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Plant and soil microbe interactions in controlled conditions: rhizosphere protozoa and bacterial community structure

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IMPRIMATUR POUR LA THESE

Plant and soil microbe interactions in controlled conditions : rhizosphere protozoa and bacterial community structure

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UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

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Summary

Plants influence the soil system by the large proportion of photosynthesized matters translocated to the roots and secreted into the soil. This root exudation provides an abundant energy source for rhizosphere living microorganisms. Plants are also strongly affected, positively and negatively, by the presence of soil microbiota, particularly bacteria, protozoa and fungi. Throughout the experiments conducted in this work, we aimed to better understand the influence of protozoa on plant growth.

The first part of this work focused on the development of a microcosm method. Firstly, physical soil sterilization methods (autoclaving (A) gamma-ray irradiation (i) and both successively (AI)) were tested to eliminate the soil microbiota and their resistance form (spores and cysts). Although all sterilization methods tested were efficient to eliminate protozoa, AI was the only efficient method to eliminate aerobic heterotrophic cultivable bacteria without changing the soil pH. However the release of NH_4^+ in the soil after AI sterilization was higher than for other methods. Secondly, a procedure to re-inoculate the sterilized soil with a complex microbial community without protozoa was developed. The protozoa-free bacterial suspension was obtained from rhizosphere soil by subsequent filtering steps to exclude protozoa. The structure of bacterial communities characterised by 16SrDNA PCR-DGGE in the protozoa-free bacterial suspension was similar to that of the native soil. Diversity (Shannon) and evenness indexes increased with time in the sterile soil inoculated with the protozoa-free bacterial suspension. However the final bacterial community composition after 2 months of incubation in the re-inoculated soil presented a lower diversity as compared to the native soil.

The second part of this work focused on the plant-microbiota interactions and on protozoa effects on plant growth. The microcosms developed in the first part of the work were re-inoculated with either sterile water or bacterial protozoa-free suspension or bacterial protozoa-free suspension and *Acanthamoeba castellanii* or with native soil suspension. The growth of *Arabidopsis thaliana* was clearly influenced by the inoculum and was particularly increased in presence of protozoa. Plants cultivated in presence of protozoa presented higher nitrogen content in leaves.

The effect of leaf clipping (simulating herbivore damage) and nitrogen fertilization on soil microorganisms (bacteria, protozoa and nematodes) associated to the rhizosphere of barley was investigated in a pot experiment. The roots-shoots ratio decreased during the plant growth and was lower in the leaf clipping treatment. The abundance of bacteria was not significantly affected by leaf clipping and was higher in the high nitrogen-treatment. The abundance of bacterial-feeders (i.e. protozoa and nematodes) in the rhizosphere of 2, 4 and 6 weeks old plants was marginally affected by the nitrogen treatment as well as by leaf clipping.

The role of protozoa in controlling the structure of bacterial community was investigated in the different experiment. The presence of protozoa did not change significantly the richness (numbers of bands) and the diversity (Shannon index) of the DNA-based DGGE fingerprints. The structure of the “total” bacterial communities was significantly changed in response to the functional group of protozoa (amoeba, ciliates and flagellates) inoculated as compared to the control (bacteria inoculum).
The presence of protozoa did not change significantly the richness and the diversity of the RNA-based DGGE fingerprints. The structure of the active bacterial communities was significantly influenced by amoebas.

Chapter 1
General Introduction

Chapter 1

General introduction

Ecosystems are always undergoing alterations to their biotic (plants, animals and microorganisms) and abiotic (environmental factors) components with numerous and complex interactions. In terrestrial ecosystem, soil nutrients up taken are incorporated in numerous steps following the food chain process into the different biotic components. The food chain starts by the primary producers (autotroph plants) then the primary consumers (herbivore animals), the secondary consumers (carnivore animals), the tertiary consumers (carnivore animals) etc. Organisms are consequently linked in terms of competition of nutrient acquisition and predation which influence the growth and the abundance of their populations. The biomass (partial or total) of organisms returns to the soil in form of detritus: leaves, faeces and dead animals. Soil nutrients are then released, more or less rapidly during the process of decomposition and are thus again be used by living organisms.

The elucidation of the relationships between the different components of terrestrial ecosystem is essential to better understand the functioning and management of this ecosystem. However it represents a real scientific challenge with the different field investigation: geology, chemistry, ecology, microbiology, plant physiology, zoology, climatology… In a large scale view, this thesis falls within the framework of the terrestrial ecosystem functioning and more particularly aims to knowledge about protozoa effects on plant growth. This chapter describes the main facts (Fig 1-1) of this interdisciplinary work.

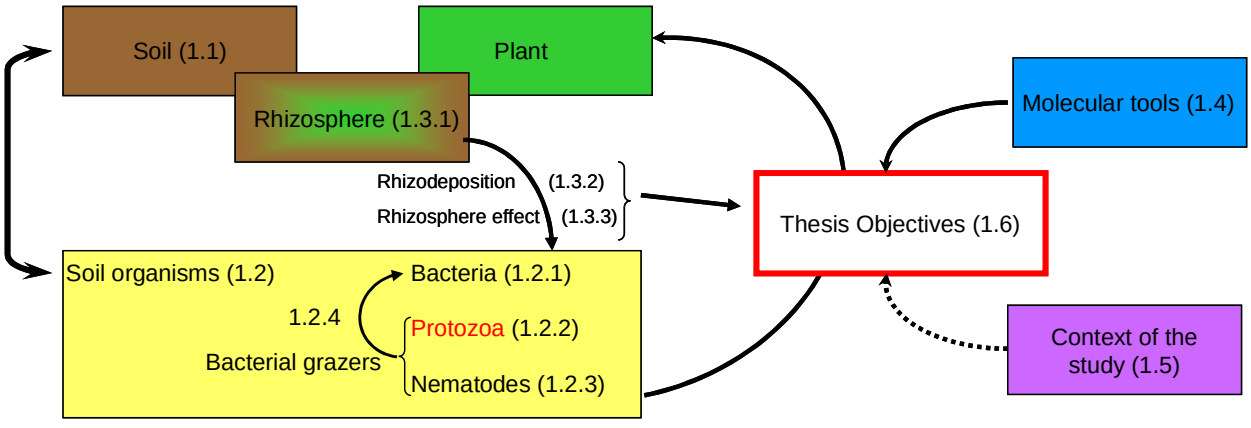


Fig 1-1: Diagram of the general introduction.

1.1 Soil system

Soil comes from slow processes of bedrock degradation, organic material accumulation, differentiation and migration of elements leading to the succession of layers (soil horizons) with different soil properties (colour, texture, structure...). It has different degree of development. The arrangement of these horizons is called soil profile and allows different systems of soil classification (FAO soil classification, USDA soil taxonomy ...). Soil is considered a three phase system (Fig 1-2), consisting of solid (mineral and organic matter, including living organisms), liquid (soil solution) and gas (N_2, O_2, CO_2).

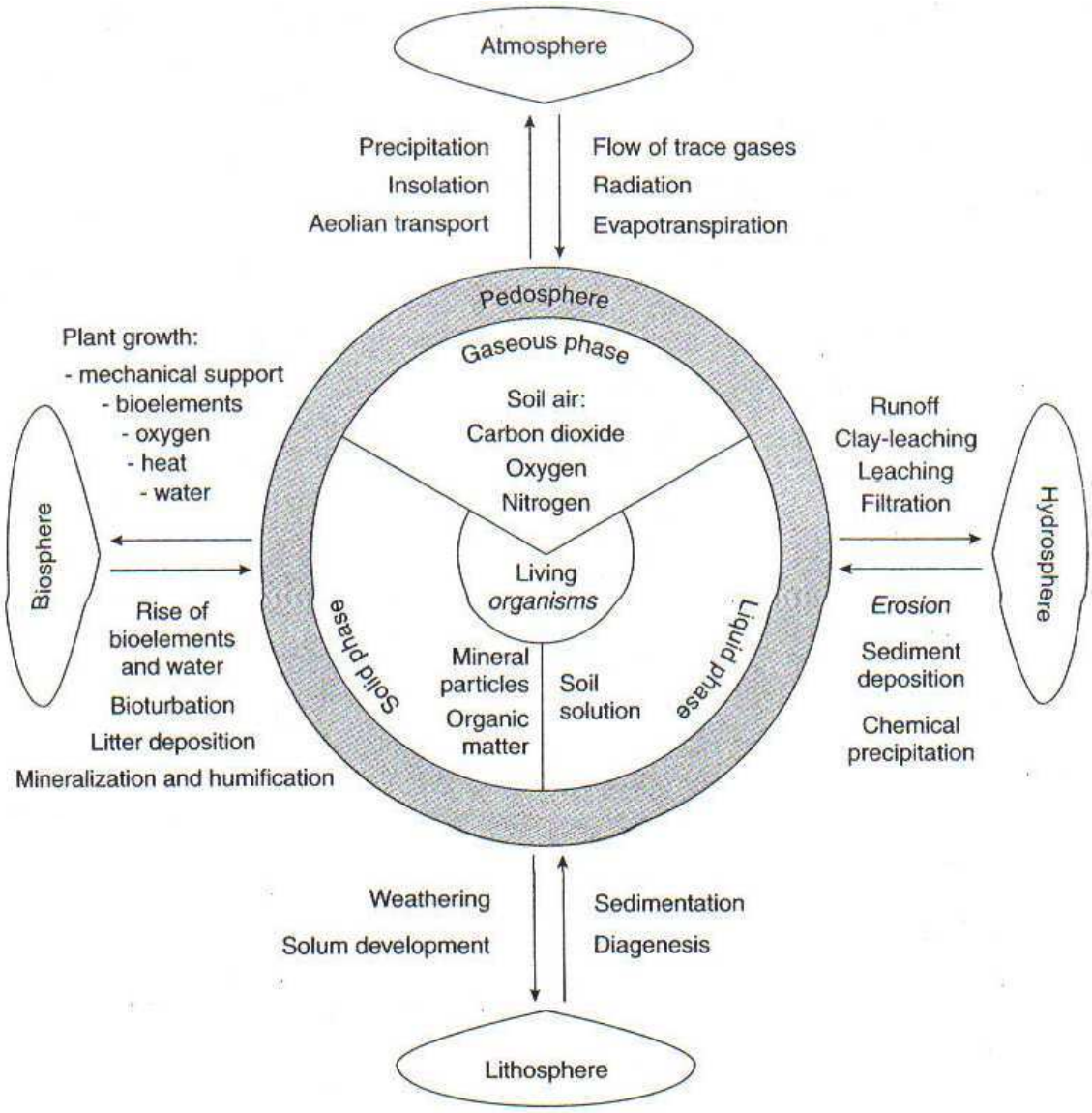


Fig 1-2: Three phases system of soil and external interaction (after Gobat *et al.,* 2004).

The texture of the soil refers to the relative proportion of sand (50-2000µm), silt (2-50µm) and clay (<2µm) size particles. A soil texture triangle is used to classify the texture class defined by USDA (1975-1999) (Fig 1-3). The texture influences the structure, porosity and permeability of soil.

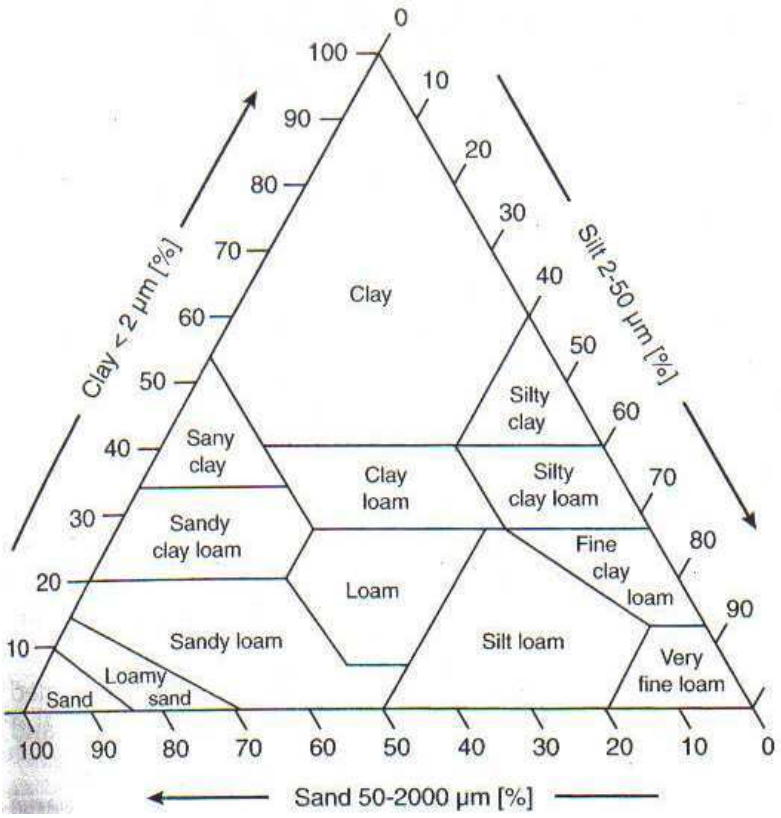


Fig 1-3: Soil texture triangle (after Gobat *et al.*, 2004).

Soil is structured by the arrangement of aggregates. The formation of soil aggregates depends on abiotic and biotic factors (Tisdall and Oades, 1982) in particular physical and chemical reactions (flocculation, ionic bridging, ions precipitation, clay-humic complex formation) between soil particles and compounds excreted by plants, microorganisms and animals (Bronick and Lal, 2005). Soil structure influences aeration, the movement and retention of water, erosion, nutrient recycling and root penetration (Diaz-Zorita *et al.,* 2002; Bronick and Lal, 2005). The spatial arrangement of the solid particles results in a complex and discontinuous pattern of pore spaces of various size and shapes that are more or less filled with water or air, forming a multitude of different soil micro-habitats for microorganisms (Fig 1-4; Chenu and Stotzky 2002).

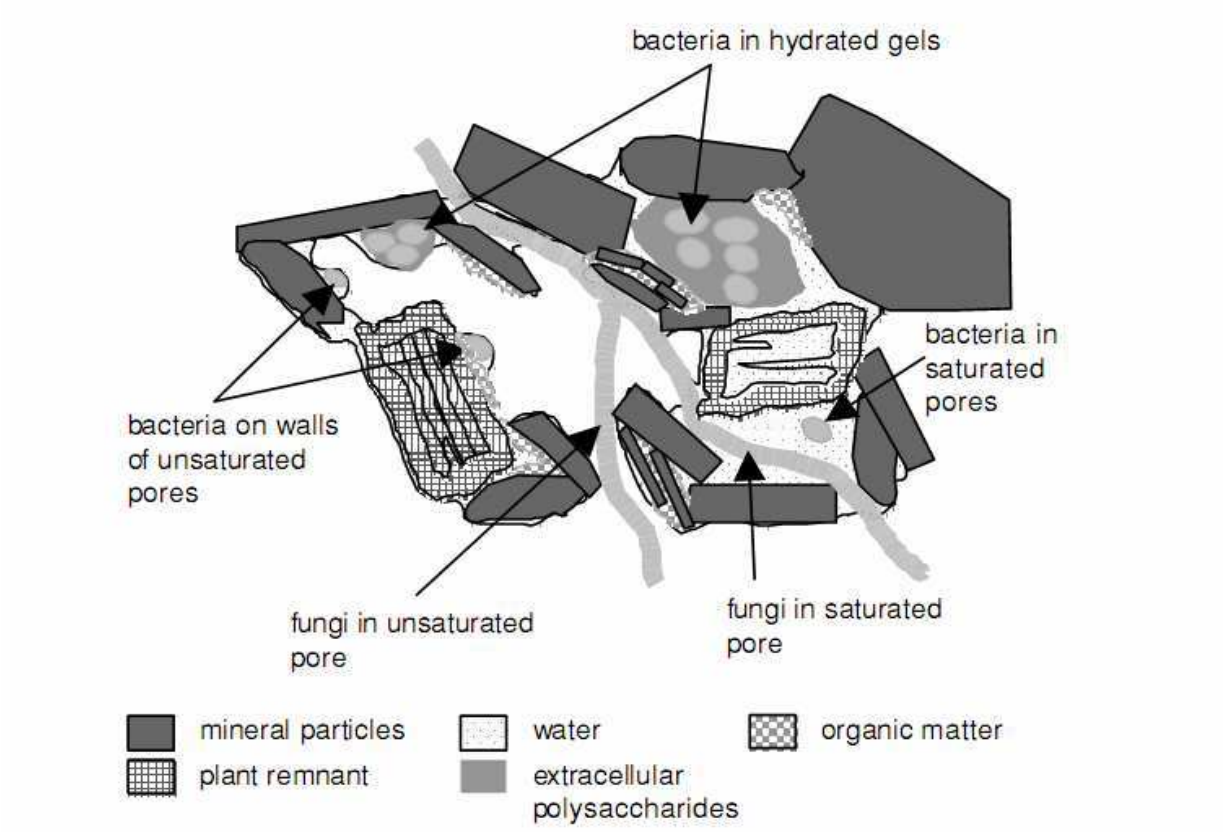


Fig 1-4: Soil micro-habitats (after Chenu and Stotsky, 2002).

1.2 The living soil

Soil is a heterogeneous system where the mineral composition, salinity, pH, nutrient availability, organic input, temperature, water content, climate, geographical and anthropogenic influences determine which ecological niches are available (Liesack *et al.*, 1997). Zone in soil where organisms activity is increased are defined as hot spots (Sexstone *et al.*, 1985) for example the litter (Krivtsov *et al.,* 2007) or the rhizosphere (Kuzyakov, 2002; see § 1.3.3). Significant soil organisms include bacteria, protozoa, fungi, nematodes, collembolla and earthworm and span a wide range in size (Fig 1-5). Soil organisms play a key role in major biogeochemical cycle, organic matter transformation and mineralization process (Swift and Anderson, 1993) and lead actively to soil development and maturation. Despite their tiny sizes, bacteria, protozoa and nematodes in soil are important in term of abundance and function. Indeed, a single gram of soil contains 10^6-10^9 bacteria, 10^4-10^5 protozoa, 10^2-10^3 nematodes. The function of these soil microorganisms are described in the four next subsections.

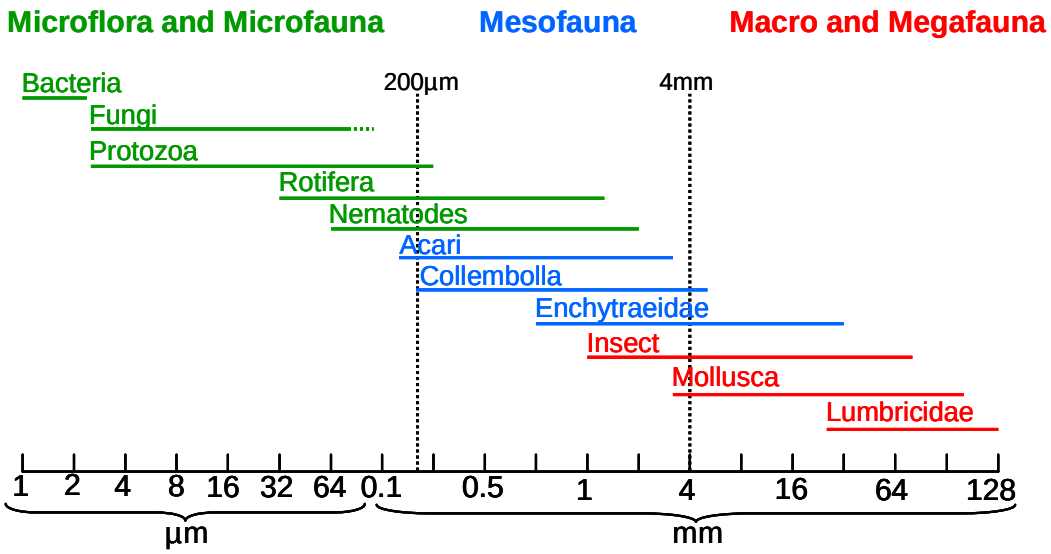


Fig 1-5: Size classification of soil organisms after Swift *et al.*, (1979).

1.2.1 Bacteria

Bacteria are unicellular microorganisms. They belong to the prokaryote group: organisms that have neither a membrane-bounded nucleus nor usually other membrane-bounded organelles and have chromosomes composed of a single closed DNA circle inside the cytoplasm. Bacteria are ubiquitous in every habitat on earth including hostile and extreme conditions of life (Pikuta *et al.*, 2007). The number of prokaryotes on earth was estimated to be $4\text{-}6.10^{30}$ cells (Whitman *et al.*, 1998). Bacterial cells are about 0.5–5.0 µm in length and display typically one of 3 shapes (Fig 1-6): rod (bacilli), sphere (cocci) and spiral (spirilla). The characteristic shape is maintained by the structure of the bacteria cell walls. There are two majors type of cell walls called Gram-positive (containing many layers of peptidoglycan and teichoic acids) and Gram-negative (containing few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins) in response to a differential stain of cells (Gram stain).

Bacteria have an asexual reproduction by cell division. However some bacteria can take up exogenous genetic material by different processes like transformation, transduction and bacterial conjugation. Bacteria exist simply as single cells or may form arrangement based on their plane of division (Fig 1-6). Some bacteria produce resistant form (spore) to survive through period of environmental stress.

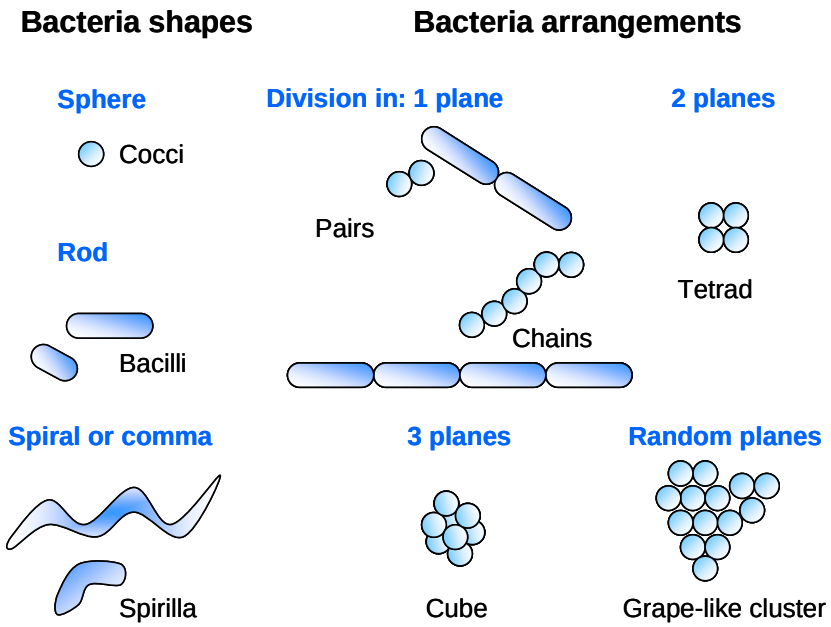


Fig 1-6: Schematic representation of bacteria shapes and arrangements.

Bacteria present a wide variety of metabolic types. Bacteria require a source of energy, an electron donor and a source of carbon for their growth. The source of energy could be chemical (chemotroph), or from sunlight (phototroph). The electron donor could be inorganic compounds (lithotroph) or organic compounds (organotroph). The source of carbon could be inorganic (autotroph) or organic compounds (heterotroph).

The metabolic traits and the gram stain was long time employed for the classification of bacteria species. Currently the classification is based on genetic analysis by molecular techniques: genome hybridization, sequencing of rRNA gene (Olsen *et al.,* 1994). Although the term bacteria traditionally included all prokaryotes, these new approaches in classification lead to divide prokaryotes in two domains: archaea and bacteria (Woese *et al.*, 1990). Sequencing of 16S rRNA genes from environmental samples has revealed the vast diversity of bacteria on earth (Schloss and Handelsman, 2004) (see also § 1.4). Several studies have evaluated the bacterial species richness on earth ranged to 10^7-10^9 (Curtis *et al.*, 2002; Dykhuizen, 1998).

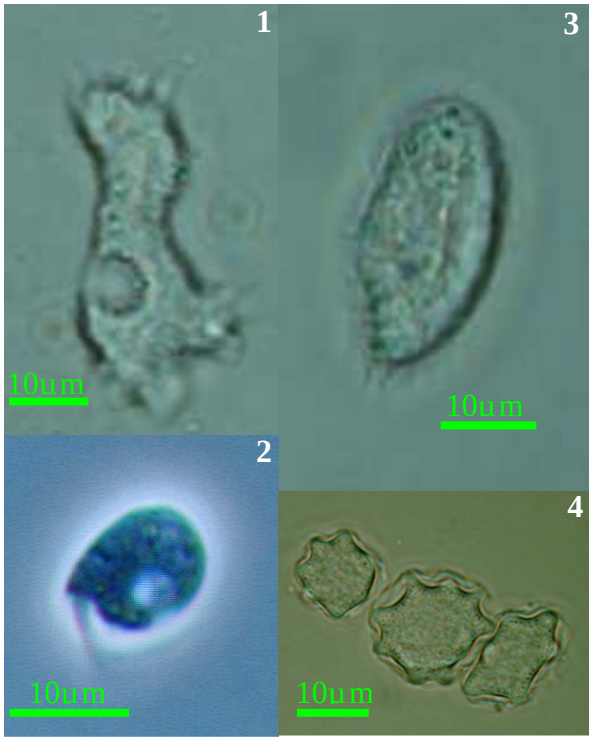
Soil bacteria could be divided in four functional groups. Most are decomposers that consume simple carbon compounds from the litter and root exudates and participate in organic mater decomposition and nutrient cycling (Hättenschwiller *et al.*, 2005). The second group is the mutualists that form partnerships with plant (symbiotic or not) and promote the plant growth. Theses bacteria are called Plant Growth-Promoting Rhizobacteria (PGPR) and improve germination rates, root growth, yield, leaf area, chlorophyll content, hydraulic activity, tolerance to drought, shoot and root weights

(Lucy *et al.*, 2004). The third group is the deleterious for plant growth and plant pathogen bacteria (Suslow and Schroth, 1982, Nehl *et al.,* 1997). The fourth group is the chemo-litho-autotroph bacteria that obtain its energy for example from nitrogen, sulfur, iron mineral compounds. These bacteria play a key role in biogeochemical cycling.

1.2.2 Protozoa

Protozoa belong to the kingdom of Protista and are a paraphyletic group. They are unicellular eukaryotic microorganisms, widespread distributed in many different ecosystems (ocean, lake, soil…). However in their environment they require a water film for locomotion and feeding. Their sizes vary between 3 µm-250µm but some protozoa can exceed 1 mm in diameter (Westphal and Mühlpfordt, 1976).

Four morphological types of these unicellular eukaryotes occur commonly in soil: naked amoebas, testate amoebas (not illustrated), flagellates and ciliates (Fig 1-7). Soil protozoa are also characterized by their ability to form resistant cysts which permit them to survive to dryness or other adverse conditions.



1: Naked Amoeba*:*
Acanthamoeba castellanii, 12-40µm in length.

2: Flagellate: *Goniomonas*
probably *Goniomonas truncata* 8-12µm long.

3: Ciliate: *Colpoda steini* 10-60 µm long.

4: Protozoa cysts: *Acanthamoeba sp*.

Fig 1-7: Morphological types of soil protozoa.

Their activities in soil are limited to the water-filled pore space. Most species occur in the upper 10 cm of soil (Janssen and Heijmans, 1998; Ekelund *et al*., 2001). The size of

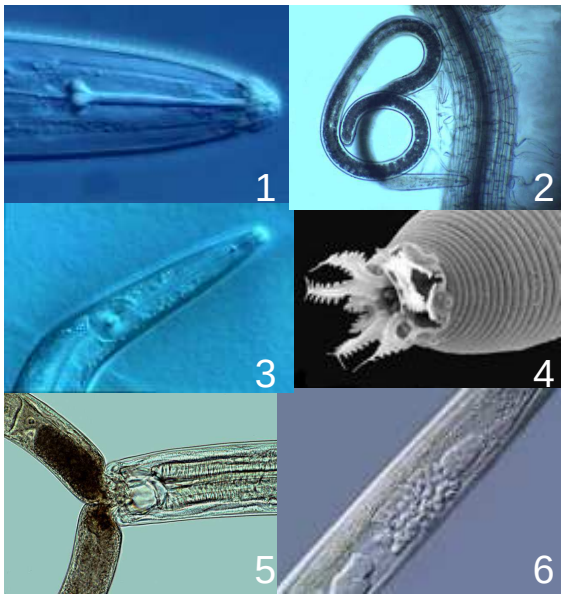
soil pores determines the distribution of the protozoa. They can be transported by the roots, by movements of fauna, ingestion where the cysts resist and are deposited in the faecal dejections (Bamforth, 1988). The density and the mortality of the protozoa vary considerably in space and time (Schönborn, 1992). There are periods of moisture and dryness in soil, which result in activity and inactivity of the microorganisms, and more particularly for the protozoa by alternations of cysts formation and germination. Pratt and Cairns (1985) classified the feeding habits of freshwater protozoa into six groups: photosynthetic, autotrophs, bacterivores/detritivores, saprotrophs, algivores, non-selective-omnivores and predators. This classification could be adapted by adding a mycophagous group to form the basis of a preliminary system for soil protozoa (Couteaux and Darbyshire, 1998; Ekelund, 1998). All these trophic groups can be found in soils but a large proportion of protozoa are bacterivores.

Identification of protozoa is based on their locomotion and morphological structures and requires sometimes transmission electron microscopy. Approximately 25000 species were described in 1976 (Westphal and Mühlpfordt, 1976) and 50000 currently (Lee *et al.,* 2002; Gobat *et al.*, 2004). A high part of protozoa species are unknown. Indeed Foissner (1997) estimated the part of unknown ciliate at 70-80% and global soil ciliate diversity amounts to at least 1330-2000 species, and recently to 1900 species according to Chao *et al.* (2006). Examination of the protozoan diversity is not straightforward in particularly in soil sample. There are many methods to evaluate the richness of protozoa: direct observation of soil suspensions, soil extraction, incubation of serially diluted soil suspensions with or without nutrient enrichment and colonisation of glass slides or chambers (Darbyshire *et al.*, 1996) but no single method that can be applied to all taxa (Couteaux and Darbyshire, 1998). The enumeration of the soil protozoa is generally carried out by the technique of the MPN (Most Probable Number) described by Darbyshire *et al.*, (1974). This method remains used in many studies because of its simplicity (Ronn *et al.*, 1995; Christensen *et al.*, 1995; Müller, 2001, Holze *et al.*, 2003). Techniques of direct counting were applied with epifluorescence microscopy (Adl and coleman, 2005; Tso and Taghon, 1997; Stevik *et al*., 1998; Berthold *et al*., 1999; Griffiths *et al.*, 1988). For a complete review on Fluorescence microscopy for visualization of soil microorganisms see Li *et al.* (2004). Due to they form a paraphyletic group, several specific primers for protozoan group were developed to assess protozoa diversity in environmental samples (Puitika *et al.,* 2007; Lara *et al.*, 2007; Rasmussen *et al.*; 2001 a, b; Grimm *et al.*, 2001, Schroeder *et al.*, 2001).

1.2.3 Nematodes

Nematodes belong to the phylum Nematoda. Nematodes are multicellular organisms, unsegmented worm, bilaterally symmetric and have a simple nervous system, a complete digestive system and no respiratory and circulatory systems. Nematodes have a sexual reproduction and a basic life cycle consisting of an egg stage, four juvenile stages where after each stage a molt is occurred, and an adult stage (Byerly *et al.*, 1976). There are parasitic species with a size ranged from 1 mm to 7 m in length (infesting plant, animals and human) and free-living species with a size ranged from 50 µm to 1-10 mm (Maggenti 1981). Nematodes are found in different ecosystem including marine and terrestrial habitats where they require a water film for locomotion. About 26600 species have been described (Hugot, 2002) and the number of estimated living species in the Nematoda phylum was evaluated at 500 000 by Hammond (1992).

In soil, free-living nematodes represent a large part of soil fauna in particular in temperate grasslands and deciduous forest (Sohlenius, 1980) and are found mainly in the first top 10 cm of soil (Yeates *et al.*, 1984). Soil nematodes have different feeding habits and consequently different role in soil food webs. They can be grouped according to their diet: Herbivore, fungivore, bacterivore, predator and Omnivore (Yeates *et al.*, 1993). This classification is based on the morphology of their mouthparts and pharynx that is characteristic of their diet (Fig 1-8). The phylogeny of nematodes based on 18SrRNA gene showed 5 mains clusters independent of their feeding habits (Blaxter *et al*., 1998).



