POPULATION DYNAMICS OF BACTERIA INTRODUCED INTO BENTONITE AMENDED SOIL

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Aan Gé
Aan mijn ouders

Het drukken van dit proefschrift werd mede mogelijk gemaakt door het LEB-fonds.

STELLINGEN

1. The number of different hypotheses proposed to explain a given biological phenomenon is inversely proportional to the available knowledge.
   Slightly modified after J.L. Monteith, Plant and Soil, 1981.

2. Het toevoegen van het kleimineraal bentoniet aan zwak lemig zand is, in modelsystemen, uitstekend bruikbaar voor het verbeteren van de overleving van geïntroduceerde bacteriën.

3. De bodemstruktuur, en met name het volume ingenomen door poriën met een hals diameter kleiner dan 6 µm, heeft een grote invloed op de overleving van in de bodem geïntroduceerde bacteriën.

4. De vorm van de poriën en de continuïteit van het watergevulde poriënsysteem bepaalt in grote mate de kolonisatie door geïntroduceerde bacteriën van beschermde microhabitats.

5. Het nuttig gebruik van Personal Computers neemt, na een zeker persoonsafhankelijk optimum, af met het toenemend aantal geïnstalleerde software pakketten.

6. Afdoende reduktie van "Broeikas Gassen", met name CO_2, kan alleen bereikt worden door een drastische verandering van levenswijze.

7. Het feit dat er binnen onze maatschappij nog steeds verwacht wordt dat de vrouw het grootste deel van de verzorging van de kinderen op zich neemt, gecombineerd met een groot gebrek aan plaatsen in de kinderdagverblijven, heeft tot gevolg dat het nog erg lang zal duren voordat er een gelijke vertegenwoordiging is van vrouwen en mannen in de hogere functies.

8. Microcosmos experimenten uitgevoerd onder geconditioneerde laboratorium omstandigheden, zijn een goede richtlijn voor het te verwachten verloop van de populatie dynamiek van geïntroduceerde bacteriën in het veld.
   Thompson et al., 1992.

9. De hoeveelheid tijd die de afgelopen 5 jaar noodgedwongen door onderzoekers en onderzoeksmedewerkers besteed is aan discussies over mogelijke verhuizingen van groepen en instituten, zou uitgedrukt moeten worden in een aantal niet-geschreven publicaties. Dan zou men kunnen concluderen dat beleidsmakers vanwege hun trage besluitvorming weinig efficiënt met de capaciteiten van het wetenschappelijk personeel van onderzoeksinstituten omgaan.

10. Cryo Scanning Elektronen Microscopie is een goed bruikbare methode om de struktuur van de bodem onder vochtige omstandigheden, met een minimum aan artefakten, te bestuderen op een schaal die voor microorganismen van belang is.

11. De uiteindelijke toepasbaarheid van geïntroduceerde bacteriën in de bodem zal in sterke mate bepaald worden door de vraag of er voldoende hoge aantallen aktieve bacteriën, nodig om de gewenste resultaten te verkrijgen, gedurende een bepaalde periode overleven.

12. Werk om te leven in plaats van dat je leeft om te werken.

Stellingen behorende bij het proefschrift "Population dynamics of bacteria introduced into bentonite amended soil" van Caroline Heijnen. Wageningen, 10 juni 1992.

CONTENTS

PUBLICATIONS

Several colleagues contributed to the work described in this thesis, and served as author in one or more of the chapters. The chapters 2 through 9 were or will be published as separate papers, with some modifications. References are given below (numbers refer to the chapters).

2. Van Elsas JD and Heijnen CE (1990) Methods for the introduction of bacteria into soil: A review. Biology and Fertility of Soils 10:127-133.

3. Heijnen CE, Van Elsas JD, Kuikman PJ and Van Veen JA (1988) Dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil; the effect of bentonite clay on predation by protozoa. Soil Biology and Biochemistry 20:483-488.

4. Heijnen CE, Hok-A-Hin CH and Van Veen JA (1991) Protection of *Rhizobium* by bentonite clay against predation by flagellates in liquid cultures. FEMS Microbiology Ecology 85:65-72.

5. Heijnen CE and Van Veen (1991) A determination of protective microhabitats for bacteria introduced into soil. FEMS Microbiology Ecology 85:73-80.

6. Heijnen CE, Chenu C and Robert M. Micro-morphological studies on clay- and unamended loamy sand, relating survival of introduced bacteria and soil structure. Submitted to: Geoderma.

7. Heijnen CE, Burgers SLGE and Van Veen JA. Metabolic activity and population dynamics of bacteria introduced into unamended and bentonite amended loamy sand. Submitted to: Applied and Environmental Microbiology.

8. Heijnen CE, Hok-A-Hin CH and Van Veen JA. Improvements to the use of bentonite clay as a protective agent, increasing survival levels of bacteria introduced into soil. Soil Biology and Biochemistry, in press.

9. Heijnen CE, Hok-A-Hin CH and Van Elsas JD. Root colonization by *Pseudomonas* introduced into bentonite amended soil. Submitted to: Soil Biology and Biochemistry.

CHAPTER 1

INTRODUCTION

General scope and aims

Bacteria have frequently been introduced into the soil environment for different purposes, such as enhancement of symbiotic or associative N_2 fixation, promotion of plant growth, biological control of soil-borne plant pathogens, biological control of frost injury and degradation of xenobiotic compounds (Chapter 2, this thesis). The effectiveness of introduced bacteria is however very variable, mostly since their survival, i.e. the maintenance of viability, is often poor and unpredictable. In soil, survival of introduced bacteria is influenced by abiotic factors, such as temperature, pH, salinity, moisture content, water potential, structure and texture, substrate availability, and by biotic factors, such as protozoan predation, bacteriophages, antagonists and competitors (Beringer and Bale, 1988). An example of an abiotic soil factor influencing bacterial survival is that decreasing water potentials coincided with decreasing population sizes of introduced rhizobia (Mahler and Wollum, 1981). An example of the influence of a biotic soil factor on bacterial survival is given by Danso *et al.* (1975) and Chao and Alexander (1981) for protozoa. They demonstrated that protozoa were responsible for the decline of bacteria introduced into soil; an increase in the numbers of protozoa was found to coincide with a decline in the numbers of introduced bacteria. For a proper assessment of the relative importance of abiotic and biotic factors to the survival of introduced bacteria, it is relevant to consider the combined effects of abiotic, in particular soil structural, and biotic factors, i.e. interactions with indigenous organisms. For example, the influence of soil texture on survival was demonstrated by Van Elsas *et al.* (1986) for *Bacillus subtilis* and *Pseudomonas fluorescens*. Both bacterial strains were found to survive better in a silt loam than in a loamy sand, probably due to a better protection of the bacteria against predation in finer textured than in coarser textured soils.

In an attempt to increase the effectiveness of introduced bacteria in the various applications mentioned above, much research has been carried out with the aim to enhance bacterial survival levels. Various different introduction methods were

developed, usually involving the use of carrier materials. A review on this subject is given in Chapter 2 of this thesis.

In recent years more and more attention is being given to possible releases of genetically engineered microorganisms (GEMs) into the soil environment. The use of GEMs is advocated in view of the great potentials to improve the effectiveness of bacterial strains for purposes similar to the ones mentioned above. However, since the use of GEMs may induce certain risks for man and its environment, a proper risk assessment is required. For such assessment in soil, i.e. a complex and strongly heterogenous environment (Hattori and Hattori, 1976), information on the influence of soil factors on e.g. survival of introduced cells, their population dynamics, and related processes such as gene transfer, is urgently needed.

In this thesis the main attention is focused on the influence of soil structure on population dynamics of bacteria introduced into soil. To obtain soil structural differences between various treatments, the soil texture is changed by clay additions, thereby largely influencing the moisture characteristics and the pore size distribution of the soil. The aim of the research was to determine how these structural factors influence survival. While a direct application of the research findings is not envisaged for the near future, the information obtained will certainly be valuable when developing suitable carrier materials for introducing bacteria into soil.

Outline of this thesis

In this thesis the population dynamics of rhizobia introduced into loamy sand was taken as a reference for studying the effects of clay amendments to soil. The use of clay amended soil instead of natural clayey soil, made it possible to study the influence of the presence of clay only, without having to pay much attention to other differences in e.g. the organic matter content. The main attention was focused on the changes in survival of introduced bacteria after amending the soil with bentonite clay. Bentonite is a smectitic clay mineral with a large swelling capacity and a high cation exchange capacity (Marshall and Holmes, 1980). A smectitic clay was chosen because clay minerals from this group were previously found to have a large influence on bacterial processes in soil. This relates to: the protection of fast-growing *R. leguminosarum* groups by montmorillonitic clay against the effects of desiccation (Bushby and Marshall, 1977); the increased survival of *Rhizobium* introduced at moderate levels into a montmorillonite-amended sandy soil (Marshall and Roberts, 1963). Moreover, the lag-phase during growth was shortened by montmorillonite additions to bacterial cultures (Stotzky and Rem, 1966) and bacterial respiration was stimulated by montmorillonite (Stotzky, 1980). In addition, gene transfer between bacteria in the presence of montmorillonite was shown to be stimulated as well (Stotzky, 1986; Van Elsas *et al.*, 1987). Some experiments described in this thesis

were also performed with the clay mineral kaolinite. Kaolinite has a low cation exchange capacity and a negligible swelling capacity (Marshall and Holmes, 1980), and is therefore expected to influence survival in different way compared to bentonite. This was demonstrated by Marshall (1964) who found that *R. trifolii* was protected from the effects of exposure to high temperatures by the addition of montmorillonite to a sandy soil but not by kaolinite additions.

Rhizobium leguminosarum biovar *trifolii* was used as a model organism for studying the population dynamics of bacteria introduced into soil. *R. leguminosarum* biovar *trifolii* was selected for two reasons. Firstly, *R. leguminosarum* biovar *trifolii* can be detected easily in soil. It contains stable antibiotic resistance markers, and can be stained very selectively using a fluorescent dye in association with an antiserum. Thus, survival could be studied using selective plate counts, as well as immunofluorescence detection techniques. A possible occurrence of viable but non-culturable cells (Colwell *et al.*, 1985) could therefore be recognized. Secondly, *R. leguminosarum* biovar *trifolii* survived reasonably well in a loamy sand, the control soil used to study the extent of the effects of bentonite amendments to soil. This was essential since survival experiments were usually meant to last approximately 60 days, and survival data of introduced bacteria for the control soil would preferably have to lie above the detection limit for this entire time period. The capacity of *R. leguminosarum* biovar *trifolii* to nodulate white clover (*Trifolium repens*) was not a criterium for choosing *R. leguminosarum* biovar *trifolii*.

Various methods used for introducing bacteria into soil and their applications were reviewed (Chapter 2). It was stressed that further consequences of the introduction of microorganisms into the environment, in particular with regard to bacterial growth, survival, genetic stability, spread, and activity should be investigated. Experiments in which the influence of bentonite clay and the presence of protozoa, often found to be responsible for the decline of bacterial numbers in soil (Habte and Alexander, 1977; Steinberg *et al.*, 1987), on the survival of introduced *R. leguminosarum* biovar *trifolii* in soil are described in Chapter 3. Possible detrimental effects of bentonite clay on protozoa were studied in Chapter 4. Experiments were done in liquid cultures only, omitting soil. Thus, bentonite effects could be studied without the interference of soil factors, such as soil structure and texture or organic matter content. Since bentonite clay appeared to have no detrimental effects on protozoa, bentonite effects on rhizobial population dynamics were hypothesized to be influenced by changes in soil structure. In Chapter 5, a possible relationship between rhizobial survival and the pore size distribution in soil was investigated. Kaolinite amendments to soil were included in this study, as well as in the micro-morphological studies using Cryo Electron Microscopy (Chapter 6), visualizing the changes in soil structure as a result of clay amendments. Chapter 8 deals with effects of the state of the inoculum (freshly grown cells, starved cells, soil adapted cells) and whether mixing of inoculum and bentonite prior to the introduction into soil or separate

additions of bentonite and inoculum to soil, influenced the population dynamics. Metabolic activity and root colonization, both important for determining the effectiveness of bacteria introduced for various different purposes were studied in Chapters 7 and 9 for bacteria introduced into bentonite amended or unamended loamy sand, respectively.

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CHAPTER 2

METHODS FOR THE INTRODUCTION OF BACTERIA INTO SOIL: A REVIEW

Summary

Literature on the use of microbial inoculants to increase crop yields, to control soil-borne plant diseases, or to degrade pollutants is reviewed. Established inoculant technology based on *Rhizobium*/peat inoculants is summarized. Special emphasis is placed on the use of carrier materials for the delivery of microbial inoculants. Some new developments, e.g. the use of synthetic carriers, are highlighted. The fact that not only inoculant survival in carrier materials should be studied, but also the ecological consequences of the introduction of bacteria, is stressed.

Introduction

For several decades bacteria have been introduced into soils to improve the growth of crops through soil inoculation or bacterial coatings on seeds, roots or tubers (a process termed bacterization) (Brown, 1974; Cooper, 1959; Date, 1970; Gaskins *et al.*, 1985). Even now, there is a still growing interest in the application of beneficial bacteria to soil, in particular given the vast possibilities for strain improvement offered by modern biotechnology. Among the main objectives are the enhancement of symbiotic or associative nitrogen fixation (Stacey and Upchurch, 1984; Alexander, 1984), plant growth promotion (e.g., through the production of plant hormones; Burr and Caesar, 1984; Gaskins *et al.*, 1985), biological control of soil-borne plant pathogens (Watrud *et al.*, 1985), biological control of frost injury (Okon and Hadar, 1987), and the degradation of xenobiotic compounds (Brunner *et al.*, 1985).

Bacterization work performed with *Azotobacter*, *Bacillus*, *Clostridium* and *Pseudomonas* spp. has been reviewed by Cooper (1959) and Mishustin and Naumova (1962). Bacterization with *Rhizobium* spp., which has become a regular agricultural practice in many countries, has been extensively described elsewhere

(Thompson, 1980; Roughley, 1970). In addition, some new developments concerning *Rhizobium* and *Frankia* inoculants have been recently summarized (Diem *et al.*, 1988). Although many successes have been reported concerning the effectiveness of introduced bacteria in soil (Mishustin and Naumova, 1962; Dunigan *et al.*, 1983; Giddens *et al.*, 1982; Diem *et al.*, 1988; Sougoufara *et al.*, 1989), the results of bacterization have been extremely variable (Burr and Caesar, 1984; Mishustin and Naumova, 1962), forming a major obstacle to a reliable and effective inoculant technology. The main problem is that soil forms a strongly heterogeneous and unpredictable environment for the introduced organisms (Hattori and Hattori, 1976; Richards, 1987). Introduced bacteria may therefore have great difficulties in finding suitable niches in soil in which to survive over longer periods, making it extremely hard to predict the final effects of bacterization.

