *Pseudomonas* spp.

In the present study, dominant *phl*D alleles were found in different fields, suggesting a low diversity of the *phl*D gene pool in the studied conditions. Flooded field condition during the rice season could lower the level of *Pseudomonas* spp. population, affecting *phl*D diversity. Also, continuous rice-wheat rotation for twenty-five years and the use of the same wheat cultivar in these fields might have created homogenous conditions, similar to monoculture practices.

The two-step PCR amplification, which permits detection and isolation of *phl*D gene pools from environmental samples, opens the door to time- and scale-level follow-up of the phloroglucinol-producing guild. The next step is to carry out studies determining the relations between the development of root pathogens and the *phl*D allelic diversity not only assessed from fluorescent pseudomonads but also from the environment. The allelic polymorphism of the *phl*D gene could then be used to monitor the evolution of agricultural systems using an integrated pest management.

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