1: Herbivore

2: Herbivore feeding on root

3: Fungivore

4: Bacterivore

5: Predator feeding on another nematode

6: Omnivore

Photos: www.mactode.com/Pages/Nemapix.html

Fig 1-8: Morphology of nematode's mouthparts and pharynx according to the diet.

1.2.4 Importance of bacteria grazer

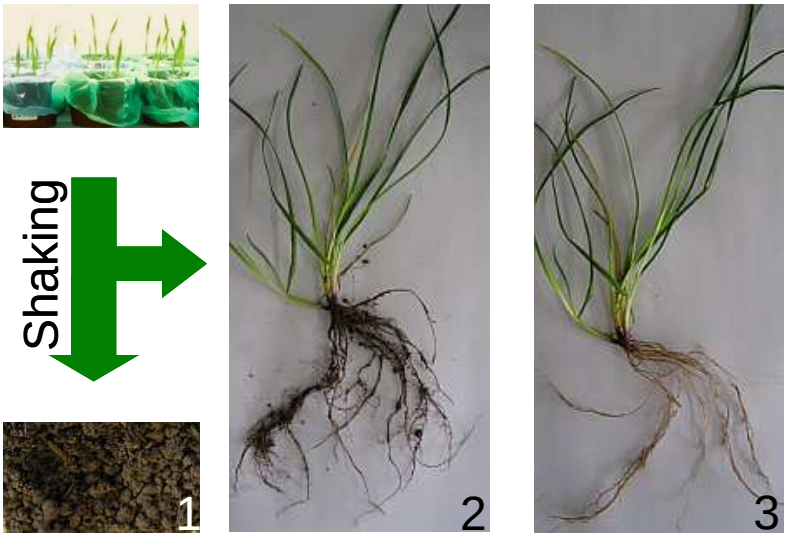
Bacteria grazers (protozoa and nematodes) are better indicators of microbial activity than microbial biomass (Andren *et al.*, 1988; Christensen *et al.*, 1996). They have been used as indicators of soil conditions in particular nematodes because they are easy to sample (Neher, 2001; Yeates, 2007). Grazing allows to control bacterial biomass, increases its turnover and altogether allows redistribution of mineral nutrients to the plant (Bonkowski, 2004; Ingham *et al*., 1985; Becker *et al*., 2001; Laasko and Setälä, 1999; Hodge *et al.*, 2000). This results in plant growth stimulation and in an increase mineralization (Alphei *et al.*, 1996; Brimecombe *et al.*, 1999; Foissner, 1999; Jentschke *et al.*, 1995). In particular, bacterial predators alter the balance in nitrogen competition between plant and bacteria and increase plant nitrogen uptake (Clarholm, 1985 and 1989). Changes in root architecture accompanied by an increase of soil IAA content (Indole 3-Acetic Acid) - a phytohormone involved in the build-up of additional lateral roots - were observed in presence of protozoa (Kreuzer *et al.*, 2006; Bonkowski and Brandt, 2002) or nematodes (Mao *et al.*, 2006, Mao *et al.*, 2007).

The importance of microfauna, on the control of microbial communities structure was postulated long ago. However, most studies were performed either in microcosms or in aquatic environments (Blanc *et al.,* 2006; Djigal *et al.,* 2004; Griffiths *et al.*, 1999; Hahn and Höfle, 2001; van Hannen *et al.*, 1999; Jürgens *et al.*, 1999). All bacterial populations are not equally submitted to predation. Some species appear more “attractive” (or edible) than others for protozoan grazers (Ayo *et al.*, 2001). Protozoa have a selective bacterial predation contrary to nematodes where the selection is imposed by the dimension of their mouthpart. Pernthaler *et al.*, (1996) have shown the importance of cell dimensions for the susceptibility to grazing: small (< 0.4 µm) and big (> 1.6 µm) cells are less susceptible to grazing than medium-size cells. Other factors, like humidity, pH and motility, may influence grazing (Hahn and Höfle, 1999). Moreover, bacterial cells attached to soil aggregates are less vulnerable to grazing.

1.3 Rhizosphere

1.3.1 Definition of the rhizosphere

The rhizosphere is defined as the volume of soil under the influence of root, as well as the root itself (Hiltner, 1904). Marilley *et al*., (1998) described three different experimental fractions (Fig 1-9) depending on distance to the root: the Bulk Soil (BS) or Non-Rhizosphere Soil (NRS) corresponding to soil that is not influenced by the roots, the Rhizosphere Soil (RS) defined by the soil that adheres to the root when the root system is shaken manually, and the Rhizoplane-Endorhizosphere (RE) corresponding to the surface and the interior of the root.



1 = NRS Soil detached from the roots after shaking

2 = RS Soil attached to the roots after shaking

3 = RE Washed roots

(Photo 2-3 : Maryline Jossi)

Fig 1-9: Three experimental rhizosphere fractions.

1.3.2 Rhizodepostion

Rhizodeposition corresponds to the organic compounds translocated through the plant and released by roots during the plant life. Indeed a large proportion of photosynthetized matters (between 10 and 50% of total photosynthates) are secreted into soil by the root (Fig 1-10) in the form of soluble exudates, secreted polymers, detached cells and lysates (Nguyen, 2003; Gobat *et al.*, 2004). The biochemical nature of compounds liberated by roots is very diverse: simple and complex sugars, amino acids, organic acids, phenolics, alcohols, polypeptides and proteins, hormones and enzymes (Nguyen, 2003). Exudates in soil are known to have very short turnover times (Boddy *et al.,* 2007; Jones *et al.*, 2004). Rhizodeposition depends of different biotic and abiotic factors (Fig 1-11, Jones *et al.*, 2004).

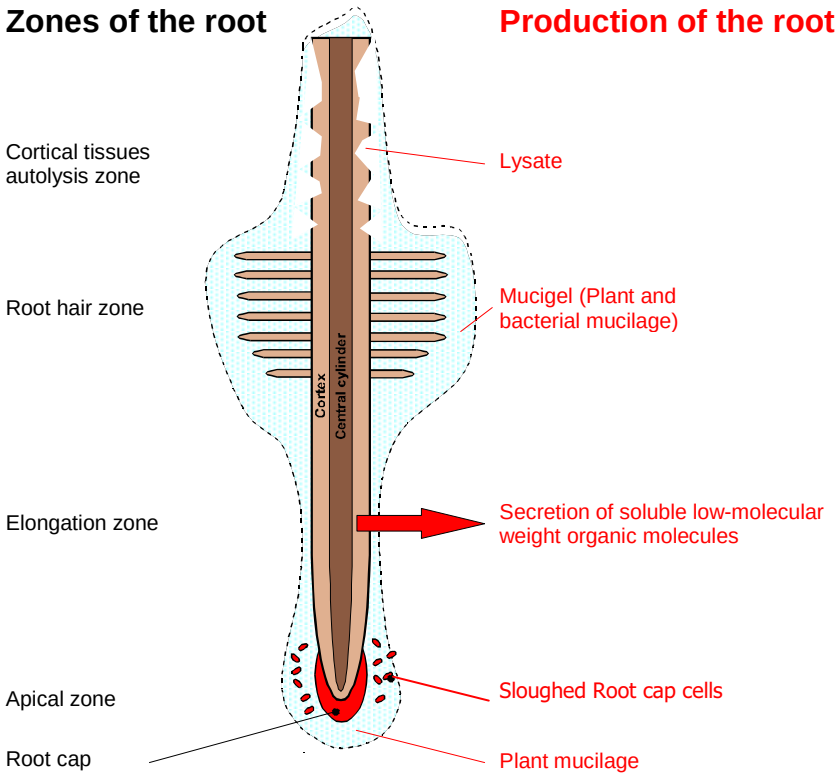


Fig 1-10: Diagram of the root (source Michel Aragno).

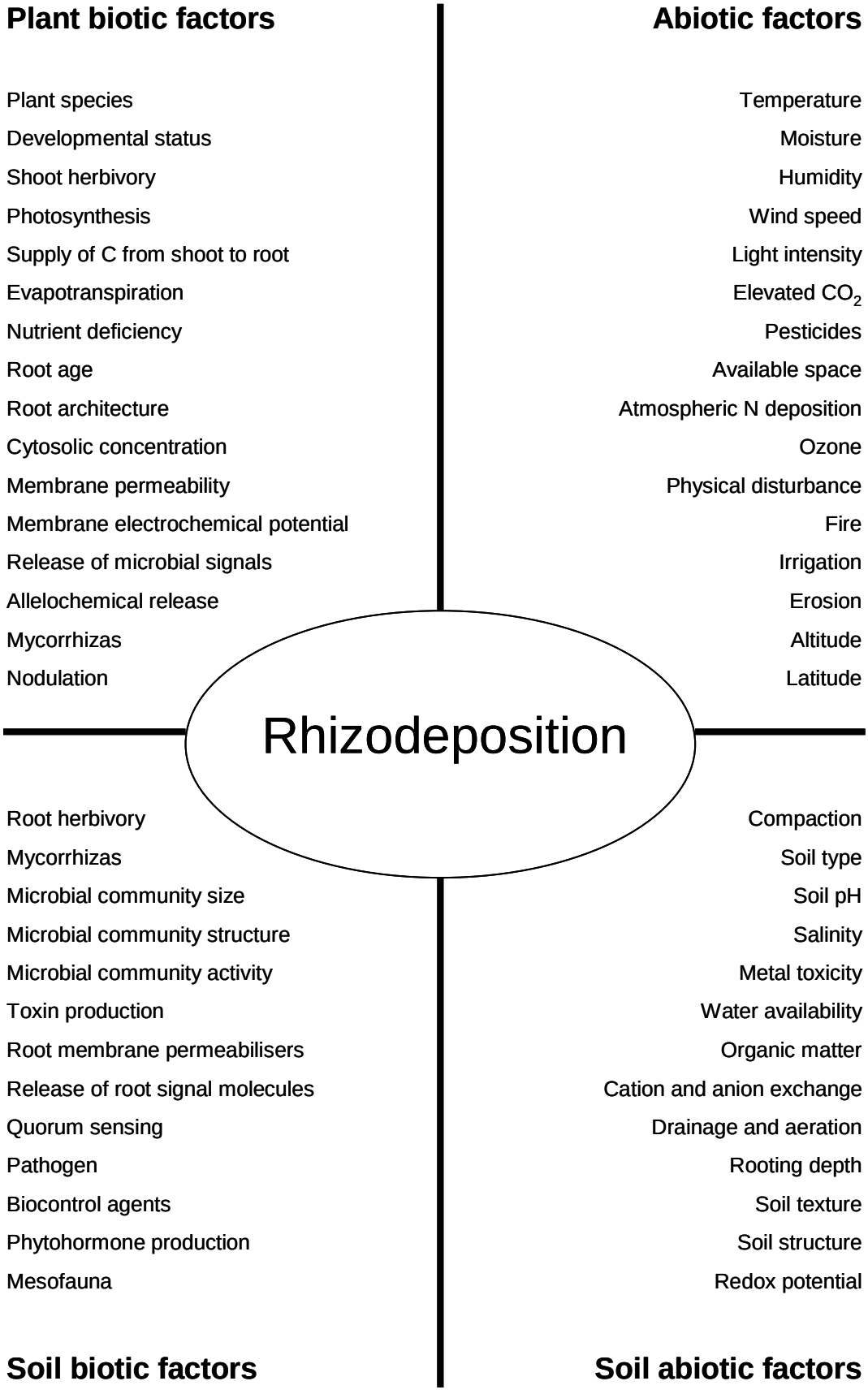


Fig 1-11: Schematic representation of factors that influence rhizodeposition (after Jones *et al.*, 2004).

1.3.3 Rhizosphere effects

Contrary to the soil that is generally poor nutrients and energy sources (Nannipieri *et al.*, 2003), the rhizosphere is an active interface between soil and plant where a higher number of microorganisms are found due a strong nutrient flow brought up by rhizodeposition (Whipps and Lynch, 1986, Tyagi 2007) and is probably the greatest hot spots in soils (Kuzyakov, 2002). Consequently microbial growth in the rhizosphere is not limited by substrate availability (kastovska and Santruckova, 2007). A gradual rhizosphere effect is created by the particular growth conditions found in proximity to roots (Hinsinger *et al.*, 2005) and leads to the selection of the microbial populations modifying the diversity of bacteria (Latour *et al.*, 1996, de Boer *et al.,* 2006). Indeed in the rhizosphere, microbial populations which present a carbon metabolism and energetics allowing them to benefit of the exudates released by the plant are mainly favoured (Latour and Lemanceau, 1997). Successful colonization of the rhizosphere environment by the bacteria depends on their rhizosphere competence (Weller 1988) that is the bacterial capacity to colonize and maintain at the root proximity.

The O_2 and CO_2 partial pressure vary in function of root respiration (Hinsinger *et al.,* 2005). Hojberg and Sorensen (1993) reported a significant lower oxygen concentration near the root. The rhizosphere environment could therefore favour anaerobic bacterial processes. For example, the ability to use nitrate as alternative electron acceptor could be a competitive advantage for bacteria in the rhizosphere, where oxygen is limiting (Ghiglione *et al.*, 2000).

The presence and function of microbial grazers in the rhizosphere is essential, although this topic was most often neglected in microbial diversity studies. Rhizosphere bacteria grazers are part of a secondary food chain originating from the rhizodeposition. Protozoan populations are denser in the rhizosphere than in the bulk soil (Zwart *et al.*, 1994; Christensen *et al.*, 1995) and protozoa reduce the abundance of some species of rhizosphere bacteria (Zwart *et al.*, 1994). However, it is not clear if there is a relationship between rhizosphere competence of bacterial population and their resistance to grazing (Jjemba and Alexander, 1999; Jjemba, 2001). Beneficial effects of protozoa on plant growth have been assigned to nutrients released from consumed bacterial biomass. This mechanism, known as the “microbial loop in soil” (Clarholm, 1985; Bonkowski, 2004) is triggered by the release of root exudates from plants that increase bacterial growth in the rhizosphere. Without grazing, the high carbon- and energy flow provided by the

rhizodeposition would result in the accumulation of bacterial biomass which would then be limited by the other soil nutrients, cutting off plant nutrition.

1.4 Molecular biology tools in microbial ecology

Bacteria are difficult to culture and very few of the total number of microbial species can be isolated and cultured on laboratory media (Torsvik *et al.*, 1990). In soil sample, only 1-2% of soil bacteria could be cultured (Amann et *al.*, 1995). Woese and Fox (1977) proposed the use of the small-subunit ribosomal gene as a phylogenetic tool to describe the evolutionary relationships among organisms. Ribosomal RNAs are essential to protein synthesis and are ubiquitous to all organisms. They contain variable and highly conserved regions in both primary and secondary structure and they appear to change in sequence very slowly. Since the mid-1980s, the use of ribosomal RNA based techniques in particular thanks to the development and application of the polymerase chain reaction (PCR, Saiki *et al.*, 1988) has facilitated a culture independent approach to investigate microorganism diversity as it occurs in nature (Olsen *et al.*, 1986).

Technical developments in molecular biology have found extensive applications in the field of microbial ecology. Indeed a range of molecular techniques based on PCR approach are available to assess microbial diversity (small-subunit ribosomal gene) or microbial function (specific gene) in term of population: RFLP (Restriction fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis) or in term of total community: DGE (Denaturing gel electrophoresis); SSCP (Single strand conformation polymorphism), T-RFLP (Terminal Restriction fragment length polymorphism) RISA (Ribosomal intergenic spacer analyses). For a complete review on methods of studying soil microbial diversity see Kirk *et al.,* (2004). Fingerprint methods such as DGE was developed and used to assess the diversity of microbial communities and how microorganisms evolve in their environment. These methods permit the analysis of the whole bacterial communities (Muyzer *et al.,* 1993). DGE allows the separation of small polymerase chain reaction products (400 base pairs) in function of their different G+C content and distribution. Consequently, the fingerprinting pattern or profile 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). PCR fragments generated from single population (same specie) display identical

electrophoresis mobility (band).

Fromin *et al.,* (2002) reviewed the statistical analysis of DGE fingerprint patterns. The main analyses described are summarized below. The fingerprint pattern are analysed according to the presence, migration length and relative intensity (related to the population density) of each band. Comparison of profiles allows to analyse the evolution of microorganisms in their environment by the observation of the possible changes in the presence/absence or in the variation of intensity of a single band after a treatment. Changes in the dominance of population could be determined with diversity (Shannon) and evenness indexes calculated with the relative intensity of the set of bands displayed on the whole profile. The presence/absence of band and the relative intensity of each band permit also to build similarity matrix and consequently perform clustering analysis to display sample presenting similar pattern. 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 such as principal component analysis (PCA) and correspondence analysis (CA) (Legendre and Legendre, 1998). 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). Multivariate statistical analysis as canonical correspondence analysis (CCA) integrates environmental data (pH, temperature, nitrate concentration…) in the fingerprint pattern analysis. This method allows to highlight the environmental factors that influence the microbial communities structure.

1.5 Context of the study

Research on rhizosphere microbial ecology at the Microbiology department of the University of Neuchâtel was initiated in 1995 after an invitation by prof. J. Noesberger (ETH-Z) to participate to the Swiss Free Air CO_2 Enrichment project on the responses of prairie ecosystems to an elevation of atmospheric pCO_2 (Tarnawski and Aragno, 2006). These researches were supported by the Swiss National Fund since 1995. During ten years the rhizosphere's team focused on:

-The characterization of rhizosphere bacterial communities (Marilley, 1999 and Marilley *et al.*, 1999; Hamelin 2003) by PCR-DGGE of 16S-rDNA/rRNA (Jossi *et al.*, 2006) and the analyses of DGGE fingerprint by statistical analyses (Fromin *et al.*, 2002).

-The characterization of rhizosphere bacteria population (Marilley *et al.*, 1998) and population involved in nitrogen cycle: denitrification (Roussel-Delif *et al.*, 2005, nitrogen-fixation, Hamelin *et al.*, 2002), and environmental *Pseudomonas* strains (Tarnawski *et al.*, 2003 , Tarnawski, 2004; Locatelli *et al*., 2002).

-The characterization of rhizosphere bacteria associated to wheat and their influences on the soil quality and productivity (Roesti, 2005; Roesti *et al*., 2006). This topic was supported by Indo Swiss Collaboration in Biotechnology.

The initial research on this thesis project was done by Laurent Locatelli (PhD student), at the laboratory of Microbiology, University of Neuchâtel (LAMUN). However, he resigned from the project in June 2002. I accepted to continue this project and benefited for two years (2003-2005) from as Swiss COST credit, in the frame of COST 627 action on "Carbon Storage in European Grasslands". The two last years of the thesis was supported by the Swiss National Fund.

1.6 Thesis objectives

The overall objective of this work was to better understand the function of protozoa in the rhizosphere. The starting point of this thesis was the microbial loop in soil (Fig1-12).

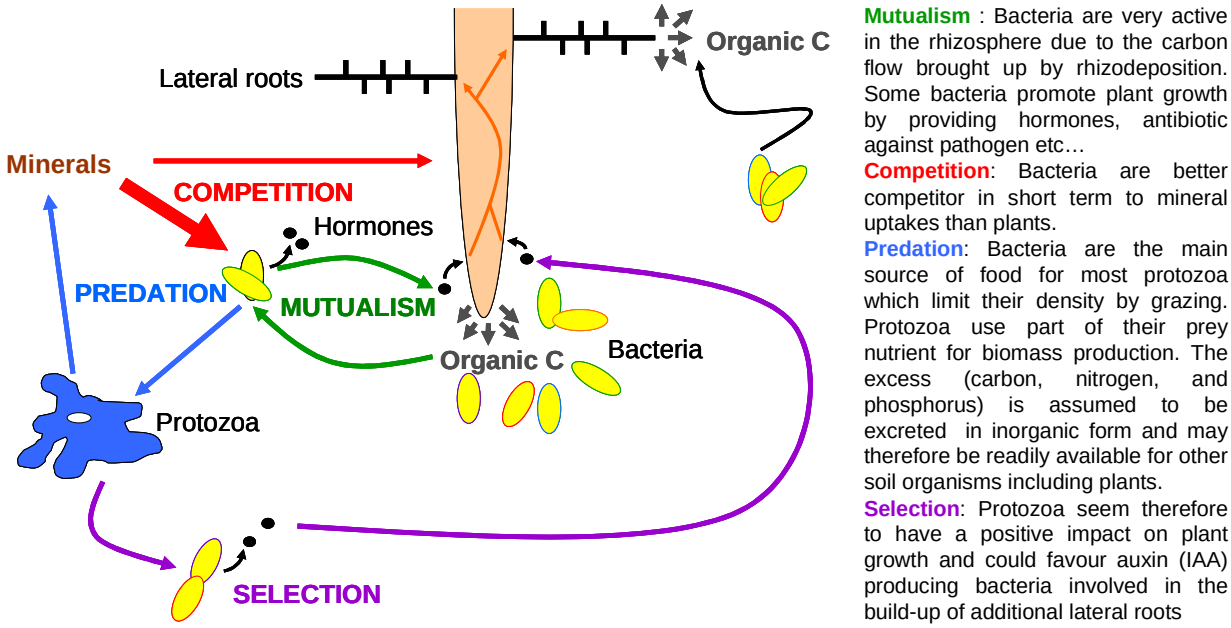


Fig 1-12: Plant-microbe interaction in the rhizosphere, the microbial loop.

Rhizosphere is a complex system with numerous interactions between plants, microorganisms, soil fauna and abiotic factors (cf 1.3). In order to limit theses interactions, we decided to work with a simplified system in controlled conditions to study the impact of protozoa on the plant growth and bacterial communities structures.

We chose 3 conditions of plant growth:

- in sterilized soil = Sterile condition
- in sterilized soil inoculated with soil bacteria = Bacteria condition
- in sterilized soil inoculated with soil bacteria and protozoa = Protozoa condition

The first objective of this thesis was to develop microcosm in order to study plant-microbiota interactions in controlled conditions (Chapter 3). The first step was to choice an effective soil sterilisation method. The second step was to produce a bacterial protozoa-free suspension as inoculum in order to control the diversity of bacterial community present in the different treatment. The last step was the purification of protozoa culture in axenic condition to limit perturbations on the reassembling complex microbial community.

The second objective was to build up microcosms with the methods developed and determine the impact of protozoa on plant growth in controlled conditions (Chapter 4). The plant growth was evaluated by the description of plant stage development, the measure of plant biomass and plant C/N ratio.

The third objective was to determine the impact of grazing by protozoa on the bacterial communities structure (Chapter 5). Firstly the effect of one amoeba specie (Acanthamoeba castellanii) on total bacterial communities structure (DNA approach) and secondly the effect of different protozoan species from different group (flagellates, amoebae, ciliates) on the total and active bacterial communities structure (DNA and RNA approach) were analysed.

The fourth objective was to study, in pot experiment without soil sterilization treatment, the effect of plant perturbation (simulated herbivore attack) on soil bacteria and microfauna (protozoa and nematode) associated to the plant roots (Chapter 6).

Chapter 2

Material and methods

Chapter 2

Material and Methods

This chapter describes the general material and methods used in the different experiments.

2.1 Soils

Soil Corcelles-Consise (Soil CC)

A chromic luvisol soil (5-30 cm, Ap horizon) was collected from an agricultural wheat field in Corcelles-Concise (VD, Switzerland). Soil characteristics are described in Le Bayon *et al.*, (2006): This soil contained 50% sand, 30% silt, 20% clay, 0.97% organic carbon, 0.31% mineral carbon, C/N ratio: 11.8, $pH_{[H2O]}$= 7.8, $pH_{[KCl]}$= 7.02, 1407.8 (±74.2) $mg.kg^{-1}$ total P (colorimetrically measured following a Kjeldahl oxidation). The soil was air-dried after collecting in the field and sieved through a 2 mm mesh and stored. The soil was remoistened at 15% (w:w) and sieved at 4 mm before use (Chapter 3, 4 and 5).

Soil Roßdorf (Soil R)

Soil (5-20 cm depth) was collected from a meadow near Roßdorf (Germany), air-dried and sieved through a 2 mm mesh. The characterisation of this soil (classification, texture, pH…) has not yet been performed (Chapter 5).

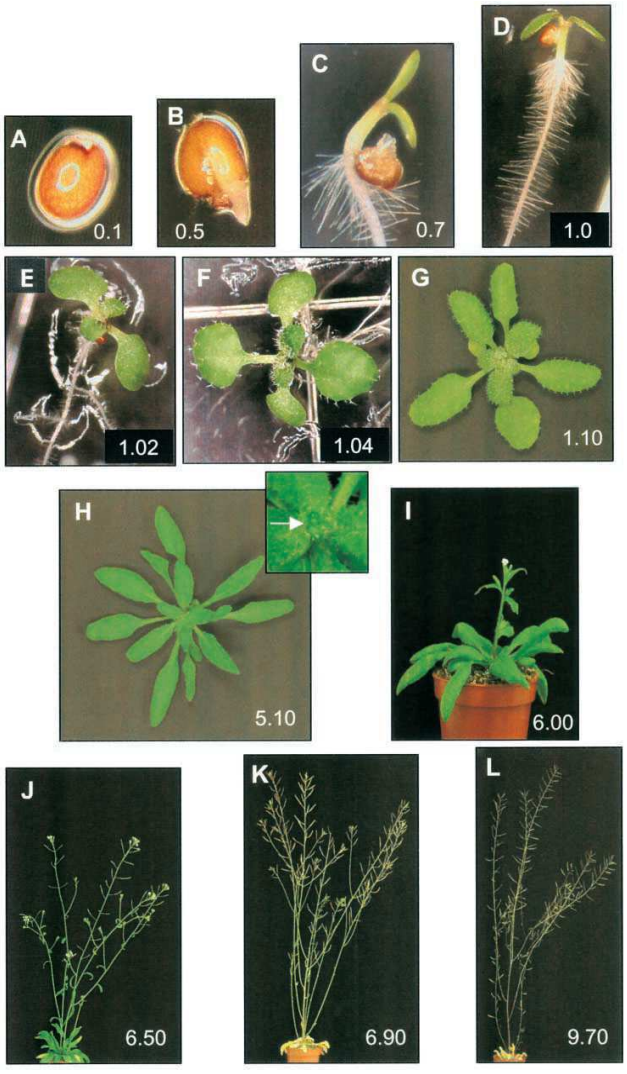
Soil Tåstrup (Soil T)

The clay loam soil (16% Clay, 32% Silt, 50% Sand; 1.4% Organic matter; $pH_{[H2O]}$= 6.5) from an agricultural site located at Tåstrup in Denmark was collected in September 2003. The soil was air-dried, sieved through a 4 mm mesh and stored at room temperature. No NPK fertilization was done since 1995 (last amendment in P and K in 1965). For the chapter 6 experiments, we mixed 67.5% of this agricultural soil with 22.5% of quartz sand (0.3-0.6 mm) to facilitate root sampling and with 10% of garden soil as inoculum passed through a 4 mm mesh sieve.

2.2 Model plants

2.2.1 *Arabidopsis thaliana*

Arabidopsis thaliana is a small annual flower plant belonging to the Brassicaceae family and is widely use as a model organism in plant biology particularly in genetic and molecular biology. The complete genome of *Arabidopsis thaliana* has been sequenced. The rapid life cycle of *Arabidopsis thaliana* (6 weeks from germination to mature seeds) is advantageous in plant growth studies (Fig 2-1).



Stage 0: Seed germination
0.1 Seed imbibition (A)
0.5 Radicle emergence (B)
0.7 Hypocotyl and cotyledon emergence (C)

Stage 1: Leaf development
1.0 Cotyledon fully opened (D)
1.02 2 rosette leaves > 1 mm in length (E)
1.04 4 rosette leaves > 1 mm in length (F)
1.10 10 rosette leaves > 1 mm in length (G)

Stage 3: Rosette growth

Stage 5: Inflorescence emergence
5.10 First flower buds visible (H)

Stage 6: Flower production
6.00 First flower open (I)
6.50 50% of flower to be produced have opened (J)
6.90 Flowering complete (K)

Stage 8: Silique ripening

Stage 9: Senescence
9.70 Senescence complete (L)

Fig 2-1: *Arabidopsis* growth stage description. Photos and plant growth description adapted from Boyes *et al.*, 2001.

Arabidopsis represents also a simpler and advantageous system because it does not form arbuscular mycorrhizal roots. Mycorrhizae use directly organic carbon provided by plants

and improve plant nutrition by nutrient translocation (mostly P and N) to the plant (Smith and Read, 1997). The absence of mycorrhizae allows to determine the direct impact of soil protozoa on nutrient plant uptake and plant growth.

Seeds sterilization and plant germination

Arabidopsis thaliana seeds were incubated overnight under a fume hood in a dessiccator containing 100 ml of calcium hypochlorite 3% and 3 ml of concentrated HCl in a beaker. After adding 1.5 ml of sterile agarose 0.1%, the seeds were let in the dark at 4 ℃ for three days in order to allow a synchronisa tion of germination. They were then spread onto 1/10 Tryptic Soy Agar (TSA 3g.l^{-1}, BioMérieux, Marcy l'étoile, France) medium and incubated vertically in an illuminated growth chamber at 20℃ lighted during 16h a day.

2.2.2 Barley

Barley (*Hordeum vulgare*) is an annual cereal plant belonging to the grass family Poaceace which serves for food (animal and human) and beer production. The growth cycle of barley (Fig 2-2) has the following divisions: germination, seedling establishment and leaf production, tillering, stem elongation, pollination, and kernel development and maturity (Large, 1954).

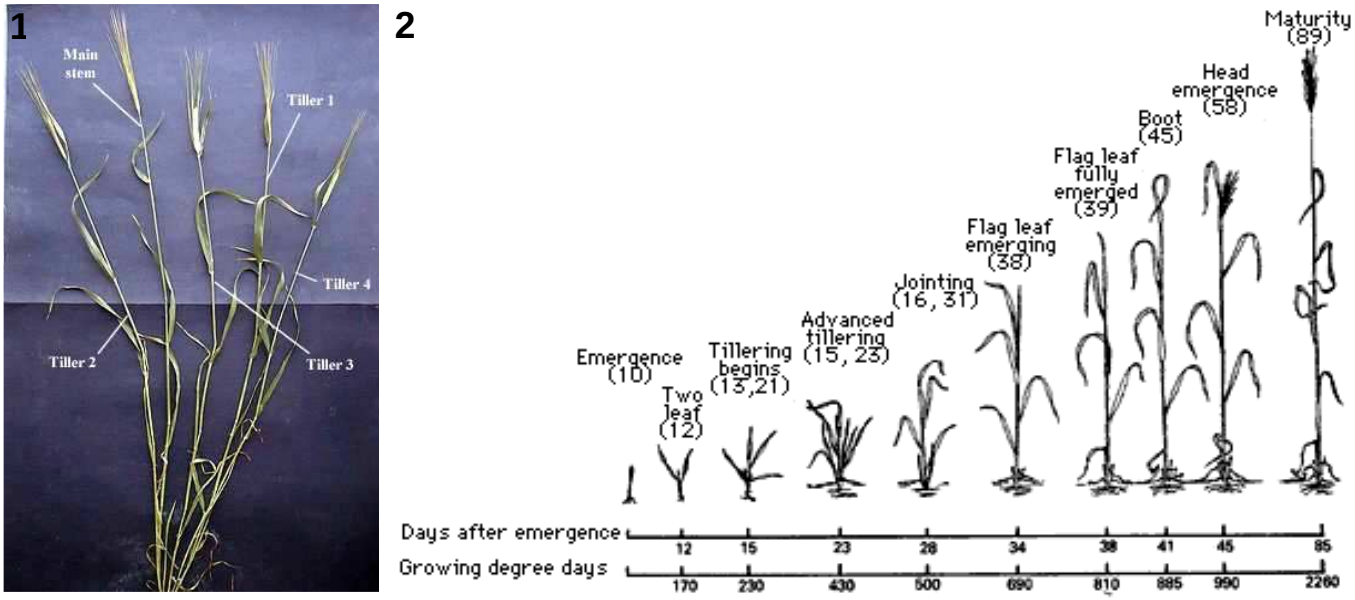


Fig 2-2: Barley growth stage according to the Zadok scale.