The present review summarizes potential and current applications of bacteria to soil and, in particular, gives an overview of the use of carrier materials for soil and plant tissue or seed inoculation. In addition, data on bacterial survival after introduction into soil and the difficulties in extrapolating these data to field applications are discussed.

Potential for application of bacteria in soils

Nowadays, there are vast possibilities in the application of bacteria for beneficial purposes; the potential of tailoring organisms to specific tasks using genetic engineering techniques has certainly contributed to this. A listing of currently used beneficial bacteria and their potential applications is given in Table 1. Perhaps the most widely used organisms today are *Rhizobium* and *Bradyrhizobium* spp., which are applied for their nitrogen fixing capacity in symbiosis with leguminous plants, thereby increasing the amount of available nitrogen for uptake by plants (Alexander, 1984). In addition, strains of *Azospirillum* have now been tested in field trials to study their effect on plant growth (Okon, 1985; Kapulnik *et al.*, 1985; Michiels *et al.*, 1989). This effect, which was originally attributed to associative nitrogen fixation by *Azospirillum*, may more likely be caused by the production of plant (root) growth stimulatory substances (Okon, 1985; Harari *et al.*, 1989). Furthermore, for cereals and forage grasses *Frankia* spp. may also be applied to induce nitrogen fixation in certain plants, e.g. in *Alnus* spp. (Sheppard *et al.*, 1989). Also, a positive response to inoculation with *Frankia* spp. was observed on field-grown *Casuarina equisetifolia* by Sougoufara *et al.* (1989).

In addition to the nitrogen status, the plant phosphorus status may also be improved by applying certain phosphorus-solubilizing bacteria, such as *Bacillus polymyxa*, *B. megaterium* or *Pseudomonas fluorescens* (Brown, 1974). Phosphorus occurs in soil both in the organic and inorganic form (phosphate), but only a small

fraction of the total soil phosphate is directly available for plants, and cycling of phosphorus by bacteria may enhance its availability. However, the conditions required for bacterial P cycling to provide a substantial contribution to the P nutrition of plants are not known (Brown, 1974).

Table 1: Examples of applications of genetically-engineered or non-engineered bacteria in soils

Bacteria	(Potential) application	References
Rhizobium spp.	Symbiotic nitrogen fixation in legumes	Stacey and Upchurch (1984); Stacey (1985); Alexander (1984)
Azospirillum spp.	Associative nitrogen fixation in gramineous plants; plant growth stimulation	Okon (1985); Kapulnik *et al.* (1985)
Bacillus polymyxa, B. megaterium	Dissolution of phosphorus	Brown (1974)
Agrobacterium radiobacter K84	Prevents *A. tumefaciens* infection (crown gall disease)	Moore (1985); Burr and Caesar (1984)
Pseudomonas spp., *Azotobacter* spp.	Plant growth promotion	Schroth *et al.*(1984); Cooper (1959)
Pseudomonas spp., *Bacillus* spp.	Biological control	Gaskins *et al.* (1985)
B. thuringiensis, *Bacillus* spp.	Selective elimination of insect pests	Aronson *et al.* (1986)
Bacillus spp., *Acinetobacter, Alcaligenes, Arthrobacter* spp.	Removal of toxic waste (organochlorides)	Brunner *et al.* (1985)
P. syringae (ice-minus)	Biological frost control in plants	Lindow and Panopoulos (1988)
Pseudomonas spp. containing *B. thuringiensis* toxin gene	Selective killing of soil-borne insect larvae	Watrud *et al.* (1985); Trevors *et al.* (1990)
Thiobacillus ferrooxidans	Bacterial leaching	Brierley (1985)
Frankia spp.	Symbiotic nitrogen fixation with *Alnus* spp. and *Casuarina equisetifolia*	Smolander *et al.* (1988); Sheppard *et al.* (1989); Sougoufara *et al.* (1989)
P. fluorescens	Dissolution of phosphorus	Brown (1974)

A wide variety of bacterial genera (Table 1) is applied for their effects on plant development (Brown, 1974; Burr and Caesar, 1984; Davison, 1988; Diem *et al.*, 1988). These bacteria, including representatives of such genera as *Pseudomonas*, *Azospirillum*, *Azotobacter* and *Bacillus* (Brown, 1974), have been termed plant growth promoting rhizobacteria (Burr and Caesar, 1984). Many of these bacteria probably exert their action by the production of plant growth hormones such as indoleacetic acid (IAA), gibberellins, or cytokinins in the rhizosphere of plants at the seedling stage (Brown, 1974; Nieto and Frankenberger, 1989). Environmental conditions (soil type, soil temperature, humidity and pH, plant species, light intensity) probably all determine the magnitude of the growth promoting effect. Fluctuations in these factors may explain the unpredictability and unreliability of plant responses to bacterizations with plant growth promoting rhizobacteria (Burr and Caesar, 1984).

Another useful property is the capacity of some bacteria to control pathogens (biological control), by antagonism or competition (niche exclusion). This subject has been reviewed elsewhere (Burr and Caesar, 1984; Gaskins *et al.*, 1985; Schroth *et al.*, 1984; Davison, 1988). Many different bacteria, in particular *Pseudomonas* and *Bacillus* strains, have been shown to be able to control pathogenic agents, especially fungi. Of particular interest is the use of pathogens, modified in such a way that the pathogenic character is attenuated. An example is the application of *Agrobacterium radiobacter* strain K84, which acts against *A. tumefaciens*, the causative agent of crown gall disease, by producing agricin 84, a toxin-like substance (Moore, 1985). Biological control agents may also act by depriving pathogens of iron in the rhizosphere, thereby limiting their development (Kloepper *et al.*, 1980). In particular, fluorescent pseudomonads are well known for the production and excretion of siderophores, iron-chelating agents (Hemming, 1986). In addition, a plant-protective action of biocontrol agents has also been ascribed to antagonism against yet unknown minor pathogens, i.e. organisms that are harmful to healthy plant development, without causing well-defined symptoms of disease (Schroth *et al.*, 1984).

The use of formulations of *Bacillus thuringiensis* and other *Bacillus* spp. for the specific control of certain insects has been reported elsewhere (Aronson *et al.*, 1986). A specific protein, the δ-endotoxin or crystal protein, which occurs as a parasporal body associated with the sporangia present in sporulated *B. thuringiensis* cultures, is responsible for the selective killing of larvae of (in particular) lepidopteran insects. Unfortunately, the spore formulations used have been shown to be sensitive to environmental conditions, such as ultra violet radiation, strongly reducing the applicability of the toxin.

Genetically-engineered *Pseudomonas* spp. containing the *B. thuringiensis* δ-endotoxin gene have been proposed as microbial pesticides against specific lepidopteran pest insects in soil (Watrud *et al.*, 1985). These and other engineered soil organisms offer potential advantages over *B. thuringiensis* spore formulations since the insertion and expression of the toxin gene in an ecologically adapted

organism introduced into the soil environment offers circumstances in which survival or even growth (= an increase in the amount of toxin) might be possible. Another example of genetically engineered microorganisms is *Pseudomonas syringae* ice$^-$, which, by niche exclusion, could inhibit the development of the ice$^+$ *P. syringae* strains responsible for ice crystal formation and frost damage on young (potato, tomato) plants at moderately low temperatures (Lindow and Panopoulos, 1988). These and other applications of genetically engineered microorganisms have been reviewed by Lugtenberg *et al.*, (1988).

Further potential applications for bacteria in terrestrial systems lie in toxic waste management. The increasing discovery of progressively more strains with biodegradative capabilities, in particular *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Arthrobacter* spp. has opened possibilities for application in polluted sites (Brunner *et al* 1985; Nicholas, 1987; Golovleva *et al* 1988; Shirkot and Gupta, 1985). In addition, novel biodegradative strains are being developed using genetic engineering.

Bacterial leaching, or the dissolution of certain metals (e.g. Cu, Au) from ores, is another area in which bacteria, in particular *Thiobacillus ferrooxidans*, may be used (Brierley, 1985). A reported attempt to increase the dissolution of copper in a test dump by introducing *T. ferrooxidans*, however, failed in its objective, although the introduced *T. ferrooxidans* strain seemed to have established well (Brierley, 1985). In smaller-scale introductions, however, the lag time of leaching was decreased.

The use of carrier materials for soil and plant tissue inoculation

Bacterial inoculants are brought into the environment for several purposes (Table 1). There are various ways of producing a suitable inoculum, which is effective and can survive in the soil environment. According to Paau (1989), a simple strategy to develop an improved *Rhizobium* inoculant for soy bean is to choose an indigenous strain which is dominant, competitive and specific to a certain geographic region. Subsequently, a mutant of this strain, which is still competitive but has an increased nitrogen fixing ability should be selected, and used as an inoculant in the geographic region where the parental strain was isolated from. A similar strategy is being followed in our laboratory and in others (Watrud *et al.*, 1985) for the development and use of microbial pesticides, e.g. soil isolates modified to contain and express a *Bacillus thuringiensis* insect toxic gene.

For a bacterial inoculant to be effective in soil, optimal conditions for its survival after release must be provided. A wealth of information has been obtained on bacterial survival after introduction into soil, in laboratory soil microcosms or in the field. In many cases, a progressive decline of bacterial numbers is observed upon introduction (e.g. Van Elsas *et al.*, 1986; Heijnen *et al.*, 1988; Postma *et al.* 1988), resulting in speculations that introduced bacteria may stand a chance of surviving in

soil mainly if they are capable of finding and entering protective microhabitats. Another widely accepted generalization is that the dynamic abiotic soil factors such as temperature, moisture content, pH and nutrient status all affect bacterial survival. There is ample evidence supporting this contention (Moffett *et al.*, 1983, Richardson *et al.*, 1988; Alexander, 1984). On the other hand, the more static abiotic factors such as soil structure and texture are also important. For example, introduced fluorescent pseudomonads have been shown to survive better in a heavier-textured soil (silt loam) than in a lighter (loamy sand) soil (Van Elsas *et al.*, 1986). In addition, amendment of the loamy sand soil with the clay mineral bentonite substantially improved the survival of introduced rhizobia (Heijnen *et al.*, 1988), possibly by protecting the introduced bacteria from predation by protozoa. Obviously, this biotic factor and others such as antagonism and competition are also crucial determinants of bacterial survival in soil (Campbell and Ephgrave, 1983; Postma *et al.*, 1990).

There still is a gap between most of the more fundamental data on bacterial survival in soil, and the data and protocols used by the inoculant (mainly *Rhizobium*) industry concerning bacterial survival in the various carrier materials. This is mainly due to the use of carrier materials in commercial formulations. There is a particular ignorance about the effect on inoculant survival of the transition from the relatively protected carrier to the harsh soil environment, even though much is known on bacterial survival inside carriers; the introduced bacteria may face exposure to soil conditions upon carrier degradation or disruption in soil. In contrast, some carriers (e.g. peat- or clay-based ones) might provide new protective habitats for the inoculant, by modifying the soil structure, resulting in a higher survival potential. Also, once the soil structure has been positively modified, future inoculations might prove to be more successful.

Bacterial inoculants may be established directly in the soil, or may be inoculated on plant tissues or on seeds. Soil inoculation may be preferable when direct inoculation on plant tissue causes deleterious effects to the inoculum, due to the presence of, for example, inhibiting compounds or antagonistic microorganisms (Gindrat, 1979). Also, unfavourable circumstances (high temperatures) during storage of the inoculum may occur, strongly decreasing the number of viable cells in the inoculant material (Chao and Alexander, 1984). In both soil and plant tissue/seed inoculation, carrier materials may be used. With seed inoculation, using a carrier material, adhesives which enhance seed-carrier contacts and might protect bacteria from desiccation may also be used (Elegba and Rennie, 1984).

The inoculum strategy chosen is often of importance for the effectiveness of the inoculant, probably because the physicochemical environment in which the bacterial cells are contained largely determines their fate and survival. This fact was recognized early during the development of the *Rhizobium* inoculant industry (Thompson, 1980; Date, 1970), and most work has been devoted towards developing an optimal carrier material for *Rhizobium* inoculants. Carrier materials used for both soil and plant tissue

inoculation may be largely similar. Early research efforts which compared the effectiveness of agar cultures, broth cultures, dried cultures, freeze-dried cultures or powdered carrier inoculants showed that powdered organic carrier materials, mainly based on peat (Thompson, 1980; Strijdom and Deschodt, 1976) were most effective for rhizobial inoculants. Although peat-based carrier materials have since then been widely accepted as being superior for use with *Rhizobium* inoculants, several problems, such as the sensitivity of peat inoculants to high temperatures or drought and the variability in peat quality (Chao and Alexander, 1984), are still associated with their use. In addition, peat may not always be the most successful carrier material for all possible applications of beneficial bacteria. For example, the peat used for inoculant formulation was recently shown to be deleterious to wheat plants that required protection from take-all, and even increased take-all severity (Huber *et al.*, 1989). Alternative carriers are therefore still being investigated, and improvements are being sought in peat inoculant technology. The use of peat-based carriers and of carriers containing materials such as coal, charcoal, charcoal/composted straw, soil/compost mixtures, ground plant material, cellulose powder, bagasse, soy bean meal, lignite, bentonite clay, talc and vermiculite have already been reviewed before (Thompson, 1980; Burton, 1976; Strijdom and Deschodt, 1976; Date, 1970; Roughly, 1970).

Given the fact that peat still is the carrier of choice for the *Rhizobium* inoculant industry, some generalizations on its use will be made here. First, since peat is an undefined, complex material, for each inoculant the peat type which allows for the best survival and effectiveness should be selected. In addition, neutralization of peat using finely divided $CaCO_3$ (pH 6.5) improves inoculant survival (Roughly, 1970). Survival is further enhanced when the neutralized peat is dried at a temperature below 100°C (preventing toxic compounds from being produced), ground, and then sterilized using 5 MRad γ rays. Bacterial cells from a freshly-grown culture should then be mixed into the peat, establishing 45-50% moisture content, and the mixture should be left to mature (4 days at 20-25°C). Survival of the inoculant cells in the peat can be prolonged to over 6 months; critical factors determining survival are the peat sterility, the moisture content, the storage temperature (ideally 5-10°C) and aeration. Additional compounds used to improve bacterial survival and to enhance seed-peat contact (coating with protective adhesives) are gum arabic at 40%, and lime pellets (to neutralize acids). Probably optimal results have been obtained using lime-pelleted peat-based carriers and gum arabic as a protective adhesive (Table 2). Table 3 lists some alternative carriers that offer potential advantages over peat.

Mineral soil (a neutral silt loam) is an adequate, easily available, and inexpensive carrier for desiccation-resistant rhizobia (Chao and Alexander, 1984). The silt loam used was amended with 0.5% $CaCO_3$ and sterilized. Bacterial survival in this carrier when dried at 35°C was even better than in peat. However, the rate of drying was

critical to survival; slow drying resulted in a survival level 3 to 4 orders of magnitude higher than fast drying.

Table 2: Survival of *Rhizobium leguminosarum* biovar *trifolii* on inoculated[a] subterranean clover seed at 25°C

Form of inoculum	Log no. of surviving rhizobia per seed after (days)			
	1	7	14	21
Agar	1.2	[0][b]	[0]	[0]
Freeze-dried	1.5	[0]	[0]	[0]
Peat slurry	2.7	1.4	0.6	0.3
Peat/lime pellet	3.8	3.7	3.4	3.2

a: Initial amount 10^4 cells/seed. Adapted from Date (1970).

b: below detection.

Pre-growth of bacterial cells (*Rhizobium meliloti*) in cheese whey, a by-product of the cheese industry, improved the drought resistance of a *R. meliloti* peat-based inoculant (Bissonnette and Lalande, 1988). The increased drought resistance was attributed to an alteration in the composition of the cells grown in the whey culture.

Oil-based *Rhizobium phaseoli* inoculants have been shown to offer better bacterial survival than peat-based ones, particularly under conditions of high temperature and drought (Kremer and Peterson, 1982). Lyophilized cells were taken up in soy bean or peanut oil, unamended or amended with finely ground charcoal. This formulation provides inoculants with a potential for use in the tropics, where hot and dry conditions often prevail.

The use of synthetic carrier materials might resolve the problems inherent in the use of compounds of variable quality such as peat. Dommergues *et al.*, (1979) showed that polyacrylamide gel was a suitable carrier for *Bradyrhizobium japonicum*. Survival of wet gel-entrapped cells at 35°C was better than peat-based cells; however, gel drying was deleterious for survival. Nodulation using gel-entrapped inoculant was comparable to that with peat-based inoculant.

Alginate-based synthetic carriers have been applied by Fravel *et al.*, (1985) and Bashan (1986a). Only 1% survival of *Pseudomonas cepacia* was found after entrapping cells in Ca-alginate/clay propagules (Fravel *et al.*, 1985). However, the bacterial viability rate remained high over a 12-week period. Bashan (1986a) induced growth of *Pseudomonas fluorescens* and *Azospirillum brasilense* cells following entrapment in Ca-alginate beads and obtained high bacterial numbers per alginate bead. The survival rate of organisms inside wet beads stored at room temperature was also

high. Introduction of beads to soil during sowing was effective in establishing the inoculum on wheat roots. Cells were also shown to be released slowly, as the alginate beads were biodegradable in soil. The addition of the clay mineral kaolinite to alginate was shown to improve the effectiveness of alginate-based *Frankia* inoculants on *Casuarina glauca* (Dommergues *et al.*, 1990).

Table 3: Carrier materials for microbial inoculants[a]

Carrier material	Organism(s)	Potential advantages/problems	Reference(s)
Peat based	*Rhizobium*, *Azospirillum*	Peat quality variable; poor organism survival under hot or dry conditions	Thompson (1980)
Mineral soil (silt loam)	*Rhizobium* spp.	Inexpensive, easily available	Chao and Alexander (1984)
Montmorillonite	Cyanobacteria	Increased inoculant survival	Kotb and Angle (1986)
Soy bean/ Peanut oil	*R. phaseoli*	Increased survival under dry and hot conditions	Kremer and Peterson (1982)
Coal	*R. phaseoli*		Packowski and Berryhill (1979)
Whey grown cells in peat	*R. meliloti*	Increased drought resistance	Bissonnette and Lalande (1988)
Alginate beads	*Pseudomonas fluorescens*, *A. brasilense*, *Frankia* spp.	Constant carrier quality, slow release	Bashan (1986a); Diem *et al.* (1988) Sougoufara *et al.* (1989)
Alginate/clay	*P. cepacia*	As above	Fravel *et al.* (1985)
Polyacrylamide	*Bradyrhizobium japonicum*	Easy root penetration	Dommergues *et al.* (1979)

a: Either peat-based compounds, or materials offering new alternatives to peat, are listed.

Synthetic carriers may offer interesting opportunities for large-scale use of inoculants in agriculture. However, although bacterial cells in carrier materials introduced into soil may show enhanced survival and effectiveness, additional soil treatments intended to cause shifts in the soil microbiological balance will often be necessary to improve effectiveness further. These treatments may include organic amendments, fertilizers, suppressive soil use or soil steaming. In addition, the use of anti-fungal and/or anti-bacterial substances in the inoculation procedure, which produces a temporary depression of the antagonistic or competing microflora

(Bashan, 1986b), may result in better survival and colonization of the inoculated strain resistant to these agents (Bashan, 1986b; Mendez-Castro and Alexander, 1983).

An additional problem is often the lack of active or passive translocation of introduced bacterial cells from the site of inoculation to other sites (e.g., from the coated seed along the growing root to deeper layers). The potential for active transport, which depends on the presence of flagella on the bacterial cell, varies between species, e.g. *Rhizobium* and *Pseudomonas* spp. are potentially motile in soil, whereas *Frankia* spp. are generally non-motile. However, active transport, e.g. in response to a chemotactic stimulus from the roots, probably only occurs over short distances (several centimetres) in soil (Bashan, 1986c). If an optimal distribution of inoculant cells over the root system is required, water applied by irrigation to soil containing coated seeds may induce active or passive vertical transport, resulting in a more even distribution of the inoculum over the root system (Parke *et al.*, 1986; Trevors *et al.*, 1990).

Concluding remarks

Most work on the inoculation of bacteria in soil is concerned with the survival of bacteria in various carrier materials and with the effectiveness of an inoculum, usually based on the height of crop yields. Although peat has been developed into an effective carrier material for *Rhizobium* spp, providing good inoculant survival and effectiveness, the recently developed synthetic carriers (e.g. alginate) have great potential for inoculant protection; in particular, *Frankia* spp have been successfully released into the field using these carriers. Provided optimal conditions are sought for survival and effectiveness of each organism to be used as an inoculant, these carriers probably have greatest potential of being developed into universal carriers, since they offer the advantages of constant quality, better homogeneity and a better defined environment. Future research should be focused on the further development of synthetic carriers, including possibilities for increasing the survival of bacterial cells inside the carrier, e.g. by inducing growth, by modifying cellular physiology through the addition of substrate, or by regulating slow release by manipulating the carrier biodegradability. The addition of alternative protective substances, either organic (e.g. glycerol, amino acids/oligopeptides, sugars, carbohydrates) or inorganic (e.g. clay minerals) to improve bacterial survival in the carriers should also be studied.

To date, long-term soil ecological consequences are rarely studied when investigating the possibilities of carrier materials. However, given the modern developments in the field of genetic engineering of microorganisms and their potential uses as, for example, microbial pesticides (Watrud *et al.*, 1985), it becomes very important to understand how the genetically engineered microbial populations develop after introduction into soil with or without the use of a carrier material. Ecological studies of

introduced bacteria should therefore be one of the research priorities. For example, 10% bentonite clay added to loamy sand caused a population of introduced *Rhizobium leguminosarum* biovar *trifolii* to remain constant throughout an incubation period of 60 days at a level of roughly 10^7 cells/g dry soil. In the absence of bentonite clay cell densities declined from around 10^7 to about 3×10^5 cells/g dry soil (Heijnen *et al.*, 1988). Van Elsas *et al.* (1987) showed that a better survival influenced gene transfer in soil, since bentonite clay added to non-sterile soil caused the plasmid pFT30 to be transferred from *Bacillus cereus* to *B. subtilis* at a frequency of 0.9×10^{-7}, whereas essentially no transconjugants were obtained in the absence of bentonite clay. This means that bentonite clay, which has the potential to serve as a carrier for introducing bacteria into soil, caused prolonged survival of an introduced bacterial strain, and, perhaps even more important with regards to risk assessment studies, caused plasmid transfer to occur at higher frequencies. When working with genetically altered microorganisms, the survival aspect of introduced bacteria needs to be approached from two different angles. First, the organism will have to survive over a sufficiently long period in which it can perform its desired task. Second, the organism should ideally be eliminated from the environment after completing its task. A possibility for this so-called biological containment is the use of a host killing gene system (Molin *et al.*, 1987). Also, it is often necessary to determine whether the introduced organism disappeared from the soil. New methods, such as the polymerase chain reaction (Steffan and Atlas, 1988) have potentially low limits of detection and could prove to be very useful in risk-assessment studies.

We therefore stress that research on carrier materials should not only be concerned with the assessment of inoculant survival (shelf life) and effectiveness, but should also look at the further consequences of the introduction of microorganisms into the environment, in particular with regards to bacterial growth, survival, genetic stability spread and activity.

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CHAPTER 3

DYNAMICS OF *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* INTRODUCED INTO SOIL; THE EFFECT OF BENTONITE CLAY ON PREDATION BY PROTOZOA

Summary

Population dynamics of *Rhizobium leguminosarum* biovar *trifolii* after introduction into a loamy sand was followed using selective plating and immunofluorescence detection techniques. Cell numbers declined steadily during the 60 days of the experiment. Upon introduction of *R. leguminosarum* biovar *trifolii* into loamy sand amended with 10% bentonite clay the population size remained constant throughout the incubation. In sterile loamy sand with or without the addition of 10% bentonite clay numbers of *R. leguminosarum* biovar *trifolii* initially increased and then remained stable. Inoculation of sterile soil with a soil suspension or with soil protozoa resulted in a decrease in rhizobial cell numbers. However, in both cases, survival was significantly improved when soil was amended with bentonite clay. These results suggested that protozoa might be, at least partly, responsible for the decline of rhizobial numbers in loamy sand, and that bentonite clay conferred partial protection of introduced rhizobial cells against predation by protozoa.

Introduction

Population dynamics, ecology and activity of microorganisms in soil are influenced by the presence of clay minerals (Stotzky, 1980; Stotzky, 1986; Van Veen and Van Elsas, 1988). Microbial growth, respiration and metabolism are among the physiological factors which are affected by the presence of montmorillonite (Stotzky, 1986). Amendment of soil with clay minerals, in particular montmorillonite, had a considerable influence on the fate of both native and introduced bacteria (Bushby and Marshall, 1977; Marshall, 1964; Marshall and Roberts, 1963; Stotzky, 1980; Stotzky, 1986; Van Veen and Van Elsas, 1986). Survival of *R. leguminosarum* biovar *trifolii* in a field experiment in sandy soil, as determined from nodulation, was shown

to be greatly improved by the addition of montmorillonite (Marshall and Roberts, 1963). Montmorillonite added to a sandy soil resulted in an increase in the survival of introduced *R. leguminosarum* biovar *trifolii* cells after exposure to temperatures of 70°C (Marshall, 1964). In addition, powdered montmorillonite added to sandy soil protected *R. leguminosarum* from effects of desiccation (Bushby and Marshall, 1977). Clay minerals were also shown to protect rhizobia against the antagonistic effects of toxin-producing microorganisms (Campbell and Ephgrave, 1983; Habte and Barrion, 1984).

Numbers of rhizobial cells may vary under field conditions. Populations may consist of fewer than 10 but occasionally exceed 10^6 cells/g dry soil (Alexander, 1984). Numbers of rhizobial cells introduced into soil mostly declined soon after introduction; independent of the inoculum size, cell numbers dropped to 10^3-10^4 cells/g soil (Crozat *et al.*, 1987). Cell numbers of introduced rhizobia did not decline in sterile, moist, non-acid soils at moderate temperatures but were considerably reduced in non-sterile soil (Danso *et al.*, 1975). As the abundance of *Rhizobium* in soil decreased, the protozoan numbers were found to increase (Danso *et al.*, 1975). Chao and Alexander (1981) suggested that not only the protozoan numbers, but also the classes of protozoa present, largely determined the survival of *Rhizobium* in soil.

We describe experiments in which the influence of bentonite clay and the presence of protozoa on the survival of introduced *R. leguminosarum* biovar *trifolii* in soil was studied. Until now, the protective characteristics of bentonite clay have mostly been studied under extreme conditions such as high temperature and desiccation stress. Our experiments were carried out at 15°C and 18% moisture content, conditions which occur regularly in Dutch soils in summer (Van Elsas *et al.*, 1986).

Materials and methods

Bacterial strains

A kanamycin (Km)-resistant derivative of *R. leguminosarum* biovar *trifolii* strain R62, produced by transposon Tn5 mutagenesis according to Simon *et al.* (1983) was employed. Stock cultures were maintained on slants of yeast extract mannitol agar (YMA: yeast extract: 1 g; mannitol: 10 g; $MgSO_4.7H_2O$: 0.5 g; K_2HPO_4: 0.5 g; NaCl: 0.1 g; $CaCO_3$: 1.5 g; agar: 15 g; Fe-EDTA: 26.9 mg; trace elements: 1 ml; demineralized water: 1 litre; pH 7.2) supplemented with 25 µg Km/ml. The trace element solution consisted of: $MnSO_4$: 1 g; $ZnSO_4.7H_2O$: 0.25 g; $CuSO_4.5H_2O$: 0.25 g; H_3BO_3: 0.25 g; $Na_2MoO_4.2H_2O$: 50 mg and demineralized water: 1 litre. Before use for survival studies, bacteria were cultured for 2 days in yeast extract mannitol broth (YMB, as YMA but omitting $CaCO_3$ and using 0.125 g K_2HPO_4 and 0.125 g $CaCl_2$-$.2H_2O$) at 29°C on a rotary shaker.

Characteristics of bacteria

Growth rates of exponentially-growing cultures of *R. leguminosarum* biovar *trifolii* R62 and *R. leguminosarum* biovar *trifolii* R62::Tn5 in YMB were compared by OD_{645} measurement as well as by dilution plating on YMA. The Tn5 mutant was found to have approximately the same growth rate as the wild-type strain.

The stability of the Km resistance of the Tn5 mutant was tested by dilution plating of batch culture grown cells on non-selective YMA followed by replica-plating on YMA + Km (50 μg/ml), and was found to be stable for at least 50 generations when grown without selective antibiotic pressure.

Soil

A loamy sand, common in eastern parts of the Netherlands, was used. The soil characteristics are shown in Table 1 (Van Elsas *et al.*, 1986). The soil had been kept in a 6 x 6 m experimental field micro-plot at Ital for 3 years. The soil was sieved (< 4 mm) and stored in plastic bags at 4°C and 20% moisture content. One week before commencing an experiment the soil was air-dried to about 10% moisture content and kept at room temperature. For some experiments 10% bentonite was added and mixed thoroughly through the soil. When appropriate, soil was sterilized by irradiation with 4 Mrad using a ^{60}Co-source.

Table 1: Soil characteristics

Characteristic	
Texture	Loamy sand
Particle size distribution (%)	
Sand	84.8
Silt	12.2
Clay	3.0
pH-KCl	6.2
Organic matter	3.5
Total N (%)	0.13
$CaCO_3$ (%)	0.1
CEC (m.e. 100/g)	9.0

From: Van Elsas *et al.* (1986)

Soil studies

In all experiments *R. leguminosarum* biovar *trifolii* was added to soil to an initial population size of approximately 10^7 cells/g dry soil, thereby establishing a moisture content of 17-18%.