Websource accessed in 2008:

1: http://oregonstate.edu/instruct/css/330/five/BarleyOverview.htm

2: http://www.extension.umn.edu/distribution/cropsystems/DC2548.html

2.2.3 Rice

Rice (*Oryza sativa*) (Fig 2-3) is annual cereal plant belonging to the grass family Poaceace. It is among the largest world crop for human alimentation particularly in Asia.
The growth cycle of rice (110-130 days) has the following division germination, seedling tillering, stem elongation, booting, heating, milk stage, dough stage and mature grain. (http://www.knowledgebank.irri.org/RP/growthStages/growthStages.htm)

Fig 2-3: Rice plant *Oryza sativa*.
(Photo Damien Boilley, source : http://commons.wikimedia.org/wiki/Image:Rice_plant_with_grains.jpg)

Seeds sterilization and plant germination

Rice seeds (*Oryza sativa* cv. Zhonghua11, a precocious Japonica rice variety) were kindly provided by Dr. Xin Ke (Institute of Crop Breeding and Planting, Chinese Academy of Agricultural Sciences, China). The seeds were dehusked by grinding lightly with a pestle in a mortar, and weighed. Seeds with a weight between 20 and 21 mg were surface sterilized by washing with 50% vol. ethanol for 1 min, 70% vol. for 2 min and 5 % NaOCl for 10 min (Kreuzer *et al.,* 2006). Sterilized seeds were transferred for germination into separate wells of 96-wells microtiter plates, each one containing 300 µl nutrient broth (0.8g.l^{-1}, Oxoid UK) in Neff's Modified Amoeba Saline (Page 1988) medium (NB-NMAS) to check the seed sterility until germination.

2.3 Microcosms

Two units of Magenta® GA-7 vessel (Sigma-Aldrich), 77mm x 77mmx 97mm (W x L x H) were joined with Magenta® vessel coupler (Sigma-Aldrich) and served as experimental microcosms (Fig 2-4). They were equipped at the bottom with a 10 mm thick drainage mat (Enkadrain ST, Schoellkopf AG, Zurich, Switzerland). Microcosms were filled with about 300 g of soil CC (see § 2.1, bulk density 0.90g cm^{-3}).

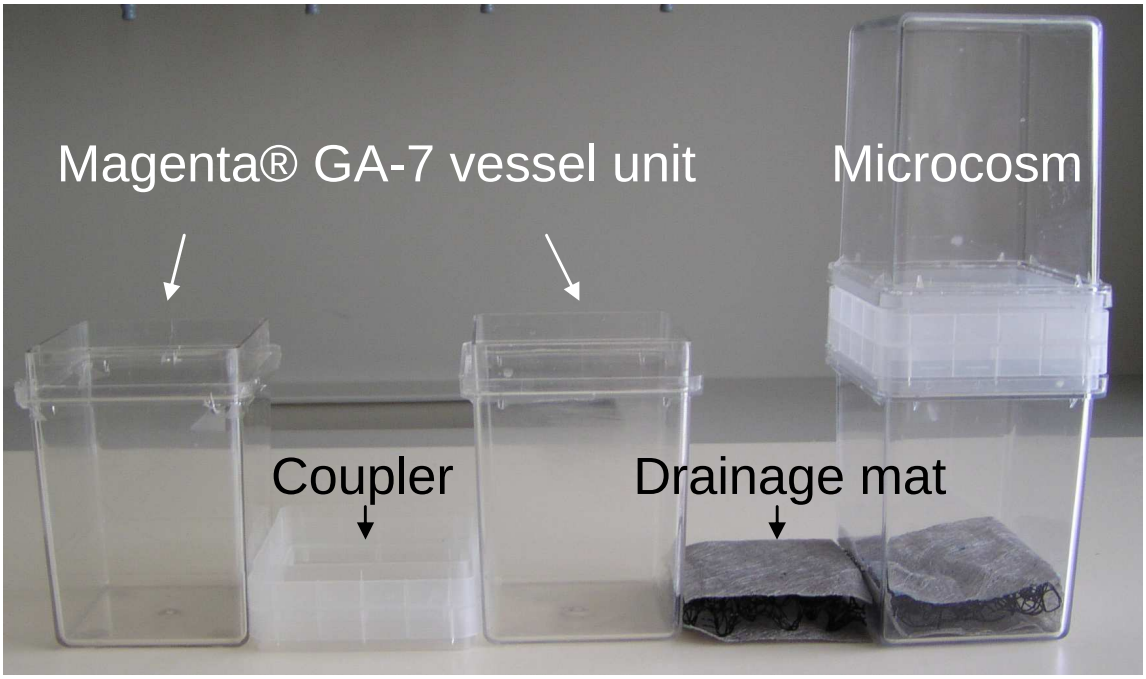


Fig 2-4: Microcosm GA-7 vessel

2.4 Microorganism counts

2.4.1 Bacteria

In order to determine the number of aerobic heterotrophic bacteria present in microcosms after the different soil sterilization treatments (Chapter 3), about 2 g of soil taken from the core of a microcosm were put in a sterile mortar and mixed with 20 ml of sterile modified Neff's amoeba saline solution (NMAS, Page 1988). This soil suspension was ten-fold serially diluted in modified Neff's amoeba saline and spread in triplicate onto Angle agar dishes (Angle *et al.*, 1991). Colony Forming Units (CFU or number of bacterial

colony per gram of dry soil) of aerobic heterotrophic bacteria were counted after one week of incubation at room temperature.

In order to determine the number of aerobic heterotrophic bacteria present in the rhizosphere of *Arabidospsis thaliana* (Chapter 4) or Barley (Chapter 6), the washed roots suspensions (corresponding to the rhizosphere soil fractions) or the soil suspension 10% (w/v) of control microcosm without plant were mixed using a kitchen blender for 1 minute. The suspensions were ten-fold serially diluted in NMAS and spread onto 1/10 TSA. Colony forming units (CFU) of aerobic heterotrophic bacteria were determined after 48 H of incubation at 23℃.

2.4.2 Protozoa

The total number of protozoa present in microcosms after soil sterilization treatment (chapter 3) or in the rhizosphere soil suspension and control soil suspension (Chapter 4 and 6) was determined by a modified Most Probable Number (MPN) method using three fold dilutions of soil suspension in tryptic soy broth medium 0.1 $g.l^{-1}$ (Rønn *et al*., 1995) in microtiter plates (Darbyshire *et al*., 1974). The microtiter plates were placed in darkness at 18 ℃ and protozoa were observed and enumerated aft er 7 and 21 days of incubation with an inverted microscope. The most probable numbers of protozoa per gram of dry soil were calculated using MPN calculator® Build 23 by Mike Curiale, available at http://members.ync.net/mcuriale/mpn/index.html.

2.4.3 Nematodes

Nematodes were extracted by a modified Baermann method according to Techau *et al.* (2004) as follows: about 5 g of roots with adhering soil were put onto two layers of cotton wool filters that were placed in PVC rings (10 cm diameter) and supported by a grid with a mesh size of 2 mm. The rings with filters and grid were placed in glass bowls filled with sufficient water (approx. 100 ml) to keep the surface of the roots moist during the extraction. Extraction was stopped after 48 h and nematodes were counted immediately with a binocular microcosm. Samples were fixed in formaldehyde (4%, 80 ℃) and nematodes were assigned to feeding groups (Yeates *et al.*, 1993).

2.5 Molecular biology

2.5.1 DNA and RNA extraction

DNA extraction

DNA extraction and purification were performed on about 0.5 g of soil material or 500 µL of soil suspension or filters. A bead-beating apparatus (FP120 FastPrep™ cell disruptor, Savant Instruments, Inc., Hotbrook, NY) was used in combination with the FastDNA Spin Kit for Soil (MP biomedicals, Illkirch, France) according to the manufacturer's protocol. The final DNA extracts was stored at - 20℃ before use.

RNA extraction

From sampling until cDNA synthesis, all RNA handling was performed under RNase-free conditions. Aqueous solutions were treated with 0.1% diethyl pyrocarbonate (DEPC). Glassware was heated to 200℃ overnight and plastic material soaked overnight in a 0.1 N NaOH/1mM EDTA solution, before rinsing with RNase-free water. The working area and materials reserved for RNA handling were treated with RNase-AWAY solution (Molecular BioProducts Inc., San Diego, CA). Total RNA were extracted and purified using combination of FastRNA tubes with Green Caps (MP biomedicals, Illkirch, France) and RNeasy® Plant Kit (Qiagen AG, Basel) according to Jossi *et al.*, 2006 as follows: In each FastRNA™ tube containing about 500 mg of frozen sample, 450 ml of RLT Buffer (Qiagen) were added. The mixture was shaken for 10 s at $6ms^{-1}$ using the FastPrep™ cell disruptor. This step was repeated once after cooling tubes for 5 min on ice. The samples were put on ice between the extraction steps. The tubes were then centrifuged for 5 min at 13000 g and the supernatant was loaded on QIAshredder Spin Columns (Qiagen) and then processed as recommended by the manufacturer. DNA was removed using DNase (Qiagen) according to the manufacturer's protocol. The final RNA extracts were eluted in 100 µl of 10mM Tris pH=7.0, and stored at - 80 ℃ before use.

2.5.2 PCR

PCR of the V3 region of 16S rRNA gene from DNA templates

The forward 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse 520r (5'-ATTACCGCGGCTGCTGG-3') universal primers (Ovreas *et al*., 1997) were used for amplification of the V3 region of the 16S rRNA gene. A 40-bp GC-clamp (Muyzer *et al*., 1993) was added on the forward primer for the DGGE experiments (see § 2.5.3). The PCR reaction mix contained (final concentrations) 1X Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM of each primer (MWG Biotech AG, Ebersberg, Germany), and 0.05U μl^{-1} of Taq DNA polymerase (Promega, Switzerland). A total of 5 µl of DNA extract were added as template for the PCR. The final reaction volume was adjusted to 50 µl with nanopure sterile water. The reaction mixtures were then subjected to 31 amplification cycles. Cycles consisted of heat denaturation at 94 ℃ for 1 min, primer annealing at 65 ℃ for 30 s with a touchdown of 1 ℃ per cycle for ten first cycles, and extension at 74 ℃ for 1 min. The mixture was maintained at 74 ℃ for 10 min for the final extension. The PCR products were checked for size and yield with an electrophoresis on 1% agarose gels in comparison to the Low DNA MassLadder (Invitrogen).

Reverse transcription of total RNA

Reverse transcription reactions were performed using ImProm-II™ Reverse Transcription System (Promega, Switzerland) with random hexamer primers in a thermocycler model PTC-200 (MJ Research Inc., Watertown, MA). A total of 3.5 µl of RNA extract was mixed with 1 µl of primers (10mM), and 0.5 µl of RNasins Ribonuclease Inhibitor. This mixture was incubated at 70 ℃ for 5min for an optimal contact between RNA and primers, and chilled on ice until the reverse transcription mix was added. This mix was then combined with (final concentrations) 1X ImProm-II™ Reaction Buffer, 0.05U μl^{-1} RNasin, 6mM $MgCl_2$, 0.5mM each dNTP, 5% (v/v) ImProm-II™ Reverse Transcriptase and DEPC-treated nanopure water in a final volume of 20 µL. The reaction consisted of annealing at 25 ℃ for 5min, extension at 42 ℃ for 1 h and inactivation of reverse transcriptase at 70 ℃ for 15 min. The resulting cD NA was used immediately for PCR or stored at -20 ℃. Positive and negative control rea ctions were performed as recommended by the manufacturer.

PCR of the V3 region of 16S rRNA gene from cDNA templates

PCR amplification of the V3 region of 16S rRNA gene from cDNA templates was performed in two steps. The whole 16S rRNA gene was first amplified using the forward GM3f (5'-AGAGTTTGATCMTGGC-3') and the reverse GM4r (5'-TACCTTGTTACGACTT-3') Bacteria primers (Muyzer and Ramsing, 1995). The PCR reaction mix contained (final concentrations) 1X Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM of each primer (MWG Biotech AG, Ebersberg, Germany), and 0.05U µl-1 of Taq DNA polymerase (Promega, Switzerland). A total of 2 µl of cDNA extract were added as template for the PCR. The final reaction volume was adjusted to 20 µl with nanopure sterile water. The reaction mixtures were subjected to 26 amplification cycles in a thermo-cycler. The first heat denaturation step was performed at 94 ℃ for 4 min 30 s. Cycles consisted of heat denaturation at 94 ℃ for 1 min, primer annealing at 56 ℃ for 30 s with a touchdown of 1 ℃ every 2 cycles for a total of ten cycles, and extension at 74 ℃ for 1 min. The mixture was maintained at 74 ℃ for 10 min for the final extension. The forward 338f with a 40-bp GC-clamp and reverse 520r universal primers (Ovreas *et al*., 1997) were used for nested amplification of the V3 region of the 16S rRNA gene of the first 16S rRNA PCR product to increase the amplification yield and to obtain a fragment size suitable for DGGE analysis. The nested-PCR was prepared and performed as for PCR of the V3 region of 16S rRNA gene from DNA templates described in § 2.5.2.

2.5.3 DGGE

DGGE protocols

Denaturing gradient gel electrophoresis analysis of 16S rRNA genes and cDNA amplicons were performed using the D-code electrophoresis system (Bio-Rad Inc., Hercules, CA). About 600 ng of PCR products were loaded directly on a 8% (w:v) polyacrylamide gel (acrylamide-bisacrylamide 37.5:1) with a linear gradient from 30% to 60% denaturants (100% correspond to 40% formamide plus 7M urea). The strains used to build the reference DGGE pattern migrated as follows from top to bottom of the gel: *Pseudomonas fluorescens* ATCC 27663, *Bacillus subtilis* ATCC 14893, *Rhizobium meliloti* DSM 1981, *Flavobacterium capsulatum* DSM 30196, *Arthrobacter globiformis* DSM 20124, *Thermus filiformis* NCIMB 12588 and *Thermus thermophilus* DSM 579. The gels were run at 60 ℃ and 150 V for 5 h in 1X TAE buffer. They w ere stained with 0.01% SYBR Green

(Molecular Probes, Leiden, the Netherlands) in 1X TAE at 4 ℃ in the dark for 20 min, then UV photographed with the Multi-Analyst package (Bio-Rad).

Gel image analysis

The gel images (TIFF files 8 bits) were resized at 500 pixels height (from the top of the wells to the bottom of the gel), converted in positive and analyzed using GelCompar II software (Applied Maths, Kortrijk, Belgium). The process of analyzing a gel starting with track definition: for a gel with 16 wells, the thickness of each lane for the densitometric curves computation was defined at 15 pixels wide. For each defined lane the GelCompar compute a densitometry curve corresponding to the black intensity of pixel from top to bottom of the lane (.i.e. each band in the gel give a peak in the curve). Bands of each lane and for all DGGE gels in analysis were assigned regarding the densitometry curve, i.e. each band are referenced with a position in pixel starting from base of the well and with a pixel relative intensity express in %. This density corresponds to the surface area under the band peak in the densitometry curve relatively to the sum of surface area of all band peaks in a profile. Then to be able to compare profiles coming from different gels, all the gel images were normalized regarding the band positions of the reference patterns for each gel. The gel images were analyzed without background subtraction and bands whose intensity contribution was below 1% were discarded. The fingerprints were compared with a band position tolerance defined at 0.5% corresponding to 2.5 pixels (maximal distance between two bands to consider them identical). Possible changes in the presence/absence or in the variation of intensity of a single band were analyzed among profiles. The bands similarities between banding patterns, taken in pairs, can be expressed as a percentage value of a similarity coefficient such as Jaccard or Dice coefficient, or a distance coefficient such as Euclidean measure (Fromin *et al*., 2002). Clustering technique: Unweighted Pair Group Method with Arithmetic mean (UPGMA) was applied using the Dice coefficient with the aim of identifying the samples in a dendogram representation which generate similar patterns. The fingerprints obtained were also codified in a numerical matrix in terms of migration length and relative intensity (see above) which it used to perform statistical analyses.

Statistical analyses

Analysis of DGGE input information present some limitations fully detailed in Fromin *et al*., 2002, e.g. (i) only the most abundant population are revealed by the PCR-

DGGE method, (ii) amplified products coming from different population can co-migrate in same position in a gel or an amplified product from a single population can give two different bands. Consequently DGGE analysis do not permit an absolute diversity analysis of a community but still a powerful method to relatively compared complex community structure from different sample., biases were the same for all. We analysed DGGE patterns considering each band as a single bacterial population and the band intensity as corresponding to the relative abundance of the corresponding population in the community (Fromin *et al*., 2002).

Richness of the profiles (S) was revealed by the number of detectable bands and Shannon diversity index (H') was calculated from the numerical matrix as $H' = -\Sigma pi \ln(pi)$ where pi represents the relative abundance of one given population in the profile. The evenness index (E) was calculated as $E = H' / H'max = H' / \ln S$. Average values of indexes were calculated for the different treatments. Then differences between indexes were statistically validated using the Student's t-test.

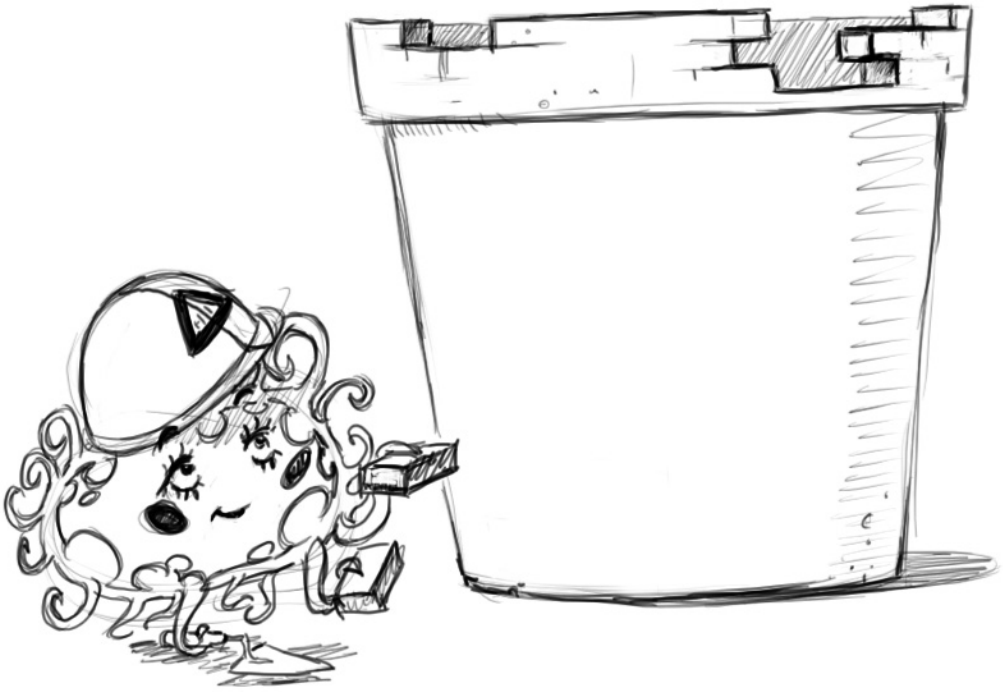
Ordinations methods were performed with the R software 2.5.0 (R development core team 2007) using vegan package. Data were initially submitted to variance partitioning analysis to display variability of the patterns by factors of interest. Two or three sets of explanatory variables were employed according to the respective treatments of the different experiments (table 2-1). The significance of the results was tested with the Monte Carlo permutation test. Data were also submitted to principal component analysis (PCA). This analysis is a mathematical technique that allows multivariate data to be characterized by a smaller number of variables. PCA generates new variables, called principal components (linear components of the original variables), which explain the highest dispersion of the samples (Fromin *et al*., 2002).

Table 2-1: Explanatory variables for variance partitioning analysis

	Explanatory variables		Description					
Chapter 3	1st	Sample:	Soil suspension	Prefiltrate	Filter	Filtrate		
	2nd	Solvent:	HMP	Water				
	1st	Incubation:	1 week	4 weeks	8 weeks			
	2nd	Solvent:	HMP	Water				
Chapter 5	1st	Inoculum:	Bacteria	Protozoa	Soil			
	2nd	Soil Fraction:	Rhizosphere soil	Bulk soil				
	1st	Inoculum:	Bacteria	Amoeba	Flagellates	Ciliates		
	2nd	Presence of protozoa:	Yes	No				
	3rd	Species:	AC	NG	BD	NJ	CX	CS
Chapter 6	1st	Nitrogen fertilization:	High	Low				
	2nd	Clipping leaves:	Yes	No				
	3rd	Growth period:	14 days	28 days	42 days			

Chapter 3

Development of microcosms to investigate plant and soil microbe interactions



Chapter 3

Development of microcosms to investigate plant and soil microbe interactions.

Preliminary to a study of microbial-plants interaction in soil, the first part of this work focused on the development of microcosms in order to study the impact of protozoa on plant growth. The first step was to find an effective soil sterilisation method. The first part of Chapter 3 presents the investigations of the effect of soil sterilisation in particular two physical methods (autoclaving and gamma ray irradiation) on soil characteristics that might influence plant and microorganisms growth. The second step was the production of a protozoa-free suspension presenting bacterial communities representative of the source soil. The second part of Chapter 3 presents the study of the bacterial community structure in the protozoa-free bacterial suspension and the establishment of the bacterial community from the protozoa-free suspension in a sterilised soil. The last step was the purification of protozoa culture in axenic condition to limit perturbation on the reassembling complex microbial community.

3.1 Evaluation of different soil sterilisation methods for microbiota-plant interactions in microcosm studies

3.1.1 Introduction

Plant development is strongly affected, positively and negatively, by the presence of soil microbiota, particularly bacteria, protozoa and fungi. To test for the effect on plant growth of pure or mixed defined microbial populations or of selected microorganisms fraction, the soil should be sterilized in a way which minimizes the deleterious effects of the treatments. In general, complete soil sterilization is only achieved by using small amounts of soil distributed in shallow layers (Trevors, 1996). However, direct soil sterilization in the microcosm allows to decrease considerably the risk of post-contamination.

There are many soil sterilisation methods described in the literature. The review by Trevors (1996) on sterilisation and inhibition of microbial activity in soil describe the most method applied: Autoclaving at 121°C, 1.1 atm; Gamm a ray irradiation at 10-70 kGy (20kGy will eliminate the majority of soil bacteria, 70kGy may be required to kill certain radio-resistant, McNamara *et al*., 2003); Microwaves 2450 MHz; Gaseous fumigation (chloroform, methyl bromide, ethylene or propylene oxide); Chemical (Mercuric chloride 500mg $HgCl_2$ /kg soil, Sodium azide 1-10%). The method applied will depend on the objective of the experiment, in a way which does not alter the experiment results.

Unfortunately numerous changes in soil characteristics after sterilisation process have been detailed. Possible effect of autoclaving are changes in soil structure (Alef and Nannipieri, 1995) and particularly a decrease of clay active surface by releasing clay-associated aluminium and potassium ions (Jenneman *et al.*, 1986). Contrary to gamma-ray irradiation, this would decrease the ion exchange and buffering capacity of clay-humic complex. Aluminium liberation is particularly susceptible to occur in acidic soils (pH 5.0) and autoclaving of such soils would not be suitable for plant growth, due to aluminium toxicity and decrease of phosphate availability by precipitation of insoluble aluminium (Rout *et al.*, 2001). This study aims to develop an efficient sterilization method eliminating soil microbiota and their resistance form (spores and cysts) and allowing to test the effect of the presence / absence of grazing protozoa (from soil community or single strain) in interaction with rhizosphere bacteria on microbial community structure and plant growth.

3.1.2 Material and Methods

Sterilisation treatments

We discarded chemical methods to avoid the impact of chemical residues on the microbial communities and plant growth. Two physical methods of sterilization were chosen: autoclaving and gamma-ray Irradiation (^{60}Co source). Autoclaving presents the advantage to be inexpensive and readily available in most laboratories. The main advantage of Gamma-ray irradiation is that minimal disturbance of soil occurs during the process (Alef and Nannipieri, 1995). The gamma-ray irradiations were performed by Studer AG Werk Hard (Däniken, SO, Switzerland).

Experimental design

Microcosms GA7 were filled with about 300g of soil CC (Chapter 2).

-Three soil microcosm replicates (noted A) were autoclaved firstly at 121℃ during 30 min with a pressure of 1.1 bar. A second autoclaving were done after 48H in the same conditions to kill spores which could germinate after the first autoclaving.

-Three soil microcosm replicates (noted I) were gamma-ray irradiated (^{60}Co source) with 35-60 kGy (unit of absorbed dose) irradiation range.

-Three soil microcosm replicates (noted AI) were autoclaved as for treatment A and then gamma-ray irradiated as for treatment I.

-Three non sterilised soil microcosms were also performed as controls (noted C).

After treatments, sterilised soils were sampled to check the absence or presence of protozoa and bacteria according to methods described in Chapter 2. Sterilized and control (not sterilized) soils were analysed for the following characteristics: soil moisture, pH [H_20], pH [KCl], Total Organic Carbon (TOC), mineral Carbon (minC), NH_4^+, NO_3^-, available P contents.

Physicochemical parameters analysis

Soil moisture content was evaluated by the loss on drying technique at 105℃. pH measurements (pH [H_20], pH [KCl]) were done.

Soils were air-dried and finely crushed with agate system. 50mg of each sample were analysed by Rock-Eval 6 (Re6) pyrolysis (Behar *et al.*, 2001) in order to characterise total organic and mineral carbon in soil samples. NH_4^+ and NO_3^- were extracted from 25g

of soil with 100 ml of KCl 15%. The soil suspension was mixed one hour in a rotary shaker and centrifuged 10 min at 3000 rpm. The supernatant was taken and the pellet was resuspended with 50ml of KCl 15% and mixed during half hour in a rotary shaker and centrifuged 10 min at 3000rpm. The two supernatants were filtrated on paper filter (17 $^{1}/_{2}$ Schleicher and Schuell AG, Riehen, Switzerland) and pooled in a 250ml volumetric flask. Filtrate solution were adjusted to 250 mL with deionised water. The distillation and the trapping of NH_4^+ and NO_3^- were done by a Kjeldahl oxidation using a Büchi B-323 system (Laboratoriums-Technik AG, Flawil, Switzerland).

Available P was analysed according to Olsen *et al.*, (1954) and was determined colorimetrically at 880 nm using the blue molybdate procedure (Murphy and Riley, 1962).

Statistical analyses

Soil data obtained from pH [H_2O], pH [KCl], TOC, minC, NH_4^+, NO_3^-, available P contents analyses of control and sterilized soil were checked for normality using Shapiro-Wilk test and for homogeneity of the variance using Bartlett test. Effects of each treatment on the chemical and physical characteristics of the soil were compared using variance analyses (ANOVA). Tukey Kramer HSD post hoc test were used to separate differences among the treatments. Statistical analyses were done with R 2.5.0 (R development core team 2007).

3.1.3 Results

Autoclaving (A), Irradiation (I) and combined (AI) treatments had differential effects on soil microbiota and chemical characteristics (Table 3-1). A few culturable bacterial cells resisted to A and I treatments, whereas the AI combination allowed a complete sterilization of the soil. Soil moisture was significantly decreased by A and AI treatments, but not by I alone. pH [H_2O] decreased significantly after A and I treatments, but not after their combination. pH [KCl] increased moderately after A and AI treatments and decreased slightly after I treatment. Whatever the treatment, soil total organic carbon content (TOC) decreased significantly, more (22%) after A treatment than after I and AI treatments (both 12%). Mineral C content was not significantly affected by any of the treatments. The most dramatic change concerned the ammonium content which was increased 12, 8, and 16-fold by A, I, and AI treatments, respectively. However the nitrate and available phosphate content was not significantly affected.

Table 3-1: Mean values of soil characteristics measured before and after sterilisation treatments.
C (control), A (autoclaved), I (irradiated), AI (autoclaved and irradiated) treatments.
Different letters (^{a,b,c,d}) indicate that sterilisation treatments are statistically different.

	Unity	C		A		I		AI	
Total aerobic heterotrophe bacteria	CFU/ g dry soil	$1.91\ 10^{7}$	$\pm 5.3\ 10^{5}$	9.15	±4.52	3.92	±3.92	0	
Total protozoa	MPN protozoa /g dry soil	$1.48\ 10^{5}$	$\pm 2.\ 10^{3}$	0		0		0	
Soil moisture	%	14.9^{a}	±0.2	11.4^{b}	±0.2	$14.2^{a,c}$	±0.1	$12.7^{b,c}$	±1.3
pH [H_2O]		8.00^{a}	±0.01	7.87^{b}	±0.03	7.76^{c}	±0.04	$7.93^{a,b}$	±0.07
pH [KCL]		7.33^{a}	±0.01	7.38^{b}	±0.01	7.31^{c}	±0.01	7.41^{d}	±0.01
Total Organic Carbon	%	0.797^{a}	±0.032	0.621^{b}	±0.027	0.700^{c}	±0.019	0.704^{c}	±0.008
mineral Carbon	%	0.179^{a}	±0.027	0.172^{a}	±0.013	0.165^{a}	±0.014	0.169^{a}	±0.009
NH_4^+	mg N (NH_4^+) / kg soil	2.6^{a}	±0.4	31.4^{b}	±1.7	20.9^{c}	±2.7	42.0^{d}	±7.0
NO_3^-	mg N (NO_3^-) / kg soil	9.0^{a}	±4.4	12.9^{a}	±7.0	11.7^{a}	±4.2	19.0^{a}	±6.6
Phosphate available	mg P / kg soil	78.9^{a}	±1.6	72.8^{a}	±2.1	81.4^{a}	±8.4	75.1^{a}	±11.0

3.1.4 Discussion

All three sterilisation methods tested here allowed to remove all protozoa but only the AI treatment efficiently killed all culturable aerobic bacteria. This study shows that autoclaving decreases the soil water content contrary to gamma-ray irradiation. In autoclaving soil, it is more suitable to adjust to desired water content after sterilization for example together with the re-inoculation.

Soil pH is an important factor for the establishment of microbial communities. Fierer and Jackson (2006) compared paired sampling locations with similar vegetation and climate but very different soil pH, and found evidence for the strong correlation between bacterial diversity and soil pH. In order to reproduce in microcosms the bacterial communities present before the sterilisation treatment, soil pH have to be closed of the control soil. Separate autoclaving treatments decreased moderately the actual acidity (pH [H_2O]), whereas the exchange acidity (pH [KCl]) varied less than 0.1 pH units. Surprisingly, the combined treatment did not affect the pH [H2O]. A decrease in pH value was shown by Salonius *et al.*, (1967), Shaw *et al.,* (1999) in a clayed soil and they attributed the pH decrease during autoclaving to the release of organic acids. On the other hand, gamma-ray irradiation could create reactive free radicals (H·, OH·), which could in turn induce the cleaving of C-C bonds (Tuominen *et al.*, 1994). As in AI treatment, irradiation followed autoclaving, it may be hypothesized that acidic substances released by autoclaving would subsequently be cleaved by irradiation.

The slight decrease in soil organic carbon after the treatments is probably not significant as far as rhizosphere studies are involved, because the organic content of soil is quite low (Nannipieri *et al.*, 2003) with respect to the high nutrient flux generated by rhizodeposition (Whipps and Lynch, 1986).

The most striking change measured was the strong elevation in NH_4^+ content. This was already noted by McLaren (1969) and by Alef and Nannipieri (1995) and may be attributed to deamination reactions from necromass and amino-acids. Most of the released NH_4^+ ions would be taken up by microorganisms upon re-inoculation. Grazing protozoa will be then the main responsible for secondary ammonium liberation (Clarholm, 1985) allowing the less competitive N-uptake plants (Hodge *et al.*, 2000) to benefit from it.