In the first set of experiments, sterilized or non-sterilized soil (loamy sand or loamy sand + 10% bentonite), corresponding to 10 g dry weight, was transferred into 30 ml screw-capped bottles.

For the second set of experiments, different soil suspension fractions were prepared by shaking 100 g of fresh soil in 200 ml sterile saline (0.85% NaCl) for 2 h. The larger soil particles were left to settle for 10 min. To eliminate the smaller soil particles the supernatant was centrifuged for 10 min a 30 x g. Part of the supernatant was filtered through a 3 μm poly-carbonate filter. Of the two fractions of soil suspension obtained, one, the filtrate, potentially contained bacteria and fungi (A) and the other, the total suspension, contained bacteria, fungi and protozoa (B) (Frey *et al.*, 1985). Either 1 ml of suspension A or a ml of B was added to 10 g (dry weight) sterile soil portions in 30 ml screw capped bottles. After 2 days, in which period the newly introduced microorganisms from the soil suspension were allowed to adjust to their new environment, *R. leguminosarum* biovar *trifolii* R62::Tn5 was introduced. Half-strength Tryptone Soya Agar plates were used for determining total bacterial numbers present in the soil samples amended with soil suspensions A or B.

In the third set of experiments, rhizobial cells and a protozoan suspension were added to sterile soil. The protozoan suspension was obtained by growing protozoa isolated from the loamy sand on water agar (agar 1.3 g, demineralized water 80 ml, amoebae saline 80 ml, pH 6.0-6.5) with *R. leguminosarum* biovar *trifolii* as a food source. The amoebae saline (AS) was prepared according to Page (1967). It was assumed that after five successive inoculations with *R. leguminosarum* biovar *trifolii*, the presence of other bacterial species on the water agar would be negligible. Protozoa were then rinsed off the agar with sterile AS. Soil portions (30 g dry weight) in 100 ml Erlenmeyer flasks, covered with aluminium caps, were inoculated with *R. leguminosarum* biovar *trifolii* and with protozoa, so as to have 300-1000 protozoa (flagellates and amoebae)/g dry soil.

In all experiments soil portions were prepared in duplicate, thoroughly mixed and held in a humid chamber at 15°C. All results given in this paper are the average of duplicate soil samples.

Enumeration of rhizobial and protozoan cells in soil

At fixed times, i.e. on days 0, 1, 2, 5, 7-8, 15-16, 30-31 and 56 or 60, the numbers of rhizobial and, when appropriate, protozoan cells were determined in the soil portions.

Total counts of the *R. leguminosarum* biovar *trifolii* population were made utilizing the slightly modified immunofluorescence (IF) technique (Schmidt, 1974) using a specific antiserum conjugated with fluorescein-iso-thiocyanate as a fluorescent dye. A recovery percentage of inoculated cells was calculated for each experiment based on the IF-count determined in the inoculum and in the soil 3 h after inoculation. The recovery percentage found was influenced by the presence of

bentonite clay (Postma *et al.*, 1988). For loamy sand the recovery of inoculated cells was found to be between 41 and 74%, for loamy sand supplemented with 10% bentonite clay the recovery was between 38 and 66%.

In addition, selective plate counts were used to determine the number of culturable rhizobial cells in soil. A 10 g soil portion was transferred into a 250 ml Erlenmeyer flask containing 95 ml of a 0.1% sodium pyrophosphate ($Na_4P_2O_7$) solution in demineralized water and 10 g gravel (2-4 mm dia) to improve dispersal of bacterial cells. Flasks were shaken for 10 min at 200 rpm at room temperature. Serial 10-fold dilutions in YMB were then plated on YMA supplemented with Km (50 µg/ml) and cycloheximide (100 µg/ml).

The most probable number method (Darbyshire *et al.*, 1974) was used to determine protozoan numbers in soil. A soil sample (5 g) was blended with 100 ml AS for 1 min at 1700 rpm so as to extract protozoa from soil. Washed *R. leguminosarum* biovar *trifolii* cells 10^6/ml) were used as a food source for the protozoa. Microtiter plates were checked for protozoan growth after 1, 3-4 and 6-7 weeks at 12°C.

Results were analyzed statistically, using regression analysis assuming a linear relationship between cell numbers and time, to determine whether the survival of introduced rhizobia differed significantly between the various treatments. Cell numbers found on days 0 and 1 were not taken into account here, since these days were seen as an adjustment period and therefore could not be noted as survival.

Results and Discussion

Numbers of *Rhizobium* cells introduced into sterile soil increased during the first week of incubation from roughly 10^7 to 3 x 10^8 cells/g dry soil and remained at this level until the end of the experiment (Fig. 1). The presence of bentonite did not significantly ($P > 0.05$) change the population dynamics of *R. leguminosarum* biovar *trifolii*. Both the results from plate- and IF-counts led to this conclusion. Van Elsas *et al.* (1987) found similar results with introduced *Bacillus* spp; no stimulation of growth was found when bentonite was added to sterile loamy sand.

In the non-sterile loamy sand, however, numbers of introduced rhizobial cells declined steadily from around 10^7 to about 3 x 10^5 cells/g dry soil (Fig. 2). These results are in agreement with those found by Crozat *et al.* (1987). However, in soil amended with bentonite, rhizobial cell numbers remained roughly constant (10^7 cells/g dry soil) throughout the duration of the experiment (Fig. 2). Survival of *Rhizobium* in non-sterile soil in the presence of bentonite clay was significantly higher (regression analysis, $P < 0.05$) than when bentonite was absent. This, and the absence of a bentonite effect in sterile soil, suggested that bentonite clay offered

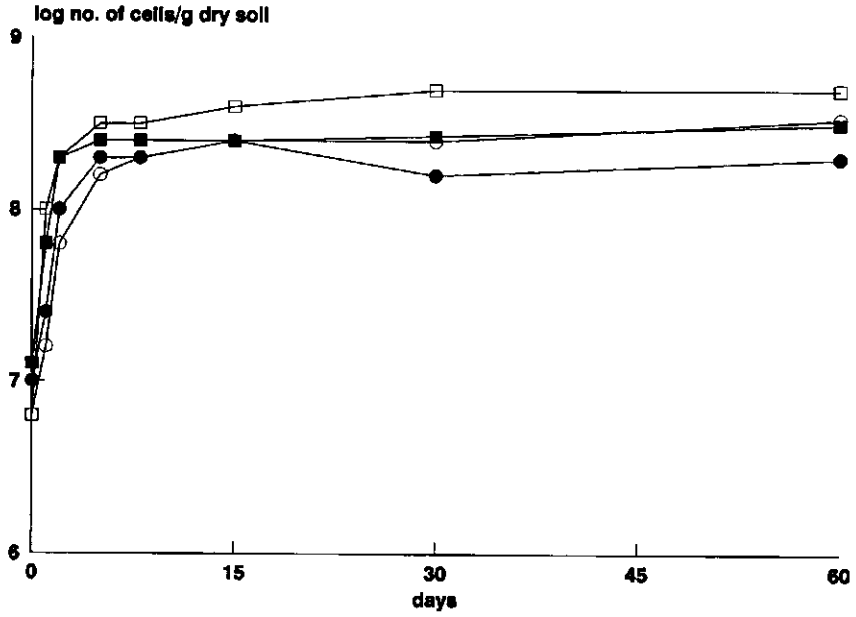

Fig. 1. Dynamics of *R. leguminosarum* biovar *trifolii* in sterile soil at 15°C and 18% moisture content. ○: IF counts, loamy sand; ●: plate counts, loamy sand; □: IF counts, loamy sand + 10% bentonite clay; ■: plate counts, loamy sand + 10% bentonite clay, IF counts were converted to 100% counting efficiency using a recovery of 53% for loamy sand and 47% for loamy sand + 10% bentonite counting efficiency using a recovery of 53% for loamy sand and 47% for loamy sand + 10% bentonite clay. Variations were within the dimension of each symbol.

some form of protection against a biological factor. In the literature conflicting data are reported for the effect of the presence of clay. Chao and Alexander (1982) showed that the survival of introduced *R. meliloti* decreased as the soil clay content increased. Van Elsas *et al.* (1986) found that introduced *Pseudomonas fluorescens* survived better under field conditions in a silt loam than in a loamy sand, suggesting that clay particles confer protection upon introduced cells. Moreover it could be shown that the addition of bentonite to non-sterile loamy sand offered some form of protection to introduced bacilli, resulting in the stimulation of gene transfer frequencies (Van Elsas *et al.*, 1987).

To test the hypothesis that bentonite clay offered protection against a biological factor, the effect of bentonite on the dynamics of introduced rhizobia in the presence of different fractions of a soil suspension added to sterile soil was studied. The fractions were chosen such that protozoan predators were either absent or present (Frey *et al.*, 1985). However, after an incubation of 2-4 weeks, protozoan predators

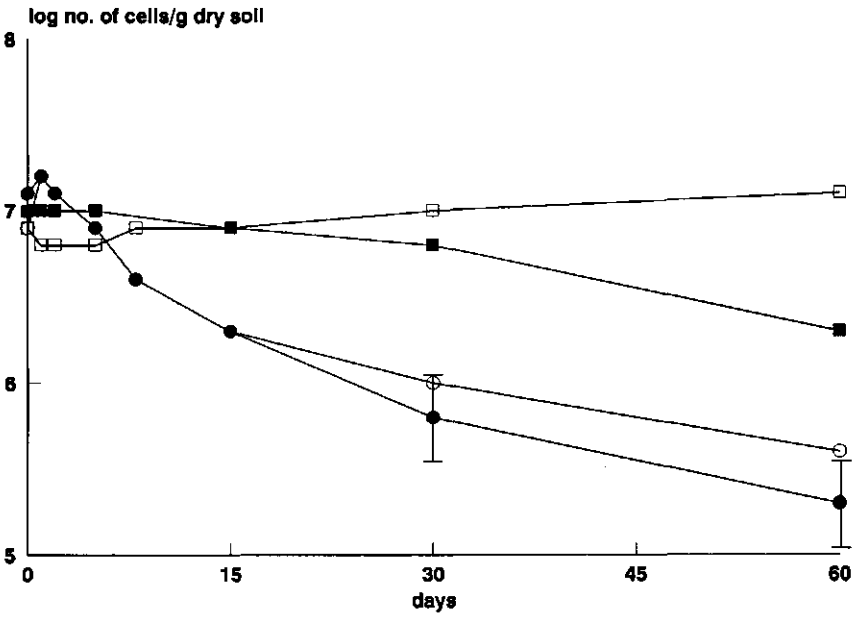

Fig. 2. Dynamics of *R. leguminosarum* biovar. *trifolii* in non-sterile soil at 15°C and 18% moisture content. ○: IF counts, loamy sand; ●: plate counts, loamy sand; □: IF counts, loamy sand + 10% bentonite clay; ■: plate counts, loamy sand + 10% bentonite clay. IF counts were converted to 100% counting efficiency using a recovery of 74% for loamy sand and 66% for loamy sand + 10% bentonite clay. Variations were within the dimension of each symbol, except for the plate counts in loamy sand on clay. Variations were within the dimension of each symbol, unless stated differently (error bars).

were detected in all soil portions, showing that the method described by Frey *et al.* (1985) had not been successful in this experiment. Thus, the results found with presumedly different soil suspensions could actually be regarded as replicate values (Fig. 3). In the presence of 10% bentonite clay rhizobial cell numbers were roughly stable, while in the absence of bentonite, rhizobial cell concentrations declined significantly ($P < 0.05$) in soil samples to which the soil suspensions were added. These results confirm the hypothesis that bentonite clay effectively protects introduced *Rhizobium* against a biological factor present in the soil suspension.

To investigate the role of protozoa and the effect of bentonite clay on their grazing activity, pre-cultured protozoa were added to sterile soil which had been inoculated with *R. leguminosarum* biovar *trifolii* cells. In the presence of protozoa, but without bentonite clay, rhizobial numbers in soil declined, after an initial growth period of about 3 days (Fig. 4). Protozoa increased from approximately 10^3 to $5x10^5$ cells/g

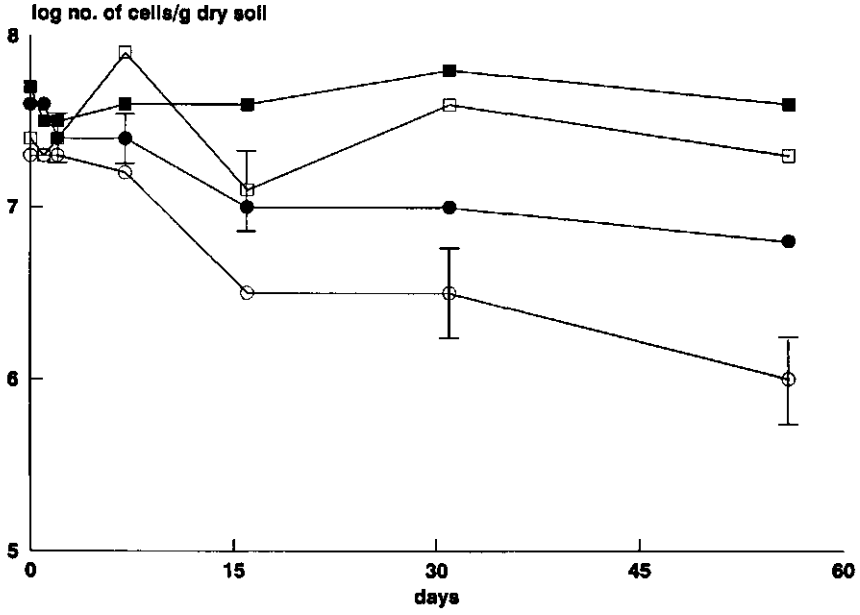

Fig. 3. Dynamics of *R. leguminosarum* biovar *trifolii* in sterilized soil samples amended with a soil suspension at 15°C and 18% moisture content. ○: IF counts, loamy sand; ●: plate counts, loamy sand; □: IF counts, loamy sand + 10% bentonite clay; ■: plate counts, loamy sand + 10% bentonite clay. IF counts were converted to 100% counting efficiency using a recovery of 70% for loamy sand and 44% for loamy sand + 10% bentonite clay. Variations were within the dimension of each symbol, unless stated differently (error bars).

dry soil during the first 15-30 days and remained at this level until the end of the experiment (Fig. 5). Only amoebae and flagellates were detected. A decline of introduced rhizobia coinciding with an increase in protozoa was also found by Danso *et al.* (1975) and Chao and Alexander (1981). In soil amended with bentonite the presence of protozoa did not lead to a decrease of rhizobial cell numbers. On the contrary, rhizobial cell concentrations increased and then remained relatively stable at a level of approximately $3x10^8$ cells/g soil. In this treatment protozoan numbers decreased slightly from approximately 10^3 to $4x10^2$ cells/g dry soil (Fig. 5) and only amoebae could be detected. The results from this experiment showed that protozoan predators were at least partly responsible for the decline of rhizobial numbers in soil, as also suggested by Chao and Alexander (1981) and Danso *et al.* (1975). Our results also suggest that the possibilities for grazing of protozoa on introduced rhizobial cells were greatly reduced by the presence of bentonite clay. Similar effects have been observed by Roper and Marshall (1978) who found that certain clays could inhibit the host-parasite interaction between *Escherichia coli* and its associated

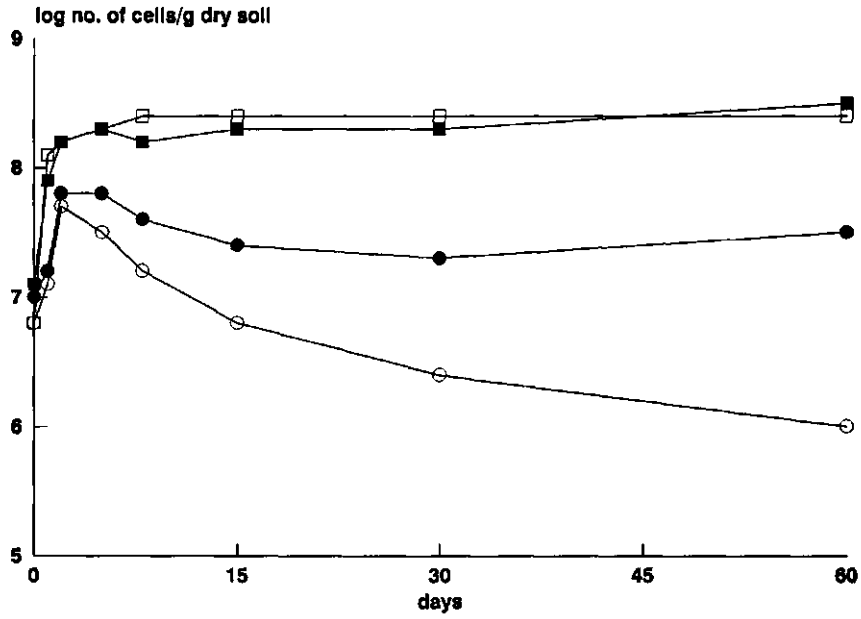

Fig. 4. Dynamics of *R. leguminosarum* biovar. *trifolii* in previously sterilized soil samples inoculated with protozoa at 15°C and 18% moisture content. ○: IF counts, loamy sand; ●: plate counts, loamy sand; □: IF counts, loamy sand + 10% bentonite clay; ■: plate counts, loamy sand + 10% bentonite clay. IF counts were converted to 100% counting efficiency using a recovery of 41% for loamy sand and 38% for loamy sand + 10% bentonite clay. Variations were within the dimension of each symbol.

bacteriophage. In the treatments in which no protozoa were added to the soil portions, plate counts were slightly lower than IF counts. Similar results were found by Rennie and Schmidt (1977) when comparing IF with most probable number counts. Moreover, Bakken and Olsen (1987) found that bacterial cells in soil, forming colonies on agar plates, represent only a small fraction of those seen by direct microscopic counts. These differences could be explained by assuming the presence of non-culturable, but still intact, viable cells (Colwell *et al.*, 1985). However, when protozoa were added to sterilized soil portions (Figs. 3 and 4, no bentonite), the IF-to-plate count ratio decreased in the course of the experiment. To explain this observation, it is suggested that protozoa, whilst moving through the soil, are able to transport rhizobial cells to niches which had not been colonized directly after inoculation. These sites possibly provide *Rhizobium* cells with extra substrate leading to growth and an increase in viable cell numbers. Moreover, the transport of rhizobial cells by protozoa could result in an increased adsorption to soil particles during the

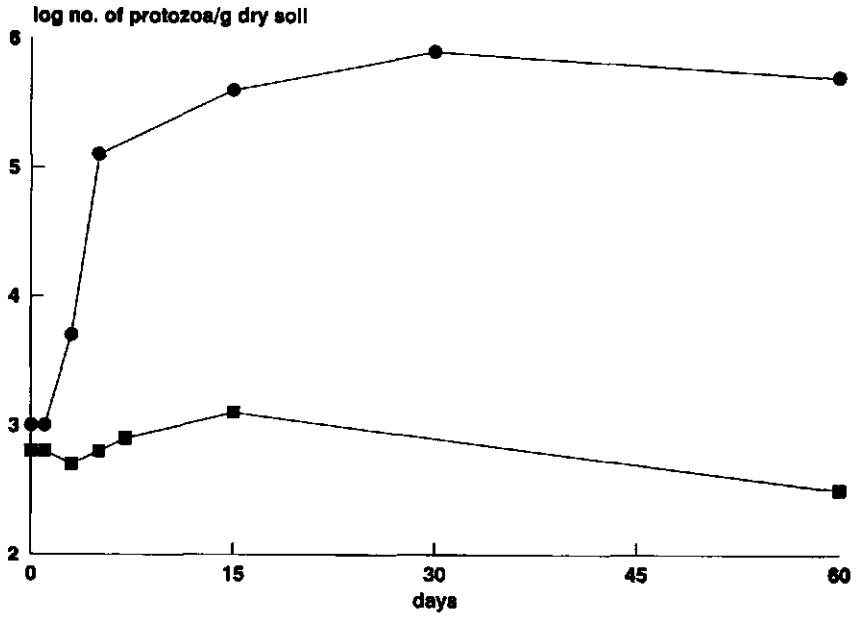

Fig. 5. Log numbers of protozoa in soil. ●: loamy sand, amoebae and flagellates; ■: loamy sand + 10% bentonite, only amoebae could be detected.

course of the experiment. This could result in a decrease in the recovery of cells with IF counts, since these more strongly adsorbed cells might not be completely released from soil particles by blending but instead might be eliminated together with the soil particles during flocculation procedures. Dilution plating techniques are less affected by adsorption phenomena, since flocculation is not necessary. Therefore, in further work recovery percentages may be determined for each sample on every sampling day, so as to be able to calculate absolute cell numbers. It is interesting to note, however, that a decrease in the IF-to-plate count ratio was only found when protozoa were added to sterilized soil in the absence of bentonite. Why it did not occur in non-sterile soil too, is as yet unknown. It seems unlikely that different numbers of protozoa present caused the ratio to change, since added protozoa reached concentrations comparable to those in non-sterile soil in about 5 days after their inoculation.

When a soil suspension was added, rhizobial numbers declined as from day 0, whereas when protozoa were added (Figs. 3 and 4), an initial growth period for rhizobia was found. Whether this is generally found is, as yet, unknown. It might suggest that apart from protozoan grazing, other organisms or processes present in the soil suspension influence the dynamics of introduced *R. leguminosarum* biovar *trifolii*. Anyhow, the present results suggest that protozoa might be the dominant

regulators of the dynamics of introduced rhizobia in soil. This is in accordance with the observations by Chao and Alexander (1981) who found that the survival of introduced rhizobia could be improved when agents toxic to protozoa were added to soil.

The large protective effect of bentonite clay on rhizobial survival might indicate that bentonite clay induces the formation of microhabitats, in which bacteria cannot be reached by grazing protozoa.

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CHAPTER 4

PROTECTION OF *RHIZOBIUM* BY BENTONITE CLAY AGAINST PREDATION BY FLAGELLATES IN LIQUID CULTURES

Summary

Bentonite clay strongly hampered the activity of the flagellate *Bodo saltans* in liquid culture, and thereby improved the survival of *Rhizobium*. A possible coating of bacteria and/or flagellates did not seem to play a part in protecting rhizobia from flagellates. Bentonite did not release any substances toxic to *B. saltans* during the incubation period. It is suggested that, in liquid cultures, bentonite clay increases the minimum level to which rhizobia can be predated upon, thereby giving rise to the presence of higher rhizobial cell concentrations at the end of the incubation period.

Introduction

For several decades bacteria have been introduced into soils to improve crop production (Date, 1970; Gaskins *et al*, 1985). A major problem however is the low survival rate of the introduced bacteria. Protozoa are often suggested to be the key organisms which control the abundance of populations of bacteria introduced into soil (Habte and Alexander, 1977; Steinberg *et al.*, 1987). It has often been observed that protozoa, which can grow by feeding on various bacterial species, are not able to reduce the density of a bacterial prey below a certain population size (Danso and Alexander, 1975; Habte and Alexander, 1978a; Habte and Alexander, 1978b; Alexander, 1981), and bacteria manage to persist at low cell densities.

The survival of *Rhizobium leguminosarum* biovar *trifolii* cells introduced into loamy sand was shown to be strongly improved by an amendment with 10% bentonite clay (Heijnen *et al.*, 1988). The bentonite severely hampered the predation by protozoa and also changed the soil texture and structure. Possible mechanisms for the protective action of bentonite clay towards bacteria grazed by protozoa may be: (i) changes in soil structure by the creation of microhabitats in which bacteria are protected from predation by protozoa, (ii) a detrimental effect on protozoa caused by

the presence of bentonite, (iii) the coating of rhizobia and/or flagellates by bentonite. Option (i) is discussed in Heijnen and Van Veen (1991) and options (ii) and (iii) will be discussed in this paper. To examine a possible detrimental effect of bentonite clay on protozoa and on their activity, the population dynamics of *R. leguminosarum* biovar *trifolii* and the flagellate *Bodo saltans* were studied in liquid cultures containing this clay mineral. Soil was omitted, so as to be able to study bentonite effects only, without interference of soil structural factors.

Materials and methods

Bacterial strain

A transposon Tn5 mutant of *R. leguminosarum* biovar *trifolii*, strain R62, resistant to kanamycin (Km) and rifampicin (Rp) was employed (Heijnen *et al.*, 1988). Stock cultures were maintained at -80°C in yeast extract mannitol broth (YMB) (Heijnen *et al.*, 1988) containing 17.5% glycerol. The bacterium was cultivated in YMB for 2 days at 29°C on a rotary shaker. Before use, cells were harvested by centrifugation (7000 x g, 15 min) and resuspended in sterile demineralized water (SDW).

Protozoa

A uniprotozoan population of the flagellate *B. saltans* (Postma *et al.*, 1990) capable of predating on *R. leguminosarum* biovar *trifolii*, was used. Flagellate cultures were maintained in SDW containing a suspension of *R. leguminosarum* biovar *trifolii* cells. A bacterial population always remained in the flagellate cultures. This background population could not be eliminated (Postma *et al.*, 1990).

Enumeration of bacteria and protozoa

R. leguminosarum biovar *trifolii* cells were enumerated by plating serial 10-fold dilutions on yeast extract mannitol agar (YMA) (Heijnen *et al.*, 1988) supplemented with 50 mg/l Km, 20 mg/l Rp and 100 mg/l cycloheximide. The latter was needed to suppress fungal growth on the agar plates. The bacterial background population was enumerated by a similar technique using half strength Tryptone Soya Agar (Oxoid, UK). The counts of the background population did not include rhizobia, because *R. leguminosarum* biovar *trifolii* was not capable of proliferating on half strength Tryptone Soya Agar. A most probable number method (Darbyshire *et al.*, 1974) was used to determine flagellate concentrations. Amoebae saline (AS) (Page, 1967) supplemented with *R. leguminosarum* biovar *trifolii* cells (approximately 10^8/ml) as a prey source was used for the determination of the number of flagellates. Microtiter plates were checked for flagellate growth after 1 week and 2-3 weeks of incubation at 15°C.

Statistical analysis

Genstat 5, Lawes Agricultural Trust (Rothamsted Experimental Station) was used for an analysis of variance on the obtained results.

Experimental systems

In experiment I the population dynamics of bacteria and flagellates were followed in 250-ml Erlenmeyer flasks which were filled with 20 ml SDW. For the first treatment rhizobia and for the second treatment rhizobia + 0.5 g bentonite (Heijnen *et al.*, 1988), a smectitic clay mineral, were added to the SDW. The third treatment consisted of SDW + rhizobia + flagellates and the fourth of SDW + rhizobia + flagellates + 0.5 g bentonite.

In a control experiment (experiment II) the influence of the bacterial background population on rhizobial dynamics was determined in a flagellate-free suspension containing the background population only. This flagellate-free suspension was obtained by adding 50 mg/l cycloheximide to a flagellate suspension, without rhizobial prey. The resulting protozoan-free bacterial background population was then added to Erlenmeyer flasks as described above together with rhizobia, and the development of the *R. leguminosarum* biovar *trifolii* population was studied. Treatments containing only rhizobia or rhizobia and *B. saltans* (and the background population) were used as controls. The concentration of bacteria belonging to the background population of the flagellate-containing suspension was similar to that in the flagellate-free suspension. Therefore, equal volumes of suspensions with and without flagellates could be added to Erlenmeyer flasks.

In order to investigate the possibility of bacterial and/or flagellate coating by bentonite clay (experiment III), rhizobia and flagellates were pre-incubated for 14 days in SDW containing 0.5 g bentonite. On day 14 an extra addition of *R. leguminosarum* biovar *trifolii* which had previously been incubated in SDW + 0.5 g bentonite for 14 days (A) was applied. This was compared with treatment B, to which *R. leguminosarum* biovar *trifolii* cells which had been incubated in SDW for 14 days without bentonite, were added. As a control (treatment C) only SDW was added. Bentonite concentrations (g/ml) were readjusted to the original level in treatments B and C, since the addition of new bacteria or SDW diluted the amount of bentonite present in the Erlenmeyer flask. The new bacteria and the SDW were added in equal amounts. The *Rhizobium* concentration was roughly doubled by the addition of new rhizobia.