3.1.5 Conclusion

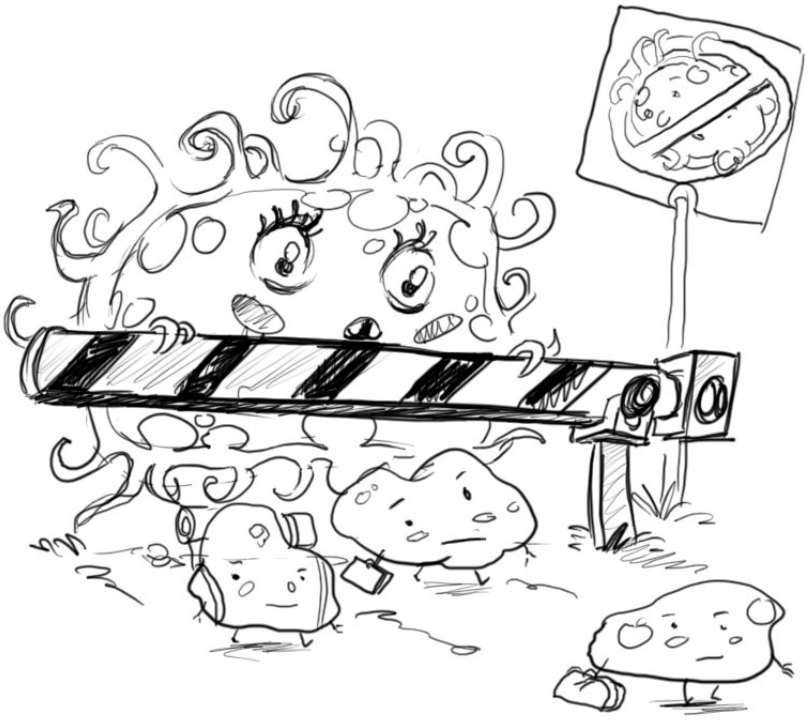
Table 3-2 present the influences of each treatment on biological and physicochemical soil characteristics compared to control soil. We defined a notation which gives one point to the respective sterilisation treatment when presence of bacteria or protozoa was observed and when the sterilisation treatment have a significant effect on the physicochemical soil characteristics compared to control soil. The best chosen sterilisation method was one which presented the lowest score.

Table 3-2: Evaluation of the soil sterilisation method. A (autoclaved), I (irradiated), AI (autoclaved and irradiated) treatments.
0= no significant effect on soil characteristics and absence of microorganisms.
1= significant effect on soil characteristic and presence of microorganisms.

TREATMENT	A	I	AI
Absence of Bacteria	1	1	0
Absence of protozoa	0	0	0
% Moisture	1	0	1
pH [H_2O]	1	1	0
pH [KCl]	1	1	1
TOC	1	1	1
minC	0	0	0
Organic Matter	0	0	0
NH_4^+	1	1	1
NO_3^-	0	0	0
Phosphorus	0	0	0
TOTAL	**6**	**5**	**4**

After these analyses, we retained autoclaved-irradiated sterilisation method to study the impact of protozoa on plant growth and rhizosphere bacterial community.

3.2 Reassembling complex microbial communities in sterilized soils to study plant-microbial interactions



3.2.1 Introduction

Plant roots continuously interact with a multitude of microorganisms and the soil food web. A mechanistic understanding of microbial interactions can only be achieved by careful manipulation of the microbial communities. Although knowledge of the factors that govern competitive interactions in natural microbial communities is sparse. Microbial communities can be re-assembled from microbial cultures. However, since only a small fraction of soil microorganisms can be cultured (Torsvik *et al.*, 1990; Amann *et al.*, 1995), this approach is of limited use. Soil defaunation procedures on the other hand, such as chloroform fumigation, target the whole microbial diversity and are non-selective. Concomitant with killing soil fauna, crucial microbial taxa, such as nitrifyers are harmed

with increasing exposure to chloroform (Griffiths *et al.*, 2000). Often one needs to conserve the maximum natural microbial diversity, for example when the competitive strength of specific microorganisms in a natural community is being assessed or when grazing preferences of bacterivores are investigated. Microbial grazers in the rhizosphere control bacterial biomass, turnover and community composition (Alphei *et al.*, 1996, Griffith *et al.*, 1999, Ronn *et al.*, 2002). Protozoan populations are denser in the rhizosphere than in the bulk soil (Zwart, 1994) and represent the second trophic level of the bacterial energy channel which is mainly fuelled by rhizodeposition. In particular protozoa have been shown to release essential nutrient for plant growth from consumed microbial biomass (Clarholm, 1985). Grazing ultimately leads to the redistribution of mineral nutrients, such as N to plant (Kuikman *et al.*, 1989; Alphei *et al.*, 1996).

A precise understanding of the functional importance of rhizosphere bacterial grazers on plant growth and on the control of bacterial community structure requires controlled condition, e.g. protozoa-free controls, which are only feasible in microcosm experiments. Ideally, the protozoa-free bacterial suspension should allow reproducing a community structure similar to that of the native soil. The aim of this study was to obtain a protozoa-free soil suspension from the native soil and to compare the bacterial community structure obtained after inoculation with that of the native soil.

3.2.2 Material and Methods

Production of bacterial protozoa-free suspension by filtration

The soil CC (Chapter 2) was mixed for 1 minute in a kitchen blender with physiological sterile water (φ-Water, 0.85% NaCl) or a 0.2% sodium hexametaphosphate sterile solution (HMP, dispersing agent) to obtain soil suspensions at 10% (w/v). The larger soil particles of the suspensions were removed by filtration on filter paper (512½, Schleicher and Schuell AG, Riehen, Switzerland). These pre-filtrates were then subsequently filtered through either 3 µm or 1.2 µm pore size filters, respectively to remove protozoa. To check for the absence of protozoa, 5 ml of the filtrates in triplicates were incubated in cell culture flasks (Nunc, Roskilde, Denmark) filled with either 10 ml Modified Neff's Amoeba Saline solution (NMAS, Page, 1988) or with 10 ml nutrient broth (Biolife, Milan, Italy) (0.8 g/l) in NMAS (NB-NMAS). Cultures were checked after 2, 7 and 21 days of incubation at 18°C in darkness.

Counts of cultivable heterotrophic aerobic bacteria of the initial soil suspensions, pre-filtrate and filtrates at 3 and 1.2 µm pore size were determined according to method described in Chapter 2. Bacteria counts were analysed with the student's test on log transformed data. Bacterial cells from the 1.2 µm filtrates were concentrated on 0.2 µm pore size membranes (Millipore, Billerica, USA). These 0.2 and 1.2 µm filters and the initial soil suspensions were submitted to DNA extraction in order to perform V3-16SrDNA PCR and DGGE analyse according to methods described in Chapter 2.

Inoculation of sterile soil

Microcosms GA7 were filled with about 300g of soil CC (Chapter 2) and were autoclaved according the treatment AI (Chapter 3.1). Sterilised soil microcosms were inoculated with 5ml of 1.2 µm φwater or HMP filtrates. The re-inoculated microcosms were sampled after 1 week, 1 month and 2 months incubation at room temperature in darkness and submitted to DNA extraction to evaluate potential changes in bacterial community structure by DGGE analyses (Chapter 2). These samples were also checked for the presence/absence of protozoa by a most probable number method according to Chapter 2.

3.2.3 Results

Checking of the presence/absence of protozoa

We checked filtrates according to the pore size of the filters and the re-inoculated soil microcosms after 1 week, 1 month and 2 months for the presence/absence of protozoa. Flagellates were observed but neither ciliates nor amoebas were found in part of the 3 µm pore size filtrate. Filtrates at 1.2 µm pore size were devoid of protozoa. Upon inoculation with this filtrate of sterile soil, no protozoa were found after 2 months of incubation period.

Bacterial counts

The prefiltration with filter paper decreased the number of CFU in the φwater and HMP prefiltrate by 2 orders of magnitude (Fig 3-1). The filtration at 1.2 µm pore size further decreased the number of CFU by 2 orders of magnitude more. The number of bacterial CFUs did not differ between the two filtered solvents.

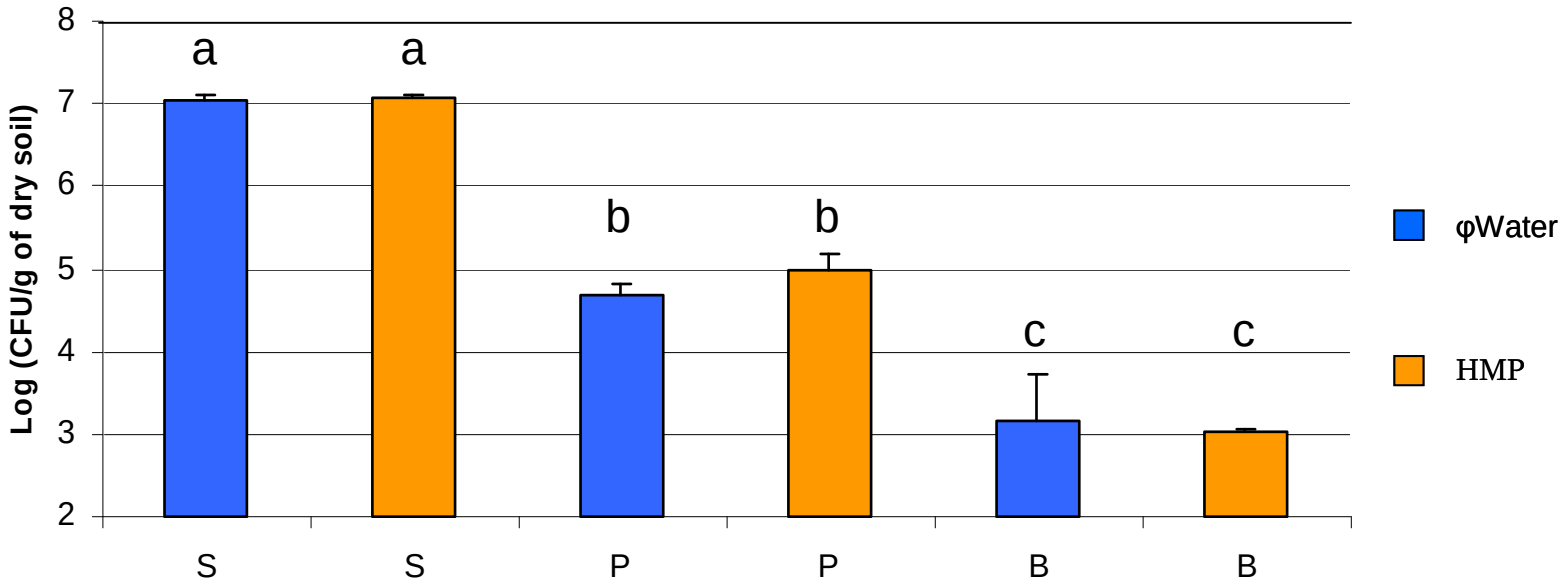


Fig 3-1: Log of CFU of total heterotrophic cultivable aerobic bacteria per g of dry soil in the different filtration steps.
S = Soil suspension, P= Prefiltrate, B= protozoa-free bacterial suspension. Bars in blue correspond to the φWater filtration samples and bars in orange correspond to the HMP filtration samples.
Different letters (a,b,c) indicate that CFU counts are statistically different.

Bacterial community structure in the protozoa free bacterial suspension

DGGE fingerprints from filtration samples presented a similarity of over 70% and were grouped in two main clusters (Fig 3-2a). The first cluster contained all soil suspension samples (S) except one. The bacterial diversity in the native soil and more extend soil suspension were similar to the soil aggregates retained on the filter paper (data not shown). The second one contains all 1.2 µm pore size filtrate samples (B). Some bands are more intense before or after filtration indicating that filtration process changed the structure of bacterial communities. Actually the bands 1, 2, 3 and 4 (Fig 3-2b) present a higher intensity in the protozoa-free bacterial suspension (B) and more extended prefiltrate (P) compared to soil suspension samples (S). The bands 5 and 6 present a higher intensity in the soil suspension (S) Prefiltrate (P) and 1.2 µm filters (F) compared to B. Filtrations did not change significantly the richness (numbers of bands ranged from 34 to 41), the diversity (Shannon index from 3.55 to 3.69) and the evenness index (from 0.98 to 0.99). The evenness index in soil suspension samples was significantly higher in HMP soil suspension samples. Variation partitioning analyses on data obtained from DGGE fingerprints from φWater and HMP filtration samples showed that 22.1% of the variability of DGGE profiles was explained by the filtration process ($p \leq 0.001$, Monte Carlo test). The solvents (φwater vs HMP) had no significant effect and explained only 5.6% of the variation.

Establishment of bacterial protozoa-free suspension in a sterile soil

DGGE fingerprints from re-inoculated soil samples presented a similarity of over 55% and grouped in a same cluster except for 2 samples corresponding to the HMP bacterial protozoa-free suspension after an incubation of 1 week (Fig 3-3a). Bands 1, 2, 3, and 4 that were intense on 1.2 µm pore size filtrate fingerprints, and Bands 5, 6 that were intense on soil suspension fingerprints, were sometimes present on the re-inoculated sterile soil samples (Fig 3-3b). Diversity index (Shannon) values (Fig 3-4) increased over the incubation period and did not differ between the two solvents. Evenness index values ranged from 0.96 to 0.98 did not differ between the two solvents and incubation periods (not illustrated). Variation partitioning analyses on data obtained from DGGE fingerprints from re-inoculated soil samples showed that 22.6% of the variability of DGGE profiles were explained by the incubation period (not significant) and 22.6% ($p \leq 0.001$, Monte Carlo test) by the solvents (φwater vs HMP). 54.8% of the variability was unexplained.

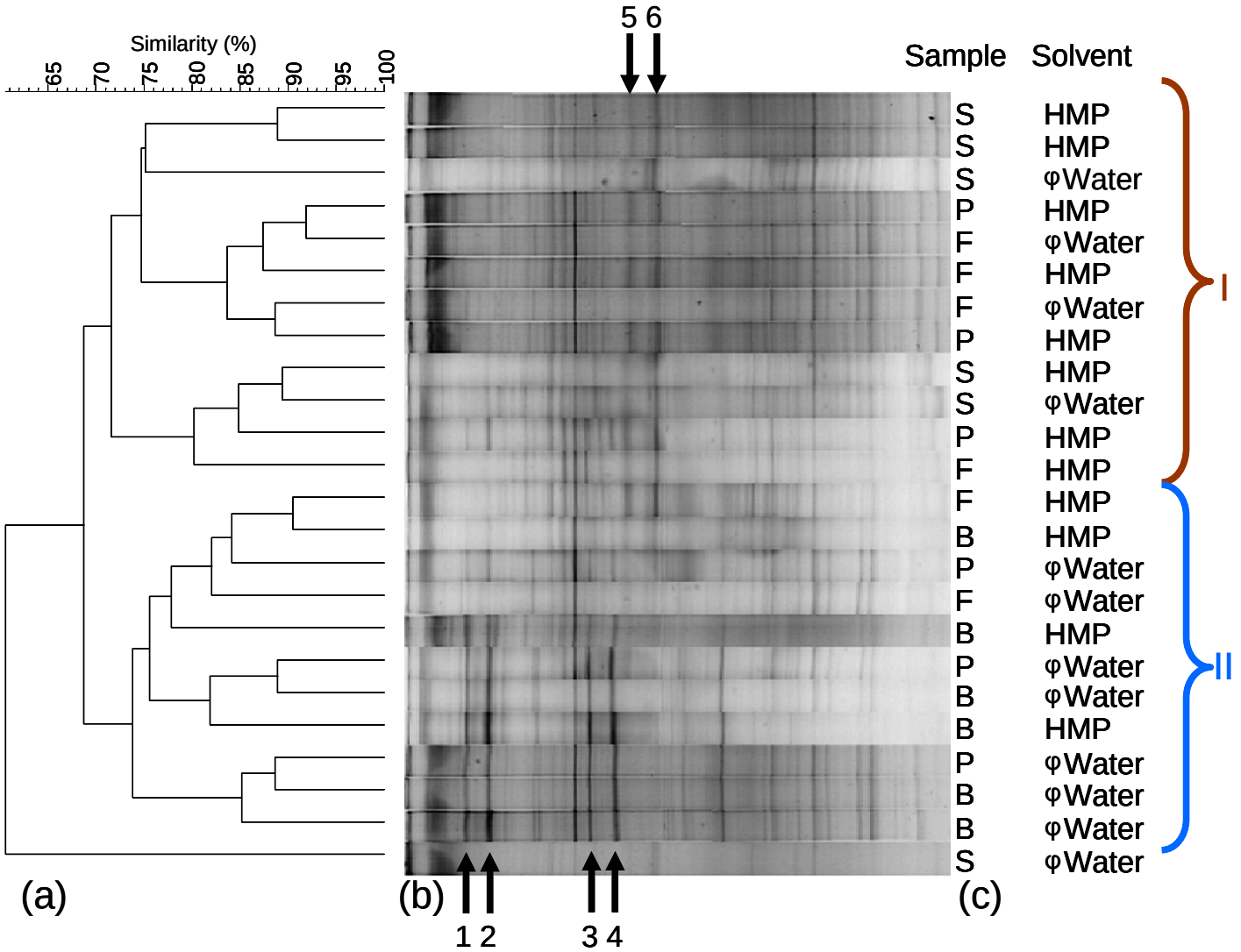


Fig 3-2: (a) Dendogram of DGGE fingerprints from filtration samples, constructed on the Dice similarity coefficient using Unweighted Pair Group Method (UPGMA).

(b) DGGE fingerprint of V3-16SrDNA gene fragment amplified from DNA extract of different filtration step samples. The bands (1, 2, 3...) marked with arrow are bands that respond differently in the different filtration steps.

(c) Sample identification table: S= Soil suspension, P= Prefiltrate, F= Filter 1.2µm pore size, B= Bacterial protozoa-free suspension, φWater= Physiological water, HMP= Sodium Hexametaphosphate.

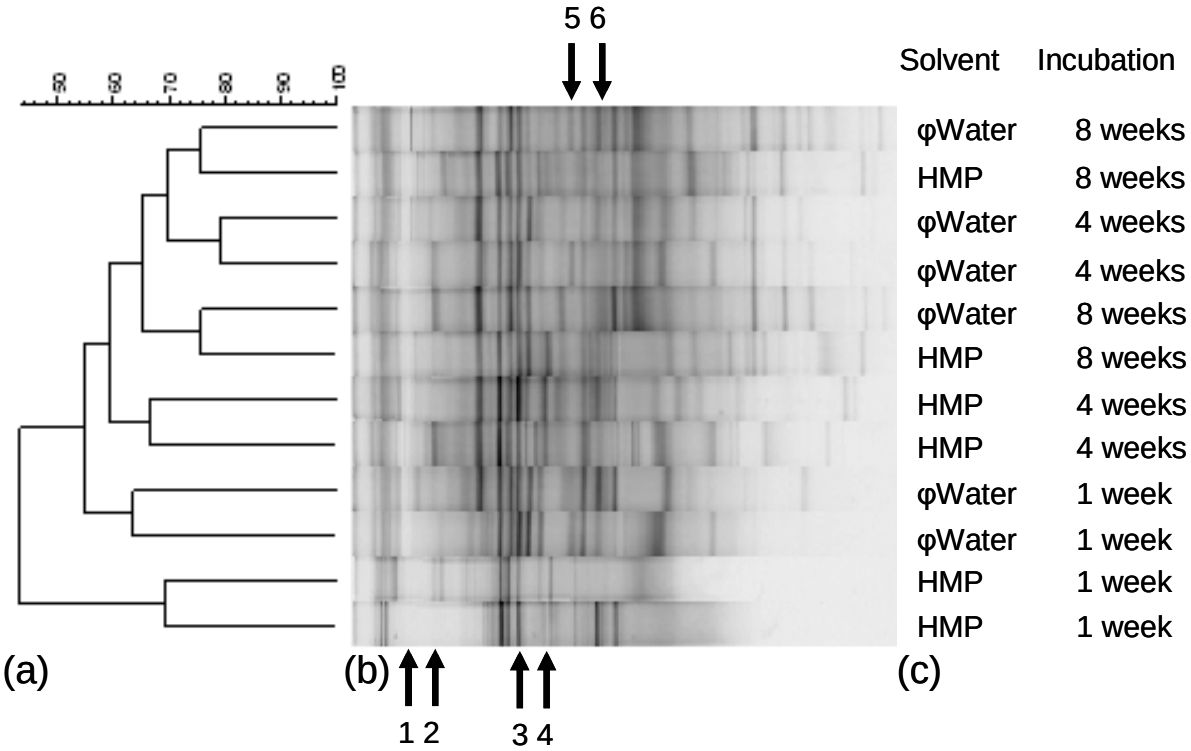


Fig 3-3: (a) Dendogram of DGGE fingerprints from re-inoculated sterile soil samples, constructed on the Dice similarity coefficient using Unweighted Pair Group Method (UPGMA).

(b) DGGE fingerprints of V3-16SrDNA gene fragment amplified from DNA extract of different re-inoculated sterile soil samples. The bands (1, 2, 3...) marked with arrow are bands that respond differently in the different filtration steps (see Fig 3-2).

(c) Sample identification table.

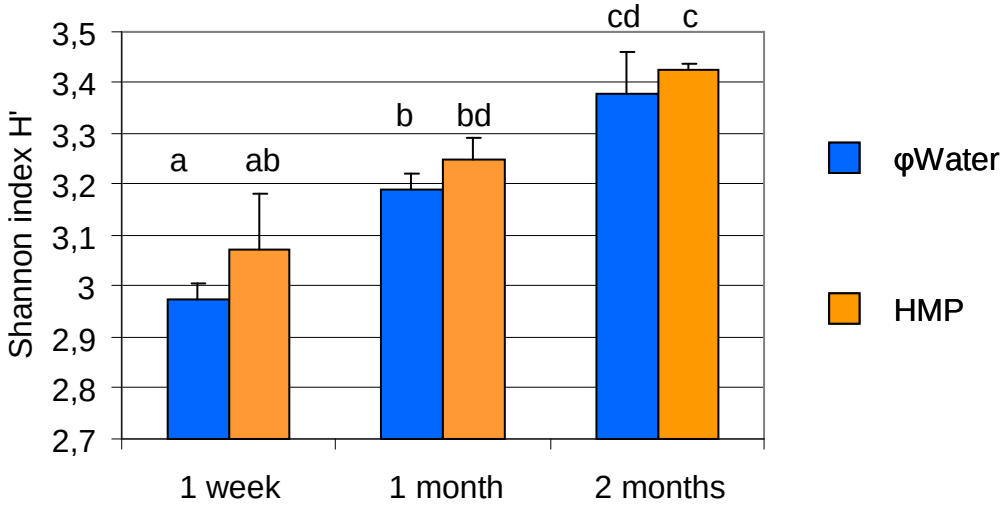


Fig 3-4: Shannon index (H'=-Σpi*lnpi) of DGGE fingerprints obtained from re-inoculated sterile soil with filtrate 1.2 µm after an incubation period. Blue bars correspond to φWater filtrate inoculum and orange bars to HMP filtrate inoculum. Different letters (a,b,c,d) indicate that Shannon index are statistically different.

Principal component analysis (Fig 3-5) showed that DGGE fingerprint of soil re-inoculated samples converged with time incubation towards soil suspension samples. Sterile soil re-inoculated with soil suspension after 3 weeks of incubation (data from Chapter 5) are closer to soil suspension than protozoa-free suspension samples after 2 months. The Shannon index (Fig 3-6a) and the richness ranged from 22 to 43 were significantly different between re-inoculated soil with the bacterial protozoa-free suspension (φwater and HMP) and re-inoculated soil with the soil suspension. The re-inoculated soil with the bacterial protozoa-free suspension (φwater and HMP) presented an evenness index (Fig 3-6b) significantly lower than source soil suspension. The diversity and richness in re-inoculated soil with the φwater bacterial protozoa-free suspension were lower than source soil suspension. No significant difference for indexes and richness was observed between source soil suspension and sterile soil inoculated with soil suspension.

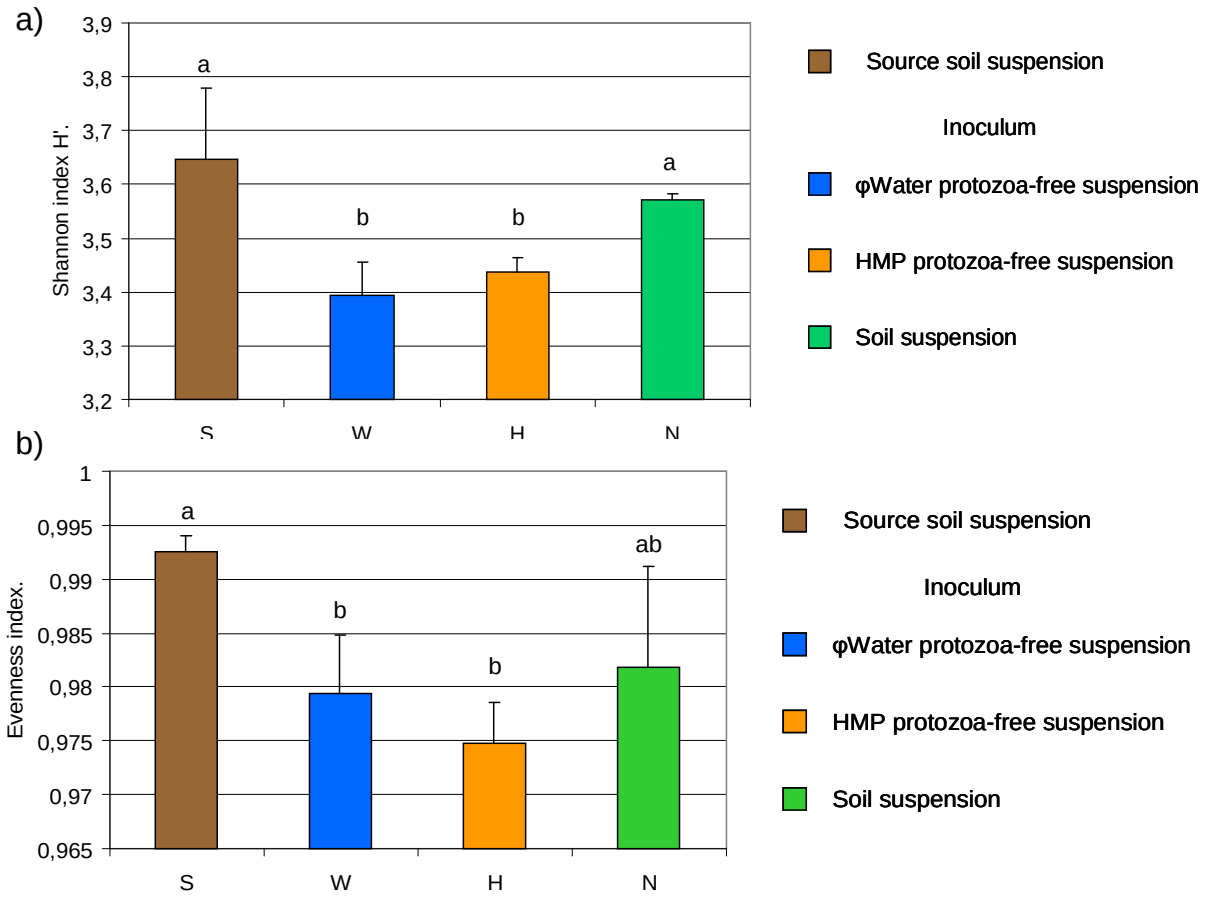


Fig 3-6a, b: Comparison of Shannon index (a) and comparison of evenness index (b) of DGGE fingerprints obtained from re-inoculated sterile soils and source soil suspensions (S). W = φwater bacterial protozoa free inoculum after 2 months, H = HMP bacterial protozoa free inoculum after 2 months, N= soil suspension inoculum after 3 weeks.

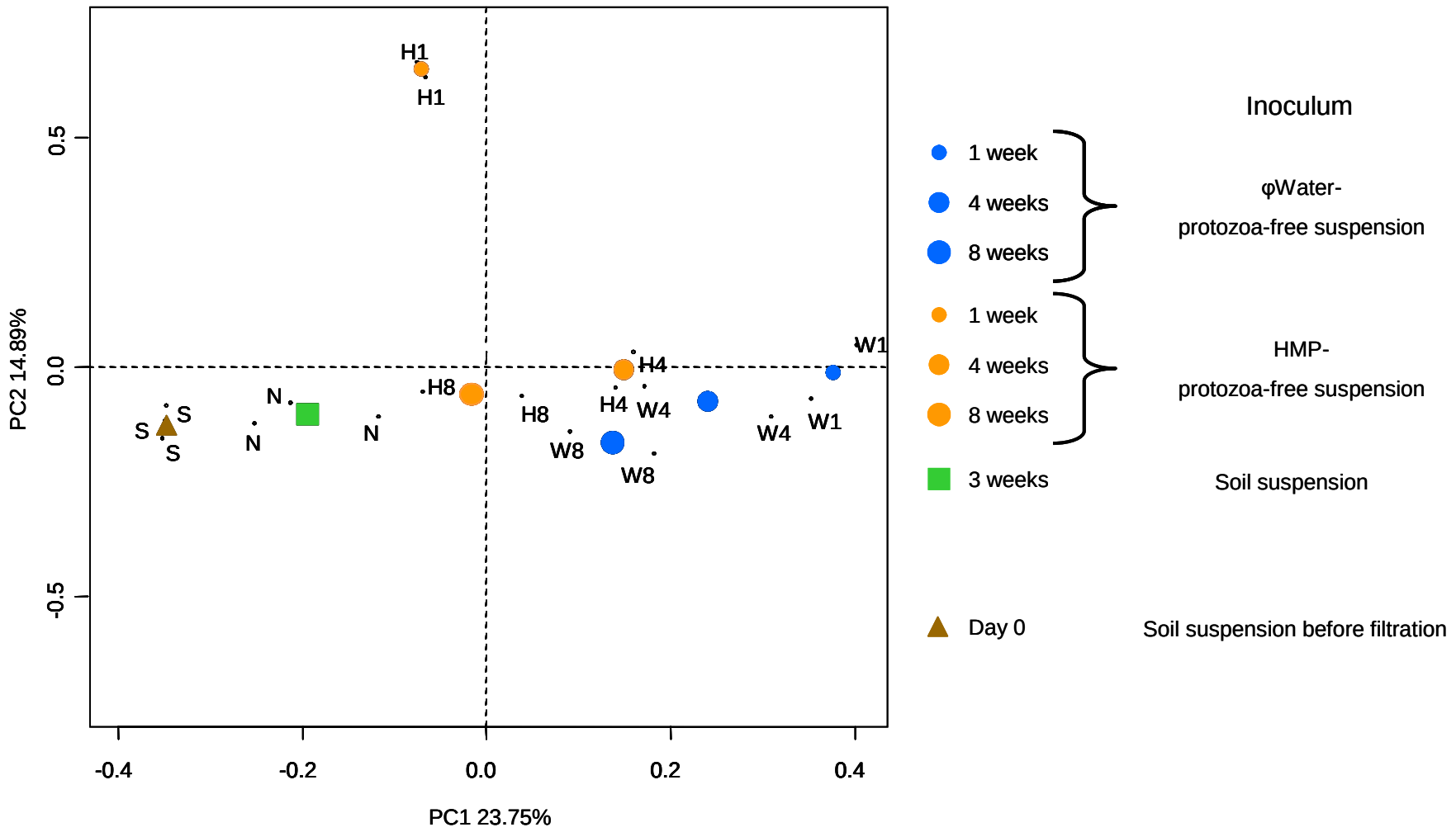


Fig 3-5: Plot of the principal component analysis (PCA) with centroïds corresponding to DGGE fingerprints from sterile soil re-inoculated with bacterial protozoa-free suspension (Circle symbols). Samples from φWater filtration are illustrated as blue circles those from HMP filtration as orange circles. Increasing size of circle symbols correspond to the incubation period of bacterial protozoa-free suspension in sterile soil (1 week, 4 weeks, 8 weeks). The square symbol corresponds to the centroïd of soil samples re-inoculated with soil suspension after 3 weeks of incubation (Data from Chapter 5). The triangle symbol corresponds to the centroïd of soil suspension (φWater and HMP) samples.

3.2.4 Discussion

Protozoa-free bacterial suspension could be obtained by the two elution methods tested (φWater soil suspension vs HMP soil suspension). The size of most soil protozoa is between 3-250µm (Gobat *et al.*, 2004) nonetheless Winding *et al.*, 1997 reported the presence of smaller flagellates (2-3 µm in diameter) in soil. Frey *et al.*, (1985) collected microfauna of a soil suspension by filtration with different pore size filter and observed only small flagellates in 3 µm pore size filtrate, small amoebas and flagellates in 5 µm pore size filtrate, and small ciliates, amoebas and flagellates in 8µm pore size filtrate. Indeed only the presence of small flagellates was observed in 3µm filtrates. To remove the soil protozoa, filtration at 1.2 µm pore size is therefore required.