In order to test whether a toxin was released by the bentonite clay during the incubation period (experiment IV), an Erlenmeyer flask containing SDW, flagellates and rhizobia was pre-incubated for 25 days in the presence of bentonite clay. The suspension was centrifuged (7000 x g, 15 min) and the supernatant was divided into two portions. One portion was reinoculated with flagellates and rhizobia (treatment E), and the other received an extra addition of bentonite before reinoculating with flagellates and rhizobia (treatment F). Flagellates were enumerated and their growth

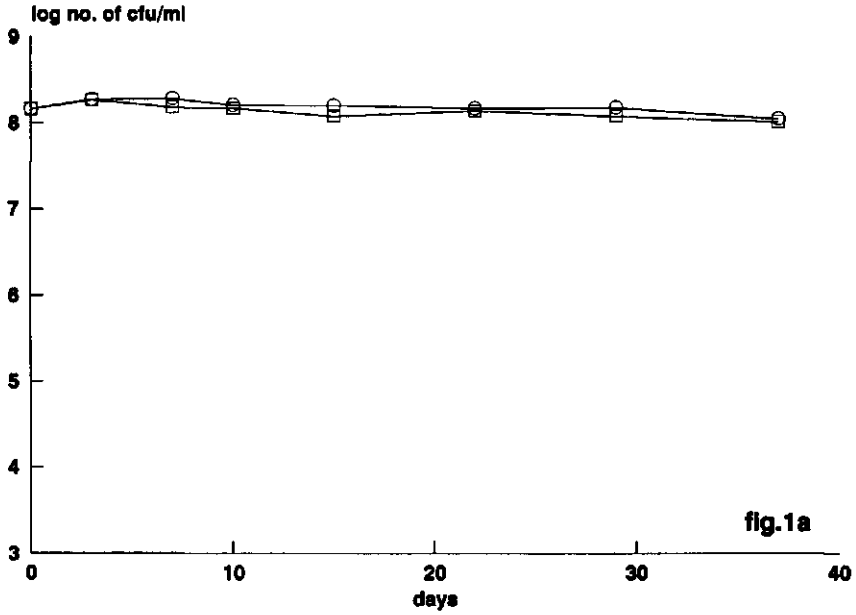
log no. of cfu/ml
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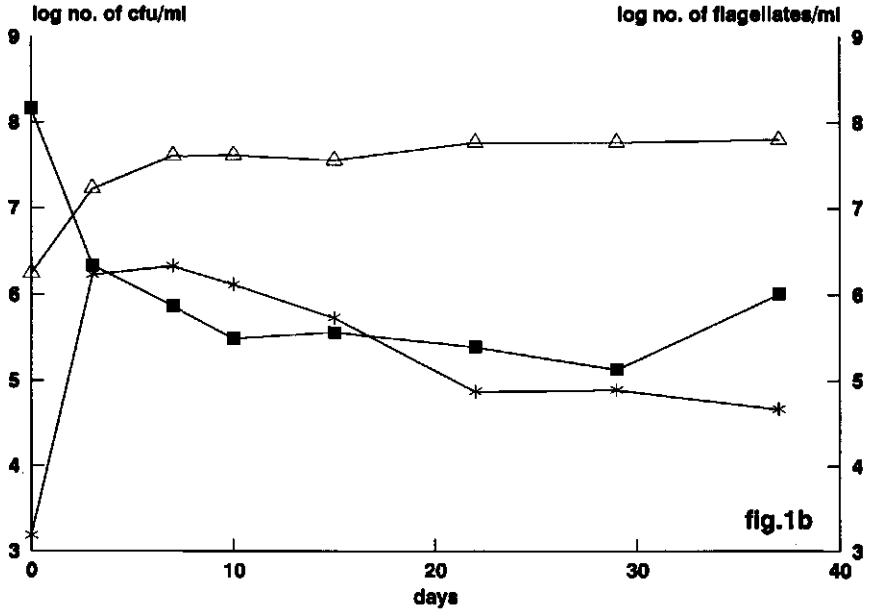
fig.1a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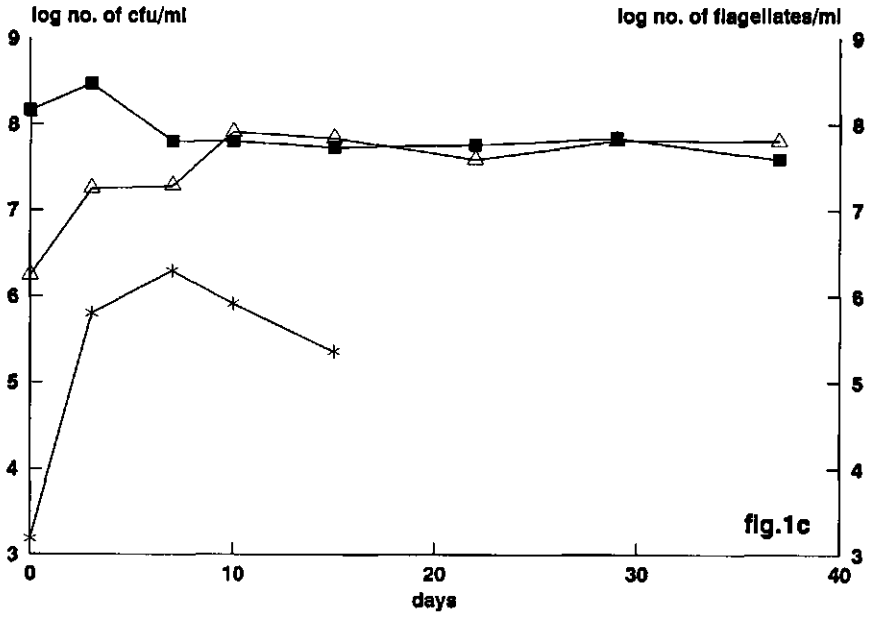

Fig. 1: (a) Population dynamics of *R. leguminosarum* biovar *trifolii* in SDW (○) and in SDW + bentonite (□). (b) Population dynamics of *R. leguminosarum* biovar *trifolii* (■), *B. saltans* (∗) and the bacterial background population (△) in SDW. (c) Population dynamics of *R. leguminosarum* biovar *trifolii* (■), *B. saltans* (∗) and the bacterial background population (△) in SDW + bentonite. No flagellates were detectable on days 22-37. LSD = 0.42 for rhizobial cell concentrations; LSD = 0.23 for the background population; LSD = 0.43 for *B. saltans*. cfu = colony forming units.

was compared with the growth of flagellates when they were inoculated into fresh SDW, also containing a rhizobial food source (treatment G).

In experiments I-IV initial rhizobial and flagellate densities were around 10^8 cells/ml and between 10^3 and 10^4 cells/ml, respectively. The Erlenmeyer flasks were incubated at 15°C. All treatments consisted of triplicate samples. The rise in pH caused by the addition of the bentonite clay was accounted for by adding approximately 75µl 1N HCl to the samples. The pH remained stable for all treatments during the entire incubation period.

Results

In experiment I rhizobial cell densities remained at a level of 10^8 cells/ml in SDW with or without added bentonite clay (Fig. 1a). The presence of bentonite clay had no

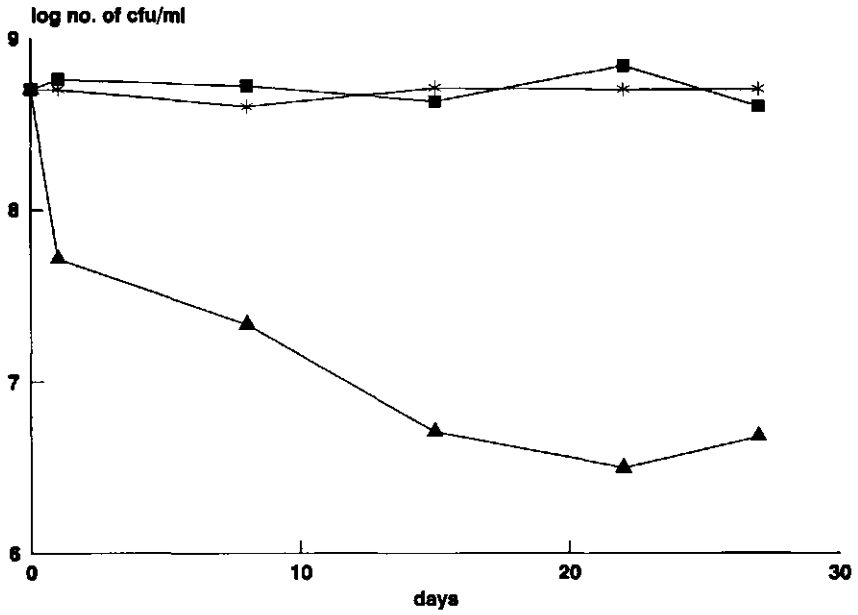

Fig. 2: Log no. of *R. leguminosarum* biovar *trifolii* cells (cfu's = colony forming units)/ml SDW in the presence of the bacterial background population (✳), in the presence of flagellates (▲) or without flagellates and without the bacterial background population (■). All treatments were without bentonite additions. LSD = 0.39.

significant influence on rhizobial cell concentrations. In the presence of flagellates and without bentonite, *Rhizobium* densities decreased significantly ($p < 0.05$) by about 2 to 3 log units in about 10 days as compared to the results in Fig. 1a. The flagellate increased from 10^3 to approximately 10^6 cells/ml during the first 3-7 days, and then slowly decreased to a level of around 10^5 cells/ml by day 37 (Fig. 1b). The bacterial background population increased to a level between 10^7 and 10^8 cells/ml (Fig. 1b). In the presence of bentonite clay rhizobial numbers were not influenced by the presence of protozoa (Figs. 1a, 1c). Bentonite additions had no influence on the dynamics of the bacterial background population (Figs. 1b, 1c). During the first 7 days of the experiment, the flagellate population was not significantly influenced by the presence of bentonite (Fig. 1b, 1c). After that there was a strong decrease in protozoan numbers (Fig. 1c). Flagellates could no longer be detected after day 15. However, the presence of bentonite interfered with the microscopic observations, resulting in a detection level of around 10^3 flagellates/ml. We suspect that the flagellates may not have disappeared completely, which was actually the case in experiment III (Fig. 3) when they could be detected at low levels until day 35.

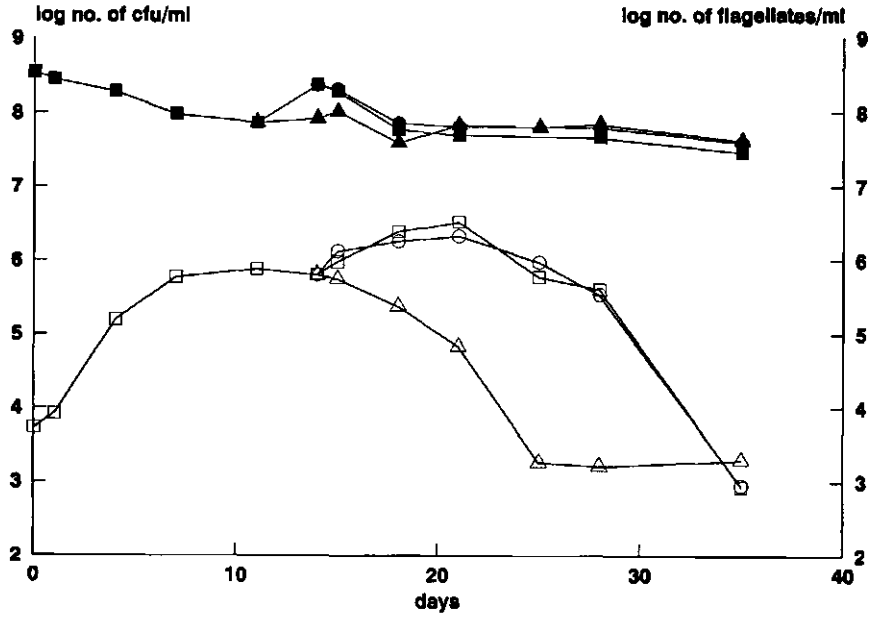

Fig. 3: Log no. of *R. leguminosarum* biovar *trifolii* (■) and *B. saltans* (□) cells/ml with an addition, on day 14, of rhizobia incubated in the presence of bentonite clay. Log no. of *R. leguminosarum* biovar *trifolii* (●) and *B. saltans* (○) cells/ml with an addition, on day 14, of rhizobia incubated without bentonite clay. Log no. of *R. leguminosarum* biovar *trifolii* (▲) and *B. saltans* (△) cells/ml without an addition of rhizobial prey on day 14. LSD = 0.70 for flagellates; LSD = 0.38 for rhizobia. cfu = colony forming units.

Experiment II (Fig. 2) showed that the background population alone had no significant influence on the survival of *Rhizobium*. In the presence of added flagellates, rhizobial cell concentrations again declined.

In experiment III it was found that the addition of fresh rhizobia, incubated in the presence of bentonite (A) or without bentonite (B) for 14 days, resulted in a significant ($p < 0.05$) increase of flagellates (day 18 - day 28, Fig. 3), as compared to the control treatment (C) to which no fresh rhizobia were added. Flagellate numbers in this control decreased to very low levels from day 15 onwards. By day 35 the flagellate concentration in treatments A and B had decreased to the same level as found in treatment C. No significant differences in flagellate concentrations were found between treatments A and B. After adding rhizobia on day 14, the rhizobial concentrations became roughly twice as high in treatments A and B as compared to C, resulting in only a small difference in the log number of colony forming units (Fig. 3).

In experiment IV, *B. saltans*, inoculated into in a supernatant of a culture from which flagellates had almost disappeared due to the presence of bentonite clay (E), grew vigorously (Table 1). Flagellate numbers were not significantly different from those in the control (G), in which flagellates were inoculated into fresh SDW with a rhizobial food source. When bentonite was also added to the supernatant (F) before reinoculation with flagellates, the flagellates did not grow, and flagellate numbers were significantly ($P < 0.05$) different from treatments E and G.

Table 1: Log no. of flagellates/ml after reinoculation into suspensions E, F and G. E: flagellates reinoculated into the supernatant of a bentonite containing flagellate culture. F: as E, with bentonite added to the supernatant before reinoculation with flagellates. G: flagellates inoculated into fresh SDW. LSD = 0.69. (N.D. = not detectable).

treatment	E	F	G
day 1	4.87	4.06	4.55
2	5.78	4.03	5.53
3	6.26	3.92	5.74
6	5.82	N.D.	5.79
9	6.09	2.88	5.73
13	6.17	3.13	5.96

Discussion

In liquid cultures bentonite had a strong protective effect on *Rhizobium* against predation by *B. saltans*. This corresponds with our previous results in soil microcosms, where we found that bentonite addition to loamy sand conferred protection to rhizobia from predation by protozoa (Heijnen *et al.*, 1988). Roper and Marshall (1979) also found a positive effect of montmorillonite on the survival of *Escherichia coli* in sea water. In our experiments the rhizobial prey was not completely eliminated by the predating flagellate, corresponding to results found by Habte and Alexander (1977) and Danso and Alexander (1975). Our results also indicated that the flagellate *B. saltans* fed mostly, and maybe even selectively, on rhizobial cells, since the background bacterial population managed to multiply while the rhizobial population decreased. Selective feeding has also been observed by Cassida (1989).

The bacterial background population had no influence on rhizobial population dynamics (experiment II). However, the cell numbers of the background population increased (Figs. 1b and 1c), even though no carbon source was added. This indicates that predation by flagellates on *Rhizobium* led to the production of metabolites, which could be utilized as a substrate by the bacterial background population. Since

the bacterial background population had no influence on rhizobial dynamics, the decline in rhizobial numbers was purely a result of predation by protozoa.