The distribution of soil microorganisms in soil is heterogeneous due to the complex spatial arrangement of soil aggregates and the variety of physical and chemical conditions (Chenu and Stotsky, 2002). The size of soil bacteria is generally close to 1 µm, but some bacteria are bigger or may form clusters like chains or microcolonies or maybe strongly aggregated to soil particles. The numbers of CFU in the prefiltrate suspension were 2 orders of magnitude lower than in the soil suspension. Filter paper retained soil aggregates and consequently a high number of bacteria cells. The addition of phosphate anions (pyrophosphate, HMP) to soil suspensions is known to better disperse bacteria and decrease the degree of bacteria retention on the surface of solid particle. (Khammar *et al.,* 2004; Chang and Yen, 1985). No difference was observed in the numbers of CFU from 1.2 µm φwater and HMP filtrates. However the filtration at 1.2 µm pore size was more difficult with the HMP prefiltrate and filter was changed several times to filtrate the same φwater prefiltrate volume. Indeed, the prefiltrate from the HMP soil suspension contains more small soil particles which block the filter pore easier than the prefiltrate from the φwater soil suspension.

Solvents (φWater vs HMP) seem to have no clear effects on the bacterial community structure in the protozoa-free bacterial suspension. No significant differences were observed for the Shannon and evenness index between filtration step samples. Nonetheless, the evenness index was significantly higher in HMP soil suspension compared to φWater due to the dispersing effect of this solvent. This index measures how equal the populations are numerically and in our case revealed that the abundance of the bacterial populations present in the different filtration step samples is similar. The diversity (Shannon index) present in the bacterial protozoa-free suspension is close to the suspension soil. However we observed few bands responding differently on the DGGE fingerprints and consequently the structure of the bacterial communities was changed.

It is particularly difficult to keep with soil sterilisation the whole chemical and physical soil characteristics (Trevors, 1996). Autoclaving releases organics acids (Salonius *et al.*, 1967), Gamma-ray irradiation creates reactive free radicals (hydrogen, hydroxyl) that could cleave carbon-carbon bonds (Tuominen *et al.*, 1994) and the combination of both increase the NH_4^+ content in soil (Chapter 3.1). Soils are generally poor in nutrient and energy sources (Nannipieri *et al.*, 2003). Furthemore we observed in Chapter 3.1 a lower Total Organic Carbon (TOC) content after soil sterilisation. In this study, no carbon was added on the sterile soil, the activity and growth of bacteria in the re-inoculated soil was limited by the availability of carbon substrate present after sterilisation. In the re-inoculated soil, one can expect that r-strategist colonize the soil first and used the most available organic compounds. K-strategists will later colonize this soil in function of their competitive activity. The establishment of the protozoa-free bacterial suspension in the sterile soil was affected by the solvents used for its production particularly at the beginnings. This difference is probably due to the complexing effect towards cations susceptible to link bacteria with soil aggregates surfaces through cationic bridge. In agreement with the works of Marschner and Rumberger (2004), the Shannon and evenness index were lower that the source soil and increased with time. Bands 1-2-3-4 which displayed a higher intensity in the 1.2 µm pore size filtrates, were sometimes found with a lower intensity on the DGGE fingerprints of the re-inoculated soil but no tendency were observed on the establishment of these corresponding populations in the re-inoculated sterile soil. Bands 5-6 which presented a higher intensity in the soil suspension and were absent or present with a low intensity in the 1.2 µm pore size filtrate were found on the re-inoculated DGGE fingerprints suggesting that the bacterial populations correlated to these bands are competitive in this soil. The diversity of bacterial communities present after 2 months in the re-inoculated sterile soil with bacterial protozoa-free suspension (φWater and HMP) was lower than the diversity present in the source soil suspension. Nonetheless, the bacterial communities present on the re-inoculated sterile soil from HMP and φWater bacterial protozoa-free inoculum seem to converge over time towards the bacterial communities of the source soil and after 2 months the difference in diversity index of bacterial communities in microcosms inoculated with HMP bacterial protozoa-free inoculum were no-longer significant. Moreover sterile soils re-inoculated with soil suspension are closer and presented a similar diversity to soil suspension samples. Theses results suggested that re-inoculated sterile soil rebuild over time their own native bacterial community structure.

3.2.5 Conclusion

The protozoa-free bacterial suspension obtained by filtration at 1.2µm pore size presents a diversity similar to that of the source soil. Used as a control bacterial inoculum, the protozoa-free bacterial suspension could allow to better understand the plant-microbiota interactions in microcosm studies. The establishment of this bacterial inoculum in sterile microcosms showed a significant lower diversity than soil suspension. However the diversity increased during the incubation period and was no longer significantly different after 2 months between sterile soil re-inoculated with HMP bacterial protozoa-free suspension and soil suspension. No significant difference of diversity was observed between solvent on the establishment of bacterial communities after 2 months. In order to limit possible perturbation due to the complexing effect towards cations susceptible to link bacteria with soil aggregates surfaces through cationic bridge, we suggested not to used HMP and instead used only φWater as a solvent for the production of bacterial protozoa-free suspension.

3.3 Purification of protozoa culture in axenic condition

The most soil common amoeba: *Acanthamoeba castellanii* was selected to study the impact of protozoa on plant growth and microbial community structure. *Acanthamoeba castellanii* culture was kindly provided by Prof. Michael Bonkowski from the University of Köln. This culture was purified on nutrient agar (NA, Biolife, Milan, Italia) in order to limit the bacterial community present in the protozoa culture (Fig 3-7). *Escherichia coli* Neu 1006 (Strains collection of the University of Neuchâtel, *E.coli*) were spread onto nutrient agar plate and incubated during 2H at 37℃. Then 10 0µl of *Acanthamoeba castellanii* maintained in NB-NMAS (Nutrient Broth-Modified Neff's Amoeba Saline solution) medium (Page, 1988) were added and spread onto the medium and incubated 48H at room temperature. An agar cube was extract from this culture and displayed on a new NA plate, previously incubated at 2h at 37℃ with a straight line of *E.coli* Neu 1006 inoculum. The agar cube face presenting amoeba culture was inoculated perpendicular to the extremity of the straight line of *E. coli* and incubated 48H at room temperature. (*Acanthamoeba castellanii* moved along the line to graze *E. coli*). After incubation, an agar cube was extracted from the culture at the opposite extremity of the inoculated site and placed on a new NA plate also inoculated with a straight line of *E.coli* Neu 1006. This procedure was repeated two times, and the last agar cube of amoeba culture was transferred onto NB-NMAS medium.

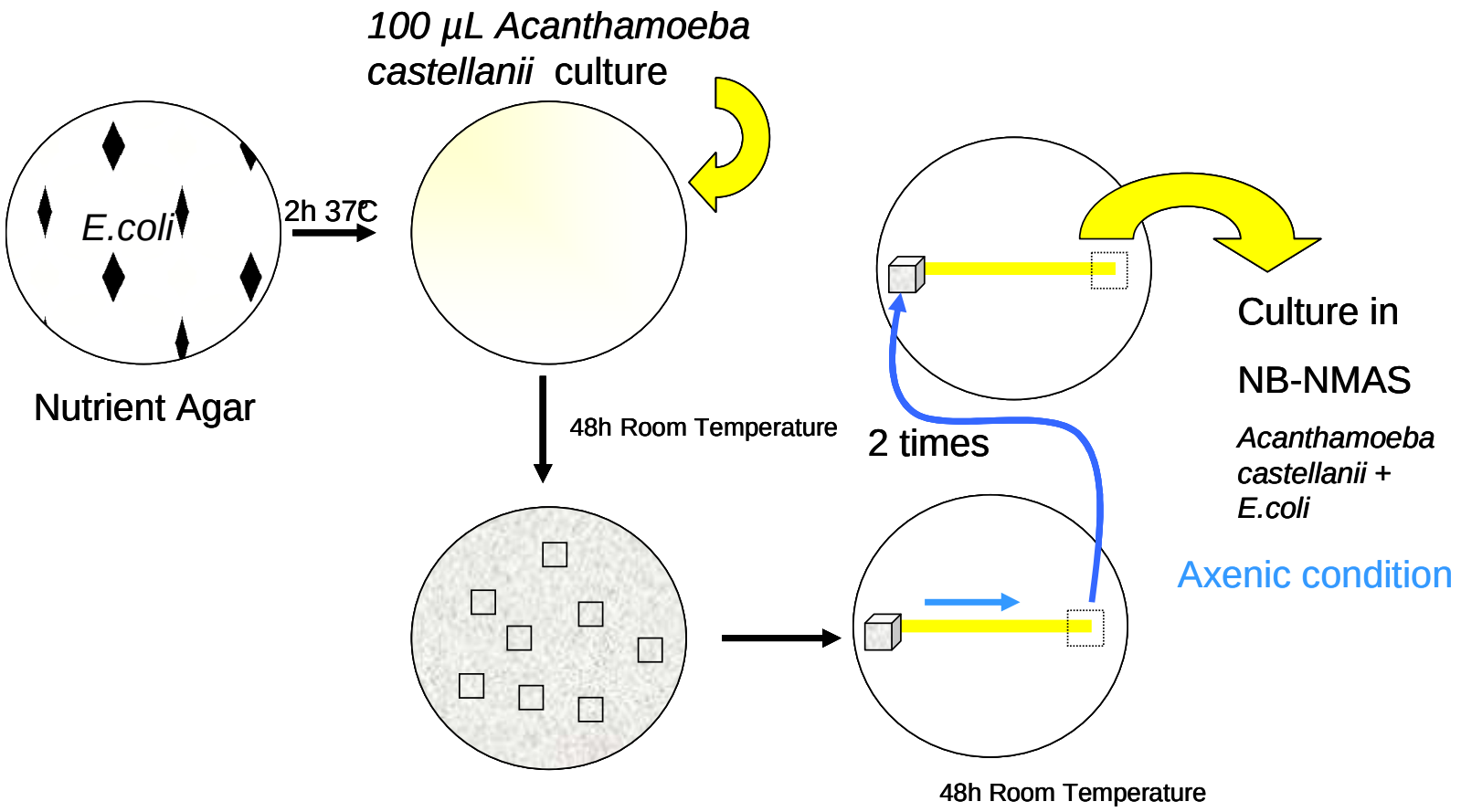


Fig 3-7 Purification of *Acanthamoeba castellanii* culture

3.4 Conclusion

In order to study plant and microorganism interactions, the different methods developed in this chapter (soil sterilistation, the production of a bacterial protozoa-free inoculum, and the purification of *Acanthamoeba castellanii* culture) allow to set up at least three different treatments.

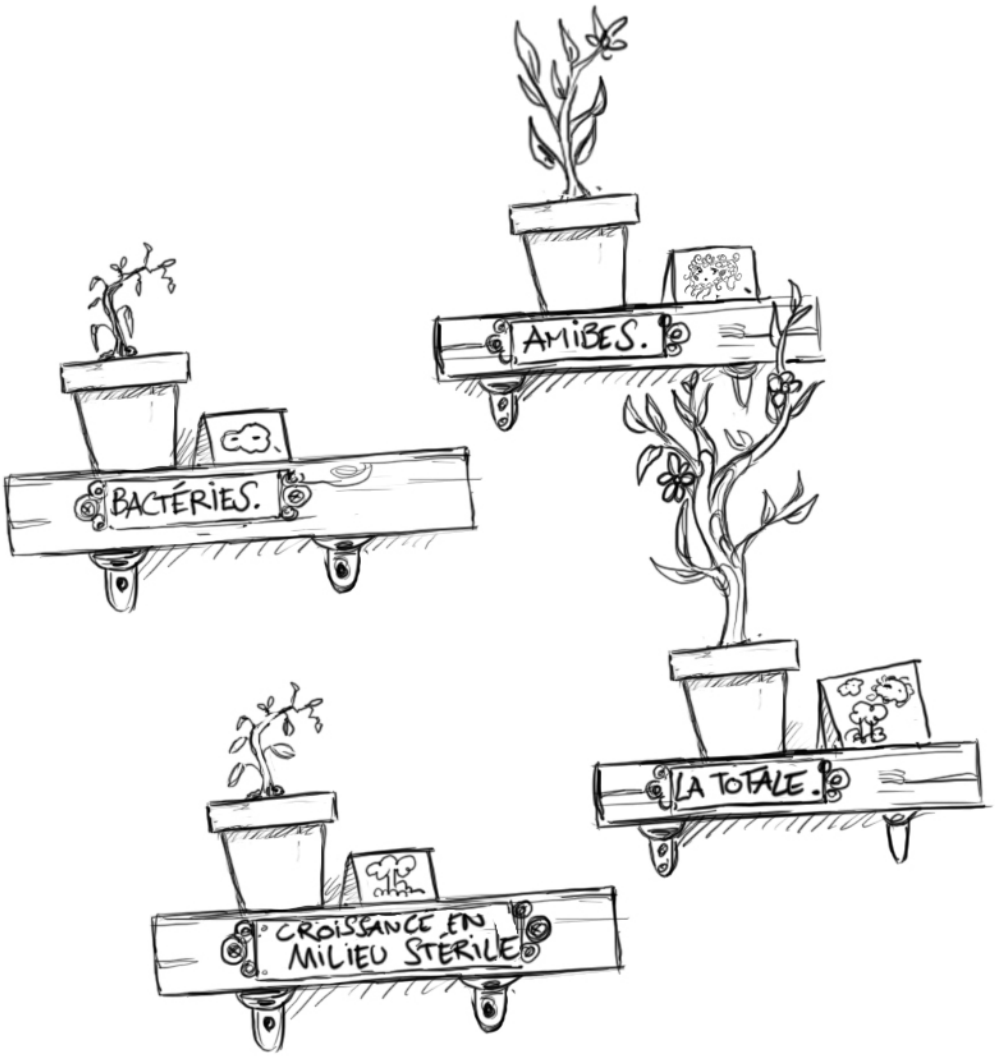
1) Sterile condition (treatment S): sterile soil.

2) Bacteria condition (treatment B): sterile soil inoculated with the bacterial protozoa-free suspension.

3) Protozoa condition (treatment P): sterile soil inoculated with the bacterial protozoa-free suspension and axenic culture of *Acanthamoeba castellanii*.

Chapter 4

Effect of *Acanthamoeba castellanii* on *Arabidopsis thaliana* growth



Chapter 4

Effect of *Acanthamoeba castellanii* on *Arabidopsis thaliana* growth

A precise understanding of the functional importance of rhizosphere bacterial grazers on plant growth requires controlled condition, e.g. protozoa-free controls, in microcosm experiments. The microcosms developed in chapter 3 were used. This chapter describes the effect of the most soil common amoeba: *Acanthamoeba castellanii* on *Arabidopsis thaliana* growth in controlled microcosms.

4.1 Introduction

Plant roots are one of the major actors in the rhizosphere dynamic, as plant derived compounds (rhizodeposition) released in soil provide abundant energy sources for rhizosphere living microorganisms and is considered as the first link of the soil food web. In mutualism feedback plant also takes benefits from some rhizobacteria (plant promoting growth rhizobacteria, PGPR) which provide hormones for growth, antibiotic against pathogen or help for nutrient acquisition (Whipps, 2001). Nevertheless bacteria are on the short term better competitors than plants for mineral uptake of nutrient in particular for nitrogen (Hodge *et al.*, 2000). Soil N content corresponds to 0.3 to 3 ‰ of dry matter (Gobat *et al*., 2004). Most of nitrogen in soil (92-97%) is present in organic forms (Stevensen, 1986) and between 3 and 20% included in microbial biomass (Williams and Sparling, 1984). Mineral forms of nitrogen in soil (nitrate and ammonium ions) are taken up by plants and are often growth limiting factors. Bacterial density is higher in the rhizosphere than in the bulk soil, and consequently competition for nutrient uptake too. However protozoa graze bacteria and control their density and turnover (Alphei *et al.*, 1996, Griffith *et al.*, 1999, Ronn *et al*., 2002). They use a part of their prey nutrient for biomass production, the excess is assumed to be excreted in soil and becoming available for plant nutrition (Clarholm, 1985, Fig 1-12), such as N to plant (Kuikman *et al*., 1989, Alphei *et al.,* 1996). Protozoa seem therefore to have a positive impact on the plant growth. The impact of the amoeba *Acanthamoeba castellanii* on *Arabidopsis thaliana* growth was assessed in controlled conditions to check if the microcosms developed allow

to study plant and microbe interactions and to evaluate the plant growth response in function of the complexity of the inoculum.

4.2 Material and Methods

Microcosms inoculation and plant growth condition

Microcosms GA7 (Chapter 2) were filled with about 300g of soil CC (Chapter 2) and autoclaved at 121℃, 30 min, 1.1 bar two times with a 48H interval for incubation and subsequently gamma-ray irradiated 60Co according to methods described in Chapter 3.

Ten sterile microcosms were inoculated with 6ml of sterile water for the S (sterile) treatment and 3 ml of soil suspension for the N (natural) treatment. 20 sterile microcosms were inoculated with 3 ml of bacterial protozoa-free suspension according to Chapter 3 for treatments B (Bacteria) and P (Bacteria and protozoa). 3ml of *Acanthamoeba castellanii* culture purified (Chapter 3) were added a week after only in P treatment. We added a total inoculum volume of 6 ml and completed it with sterile water. 4 sterile seedlings of *Arabidopsis thaliana* columbia (Chapter 2) were plant per microcosm. 3 more microcosms per treatments were prepared and not planted as control. microcoms were set up in a phytotron at 20℃ lighted during 16h a day.

Microcosms sampling

Evaluation of the plant developmental stages according to Boyes *et al.*, (2001) was observed for each plant after 22, 43 and 63 days of growth (day 0 corresponds to the seed germination on TSA/10). The applied treatments did not allow the sampling of all plants at the same developmental stage. One half of the microcosms were sampled for destructive analysis on the 43rd day of growth. The shoots were collected and dried at 30℃ during one week for dry biomass determination. Roots with adhering soil were collected then washed in NMAS solution and dried at 30℃ during a week. The washing-roots suspensions (RS fraction) were kept for bacteria and protozoa enumeration. The bacteria and protozoa counts were determined according methods described in Chapter 2 from the washing-roots suspension or soil suspension 10% (w/v) of planted and controls microcosms respectively. Bacteria and protozoa counts were analysed with the student's test on log-transformed data. Soil from planted and control microcosm was kept for determination of soil nitrogen content.

Soil and plant nitrogen content

NH_4^+ and NO_3^- were extracted from 25g of soil with 100 ml of KCl 15%. The soil suspension was mixed one hour in a rotary shaker and centrifuged 10 min at 3000 rpm. The supernatant was taken and the pellet was resuspended with 50ml of KCl 15% and mixed a half hour in a rotary shaker and centrifuged 10 min at 3000rpm. The two supernatants were filtrated on paper filter (17 1/2 Schleicher and Schuell AG, Riehen, Switzerland) and pooled in volumetric flask 250ml. Filtrate solution were adjusted to 250 ml with deionised water. The distillation and the trapping of NH_4^+ and NO_3^- were done by a Kjeldahl oxidation using Büchi B-323 system (Laboratoriums-Technik AG, Flawil, Switzerland).

Total C and N plant content of dried *Arabidopsis thaliana* leaves, previously crushed with an agate mortar, were determined using an element analyser (EA 1108, Carlo Erba instruments, Milan, Italy). Nitrogen (NH_4^+, NO_3^-) soil contents were compared using variance analyses (ANOVA). Tukey Kramer HSD post hoc test were used to separate differences among the treatments. Statistical analyses were done with R 2.5.0 (R development core team 2007).

4.3 Results

4.3.1 Plant growth

Arabidopsis thaliana growth was clearly different in microcosm in function of treatment after 22 days of incubation (Fig 4-1). Compared to Natural (N) treatment, the delay of growth is more important for the plants grown in Sterile (S) condition than plants grown in Bacteria (B) and Protozoa (P) treatments (table1).

Nevertheless the delay of plant growth in the P treatment was lower than in the B treatment. These plant growth delays were accentuated after 43 days of incubation (Table 4-1). An increase of plant mortality rate was observed between the 22nd and the 43rd day of growth in treatments S and B and to a lesser extend in treatment P compared to treatments N (Table 4-2). However the remaining living plants of the P treatment continuated their growth until the flowering stage, even if they grew slower and flowered only after 63 days as compared to plants in the N treatment. These last already presented buds or flowers on the 43rd day of growth.

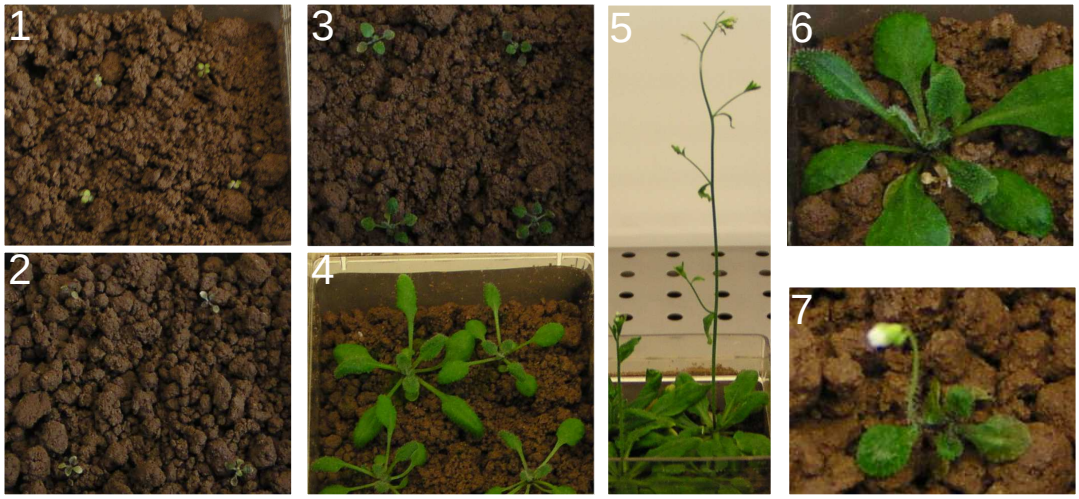


Fig 4-1: Photos of *Arabidopsis thaliana* in microcosms. Photos 1-4 correspond to *Arabidopsis thaliana* after 22 days of growth for Sterile, Bacteria, Protozoa and Natural treatments respectively. Photo 5: Flower production in Natural microcosm after 43 days of growth. Photo 6: Flower Bud emergence in Protozoa treatment after 63 days of growth. Photo 7: Small flower in bacteria treatment after 43 days of growth.

Treatment	*Arabidopsis thaliana* growth stage after 22 days									
	1.02	1.04	1.05	1.06	1.07	1.08	1.09	1.10	5	6
Sterile	100	0	0	0	0	0	0	0	0	0
Bacteria	81.8	18.2	0	0	0	0	0	0	0	0
Protozoa	56.2	39.1	3.1	1.6	0	0	0	0	0	0
Natural	0	10	0	25	2.5	27.5	12.5	22.5	0	0

Treatment	*Arabidopsis thaliana* growth stage after 43 days									
	1.02	1.04	1.05	1.06	1.07	1.08	1.09	1.10	5	6
Sterile	100	0	0	0	0	0	0	0	0	0
Bacteria	77.2	18.2	0	0	0	2.3	0	0	0	2.3
Protozoa	51.5	26.6	0	4.7	1.6	9.4	3.1	3.1	0	0
Natural	0	5	0	0	0	0	0	0	55	40

Table 4-1: Percentage of *Arabidopsis thaliana* plant in different growth stage in function of treatment after 22 and 43 days incubation. According to Boyes *et al.*, 2001: principal growth stage 1 corresponds to the leaf development. The numbers of rosette leaves superior to 1 mm in length are indicated in stage numbers e.g. 1.02 for 2 rosette leaves superior to 1 mm in length. Principal growth stage 5 corresponds to the inflorescence emergence and 6 to flower production.

Treatment	Mortality	
	22 days	43days
Sterile	0	80
Bacteria	20.45	86.36
Protozoa	14.06	74.60
Natural	0	5

Table 4-2: *Arabidopsis thaliana* mortality rate (%) in microcosm in function of treatment.

4.3.2 Bacteria and protozoa counts

Microcosms were sampled after 43 days of plant growth. Microbial analysis of the S treatment microcosms showed that 40% of them presented heterotrophic aerobe cultivable bacteria colony (data not shown). These contaminated microcosms were discarded from the experiment for further analysis. The number of heterotrophic aerobe bacteria per gram of dry weight soil (CFU/g DW soil) in the rhizosphere soil were close to 10^8 regarding of the treatment applied (Fig 4-2). The numbers of CFU/g DW soil were higher in microcosm with plant compared to control, and this was significant in N treatment.

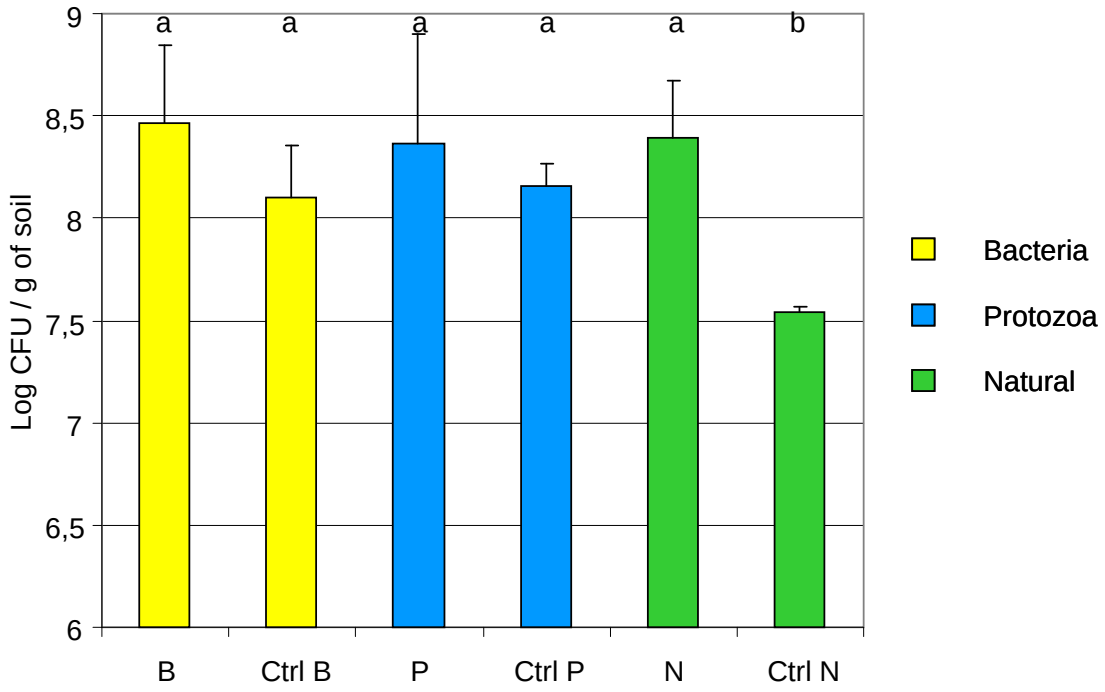


Fig 4-2: Log of CFU of heterotrophe aerobic bacteria per g of soil in function of treatment after 43 days of growth. B= Bacteria, P= Protozoa, N= Natural, Ctrl mean control microcosm without plant. Different letters (a, b) indicate that CFU counts are statistically different.

No protozoa were found in the S and B microcosms (data not shown). As expected, only *Acanthamoeba castellanii* was retrieved in the P treatment, at 10^4 Most Probable Number per g of dry weight soil (MPN/g DW soil). No significant difference was observed between planted and control P microcosms (Fig 4-3). Total protozoa MPN/g DW soil in N treatment (flagellates, ciliates and amoebas) were only 1.5 times higher than in P microcosms where *Acanthamoeba castellanii* was the sole inoculated protozoa. Finally higher protozoa MNP/g DW soil were found in planted vs. control N microcosms.

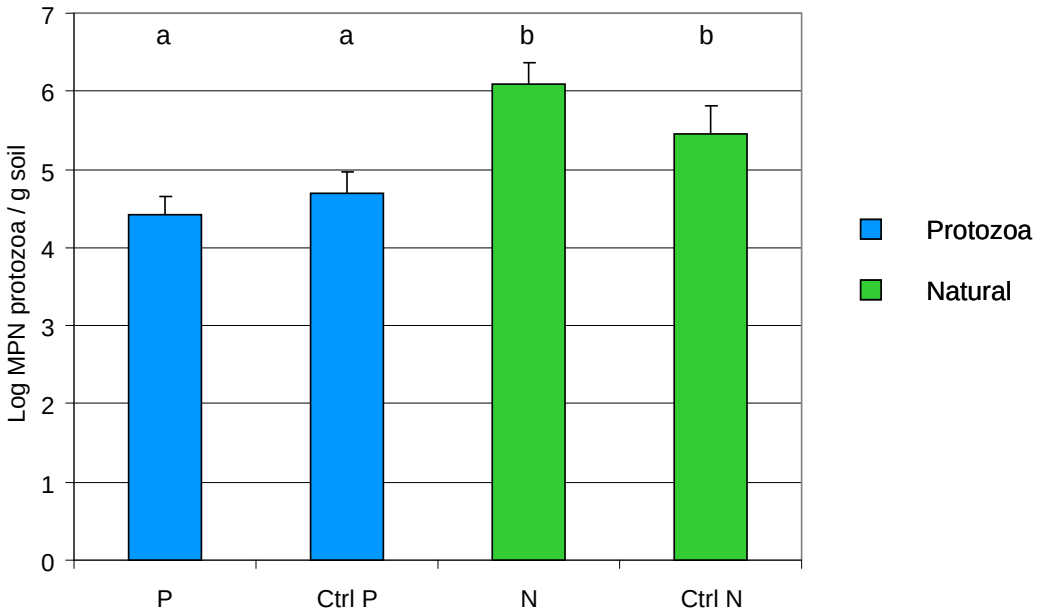


Fig 4-3: Log of Most probable number of protozoa per g of dry weigth soil in function of treatment after 43 days. P = protozoa, *Acanthamoeba castellanii*, N= Natural, total soil protozoa. Different letters (a, b) indicate that MPN counts are statistically different.

4.3.3 Soil Nitrogen content

Soil ammonium content

Whatever the treatment the soil NH_4^+ contents were similar in planted and control microcosms (Figure 4-4a). Compared to S control microcosms the NH_4^+ contents were higher in B and P treatments but not significant, and 5 times lower in N treatment.

Soil nitrate content

In S treatment the NO_3^- concentration in soil was 4 times higher in planted than in control microcosms (Figure 4-4b). This unexpected result could not be clearly explained by a high released of nitrate by plant into the soil because of their small biomass, even if 80% of the plants were dead after 43 days of growth. No differences were observed in soil nitrate content among B, P, and N treatment for both planted and control microcosms.

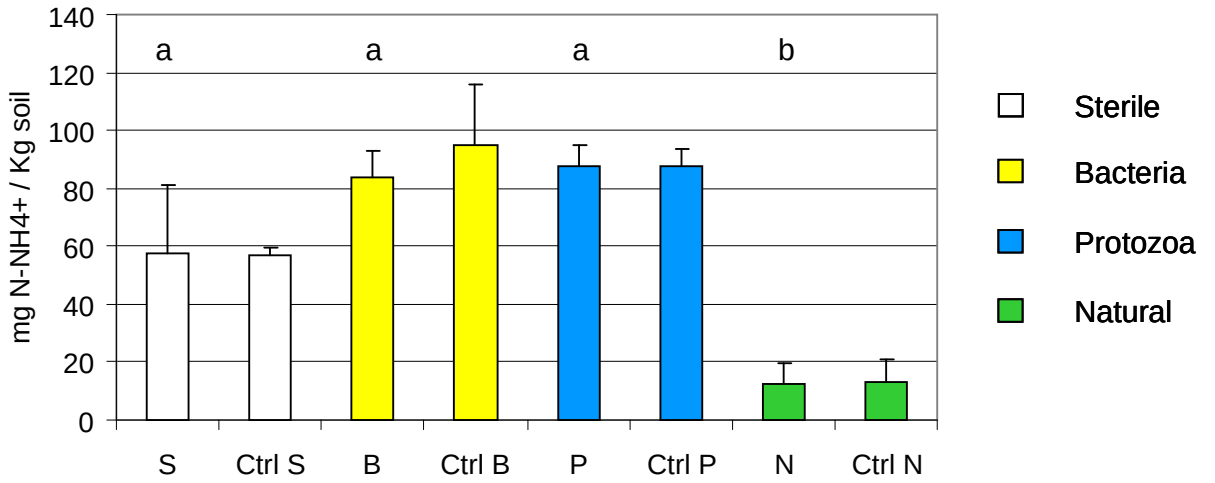


Fig 4-4a: Soil N-NH_4^+ content in function of treatment after 43 days of growth. B= Bacteria, P= Protozoa, N= Natural, Ctrl mean control microcosm without plant. Different letters (a, b) indicate that soil N-NH_4^+ content are statistically different.

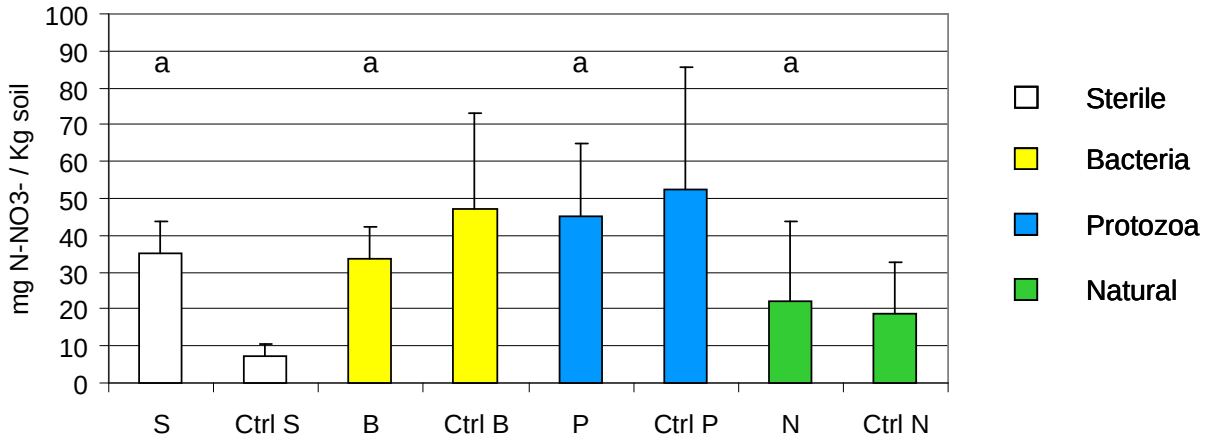


Fig 4-4b: Soil N-NO_3^- content in function of treatment after 43 days of growth. B= Bacteria, P= Protozoa, N= Natural, Ctrl mean control microcosm without plant. Different letters (a, b) indicate that soil N-NO_3^- content are statistically different.

4.3.4 Plant Nitrogen content

Total nitrogen in dried plant leaves was higher in N (5.8%) and P (5.7%) than B (2.1%) treatment (data not shown). The plant C/N ratio (Fig 4-5) was similar in both P and N treatments after 43 days of growth and significantly lower compared to B treatment. The C/N of plants measured on the same development stage in P and N treatments were also similar (flowered plants in P (P63) compared to those in N treatments (N43).

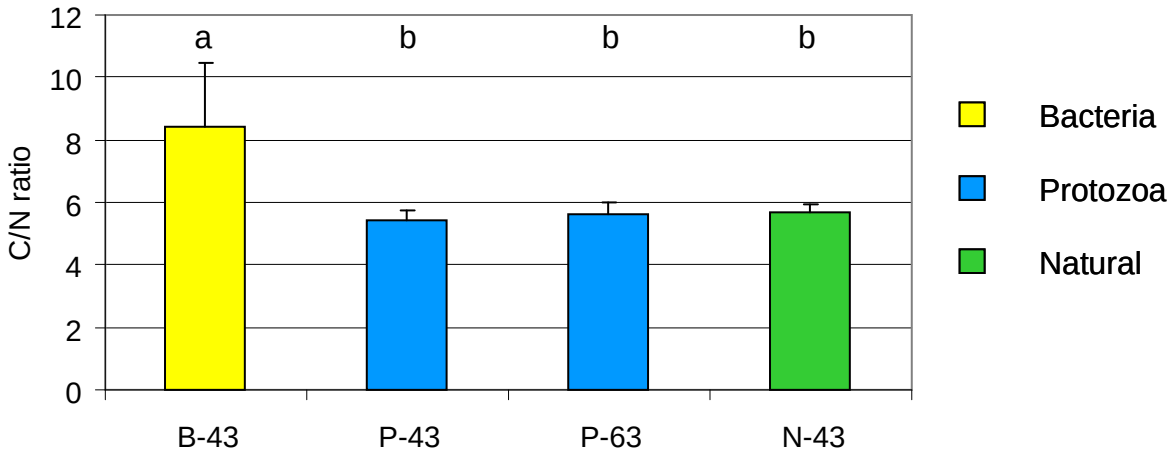


Fig 4-5: C/N ratio of dry *Arabidopsis thaliana* leaves in B, P and N treatment after 43 or 63 days of growth. Different letters (a, b) indicate that C/N ratio are statistically different.

4.4 Discussion

Arabidopsis thaliana growth was clearly different in microcosm in function of treatment. Compared to the Natural treatment, the delay of growth after 22 days is more important for the plants grown in Sterile condition than plants grown in Bacteria and Protozoa treatments. In our study, 69% of the soil mineral nitrogen was in the form of ammonium at concentration of 42 ppm after the sterilization process (Chapter 3). Hoffmann *et al*., (2007) reported that *Arabidopsis* grown with NH_4^+ as the sole N source presented growth retardation. The two most visual symptom of ammonium toxicity are the chlorosis of leaves and the suppression of growth. The toxicity results from ammonia (NH_3), which is able to diffuse through plant membranes and interfere with plant metabolism (Britto *et al.*, 2002). Seedlings were planted a week after re-inoculation and consequently in the re-inoculated microcosms a part of the mineral nitrogen was take up by bacteria during their establishment. The absence of microorganisms in the sterile treatment has clearly affected the growth of *Arabidopsis* thaliana, as the growth was stronger in other treatments. Moreover it is known that during autoclaving of soil, organic

materials could form phytotoxin molecules. Rovira and Bowen (1966) showed that inoculation of soil microorganisms (bacteria and fungi) in a sterile autoclaved soil allowed to remove phytotoxin in soil and improved plant growth. Indeed after 22 days of growth, the mortality rate of *Arabidopsis thaliana* in sterile microcosm was zero and all plants were on stage 1.02 while the others plants in Bacteria, Protozoa and Natural treatment were more advanced stages suggesting that microorganisms decreased the toxicity allowing the plants to grow better. Although after 43 days, the plant mortality rate in the Bacteria and Protozoa treatments was not negligible, a positive effect of protozoa presence on plant growth was found. Plants in the Protozoa treatment presented a higher biomass than in the Bacteria treatment (data not shown). Moreover the C/N ratio in plant leaves revealed that *Acanthamoeba castellanii* increased the nitrogen content in plant, this was also demonstrated in Bonkowski *et al.*, 2004.

The nitrate soil content was increased after 43 days of growth in the Bacteria and Protozoa treatments due probably to the nitrifying activity in soil microcosms. We observed that the ammonium content in these microcosms was higher than in the sterile control but the percentage of ammonium was unchanged. This increase of NH_4^+ in the soil could be explained by the mineralization of organic matter and the death plant tissues. Contrary to the other treatments, in the Natural treatment the plant growth was faster and the mineral nitrogen content was lower than in the Bacteria and Protozoa treatments. We observed a shift in soil nitrogen mineral content in the Natural treatment. Indeed after 43 days, 59% of the soil mineral nitrogen was in nitrate form.

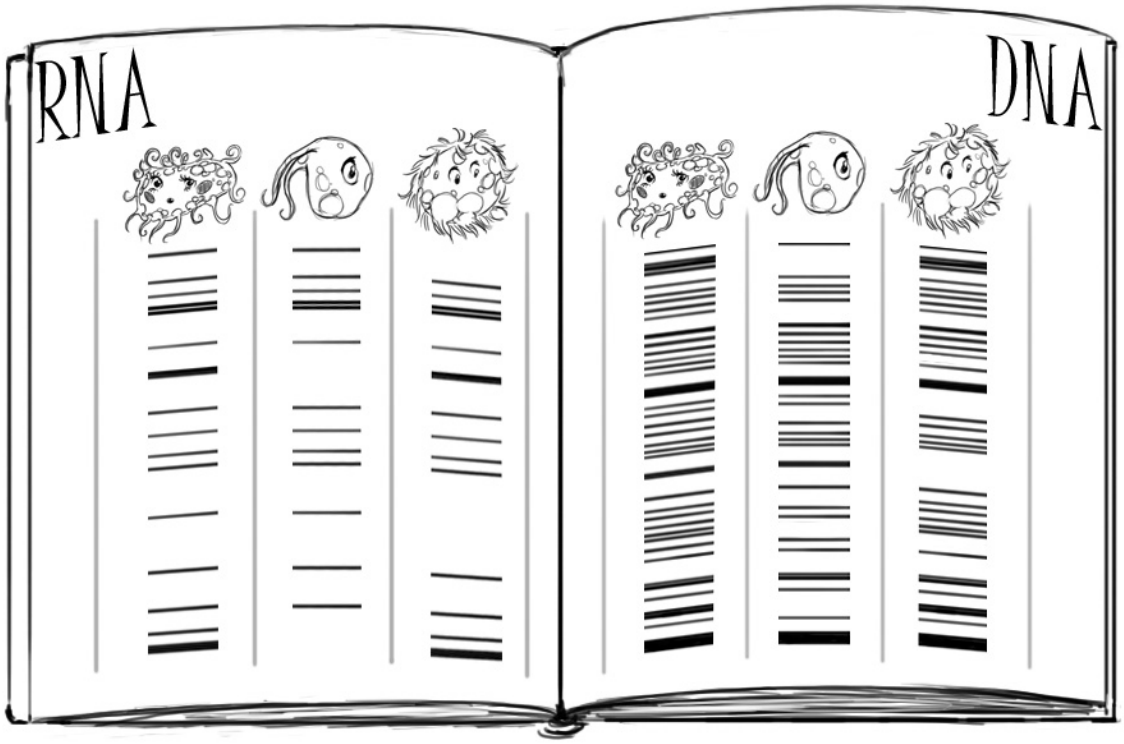
The cultivable bacteria counts in the different treatments are similar and correspond to the same order of magnitude of cultivable bacteria counts in the natural soil. This result confirmed the good colonization and maintenance of the bacterial inoculum in the previously sterilized soil. In the same way, the most probable number of *Acanthamoeba castellanii* in the Protozoa treatment showed the good colonization and maintenance of the amoeba population.

4.5 Conclusion

Use of microcosm developed in Chapter 3 allowed to study plant microbe interactions. These results showed that the growth of *Arabidospis thaliana* was clearly affected by the inoculum and positively by the complexity of the inoculum. *Acanthamoeba castellanii* did not change significantly the soil nitrogen mineral content, improved plant growth, increased biomass and leaves nitrogen content.

Chapter 5

Effect of protozoa on rhizosphere bacterial community structure



Chapter 5

Effect of protozoa on bacterial communities structure

5.1 Introduction

In the soil, protozoa are most important predators of bacteria (Zwart and Brussard, 1991, Zwart *et al.,* 1994). All rhizosphere bacterial populations are not equally submitted to protozoan predation. However, it is not clear if there is a relationship between rhizosphere competence of bacterial population and their resistance to grazing (Jjemba and Alexander, 1999; Jjemba, 2001). Studies on freshwater protozoa have shown that : i) the cell dimension (Pernthaler *et al.*, 1996, Sherr *et al*., 1992), motility and morphological change (Hahn and Höfle, 1999, Matz and Kjelleberg, 2005) ii) the gram positive wall cell structure (Weekers *et al.*, 1993) and their resistance to grazing. Other environmental factors, like humidity and pH may also influence grazing (Hahn and Höfle, 1999).

It can be postulated that protozoan species differ in their selectivity and impact on bacterial populations. Flagellates for example have been shown to be highly selective but bacterial consumption is limited by their long handling time of prey (Boenigk and Arndt, 2002). On the other hand, Ciliates have been shown to require high bacterial numbers behave more like filter feeders (Fenchel 1980, 1986). Amoebas in contrast are surface feeders able to penetrate bacterial biofilms (Parry, 2004) and may capture with their pseudopodia prey in small soil pores unavailable for other bacterivores (Zwart *et al.,* 1994). The importance of protozoa, on the control of microbial community structure was mainly studied in aquatic environments (Hahn and Höfle, 2001; van Hannen *et al.*, 1999; Jürgens *et al.* 1999) as well as in rhizosphere soil (Griffiths *et al.*, 1999, Ronn *et al.*, 2002, Murase *et al*., 2006). In the soils, protozoan grazing influence the structure, taxonomic composition and physiological status of bacterial communities (Alphei *et al.*, 1996; Griffiths *et al.*, 1999; Rønn *et al*., 2002). Moreover Kreuzer *et al.*, (2006) showed that *Acanthamoeba castellanii* affected root architecture of rice (*Oryza sativa*) and induced shift in the composition and spatial arrangement of bacterial communities. Until today it is unclear whether functional groups of protozoa predators in soil differ in their impact on

bacterial communities or if species differences are more important than differences between functional groups.

The first part of this chapter describes the effect of *Acanthamoeba castellanii* on the total (DNA-based approach) bacterial community structure associated with *Arabidopsis thaliana*. The second part of this chapter describes the effects of major protozoan group like naked amoebas, flagellates or ciliates on the total (DNA-based approach) and the active (RNA-based approach) bacterial community structure.

5.2 Effect of *Acanthamoeba castellanii* on bacterial community structure associated with *Arabidopsis thaliana*

5.2.1 Material and Methods

The samples used in this experiment are the RS fraction and soil suspension 10% (w/v) collected in the *Arabidopsis thaliana* experiment described in Chapter 4. The samples were submitted to DNA extraction, to V3-16S rDNA PCR amplification and DGGE analysis according methods described in Chapter 2.

5.2.2 Results

DGGE profiles obtained from soil samples presented a similarity up to 50% and were grouped in two main clusters (Fig 5-1). The first cluster contained all B and P treatment samples. The second one contains all N treatment samples. Treatment applied did not change significantly the evenness for DGGE fingerprint of planted microcosms (from 0.979 to 0.984). The richness (numbers of bands ranged from 24 to 35) and the diversity (Shannon index Fig 5-2) were lower in B and P compared to N treatment but not significant. B and P treatments presented a similar diversity. Variation partitioning analyses on data obtained from DGGE fingerprints showed that 31.1% of the variability of DGGE profiles was explained by treatment ($p \leq 0.001$, Monte Carlo test). The presence of plant explained only 3.3% of variation (not significant).

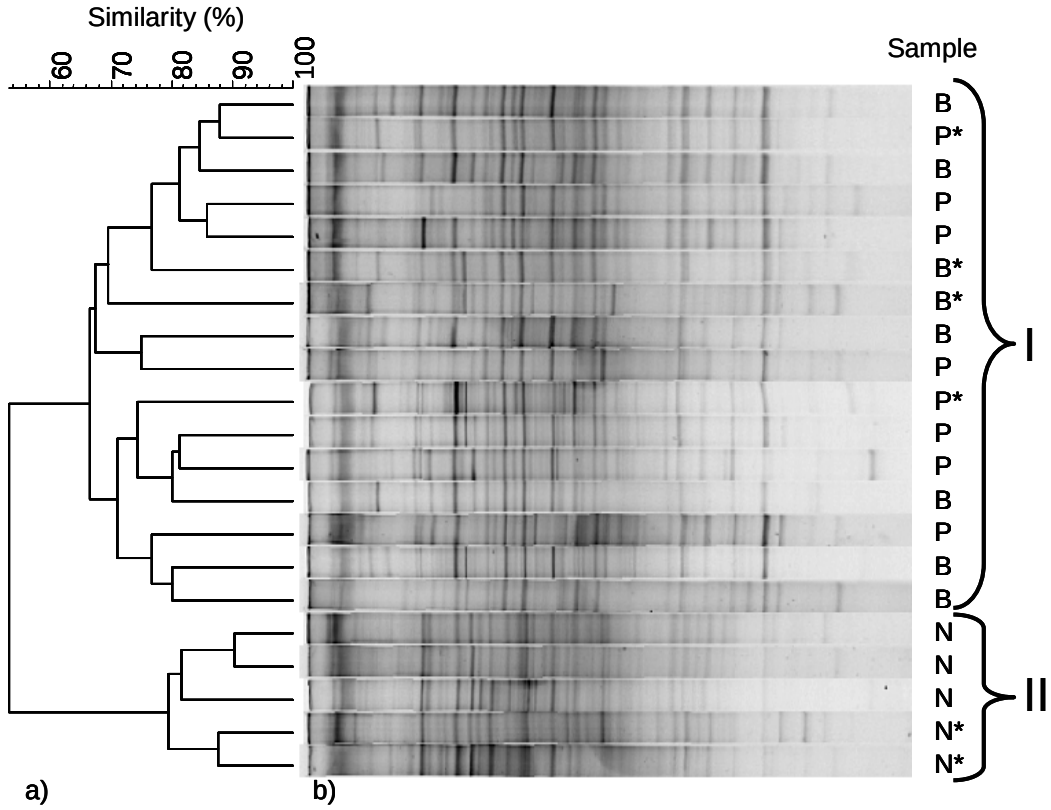


Fig 5-1: a) Dendogram of DGGE fingerprints from rhizospheric soil sample of *Arabidopsis thaliana* or unplanted microcosm (*) in function of Bacteria (B), Protozoa (P) and Natural (N) treatments constructed on the Dice similarity coefficient using Unweighted Pair Group Method (UPGMA).

b) DGGE fingerprint of V3-16SrDNA gene fragment amplified from DNA extract of different soil sample.

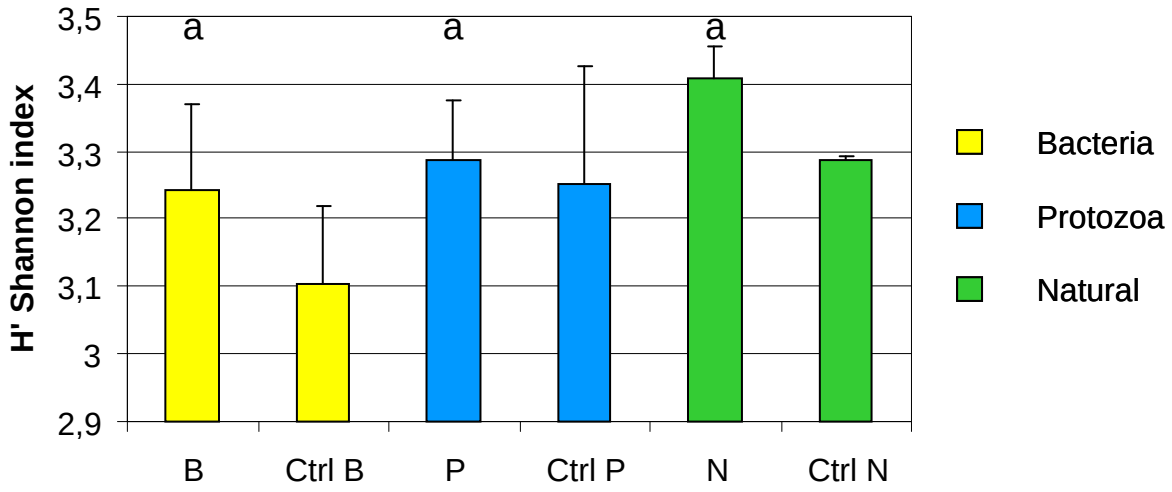


Fig 5-2: Shannon Index (H'=-Σpi*lnpi) of DGGE fingerprints obtained from rhizosphere soil of *Arabidopsis thaliana* and unplanted microcosm in function of treatment. B bacteria, P protozoa and N natural. Ctrl mean unplanted microcosms. Different letters [a, b] indicate the Shannon indexes that are statistically different.

5.3 Effect of major protozoan group (amoeba, ciliate, and flagellate) on the structure of the bacterial communities associated with rice

This experiment was realized in collaboration with René Erdman (PhD student) and Prof. Michael Bonkowski from the Terrestrial Ecology department of the TU Darmstadt and the University of Köln respectively (Germany).

5.3.1 Material and Methods

Microcosms

Soil R (Chapter 2) was mixed with sand (2:1, w/w), autoclaved for 1 hour at 121℃ 1.1 bars and washed twice with the same volume of sterile water to remove nutrients liberated from autoclaving and subsequently dried at room temperature. Glas jars served as experimental microcosms ("Frucht & Fun", V = 1 l, Leifheit AG, Nassau, Germany). The bottom of the jars were filled with 300 g dry weight of soil-sand mixture and closed with a Plexiglas lid allowing gas exchange with two band-aids. Subsequently, the microcosms were autoclaved three times at intervals of 24 h.

Protozoa cultures

Cultures of two species of amoebas (*Acanthamoeba castellanii*, *Naegleria galeacystis*), two species of flagellates (*Bodo designis*, *Neocercomonas jutlandica*) kindly provided by Dr. Fleming Ekelund (Department Terrestrial Ecology, University of Copenhagen, Denmark) and two species of ciliates (*Colpoda sp*., *Colpoda steinii*) were cultured in axenic condition with *Escherichia coli* (DSM 00498) in 1/10 NB-NMAS or in diluted NB-NMAS (1/200 Nutrient Broth, dNB-NMAS). Cultures were incubated at room temperature.

Set up of microcosm

A protozoa-free bacterial inoculum was prepared from rhizoshere soil collected on a meadow on the biology campus of the TU-Darmstadt (Germany), by filtering (1.2 µm pore size) the supernatant of a soil slurry (see Chapter 3). The bacterial inoculum was cultured in NB-NMAS medium for 10 days at room temperature and checked for absence of protozoa. All microcosms were inoculated with 5 ml of the soil bacterial protozoa free suspension and incubated for three days. Microcosms were set up with 10 replicates for each treatment, by adding 10 ml of the protozoan culture (containing approximately 2x 10^5

cells). Ten microcosms were set up by adding 10 ml of sterile water as control. Three days later, one sterile rice seedling (see chapter 2) was planted into each microcosm and incubated in a climate chamber at 25℃ lightened wi th 8 hours a day. The microcosms were destructive sampled after 27 days of growth. The rhizosphere soils were collected and freeze at -80℃ until nucleic acid (DNA and RNA) extractions in order to perform PCR-DGGE analysis as described in Chapter 2.

5.3.2 Results

DNA-based bacterial communities analysis

V3-16SrDNA-DGGE fingerprints from DNA extract of rhizosphere samples presented a high similarity of above to 85% and were grouped in two main clusters (Fig 5-3). However both clusters contained at least one fingerprint from each different protozoa inoculum.
Protozoa inoculum did not change significantly the richness (numbers of bands ranged from 31 to 36, the diversity (Shannon index from 3.44 to 3.49, data not shown). The evenness indexes in major group of protozoa microcosms (amoeba, flagellate and ciliates) were significant lower than control (bacteria). Moreover we observed significant difference of evenness index between all protozoa species inoculated and Bacteria microcosm except for *Neocercomonas jutlandica* (Fig 5-4). Redundancy analysis (data not shown) obtained from DGGE fingerprints from rhizosphere soil samples showed that 15.06% (not significant Monte Carlo test, 999 permutations) of the variability of DGGE profiles was explained by the functional group of protozoa and 31.66% by the protozoa species (not significant Monte Carlo test, 999 permutations).

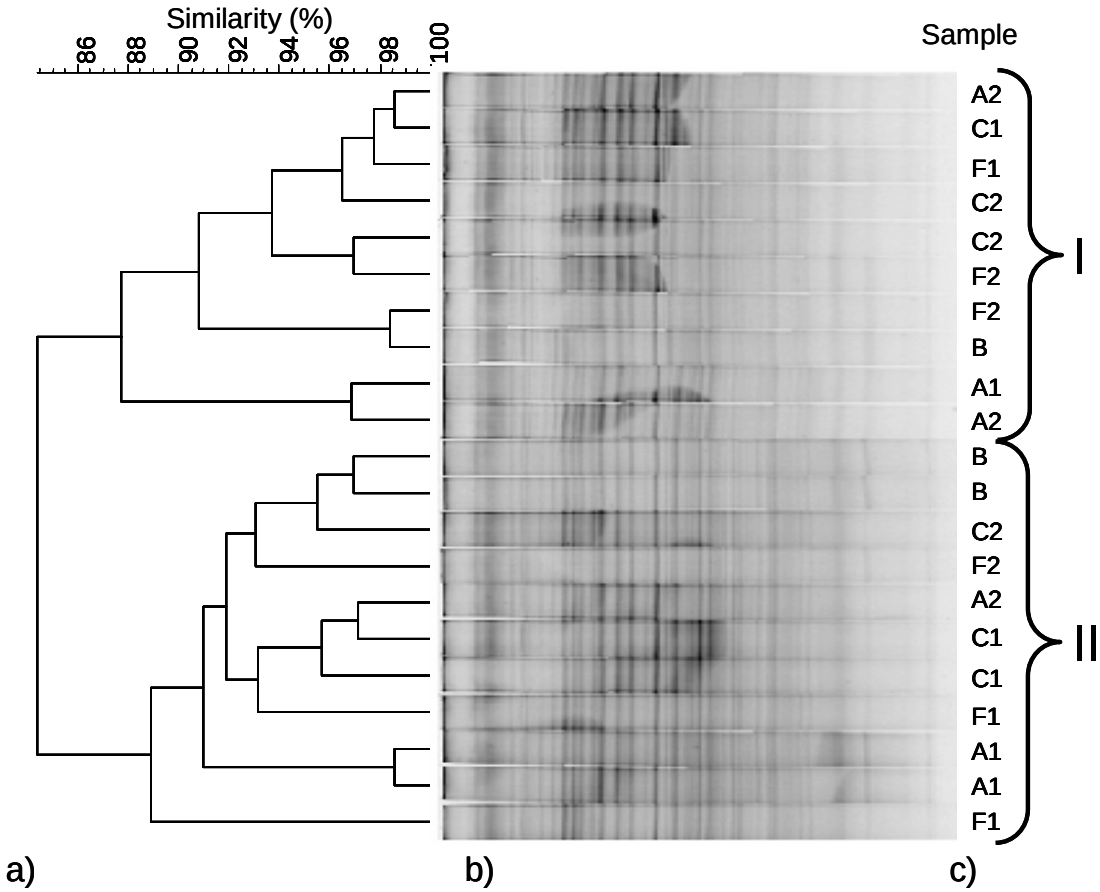


Fig 5-3: (a) Dendogram of DNA-based DGGE fingerprints from rhizosphere soil samples, constructed on the Dice similarity coefficient using Unweighted Pair Group Method (UPGMA).

(b) DGGE fingerprint of V3-16SrDNA gene fragment amplified from DNA extract.

(c) Sample identification table: B= Bacteria, A1: *Acanthamoeba castellanii*, A2 *Naegleria galeacystis*, F1: *Bodo designis*, F2: *Neocercomonas jutlandica*, C1 *Colpoda sp*, C2: *Colpoda steinii*.

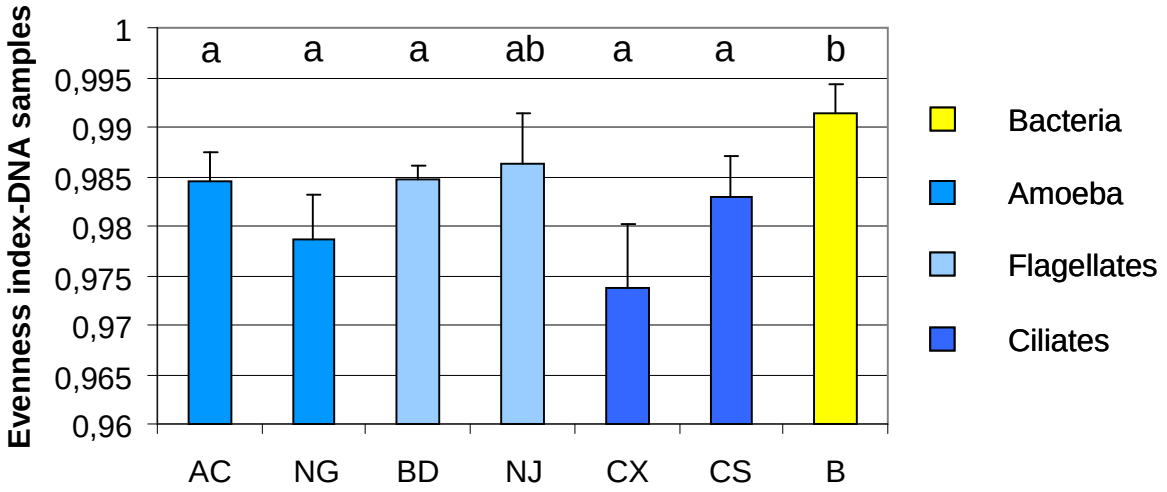


Fig 5-4: Evenness index from DNA-based bacterial communities in function of protozoa species inoculated. B= Bacteria, AC: *Acanthamoeba castellanii*, NG *Naegleria galeacystis*, BD: *Bodo designis*, NJ: *Neocercomonas jutlandica*, CX *Colpoda sp*, CS: *Colpoda steinii*. Different letters [a, b] indicate the evenness indexes that are statistically different.

RNA-based bacterial communities analysis

V3-16SrDNA-DGGE fingerprints from RNA extract of rhizosphere samples presented a similarity of above to 40%. Among the cluster obtained, we distinguished two clusters (Fig 5-5). The cluster I contained mainly RNA-based fingerprints from amoeba samples sharing 52% of similarity and the cluster II mainly from flagellates and bacteria samples sharing 58% of similarity. Ciliates samples were spread in the different clusters. Moreover PCA plot (Fig 5-6) revealed that DGGE profiles from amoeba inoculum microcosms were more separated to bacteria control than other protozoa inoculum. Protozoa inoculum did not change significantly the richness (numbers of bands ranged from 19 to 26) and the diversity (Shannon index from 2.80 to 3.19). The evenness index (Fig 5-7) did not change significantly in function of protozoa species inoculated compared to bacteria samples except for *Acanthamoeba castellanii* inoculum t-test p=0.032).

Redundancy analysis (data not shown) obtained from DGGE fingerprints from rhizosphere soil samples showed that 18.01% of the variability of DGGE profiles was explained

significantly by the major groups of protozoa (p=0.048 Monte Carlo test, 999 permutations) and 33.57% by the protozoa species (not significant p=0.09, Monte Carlo test, 999 permutations).

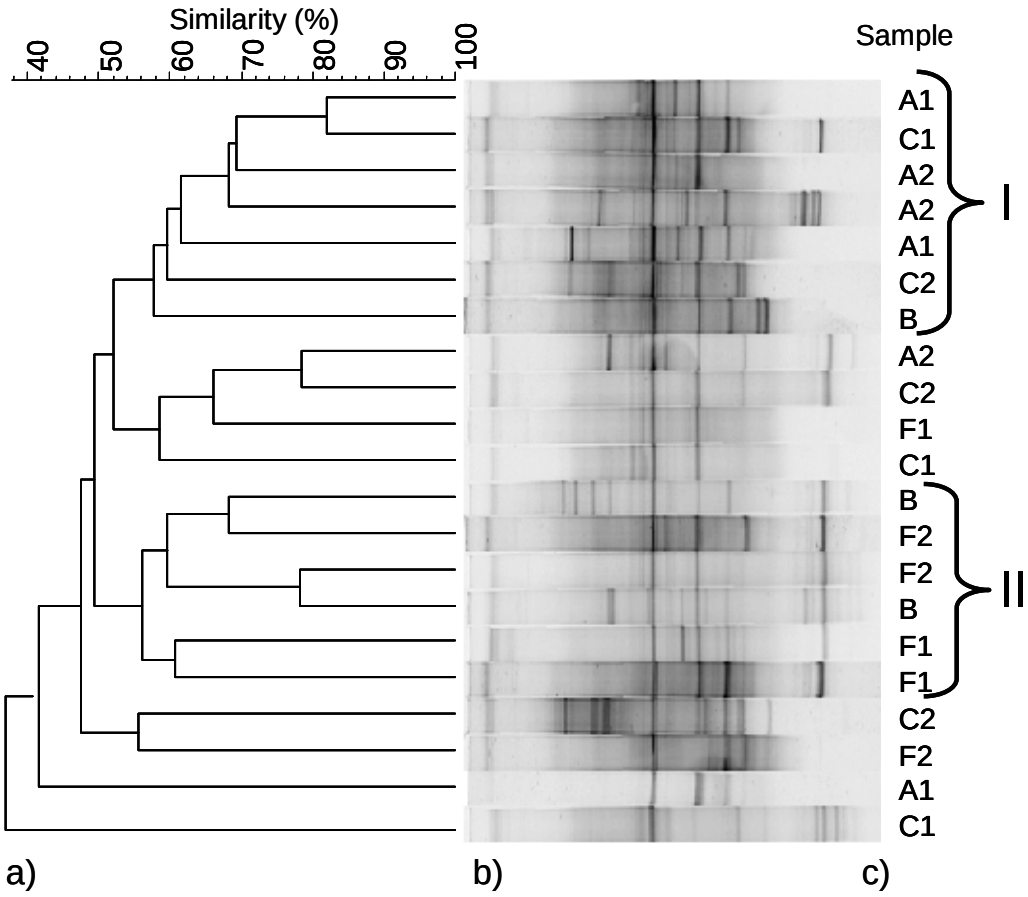


Fig 5-5 (a) Dendogram of RNA-based DGGE fingerprints from rhizosphere soil samples, constructed on the Dice similarity coefficient using Unweighted Pair Group Method (UPGMA).