In the presence of bentonite clay flagellate numbers decreased. This could be the result of a physical barrier: a bentonite coating of either the rhizobia (making them unrecognizable or inedible) or the flagellate (imposing difficulties in moving or disturbing the feeding mode by a coating of the flagella). Rhizobial coating probably did not act as a barrier for predation, because flagellates were able to reproduce when more, presumably coated, bacterial prey was added (Experiment III, treatment A). Pointing in the same direction is the similar reaction of flagellates to 'coated' and 'non-coated' rhizobia (treatments A and B) which would not have been the case if a rhizobial coating was the reason for the bentonite protection. A bentonite coating of flagellates also seemed to be unlikely, because predation continued when more rhizobia were added (treatments A and B). Marshall (1968) found strong evidence for a bentonite coating round rhizobial cells. Also, Roper and Marshall (1974) showed that *E. coli* could be coated with colloidal montmorillonite in a saline sediment. It was concluded from our results that even if a bentonite coating of rhizobia or flagellates was present, this coating did not act as a physical barrier, hampering predatory activities.

The results from experiment IV indicate that the bentonite did not release a substance toxic to flagellates. If a toxic substance had existed, it would be expected to be present in treatment E, which should then have shown a reduction in flagellate concentrations in stead of the observed increase. In agreement with our results, Amblès *et al.* (1989) found that bentonite added to a podsol soil did not release previously bound toxic lipids, and thereby reduced the toxicity of these lipids towards soil microorganisms. The combined results from experiment IV led us to conclude that the actual presence of the bentonite clay in suspensions caused flagellate growth to cease after a certain time lag.

Thus, the protective effect of bentonite clay on the survival of rhizobia in solutions might be caused by increasing the minimum level to which a prey can be predated upon. Protozoa, unable to distinguish between bentonite and rhizobia, predating in suspensions containing bentonite, will consume large amounts of metabolically inert clay (particle diameter $< 2\mu m$ (Knapp, 1979), together with rhizobia (0.5-0.9 x 1.2-3.0 μm (Jordan, 1984)). The energy gain for the protozoa will be relatively low, the number of predators will remain constant or decrease, and the bacterial survival will be at a higher level than when bentonite was absent. Doubling the amount of bacteria on day 14 in experiment III caused the energy gain through the consumption of particles, containing relatively more bacteria, to increase. Therefore, protozoan concentrations did not decline until the surplus of bacteria had disappeared through predation and the fraction of bentonite in the consumed particles had again risen above a certain critical level. Then flagellates were again found to decrease and rhizobia survived, resulting in a relatively high concentration of

rhizobial cells as compared to situations where bentonite was absent. In other words, the minimum level to which a prey can be predated upon was increased by the presence of bentonite clay. Habte and Alexander (1978b) also found that once the final minimum level of *Klebsiella* was reached through predation, the concentration of *Tetrahymena* started to decrease. Similar results were found by Danso and Alexander (1975) and by Steinberg *et al.* (1987).

The experiments described in this paper clearly show that the protection of rhizobia in liquid cultures against predation by protozoa was not caused by the release of a substance toxic to protozoa. It seems reasonable to expect that this will not occur in the soil environment either. Also a bentonite coating of rhizobia or flagellates could not explain the increased rhizobial survival. It is suggested that the presence of bentonite clay in liquid cultures increases the minimum level to which a prey can be predated upon, resulting in a higher survival level for the introduced bacterial strain.

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CHAPTER 5

A DETERMINATION OF PROTECTIVE MICROHABITATS FOR BACTERIA INTRODUCED INTO SOIL

Summary

Survival studies with rhizobia introduced into loamy sand showed that a kaolinite amendment of the soil improved the survival of *Rhizobium*, and that bentonite had a very strong positive effect on rhizobial survival. The survival level was significantly higher in soil amended with 10% than with 5% bentonite. The amount of water present in the bentonite amended soil had a significant influence on rhizobial survival; in drier soil, survival levels were highest. For the loamy sand, the loamy sand amended with 5 and 10% bentonite or with 10% kaolinite, the number of rhizobial cells surviving on day 57 after introducing 2.5-5.0 x 10^7 cells/g dry soil could be described using the distribution of pores from three size classes in a mathematical relationship. Pores with necks <3 μm and between 3 and 6 μm positively affected the survival of introduced rhizobia, whereas pores with necks >6 μm had a negative effect.

Introduction

In the past, bacteria have often been introduced into soil as a means by which to improve crop productivity, e.g. symbiotic *Rhizobium* spp. to legumes (Stacey and Upchurch, 1984). A successful application of introduced bacteria in soil requires the survival of sufficient numbers of the introduced strain in the new environment. It is difficult to predict the survival rate of introduced bacteria in soil, mainly because soil forms a very heterogeneous environment, in which it is difficult for introduced bacteria to reach and enter suitable (micro) habitats where they can survive (Hattori and Hattori, 1976; Postma *et al.*, 1989).

Crozat *et al.* (1987) found that rhizobial cell numbers dropped to 10^3-10^4 cells/g soil after their introduction into soil, regardless of the inoculum size. Predation, e.g. by protozoa, is often assumed to be responsible for the decline in bacterial cell

numbers observed after their introduction into soil (Habte and Alexander, 1977; Steinberg *et al.*, 1987). Postma *et al.* (1989) found that if the soil was relatively dry at the moment of introduction, the survival rate of introduced bacteria was higher than when cells were introduced into relatively wet soils. They suggested that under dry conditions the introduced bacteria were able to enter relatively small pores, where they would be better protected from predation than cells introduced into wetter soils. Heijnen *et al.* (1988) found that the addition of 10% bentonite clay to loamy sand greatly improved the survival of introduced rhizobia, due to a decrease in the predatory activity of protozoa. Under extreme environmental conditions such as temperatures of 70°C (Marshall, 1964) or desiccation stress (Bushby and Marshall, 1977) montmorillonite clay was also found to protect introduced bacteria in soil. Other factors affected by the presence of clay minerals related to bentonite are microbial growth, respiration and metabolism (Stotzky, 1986).

The addition of bentonite clay to loamy sand has a complex effect on the soil structure (Schahabi, 1967; Kremer *et al.*, 1970), influencing both pore size distribution and pore space. The protective effect of bentonite clay on the survival of introduced bacteria in soil previously found by Heijnen *et al.* (1988) might be caused by a change in soil structure, resulting in the creation of microhabitats, in which bacteria are protected from predation by protozoa. Another mechanism for the bentonite protection might be a direct detrimental effect of bentonite on protozoa (possibly a coating of bacteria and/or protozoa by bentonite). The first aspect is discussed in this paper, the latter in Heijnen *et al.* (1991).

In this paper we have tried to find a relationship between the amount of pores (with pore necks larger than rhizobia but smaller than most protozoa predating on them) present in soil and the survival of introduced rhizobial cells. We hypothesized that pores which could not be entered by protozoa, simply because the pore necks were too small, might serve as protective microhabitats. The volumes occupied by these so-called protective microhabitats were estimated.

Due to the changes in soil structure upon the addition of bentonite, the moisture characteristic of the soil is strongly affected (Schahabi, 1967; Kremer *et al.*, 1970). We therefore also studied the population dynamics of introduced rhizobia in different soil samples, with or without bentonite, in which all pores with the same neck diameter would be water-filled. In this paper, amendments of loamy sand with kaolinite clay were studied along with bentonite amendments, in order to make a comparison between the effect of a swelling clay (bentonite) and a non-swelling clay (kaolinite) on the survival of introduced bacteria in soil.

Materials and methods

Soil and clay minerals

A loamy sand from the Netherlands was used. Soil characteristics were given by van Elsas *et al.* (1986). The soil was sieved (4mm) and stored at 4°C. One week before starting an experiment, the soil was stored at 15°C. For some experiments bentonite, a smectitic clay mineral with a very large swelling capacity (Van Olphen, 1963) was added to the loamy sand. In other experiments the loamy sand was amended with kaolinite, a clay mineral with hardly any swelling capacity (Van Olphen, 1963).

Bacterial strain

A transposon Tn5 mutant of *Rhizobium leguminosarum* biovar *trifolii*, strain R62, devoid of vector sequences, resistant to kanamycin (Km) and rifampicin (Rp) was employed (Heijnen *et al.*, 1988). The bacterium was cultured in yeast extract mannitol broth supplemented with 25 mg/l Km for 2 days at 29°C on a rotary shaker (150 rpm). Before use, cells were harvested by centrifugation (7000 x g, 15 min.) and resuspended in sterile demineralized water.

Enumeration of introduced bacteria

Total numbers of culturable rhizobial cells were determined by transferring a 10 g soil portion into a 250-ml Erlenmeyer flask containing 95 ml of a 0.1% sodium pyrophosphate solution and 10 g gravel (2-4 mm dia) to improve dispersal of bacterial cells. Flasks were shaken for 10 min at 200 rpm at room temperature. Serial 10-fold dilutions were plated on yeast extract mannitol agar (Heijnen *et al.*, 1988) supplemented with 50 mg/l Km and 20 mg/l Rp. Cycloheximide (100 mg/l) and benomyl (50 mg/l) were added to inhibit fungal growth. Total rhizobial numbers, including non-culturable cells (Colwell *et al.*, 1985) were determined using an immunofluorescence (IF) technique with a specific antiserum as described by Postma *et al.* (1988). IF counts and plate counts were done on separate soil portions in duplicate.

Soil studies

Survival experiments with introduced rhizobia were carried out using soil microcosms containing moist soil corresponding to 10 g dry weight. The loamy sand was amended with 5 or 10% (w/w) bentonite, with 10% kaolinite or was left unamended. The soil samples were brought up to the required moisture content, less 250 µl, which amount was added together with the bacterial inoculum (2.5-5.0 x 10^7 cells/g dry soil) 4 days later. After inoculation, each soil sample was mixed and the bulk density of the soil portions was adjusted to 1.2 g/cm^3.

Moisture characteristic curves of the loamy sand amended with or without clay minerals were determined by placing saturated soil samples on tension tables at a succession of known matric suction levels. When the desired matric suction level was reached, the water content of the sample was determined. To determine the effective

diameter (d) of the largest pore to remain full of water when a matric suction (s) is applied to drain the soil, the relationship:

$$s = \Gamma/\sigma g d$$

was used (Marshall and Holmes, 1980), in which Γ is the density of water, g the acceleration due to gravity and σ the surface tension of water. The volume of water withdrawn from soil on increasing the suction from s_1 to s_2 represents the volume of pores with an effective diameter between d_1 and d_2. This relationship can only be used for water affected by capillary forces (0-100 kPa), thereby assuming the contact angle to be zero. In cases where the pore neck diameter is smaller than the pore diameter, the effective diameter d is actually the pore neck diameter.

The survival of introduced rhizobia was studied in loamy sand, in loamy sand amended with 10% bentonite and in loamy sand amended with 10% kaolinite (Experiment I), in order to determine the effect of the type of clay mineral added to the soil on rhizobial survival. The moisture content of 18% (w/w) was the same for all three treatments, corresponding to a water potential of -10 kPa for the loamy sand and the loamy sand amended with 10% kaolinite, and a water potential of -400 kPa in the presence of 10% bentonite. To investigate whether the bentonite effect found in experiment I was the result of a reduced availability of water due to the presence of bentonite clay, the water potential was kept equal to -10 kPa in experiment II. This water potential of -10 kPa is also known as field capacity and is often regarded to correspond to a favourable water content of the soil as regards to plant growth. In addition, the effect of the amount of bentonite present in the samples was studied. This means that the survival of introduced rhizobia was studied in (a) loamy sand at 18% moisture content, -10 kPa water potential; (b) loamy sand + 5% bentonite clay at 18% moisture content, -80 kPa water potential; (c) loamy sand + 5% bentonite clay at 30% moisture content, -10 kPa water potential; (d) loamy sand + 10% bentonite clay at 18% moisture content, -400 kPa water potential; (e) loamy sand + 10% bentonite clay at 30% moisture content, -100 kPa water potential and (f) loamy sand + 10% bentonite clay at 45% moisture content, -10 kPa water potential.

Soil samples were incubated for 57 days at 15°C in a humid chamber in the dark. No moisture losses from the soil portions were observed. The survival of introduced rhizobia will be defined here as the number of introduced cells present in the soil after a certain incubation time.

Statistical analysis

Genstat 5, Lawes Agricultural Trust (Rothamsted Experimental Station) was used for an analysis of variance of the obtained data and for the examination of the relationship between survival of introduced rhizobia and the volume of pores of certain size classes in the soil samples.

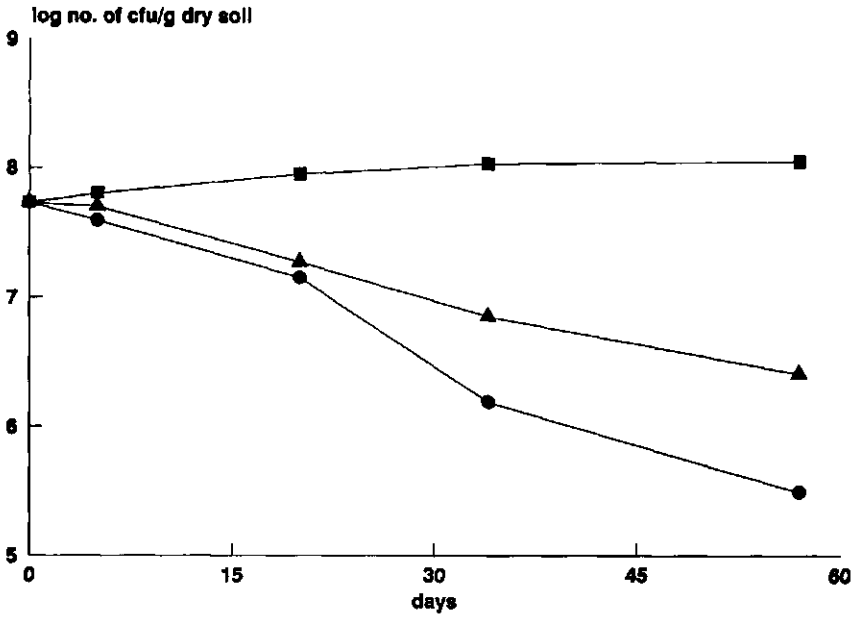

Fig. 1. Population dynamics of *R. leguminosarum* biovar *trifolii* in loamy sand (●), loamy sand amended with 10% kaolinite (▲) and loamy sand amended with 10% bentonite (■), determined using plate count techniques. cfu = colony forming units. The largest difference on a log scale found between two duplicate survival levels was 0.20.