(b) DGGE fingerprint of V3-16SrDNA gene fragment amplified from RNA extract.

(c) Sample identification table: B= Bacteria, A1: *Acanthamoeba castellanii*, A2 *Naegleria galeacystis*, F1: *Bodo designis*, F2: *Neocercomonas jutlandica*, C1 *Colpoda sp*, C2: *Colpoda steinii*.

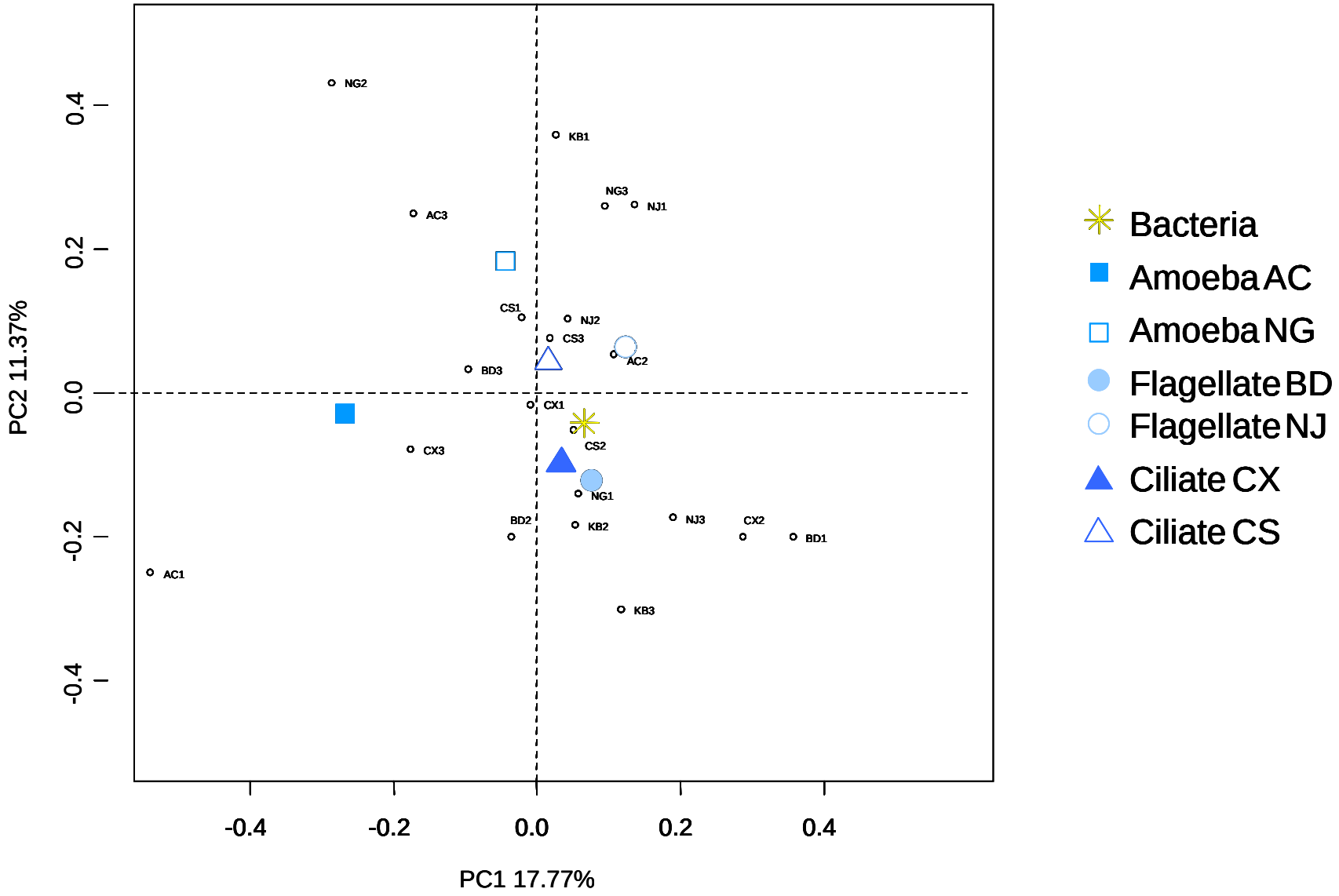


Fig 5-6: Plot of the principal component analysis (PCA) with centroïds corresponding to DGGE fingerprints from rhizosphere soil samples in function of protozoa inoculum.

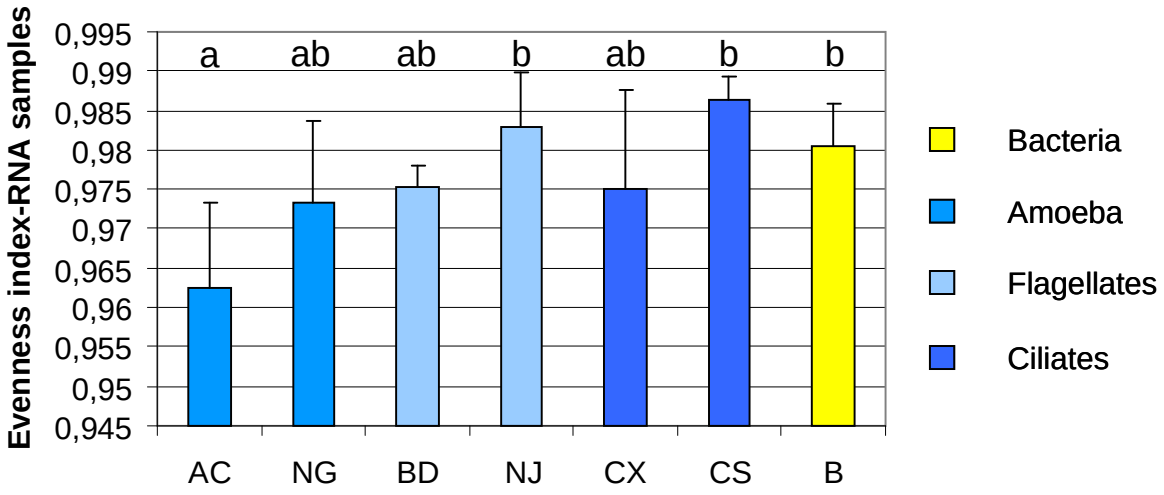


Fig 5-7: Evenness index from RNA-based bacterial communities in function of protozoa species inoculated. B= Bacteria, AC: *Acanthamoeba castellanii*, NG *Naegleria galeacystis*, BD: *Bodo designis*, NJ: *Neocercomonas jutlandica*, CX *Colpoda sp*, CS: *Colpoda steinii*. Different letters [a, b] indicate the evenness indexes that are statistically different.

5.4 Discussion-Conclusion

In the two experiments done (*Arabidopsis thaliana* or rice), reassembling complex bacterial communities present on the re-inoculated soil from bacterial protozoa-free inoculum and from bacteria-protozoa inoculum presented a high richness indicating a successful establishment and a high diversity of soil bacteria inoculated. DNA-based DGGE profiles from the rhizosphere soil of plant growth in B treatment or the different P treatments presented high similarity. The diversity (Shannon index) of the bacterial communities in the rhizosphere soil from treatments B and P was similar in the two experiments and was slightly lower but not significant than in Natural treatment in the *Arabidopsis thaliana* growth experiment. Concerning the experimental design, Bacteria and Protozoa microcosms were inoculated with the same protozoa-free bacterial inoculum while Natural microcosms were inoculated with a complete soil suspension. As described in chapter 3, even if the bacterial protozoa-free suspension present a similar diversity than the source soil, 3 weeks after inoculation of the sterile soil, complete soil suspension

inoculum presented a bacterial community closer to the source soil than protozoa-free bacterial inoculum. The presence of the plant by input of C in soil has favoured the growth of specific bacterial populations as already observed by Latour *et al.*, (1996) and subsequently decreased the difference of diversity index observed in the two bacterial inoculums (protozoa free bacterial inoculum and complete soil suspension). The evenness indexes calculated from the different DNA-based DGGE fingerprints for protozoa species and more extend to major group of protozoa was significant lower than control. This index measures how equal the populations are numerically and in our case revealed that the grazing of bacteria by the main group of protozoa has changed the structure of the total bacterial communities by decreasing some populations. Under individual protozoa predation pressure, no clear shifts of bacterial composition were observed. Ronn *et al.*, 2002 evaluated the impact of individual protozoa grazing in soil amended with different resources (sterile wheat roots or organics compounds) and showed that individual protozoan isolates caused clearly discernible differences in the DNA-based DGGE fingerprint compared to the control.

DNA-based profiles display the most abundant populations independently of their current activity whereas RNA-based community profiles highlight active bacterial populations at the time of the sampling. Protozoa grazing increase bacteria turnover and are consequently good indicators of microbial activity (Andren *et al.*, 1988; Christensen *et al.*, 1996). Consequently the bands displaying a high intensity on the RNA-based DGGE profiles correspond to the bacteria grazed populations and/or populations active in rice rhizosphere. The predation pressure was more displayed on RNA-based DGGE profiles. Indeed they presented a lower similarity, richness (number of bands), diversity (Shannon index) than the DNA-based profiles. This result is not really surprising. Koizumi *et al.*, (2003) observed higher differences in the structure of RNA-based vs. DNA-based community profiles between the upper and the lower layers of lake sediment, and Mahmood and Prosser (2006) obtained quicker, finer-scaled and more reproducible shifts following treatment in ammonia-oxidizing RNA-based community profiles than in DNA-based community profiles.

Analyses of the RNA-based data showed that the different major group of protozoa presented different predation pressure on bacterial communities. These observations confirm the necessity of RNA-based fingerprinting approaches in order to gain a more complete understanding of the protozoa grazing on bacterial communities structure and diversity. The effect of amoeba's group and in more extend *Acanthamoeba castellanii* was clearly different of the other protozoan groups. With their pseudopodia amoeba can graze

bacteria in soil pore inaccessible to other predators (Elliott *et al*., 1980). In natural soils, the interactions among protozoa may change the individual protozoa species effects. Indeed Murase *et al.*, (2006) showed an abundance of DGGE bands affiliated to flagellates in eukaryotic community of rice's rhizosphere soil. Further analyses to better understand the functional importance of protozoa and the impact of grazing on bacterial communities require complex protozoa assemblage inoculum.

Chapter 6

Simulated herbivore attack effects on rhizosphere soil microorganisms associated to barley

Chapter 6

Simulated herbivore attack effects on rhizosphere soil microorganisms associated to barley

Plants are in interaction with soil organisms (belowground system) and terrestrial animals (aboveground system). The interactions between the two systems are important to better understand terrestrial ecology. The effect of aboveground herbivory on belowground organisms is more often investigated. However Scheu (2001) reviewed the two pathways by which the belowground community may affect the aboveground system.

Contrary to the previous chapter which focused only on the belowground interactions, this chapter describes the effect of aboveground herbivory by simulated herbivore attack on belowground rhizosphere microorganisms (bacteria, protozoa and nematodes) associated to barley.

6.1 Introduction

The rhizosphere is an active interface between the soil and plant where a higher number of microorganisms are found due a strong nutrient flow brought up by rhizodeposition. This input of carbon released by roots depends of different factors: the plant species, age and stage of plant development (Meharg and Kilham, 1990), the nutritional status of the soil (Merckx *et al*., 1987), including the nitrogen availability (Liljeroth *et al.*, 1990 a, b). Aboveground herbivory can affect the quality and the quantity of carbon released by root exudation (Hamilton and Frank 2001). Vancura and Stanek (1975) found that exudation increased in defoliated bean plants but decreased after a time corresponding to a depletion of reserves. Holland *et al.*, 1996 observed a significant stimulation by herbivores of root exudation. Microbial growth in soil is strongly carbon limited. Therefore increased rhizodeposition due to herbivory should stimulate bacterial density and lead to an increased competition between plant roots and rhizobacteria for growth limiting nutrients, such as nitrogen and phosphore. Protozoa are the main predators of bacteria in soil (Zwart and Brussard, 1991). In the rhizosphere, as a consequence of grazing, the turnover rate of microbial biomass is high, keeping its density at a much lower level as without grazing. Then potentially limiting bioelements (N and P) being assumed to be excreted, they will be available to organisms with lower affinity for

uptake in particular to plant (Clarholm, 1985). Like protozoa, nematodes play an important role in the mineralization processes in soil. Moreover the importance of microfauna, particularly protozoa and nematodes, on the control of bacteria abundance may affect the structure of bacterial community (Djigal *et al.,* 2004; Blanc *et al.,* 2006; Griffths *et al.*, 1999; Ronn *et al.* 2002). Aboveground herbivory as well as clipping have been shown to affected bacteria predators abundance (Stanton, 1983; Mikola *et al.*, 2001). The objectives of this work were to evaluate the response of soil microorganisms (bacteria, nematodes and protozoa) abundance and bacterial diversity structure to aboveground herbivore simulating by clipping under two different level of nitrogen fertilization.

6.2 Materials and methods

Microcosms and experimental design

The microcosms consisted of pots (85 mm height, 130 mm top diameter and 100 mm bottom diameter) containing 1kg of sieved (4mm) soil T in a plastic bag (Chapter 2). We used two different levels of N-fertilizer: low (28,5mgN/kg soil) and high (144mgN/kg soil). We added a fertilizer solution in each microcosm (K_2SO_4, $CuSO_4$, $ZnSO_4$, $MnSO_4$, $CoSO_4$, $MgSO_4$, Na2MoO4, $CaCl_2$, KH_2PO_4 and K_3PO_4,$7H_20$ corresponding to 150, 4.2, 10.8, 21, 0.67, 90, 0.36, 150, 120 and 2,28 mg per Kg soil respectively).

Three germinated seeds of *Hordeum vulgare* were planted in each microcosm. We weighted each microcosm and added every day ultra purified water to keep soil moisture content during the experiment. Growth chamber conditions during 24 h were 8 h of darkness, 4 h of low (355 µmols/m2/s), 8 h of high (860 µmol/m2/s) and 4 h of low light intensity at 20 ◦C for 12h around the highest light intensity and otherwise 15 ◦C. Humidity was 65% in light and 90% in dark periods.

The following treatments were set up in a two factorial design with nine replicates for each treatment:

1. Low N-fertilization without clipping of leaves (L-)
2. Low N-fertilization with clipping of leaves (L+)
3. High N-fertilization without clipping of leaves (H-)
4. High N-fertilization with clipping of leaves (H+)

In order to simulated herbivore attack, we removed a half circle of 3 mm in diameter on the border of one leave (chosen randomly) per plant per day during a week before to realize destructive sample.

Sampling

Destructive samplings of 3 microcosms per treatment were realized after 14, 28 and 42 of growth. The shoots were collected and dried at 105℃ during 24H to determine the dry biomass. Roots with adhering soil were collected; about 5g was used for the nematode counting (Chapter2). The roots with adhering soil were then washed with a sufficient volume (100 to 300 ml) of Neff's modified amoeba saline (Page, 1988). The washed roots were dried at 105℃ for 24H to determine the roots biomass. The roots-washing suspensions (RS fraction) were kept for bacteria and protozoa enumeration and DNA extraction in order to perform V3-16SrDNA PCR and DGGE according to methods described in Chapter 2.

Statistical analyses

The number of bacteria, protozoa and nematodes were $\log_{10}$ transformed. Abundance of microorganisms data were tested by a two-way ANOVA. Tukey Kramer HSD post hoc test were used to separate differences among the treatments using SAS system software, (SAS institute, Cary, North Carolina, USA). The DGGE fingerprints were analysed according to the chapter 2.

6.3 Result

Barley growth

During the experiment the biomasses of shoots as well as roots increased whatever the treatment. The highest increase of plant biomass was between the first and the second sampling whatever the treatment suggesting a high nutrient acquisition during this period. Indeed plant increased during this period 5 and 10.3 times the root biomass and 5.6 and 7.8 times the shoot biomass in the low and high N-fertilization respectively. The biomasses ratio dry roots / dry shoots (R/S) decreased for plant grown with a low level of N-fertilization (Table 6-1.) indicating a stimulation of the shoot system biomass. The ratio R/S

increased up to 28th day for plant grown with a high level of N-fertilization and was significantly different at this sampling date to the low N-fertilization (P=0.011). Plants grown with a high level of N-fertilization increased first the roots biomass but after 28 days the shoot biomass was stimulated.

	Day	Root biomass		Shoot biomass		Root/Shoot ratio		Clipping intensity
L-	14	0.171^{aA}	±0.040	0.339^{aA}	±0.020	0.503^{aA}	±0.087	
	28	0.864^{aB}	±0.281	1.906^{aB}	±0.223	0.449^{abA}	±0.108	
	42	1.050^{aB}	±0.156	3.648^{aC}	±0.381	0.292^{aA}	±0.068	
L+	14	0.162^{aA}	±0.040	0.338^{aA}	±0.009	0.478^{aA}	±0.108	219,6
	28	0.497^{aB}	±0.136	1.372^{aB}	±0.180	0.358^{aAB}	±0.054	54,1
	42	0.824^{aC}	±0.079	3.347^{aC}	±0.378	0.247^{aB}	±0.009	22,2
H-	14	0.211^{aA}	±0.018	0.374^{aA}	±0.042	0.568^{aA}	±0.064	
	28	2.178^{bAB}	±0.477	2.557^{bB}	±0.388	0.818^{bA}	±0.198	
	42	2.680^{aB}	±1.532	6.996^{bC}	±0.378	0.379^{aA}	±0.206	
H+	14	0.185^{aA}	±0.096	0.394^{aA}	±0.126	0.452^{aA}	±0.097	188,4
	28	1.202^{aAB}	±0.449	2.040^{abA}	±0.146	0.589^{abA}	±0.216	36,4
	42	2.384^{aB}	±1.017	6.286^{bB}	±1.295	0.371^{aA}	±0.106	11,8

Table 6-1: Root and shoot dry weight (g) after 14, 28 and 42 days of growth.
Low N-fertilzation (L), High N-fertilization (H), without clipping (-) and with clipping (+).
Clipping intensity corresponds to the surface of leaves removed by clipping per gram of dry shoot. Different letters indicate the values that are statistically different: $^{a,\ b}$ for difference between treatments (L-, L+, H-, H+) for a given sampling date, ^{A,B} for difference between sampling dates (14, 28, 42 days) for a given treatment.

Plants clipped presented a lower ratio R/S than control. Compared to the control, the biomasses of roots and shoots of clipping plants were lower indicating that clipping affected plant growth whatever the clipping intensity. The strongest effect of clipping was observed on the 28th day. Indeed plants clipped presented a roots biomass 42.5% and 44.8% and shoot biomass 28% and 20.2% lower than control in the low and high N-fertilization treatment respectively.

Bacteria

Colony forming unit (CFU) increased in both N-treatments up to the 28th day (Fig 6-1). On the 28th and 42nd day, the numbers of CFU from the high N-fertilization are higher comparing to the numbers of CFU from low N-fertilization. After 42 days of growth, the numbers of CFU were maintained in high level of N-Fertilization while the CFU of low level of N-fertilization were decreased. The bacteria counts were not different between the controls and clipped plants.

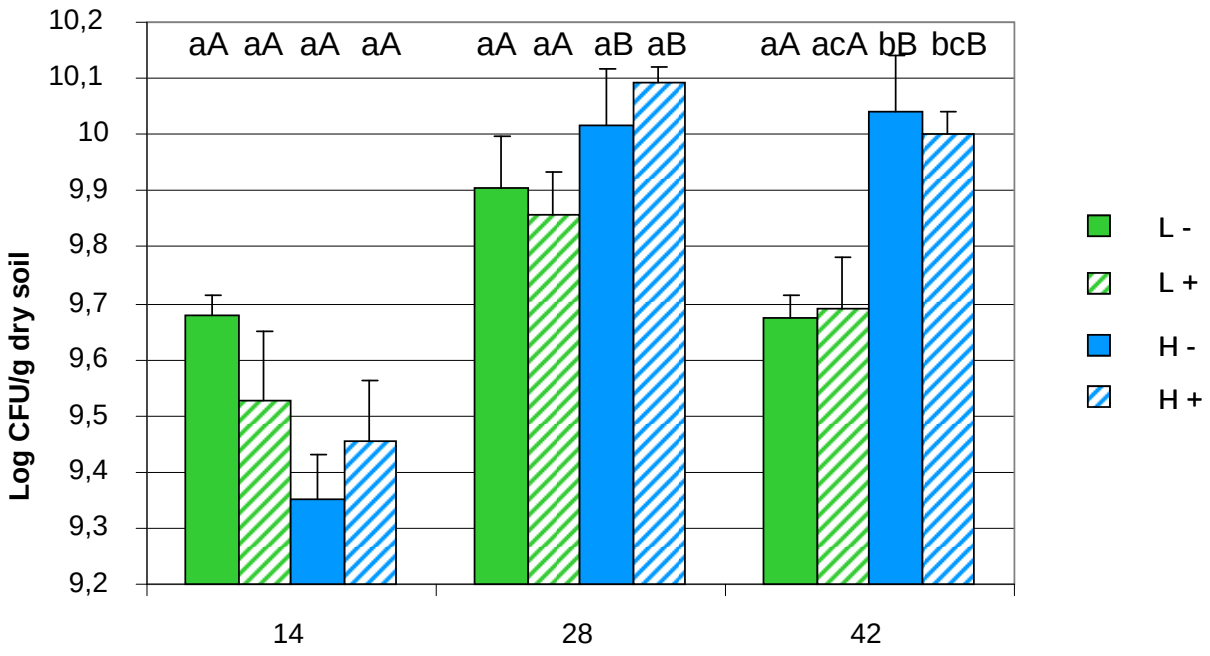


Fig 6-1: Log of CFU of heterotrophe aerobic bacteria per gram of dry soil after 14, 28 and 42 days of barley growth. Green and blue bars mean low and high N-fertilization respectively. Clipping treatment corresponds to the streaked bars. Error bars indicate standard error of the mean, n=3. Different letters indicate the values that are statistically different: $^{a, b}$ for difference between treatments (L-, L+, H-, H+) for a given sampling date, ^{A,B} for difference between sampling dates (14, 28, 42 days) for a given treatment.

Protozoa

The density of protozoa increased during the experimental period in the rhizosphere of barley. Until the 28th day the numbers of protozoa are similar whatever the treatment (Fig 6-2). On the 42nd day, protozoa counts were significantly higher in high N-fertilization. Clipping had no consistent effect on protozoa densities during the experimental period. However on the 42nd day, the protozoa counts from clipped plants were higher than control but not significant.

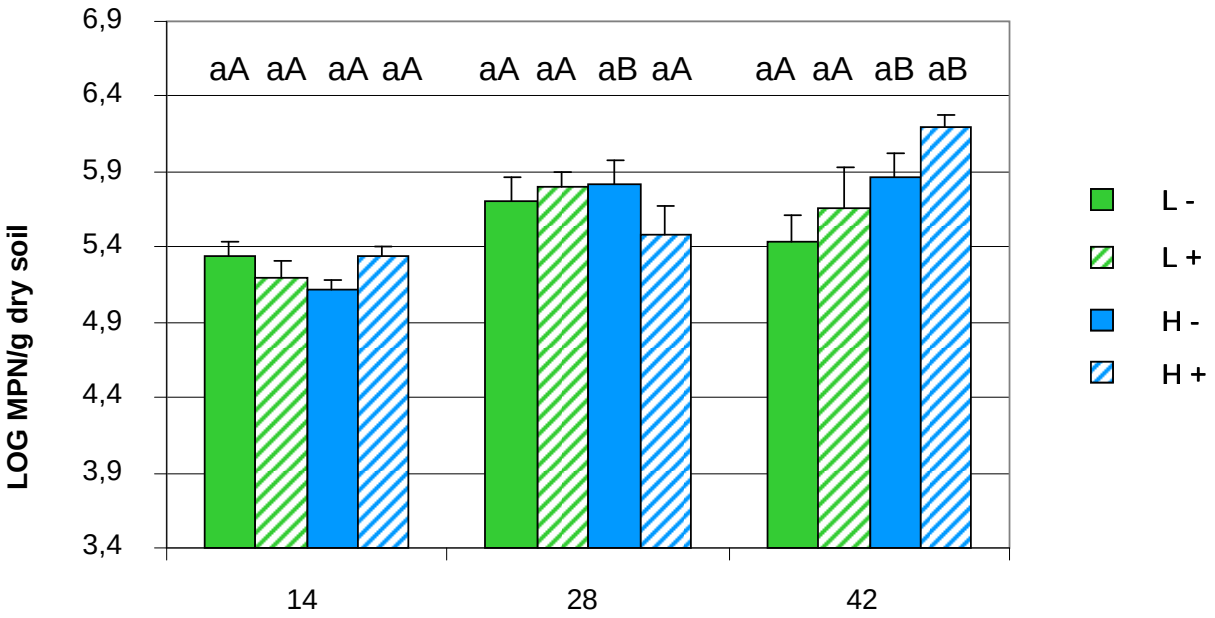


Fig 6-2: Log of Most Probable Number of total protozoa per gram of dry soil after 14, 28, 42 days of barley growth. Green and blue bars mean low and high N-fertilization respectively. Clipping treatment corresponds to the streaked bars. Error bars indicate standard error of the mean, n=3. Different letters indicate the values that are statistically different: $^{a, b}$ for difference between treatments (L-, L+, H-, H+) for a given sampling date, ^{A,B} for difference between sampling dates (14, 28, 42 days) for a given treatment.

Nematodes

The density of nematodes increased during the experimental period. Nematode feeding group distribution is summarised in Table 6-2. Among nematodes, 40-80% were identified as bacteria-feeders, 20-40% fungi feeders, 0-10% plant feeders, 0-2% others (predators, root associated, not identified). On the 28th day, the density of bacterial-feeders nematodes was significantly higher in low N-fertilization treatment. No significant

differences were found for bacterial feeders nematodes regarding the clipping treatment (Fig 6-3). However clipping increased the density of bacterial-feeders nematodes for the high N-fertilization and decreased for the low N-fertilization.

	Day	% Bacterial-feeders	% Fungal-feeders	% Plant-feeders	% Other
L-	14	63,44	27,63	7,66	1,27
	28	74,63	24,81	0	0,56
	42	79,29	19,88	0	0,83
L+	14	48,37	42,52	9,11	0
	28	68,54	30,23	1,23	0
	42	81,08	17,68	0,57	0,67
H-	14	54,7	33,32	11,98	0
	28	59,95	36,15	2,29	1,61
	42	68,13	30,81	0	1,06
H+	14	40,17	52,32	6,93	0,58
	28	74,18	25,32	0,5	0
	42	79,51	19,49	0	1

Table 6-2: Distribution of nematodes feeding group. Low N-fertilization (L), High N-fertilization (H), without clipping (-) and with clipping (+). Other group corresponds to predators, root associated and not identified nematodes.

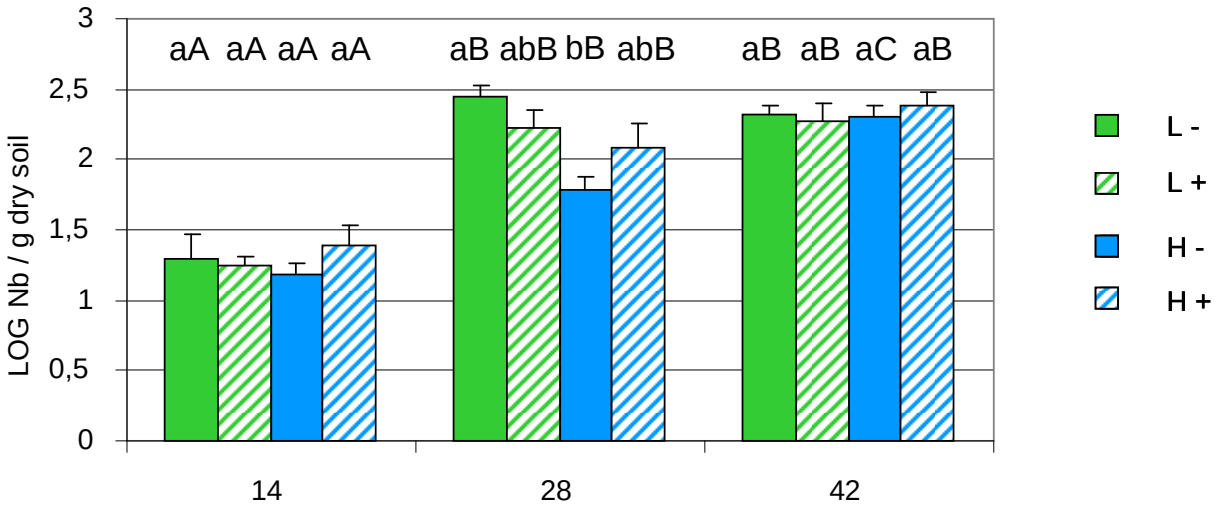


Fig 6-3: Log of bacterial-feeders nematodes (Nb) per gram of dry soil after 14, 28 and 42 days of barley growth. Green and blue bars mean low and high N-fertilization respectively. Clipping treatment corresponds to the streaked bars. Error bars indicate standard error of the mean, n=3. Different letters indicate the values that are statistically different: $^{a, b}$ for difference between treatments (L-, L+, H-, H+) for a given sampling date, ^{A,B} for difference between sampling dates (14, 28, 42 days) for a given treatment.

DGGE profiles analyses

PCR amplification of the V3 region of 16S rDNA generated no contrasted DGGE profiles. Treatments did not change significantly the richness (numbers of bands ranged from 27 to 32), the diversity (Shannon index from 3.17 to 3.34) and the evenness index (from 0.961 to 0.973) (Data not shown). The diversity of bacterial communities associated to barley was equivalent whatever the treatment applied. The principal component analysis (PCA) (Fig 6-4) showed that DGGE fingerprints from the first sampling were separated to the two later samplings. Clipping and N-fertilization level seem not to lead to high change on bacterial communities structure. Variation partitioning analyses showed that 13.4% of the variability of DGGE profiles were explained by the sampling date ($p \leq 0.001$, Monte Carlo test, 999 permutations) and 2.8% and 2.2 % by N-fertilization level and clipping respectively (Not significant). 81.6% of the variability was unexplained.

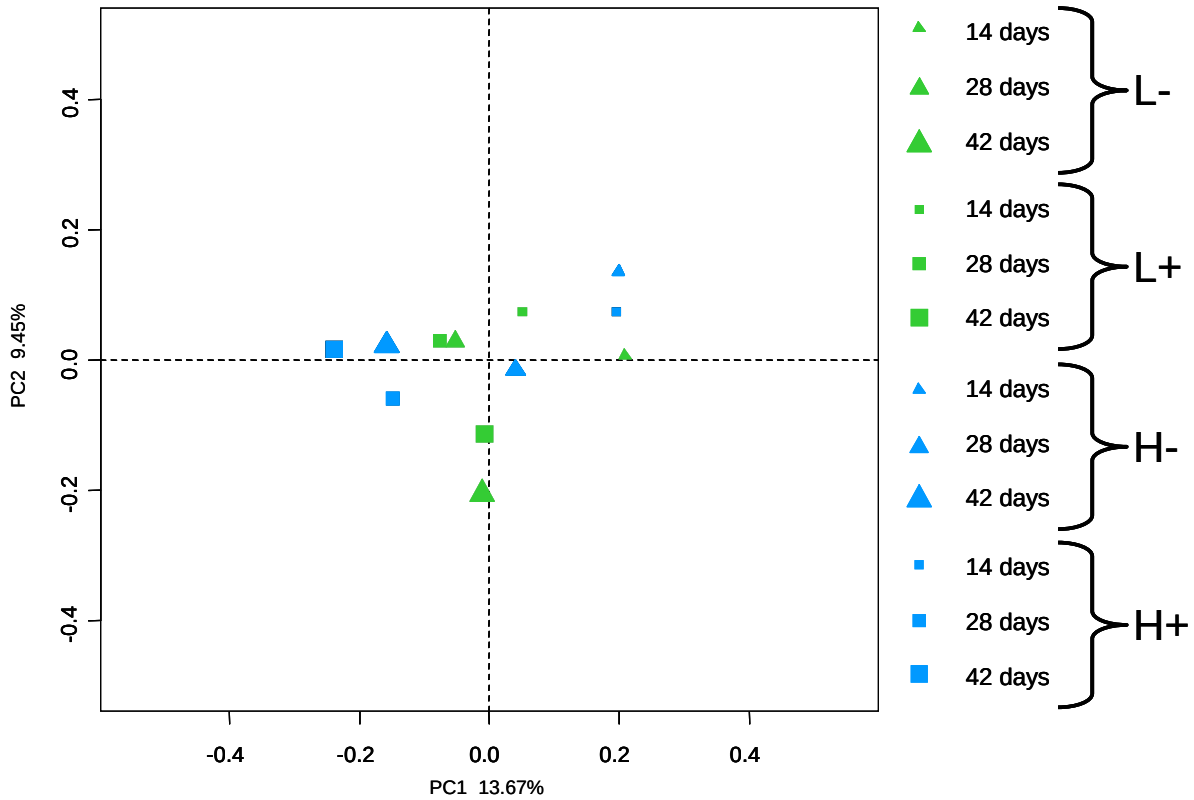


Fig 6-4: Plot of the principal component analysis (PCA) with centroïds (triangles and squares) corresponding to DGGE fingerprints from low N-fertilization in green and high N-fertilization in blue treatments. Triangle symbols correspond to the treatment without clipping and square symbols correspond to the treatment with clipping. Increasing size of the symbols correspond to sampling date (14, 28 and 42 days).

6.4 Discussion

Plants showed a positive response of high N-fertilization by increasing biomass of shoot as well as root. The highest increase of plant biomass was between the first and the second sampling whatever the nitrogen treatment suggesting a high nutrient acquisition during this period. The Root/Shoot (R/S) decreased over time in the low N-fertilization indicating that plant allocated less biomass to belowground than aboveground system. In the high N-fertilization, plants allocated proportionally less biomass on shoot than root until the 28th day. However Bardgett *et al.*, (1999) reported that N-addition decreased root biomass and increased the S/R ratio of 4 different grass species. In accordance with Liljeroth (1990 b,c), we observed that nitrogen fertilization had consistent effect on bacteria counts in particularly after 42 days of barley growth. The high number of CFU on the high N-fertilization was attributed to greater belowground C allocation due to the increasing of root biomass after 28th day. Nevertheless the diversity of bacterial communities was not affected by the N-fertilization level and to lower extend the increase of root biomass suggesting than carbon allocated in rhizodeposition did not changed in quality. High N-fertilization had a negative effect on bacteria-feeders nematodes density on the 28th day while the protozoa benefited from the increase in bacterial density on the 42nd day. For the low N-fertilization, the numbers of CFU decreased after 42 days due probably to the nitrogen limitation in soil instead of a high competition with plant. Indeed Hodge (2000) reported that plants are the inferior competitors for N-uptake particularly in the short term.

Plants showed a negative response to clipping treatment whatever the clipping intensity. Clipping decreased the R/S ratio and particularly the root biomass in accordance with Stanton (1983). Moreover several investigations on the attack by a plant-feeder insect, aphid, on different plant reported a reduction of root growth (Vestergard *et al.*, 2004; Smith and Schowalter, 2001). The strongest effect of clipping was measured on the 2nd sampling. Although in our study the clipping intensity decreased over time, the response of plant to clipping seems to be dependent of the plant stage development. Clipping did not change significantly the bacteria density as well as bacterial composition. Nevertheless on the 28th day, the abundance of bacteria in the rhizosphere of clipped plant was not significantly different of the control although their root biomass was strongly reduced. Bacteria predators responded slightly to the clipping treatment. Protozoa density was not affected by clipping treatment although the density was higher in clipped plants treatment after 42 days of growth. Clipping increased not significantly the nematodes abundance in low N-fertilization while in High N-fertilization the numbers of nematodes

decreased. This result suggests that clipping response could be mediated by soil nutrient availability. This finding was reported by Techau *et al.*, (2004) and Vestergard *et al.*, 2004 in aboveground herbivory studies.

6.5 Conclusion

Plants showed a response to both treatments probably by changing the relative allocation between below and aboveground biomass. The density of bacteria and their predators (protozoa and nematodes) in the rhizosphere of barley increased during the experimental period. The high N-fertilization led to increase the bacterial density in day 42 but simulated herbivory attack did not affect bacterial density although the root biomass was strongly reduced. Slight responses of protozoan and nematodes predators were found. The intensity clipping was maybe not sufficient and for the experiment too short to display effects on bacteria predators.

Chapter 7

General discussion and conclusion

Chapter 7

General discussion and conclusion

There is particularly a lack in knowledge in the function of rhizosphere protozoa. For the period 2000-2007: 4810 manuscripts with keyword protozoa were published, less than 10% speak about soil protozoa (397) and only 1% speak about protozoa and plant growth (57) or protozoa and rhizosphere (53) (Source Web of science). Throughout the experiments conducted in this work, we aimed to better understand the influence of protozoa on rhizosphere bacterial communities and plant growth. As few are known, a reductionism approach in microcosm allows a precise understanding of the functional importance of rhizosphere bacterial grazers. In such case the major challenge is to develop microcosms which simplify many and complex interactions but being not so far of the natural conditions.

The development of microcosms detailed in chapter 3, was used to study the growth of *Arabidopsis thaliana* in three controlled conditions: sterile, in presence of bacteria, of bacteria and protozoa. As described in the chapter 3, the most striking change measured after soil sterilization was the strong elevation in NH_4^+ content. NH_4^+ release may be attributed to deamination reactions from necromass and amino-acids during soil autoclaving. In our study, 69% of the soil mineral nitrogen was in ammonium form at concentration of 42mg/Kg soil after the sterilization process. We observed that *Arabidopsis thaliana* grown in sterile condition presented a suppression of growth. We first hypothesized this symptom could be attributed to an ammonia stress as Hoffmann *et al*., (2007) reported that *Arabidopsis* grown with NH_4^+ as the sole N source presented growth retardation. But we noted also, in several investigations that the mineral nitrogen fertilisation added is often 2 times higher: e.g. 144mgN/Kg of soil added in microcosms described in the chapter 6. Our second hypothesis was that soil autoclaving liberated some phytotoxin molecules which inhibited the plant growth. Rovira and Bowen (1966) showed that inoculation of soil microorganisms (bacteria and fungi) in a sterile autoclaved soil allowed to remove phytotoxin and improved plant growth. By the way, we expected that most of the released NH_4^+ ions would be taken up by microorganisms upon re-inoculation. Indeed in inoculated microcosms, the plant development stage was more advanced than in sterile condition in which the absence of microorganisms makes the soil an inappropriate substrate for plant growth. In order to improve microcosms and

possibilities for further studies, sand-soil mixture could be an alternative procedure to decrease the organic material content and consequently reduce the ammonium and phytotoxin released in soil during the sterilization process, but such proposition conduced again to move away from natural soil conditions.

In Bacteria and Protozoa treatments total heterotrophe aerobic cultivable bacteria counts in re-inoculated microcosms were similar (Chapter 4 and 5) and corresponded to the same order of magnitude of counts from a natural soil. This result suggests that the bacteria successfully colonized the soil and grew up to its carrying capacity. The bacterial protozoa-free suspension as inoculum for bacteria or for bacteria and protozoa treatments presented a diversity similar to the source soil (Chapter 3). We observed that the structure of bacterial communities in re-inoculated microcosms seemed to converge over time to the structure of the bacterial communities of sources soil suggesting that sterile soil re-inoculated is able to rebuild its own native bacterial community. We also observed that whatever the inoculum (N or B or P) the diversity of bacterial communities was similar in planted microcosms, and more the inoculum was complex more the plant growth was improved. To conclude, the microcosms developed allowed to study plant and microbiota interactions and particularly to show the effect of microbial inoculum complexity on plant growth.

The starting point of this thesis was the microbial loop (Fig1-11; Clarholm, 1985; Bonkowski 2004) where beneficial effects of protozoa on plant growth have been assigned to nutrients released from consumed bacterial biomass. These findings were used to build a rhizosphere model (see Appendix and Chapter 8). Although this model requires experimental design for calibration and validation, it showed a positive effect of protozoa on plant growth. Indeed without protozoa the soil system was depleted in inorganic nitrogen inducing inhibition of the plant growth. We observed (Chapter 4) as several studies (Clarholm, 1985; Kuikman *et al*., 1990) that plants grown in the presence of protozoa had larger N contents and biomass dry weights than plants grown in absence of protozoa. Nevertheless we could not conclude that this positive effect of protozoa on plant growth result exclusively to the liberation of nutrients. Indeed plants in Bacteria and Protozoa treatments was not limited by the mineral nitrogen content and we did not observed a higher ammonium content in P compared than B. The nitrate content in the P treatment was higher than B treatment but not significant. We observed a shift of the soil nitrogen mineral content in N treatment after 43 days, 59% was in nitrate form. Protozoa seem to stimulate the activity of nitrifying bacteria which transform ammonium to nitrate.

Bonkowski (2004) reported that “the view that interactions between plants and microfauna, particularly protozoa, in the rhizosphere are solely based on the liberation of nutrients from consumed microbial biomass is rather simplistic”. Somes researches also showed that plants develop an extensive and highly branched root system in the presence of protozoa due to the hormonal effects on root growth by beneficial rhizobacteria (Jentschke *et al.*, 1995; Bonkowski and Brandt 2002; Kreuzer *et al.,* 2006). There is now increasing evidence that the effects of bacteria on root architecture are controlled by protozoan grazing (Bonkowski and Brandt, 2002; Kreuzer *et al,* 2006*)* and their selection pressure on microbial communities.

Regarding the bacterial community present in the Bacteria and Protozoa treatment, no clear shift of bacterial composition were observed in DNA based analyses (Chapter 5). The importance of major protozoan group like naked amoebas, flagellates or ciliates on the control of bacterial community structure was investigated in microcosms experiment (Chapter 5). Although the different protozoa isolate did not change the diversity they changed the structure of DNA-based bacterial communities by decreasing the abundance of some bacterial populations. Analyses of the RNA-based data showed that the different major group of protozoa presented different predation pressure on bacterial active communities. The effect of amoeba’s group and specially *Acanthamoeba castellanii* was clearly different of the other protozoan groups. With their pseudopodia amoeba can graze bacteria in soil pore inaccessible to other predators (Elliott *et al*., 1980). These observations confirm the necessity of RNA-based fingerprinting approaches in order to gain a more complete understanding of the protozoa grazing and functioning on bacterial communities structure and diversity.

The elucidation of the relationships between the different components of terrestrial ecosystem is essential to better understand the functioning and management of this ecosystem. My main contribution for further related studies is the development of microcosms and methods to study interactions between microorganisms and plant.

Chapter 8

Perspectives

Chapter 8
Perspectives

8.1 Plant-microbiota interactions

The rhizosphere is defined as the volume of soil under the influence of root as well as the root itself (Hiltner, 1904). This definition seems to refer to a simple system but the rhizosphere, where important ecological processes take place, evolved in temporal and spatial scales increasing the complexity of the system. The microcosms developed allowed to study plant and microbiota interactions and particularly to show the effect of microbial inoculum complexity on plant growth. In order to better understand the function of soil protozoa in the rhizosphere, other inoculum increasing the links and interactions between plant and microorganisms should be tested for example addition of a predator to protozoa or myccorhiza.

Modelling the rhizosphere could be useful to better understand biological process. Some different rhizosphere models have been constructed in particular on the carbon and nutrient cycling (Toal *et al.*, 2000, Kuzyakov and Domanski, 2002, Moore *et al.*, 2005) and microbial population dynamics (Darrah *et al.* 1991 a,b). In my knowledge few rhizosphere model with microbial grazer were constructed. Foereid and Yearsley, in 2004 constructed a rhizosphere model including microbial grazer in rhizosphere carbon flux and showed a small effect of microbial grazer on the system. Zelenev *et al.*, 2006 modelled bacteria and their predators (protozoa and nematodes) population dynamics and investigated mineral nitrogen release during short-term organic matter decomposition in soil. A preliminary model presented in Appendix was constructed with the Stella^{TM} software (v8.1.4, ISEE^{TM} systems, 2005). This model is included in a web-course “Do It Your Soil” and describes bacterial predation effects on plant growth. This model requires experimental measurements for calibration and validation. The first step will be to build microcosm in a way that limit soil sterilization effects like NH_4^+ release for example. Sand may be a good compromise in order to control nitrogen content e.g. sterile sand microcosms amended with different level and ratio of mineral nitrogen (NH_4^+/NO_3^-). Then sterile microcosms have to be inoculated with bacteria only and bacteria plus one or more protozoa as described in chapter 3. Measurement of mineral nitrogen in sand, plant nitrogen content, plant, bacteria and protozoa biomasses could be analysed after different growth period.

8.2 Protozoa effects on nutrient flow

Soil bacteria play a key role in major biogeochemical cycle for example in nitrogen cycle by nitrogen fixation, nitrification, denitrification, in organic matter transformation and mineralization process. How the influence of protozoa on the major biogeochemical cycles (C and N) is important for the plant growth? How do protozoa influence by grazing the rhizosphere bacterial community structure particularly rhizodeposition-dependent bacteria and the carbon turnover in soil? What are the bacterial populations actually feeding on rhizodeposition?

Stable isotope probing (SIP) allowed a further insight into the functional responses of populations in a community. Stables isotopes are ideal tracers for origin, residence time and turnover processes of organic matter in the environment (Creach *et al.*, 1999; Griffiths *et al.*, 2004). A method has been developed that exploits stable isotope labelling of nucleic acids to determine the metabolic capabilities of active component of complex natural communities (Radajewski *et al.*, 2000 and 2002; Whitby *et al.*, 2001; Manefield *et al.*, 2002a, b); Lueders *et al.*, 2004). SIP is based on the incorporation of ^{13}C from labelled substrates into DNA and RNA by actively metabolizing bacteria (Ostle *et al.*, 2003). Density gradient ultracentrifugation is used to separate "heavy" ^{13}C-labeled DNA (or RNA) from the unlabeled "light" one of populations which did not used the provided substrate. PCR (or RT-PCR) can then be used to amplify genes, from the ^{13}C-enriched DNA. The resulting amplimers are then cloned and sequenced in order to identify the bacterial populations. Prosser *et al.*, 2006 highlighted the power of SIP in resolving plant-microorganisms interactions in the rhizosphere.

8.3 Protozoa effects on bacterial function

Some soil bacteria populations are known to have a specific function in soil like nitrifying bacteria that transform ammonia to nitrate, or the Plant Growth-Promoting Rhizobacteria (PGPR) that form partnerships with plant (symbiotic or not) and improve germination rates, root growth, yield, leaf area, chlorophyll content, hydraulic activity, tolerance to drought, shoot and root weights (Lucy *et al.*, 2004). What is the influence of protozoa on these populations? Do protozoa improve indirectly plant growth by stimulation of these specific populations? Are these populations less edible?

Several species of protozoa do not digest their preys immediately after the phagocytosis: Actually, the phagosomes do not fuse directly with the acid- and digesting enzymes-containing vesicles and consequently non all bacterial cells engulfed within a digestive vacuole will be killed and/or destroyed (Gonzalez *et al.*, 1990). Bacterial DNAs could be extracted from protozoan cells and submitted to SSUrDNA PCR and DGGE, to study the identity and diversity of preys and test the selectivity of protozoa towards their preys. This promising approach needs the prior development of a gradient centrifugation technique allowing separation of protozoan cells from free bacterial cells.

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Appendix

Appendix

10.1 Model assumptions

The spatial scale of the model is the rhizosphere soil at the vicinity of the root. The model was based on the following assumptions: The carbon flow brought up by rhizodeposition and the soil moisture are assumed to be constant and independent of the system. Rhizodeposition is particularly rich in organic carbon increasing the microbial density and activity in the rhizosphere but is relatively poor in nitrogen and other elements. Consequently bacteria take up nitrogen and other elements in soil minerals stock however nitrogen is the primary limiting nutrient in most terrestrial ecosystems (Vitousek *et al.*, 1991). In short term, bacteria are best competitor for nitrogen uptake than plant (Hodge *et al.*, 2000). The growth rates of the plant and bacteria are limited by the availability of nitrogen in soil and a maximal biomass. Without a control of the bacterial population, the soil system will be depleted in nitrogen and cut off the plant growth. Protozoa and to a lesser extent bacterial grazers control bacterial population by grazing. Protozoa use a part of their prey nutrient for biomass production. The excess (carbon, nitrogen, and phosphorus) is assumed to be excreted in inorganic form and may therefore be readily available for other soil organisms including plants. In particular, bacterial predators alter the balance in nitrogen competition between plant and bacteria and increase plant nitrogen uptake (Clarholm, 1985).

10.2 Model description

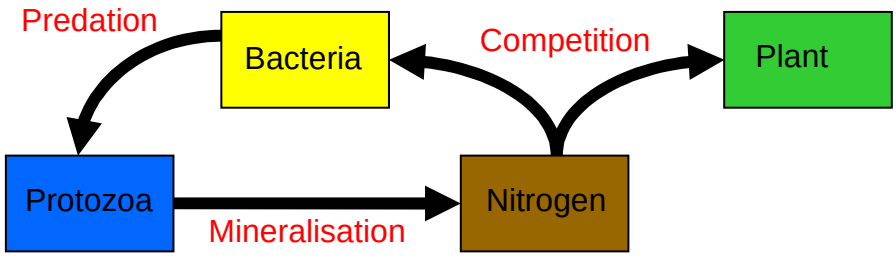

Fig 10-1: Diagram of the model.

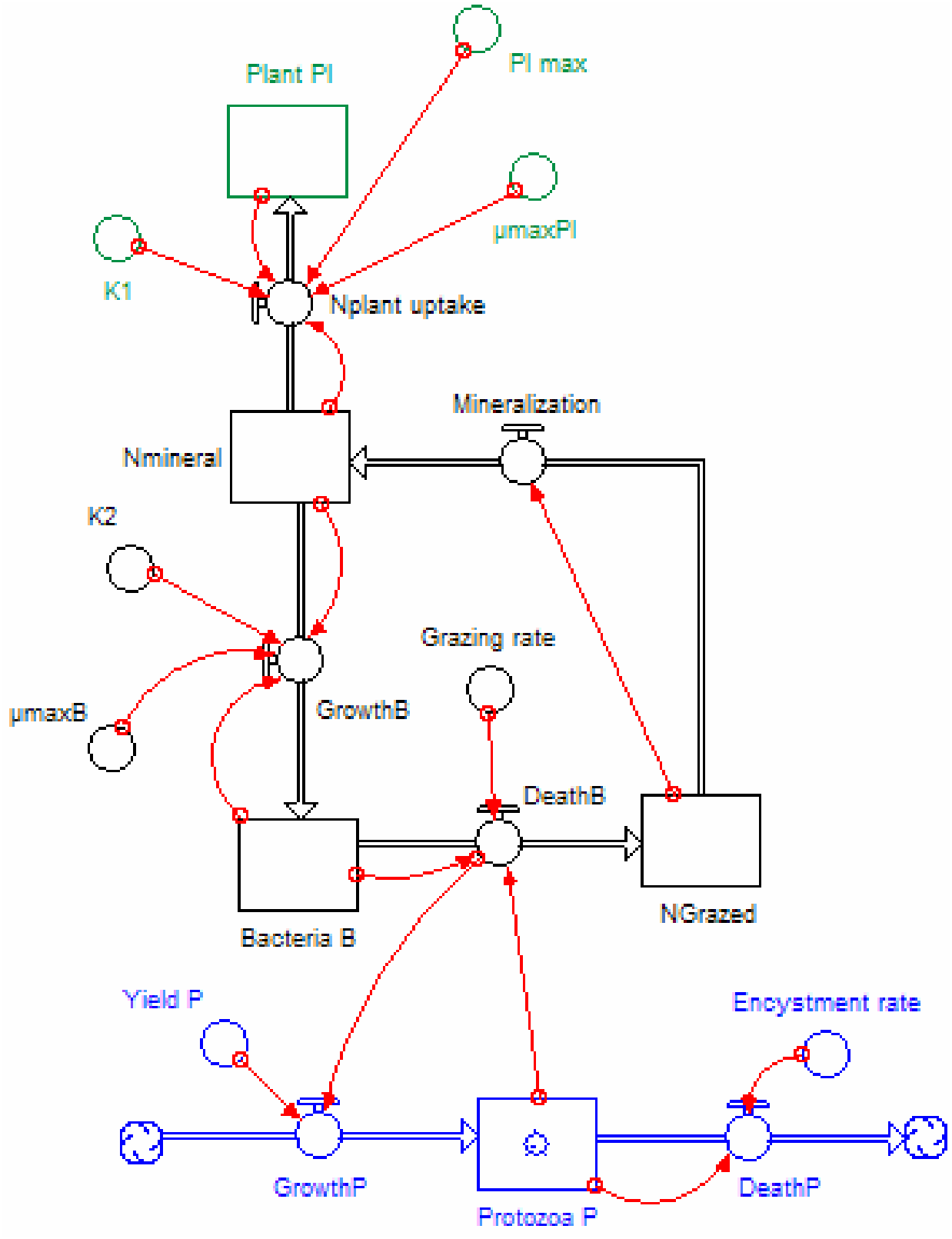

Fig 10-2: Stella model.

10.3 Model equations

Bacteria (B)

Bacteria_B(t) = Bacteria_B(t - dt) + (GrowthB - DeathB) * dt

Initial stock Bacteria_B = 1000

Bacteria N uptake is dependent on soil Nitrogen concentration according to the Monod equation:

Inflow:

GrowthB = (µmaxB*Nmineral/(K2+Nmineral))*Bacteria_B

Outflow:

DeathB = Grazing_rate*Bacteria_B*Protozoa_P

Plant (Plt)

Plant_Pl(t) = Plant_Pl(t - dt) + (Nplant_uptake) * dt

Initial stock Plant_Pl = 10

Plant N uptake rate is dependent on the soil nitrogen content according to the Monod equation and maximal nitrogen content.

Inflow:

Nplant_uptake = µmaxPl*Nmineral/(K1+Nmineral)*Plant_Pl*(1-Plant_Pl/Pl_max)

“Mineral Nitrogen”- Nmineral

Nmineral(t) = Nmineral(t - dt) + (Mineralization - GrowthB - Nplant_uptake) * dt

Initial stock Nmineral = 1500

Inflow:

Mineralization = 2/3*NGrazed

Outflows:

GrowthB = (µmaxB*Nmineral/(K2+Nmineral))*Bacteria_B

Nplant_uptake = µmaxPl*Nmineral/(K1+Nmineral)*Plant_Pl*(1-Plant_Pl/Pl_max)

Protozoa (P)

Protozoa_P(t) = Protozoa_P(t - dt) + (GrowthP - DeathP) * dt

Initial stock Protozoa_P = 0

Inflow:

GrowthP = Yield_P*DeathB

Outflow:

DeathP = Encystment_rate*Protozoa_P

“Nitrogen grazed”- NGrazed

NGrazed(t) = NGrazed(t - dt) + (DeathB - Mineralization) * dt

Initial stock NGrazed = 0

Inflow:

DeathB = Grazing_rate*Bacteria_B*Protozoa_P

Outflow:

Mineralization = 2/3*NGrazed

Parameters and Constants:

Encystment_rate = 0.3

Grazing_rate = $0.0012H^{-1}$

K1 = 10

K2 = 33

Pl_max = 850

Yield_P = 0.33

µmaxB = $0.33\ H^{-1}$

µmaxPl = $0.15\ H^{-1}$

10.4 Model Result

Scenario 1: Plants grown in sterile condition

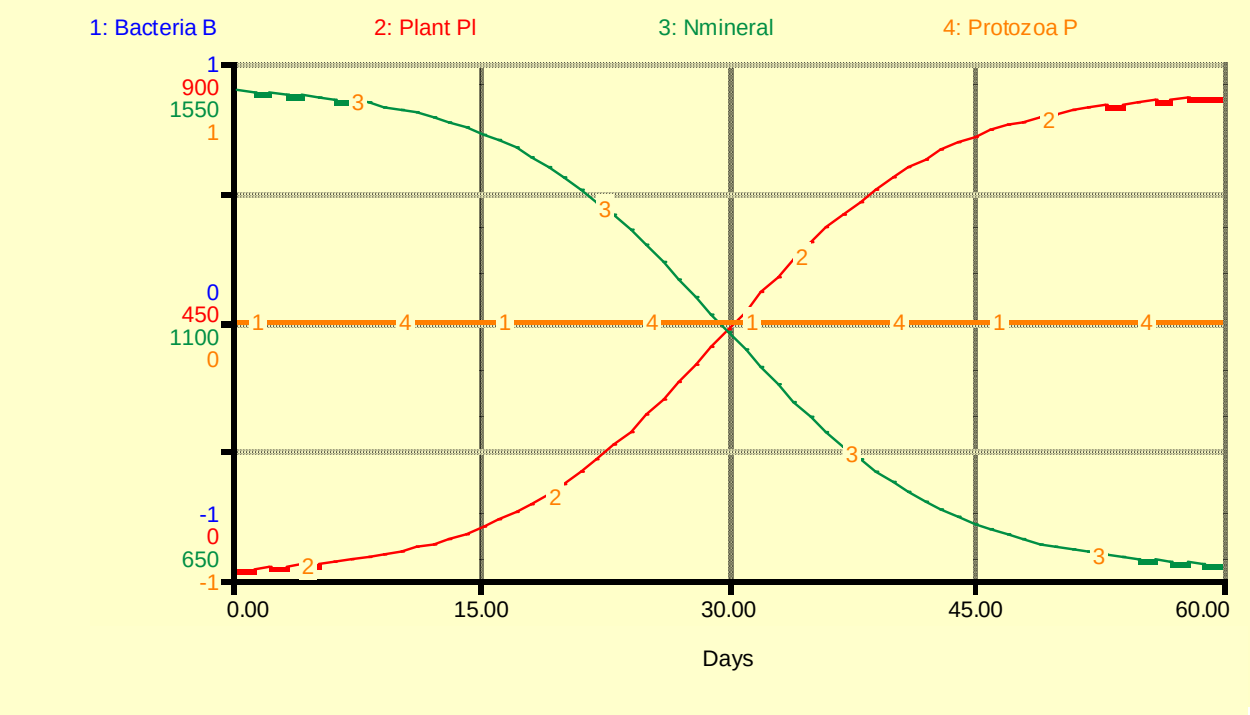

Fig 10-3: Plants grown in sterile condition.

Scenario 2: Plants grown with bacteria and without protozoa

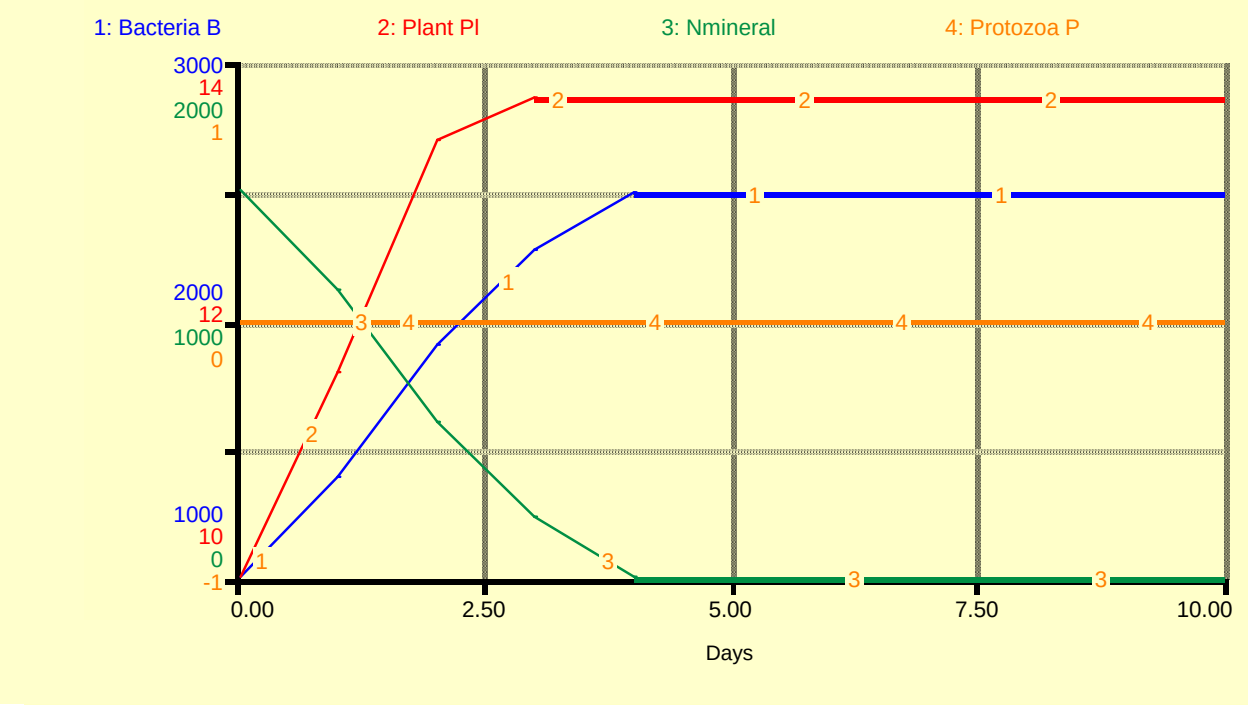

Fig 10-4: Plant and Bacteria competition for N uptake.

Scenario 3: Plants grown with bacteria and their predators (protozoa)

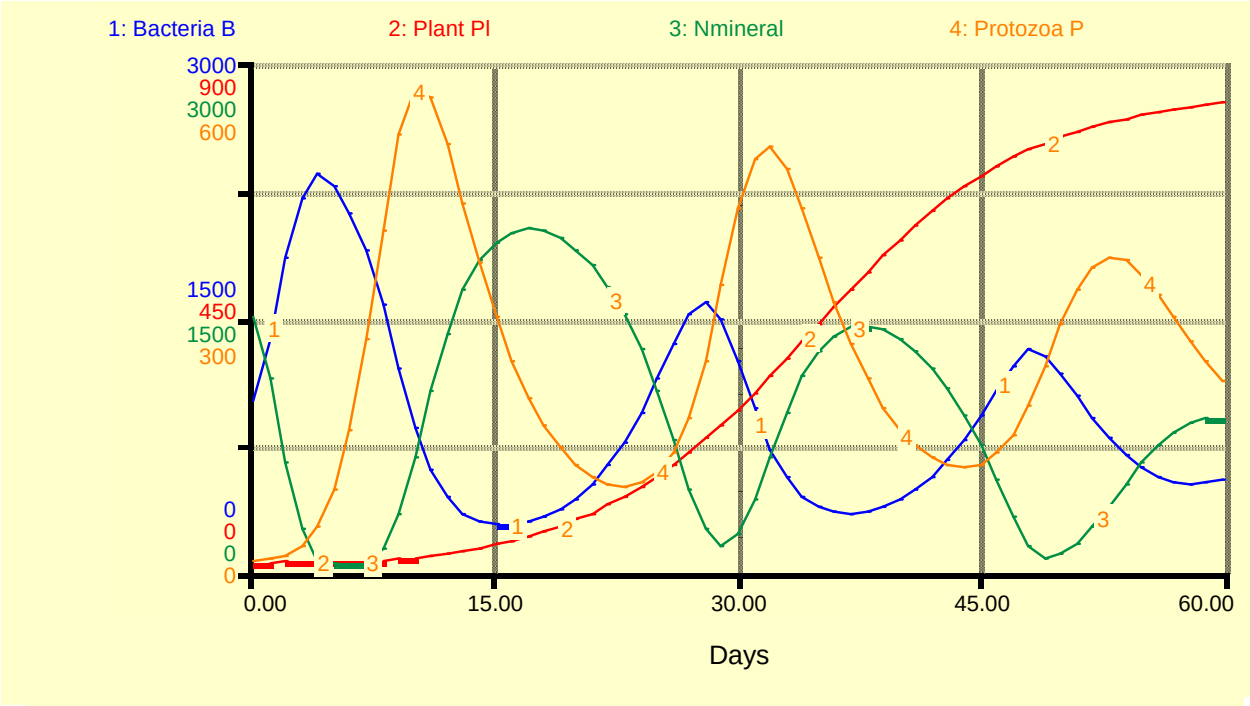

Fig 10-5: Plant grown with bacteria and protozoa.