Results and Discussion

The influence of the addition of either bentonite or kaolinite to loamy sand on the survival of introduced bacteria was studied in experiment I (Fig. 1). The number of rhizobial cells introduced into loamy sand decreased during an incubation period of 57 days by approximately 2 log units; the presence of 10% kaolinite in loamy sand significantly ($P < 0.05$) increased the numbers of cells remaining in the soil at the end of the incubation period. *Rhizobium* cells survived significantly better ($P < 0.05$) in soil amended with 10% bentonite clay than in soil left unamended or amended with kaolinite (Fig. 1). Therefore, the type of clay mineral, and not just the fact that a clay mineral had been added, determined the extent to which the survival of introduced cells was improved. Similar results with bentonite clay were previously shown by Heijnen *et al.* (1988). Stutz *et al.* (1989) also found that the type of clay mineral present was important for the survival of an introduced strain of *Pseudomonas fluorescens*; survival in vermiculite was better than in montmorillonite, which in turn improved survival as compared to illite. Marshall (1964) demonstrated that a kaolinite induced protection did not occur for rhizobia incubated in sterile sand amended with

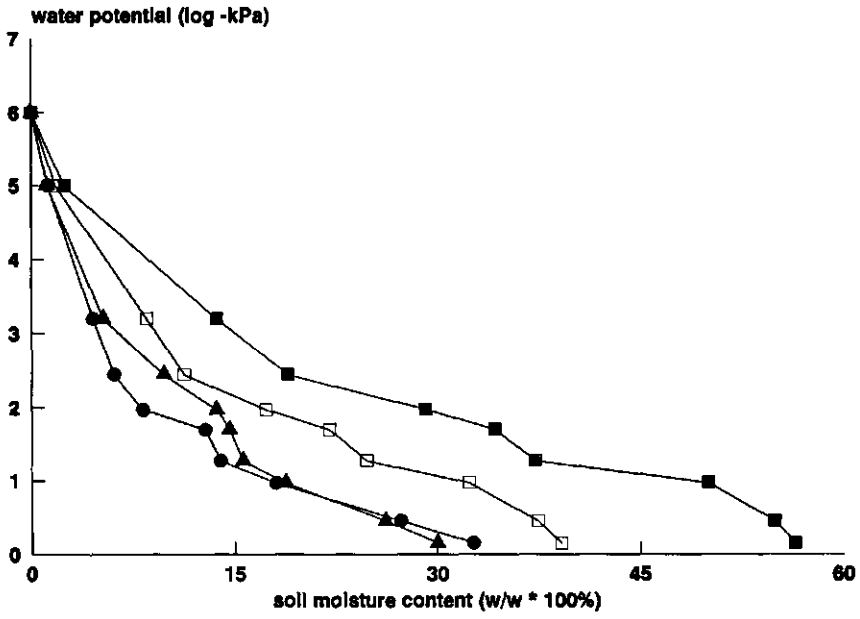

Fig. 2. Moisture characteristic curves of loamy sand (●), loamy sand amended with 10% (■) or 5% bentonite (□) and loamy sand amended with 10% kaolinite (▲). The bulk density was 1.2 g/cm^3.

kaolinite at 70°C. Bentonite however did protect the introduced rhizobia under these conditions. Dommergues (1964) found that kaolinite could protect introduced bacteria from desiccation at suction levels < -3.2 x 10^4 kPa.

A possibility for the better protection of introduced bacteria offered by bentonite clay than by kaolinite, could be that through the swelling of bentonite clay in the moist soil (Marshall and Holmes, 1980) more sites were created in which bacteria were protected from predation by protozoa than in the kaolinite (a non-swelling clay mineral) amended soil; protozoa had previously been found to be responsible for the decline of introduced rhizobia in the loamy sand used for the experiments described here (Heijnen *et al.*, 1988).

Darbyshire (1976) and Vargas and Hattori (1986) found that the activity of ciliates was strongly reduced at water potentials below -50 kPa and -120 kPa, respectively. In experiment I a moisture content of 18% in loamy sand amended with 10% bentonite clay corresponded to a water potential below -100 kPa (Fig. 2). This low water potential could therefore have inhibited predatory activities by protozoa, resulting in an improved survival of the introduced bacteria in loamy sand amended with 10% bentonite clay. In experiment II (Fig. 3) the survival of introduced rhizobia was studied

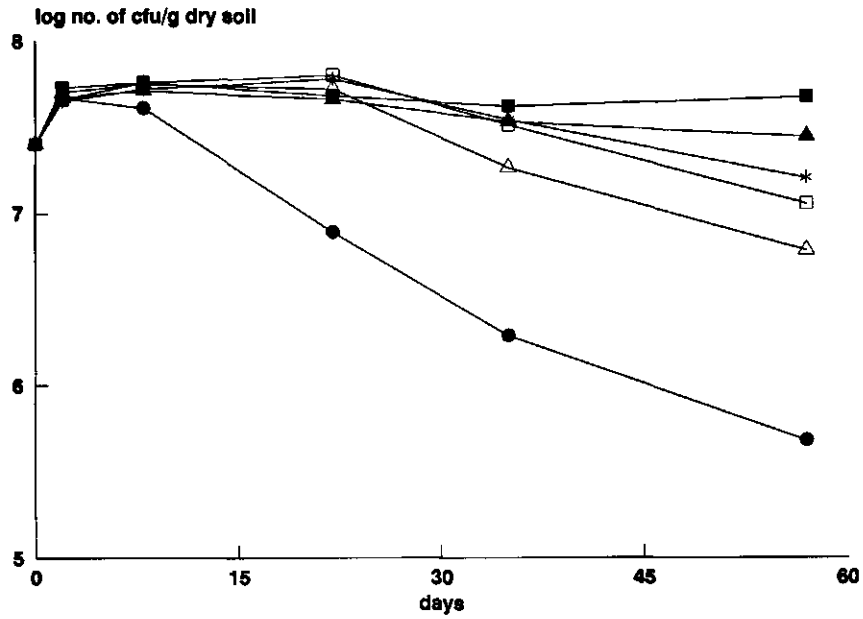

Fig. 3. Population dynamics of *R. leguminosarum* biovar *trifolii* determined using plate count techniques in loamy sand, 18% moisture content (●); loamy sand amended with 10% bentonite, 18% moisture content (■); loamy sand amended with 10% bentonite, 30% moisture content (▲); loamy sand amended with 10% bentonite, 45% moisture content (∗); loamy sand amended with 5% bentonite, 18% moisture content (□); loamy sand amended with 5% bentonite clay, 30% moisture content (△). cfu = colony forming units. The largest difference on a log scale found between duplicate survival levels was 0.20.

in loamy sand amended with 5 and 10% bentonite, incubated at different moisture contents. A comparison of the survival in soils with the same moisture content or with the same water potential was made.

At a moisture content of 18%, introduced rhizobia survived significantly better ($P < 0.05$) in the bentonite amended than in unamended loamy sand as from day 8. On day 57 the survival rate was significantly ($P < 0.05$) higher in the 10% than in the 5% bentonite treatment. At a moisture content of 30% the difference in rhizobial survival between 5 and 10% bentonite amendments was significant ($P < 0.05$) as from day 35, again with the best survival in the 10% bentonite treatment. At a given water potential, the bentonite treatments gave a significantly ($P < 0.05$) higher survival level as compared to the loamy sand as from day 22. Survival in the 10% bentonite treatment was significantly ($P < 0.05$) higher than in the 5% treatment as from day 35. The survival of introduced rhizobia in loamy sand + 5% bentonite was significantly lower ($P < 0.05$) at a moisture content of 30% than at 18% as from day 35. For the 10% bentonite treatment significant differences between the three moisture contents

occurred only on day 57; survival was best at a moisture content of 18%, and worst at 45% moisture content.

Both in Experiments I and II, no significant differences between total rhizobial counts using immunofluorescence detection and plate counts were observed on day 57 (Table 1). Therefore, it seems unlikely that large numbers of non-culturable cells were present in this experiment. Many researchers however found that certain proportions of bacterial cells in soil lack culturability (Bottomley and Maggard, 1990; Heijnen *et al.*, 1988; Kennedy and Wollum, 1988), resulting in lower plate counts as compared to IF counts.

Table 1: Log number of rhizobial cells counted on day 57 in Experiments I and II using plate count (PC) and immunofluorescence detection techniques (IF).

	PC	IF
Experiment I		
loamy sand	5.49	5.62
loamy sand + 10% bentonite	8.05	8.09
loamy sand + 10% kaolinite	6.41	6.42
Experiment II		
loamy sand, 18% moisture	5.68	5.65
loamy sand + 5% bentonite, 18% moisture	7.05	7.11
loamy sand + 5% bentonite, 30% moisture	6.78	6.69
loamy sand + 10% bentonite, 18% moisture	7.67	7.67
loamy sand + 10% bentonite, 30% moisture	7.44	7.47
loamy sand + 10% bentonite, 45% moisture	7.20	7.25

The protective effect of bentonite clay was confirmed in Experiment II. The observation of such a prominent bentonite effect on rhizobial survival at water potentials of -10 kPa, shows that the improved survival of introduced rhizobia in soil amended with bentonite at 18% moisture content was not caused exclusively by a reduced water availability in the bentonite treatments. In the bentonite amended loamy sand and in the non-amended loamy sand, both with water potentials of -10 kPa, pores with the same neck diameters were all filled with water. Theoretically, this would enable protozoa to proliferate equally in both amended and unamended loamy sand. When protozoan grazing is the main process responsible for the decline in numbers of bacterial cells introduced into soil (Heijnen *et al.*, 1988), it can be concluded that at water potentials of -10 kPa protozoan grazing was also hampered by the very presence of bentonite clay. The inability of protozoa to proliferate due to a discontinuous water film as a result of a lack of water as shown by Vargas and Hattori (1986), could explain the higher survival levels of introduced rhizobia in the

drier bentonite treatments as compared to the wetter ones.

Since bentonite clay was found to be non-toxic to protozoa (Heijnen *et al.*, 1991), the increased survival of introduced bacteria in loamy sand amended with clay minerals could be explained by assuming a change in the pore size distribution. *R. leguminosarum* biovar *trifolii* has a cell size of 0.5-0.9 x 1.2-3.0 μm (Jordan, 1984). We assumed that protozoa feeding on these rhizobia are at least 2-3 times larger than their prey; this means that if rhizobial cells enter pores with a neck < 6 μm, they would be protected from predation by protozoa, simply because most of their predators are too large to enter these small pores. Pores with a neck diameter < 3 μm should also be able to offer protection, but the introduced rhizobia might have difficulties in entering these pores themselves. These assumptions agree with Hattori and Hattori (1976), who suggested that pores with diameters between 2 and 6 μm might be favourable microhabitats for soil bacteria.

At a suction of -50 kPa, pores with an effective diameter of 6 μm will, theoretically, remain filled with water. The moisture retention curves (Fig. 2) show that there is an increase in the volume of pores with necks < 6 μm in the order: loamy sand < loamy sand + 10% kaolinite < loamy sand + 5% bentonite < loamy sand + 10% bentonite. The same order was found for pores < 3 μm (suction < -100 kPa).

Combining the results of the survival experiments in loamy sand, with or without clay mineral amendments, with the findings from the moisture characteristic curves, it shows that a positive correlation coefficient of 94% existed on day 57 between the volume of pores with neck diameters < 6 μm and the survival level of the rhizobia introduced at a level between 2.5-5.0 x 10^7 cells/g dry soil in samples with a water potential of -10 kPa. It is stressed that at water potentials of -10 kPa, the introduced bacteria would theoretically be able to enter the same range of water-filled pores in all four treatments. The high correlation coefficient indicates that pores with a small neck diameter might be very important for the survival of introduced rhizobia; they may be considered to be protective microhabitats.

We have tried to find a mathematical description to describe the log numbers of introduced rhizobia surviving in soil samples after an incubation period of 57 days. The relationship found, with 98% of the variance accounted for, was:

$$S_{d57} = 6.98 + 0.04\ P_{<3\mu m} + 0.92\ P_{3\text{-}6\mu m} - 1.92\ P_{6\text{-}15\mu m}$$

in which S_{d57} is the number of rhizobial cells remaining in soil samples inoculated with 2.5-5.0 x 10^7 cells/g dry soil on day 57 and P is the amount of soil moisture (w/w x 100%) which can be theoretically held by pores with the appropriate pore (neck) diameter size class.

Using this relationship it can be concluded that pores < 6 μm play a positive part in the survival of introduced bacteria in soil. Considering the size of rhizobia as compared to the pore diameter, the contribution of pores with a neck diameter < 3

μm is only small. The pore size class 3-6 μm, however, can easily accommodate rhizobial cells, and protozoa will probably have difficulties in entering these pores. For these reasons, pores with diameters < 6 μm can serve as protective microhabitats. According to the model, pores with neck diameters between 6 and 15 μm have a negative effect on rhizobial survival; both rhizobia and protozoa can easily enter these pores. Pores > 15 μm were not included in the model, because they made no significant contribution at a level of $P < 0.05$. All other pore size classes were significant at a level of $P < 0.05$.

Most results discussed here are based on moisture retention curves. Another possibility for quantifying pores is the use of image analysis on soil thin sections. A problem is that results obtained with moisture retention curves and image analysis tend to differ (Bullock and Thomasson, 1979) and it is difficult to say which method gives the correct results. We tried to use image analysis of soil thin sections, but were not able to give a reliable estimate of pores < 30 μm.

A disadvantage of the use of water retention curves to estimate pore volumes is that shrinkage of the soil may affect the reliability of this technique (Marshall and Holmes, 1980). The bentonite amended soil samples used for our experiments also showed some shrinkage. However, shrinkage mainly occurred at suction levels between -0.1 and -10 kPa. Since suction levels used to determine the pore sizes discussed in this paper are much lower than -10 kPa and survival studies were also done at water potentials at or below -10 kPa, shrinkage will probably not have influenced our results too much.

It can be concluded from these experiments that the survival of introduced rhizobia was improved through the addition of clay minerals, in particular bentonite. The water potential and the amount of bentonite present significantly affect the survival rate. The main reason for the improved survival in loamy sand amended with bentonite clay may be attributed to the creation of protective microhabitats, inaccessible to protozoa predating on rhizobia. Therefore, the addition of bentonite to other soil types might also improve the survival of introduced bacteria, as long as more protective microhabitats are created. Sufficient protective microhabitats should give rise to optimal survival chances for introduced bacteria in soil.

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CHAPTER 6

MICRO-MORPHOLOGICAL STUDIES ON CLAY- AND UNAMENDED LOAMY SAND, RELATING SURVIVAL OF INTRODUCED BACTERIA AND SOIL STRUCTURE

Summary

With the use of CryoSEM techniques the microstructure of bentonite, kaolinite- and unamended loamy sand was studied. A wide range of pore sizes could be recognized in all three treatments, but pore size distributions differed. The bentonite clay was present as a very porous matrix, containing pores with an average maximum length of approximately 3 μm, and an average minimum width of approximately 2 μm. Pores of similar sizes were also present in kaolinite- and unamended loamy sand, but they were much less frequent. Previous results, suggesting that the protective effects of bentonite clay on the survival of introduced bacteria in soil was caused by an increase in the number of pores with and equivalent diameter < 6 μm could be confirmed.

Introduction

Soil structure and texture have a large influence on the population dynamics, ecology and activity of soil microorganisms (Stotzky, 1986). For example, decomposition rates of organic matter are lower in fine textured, clayey soils, than in coarse textured, sandy soils (Van Veen and Kuikman, 1990). Van Elsas *et al.* (1986) found that survival of introduced bacteria was enhanced in a silt loam as opposed to a loamy sand. Heijnen *et al.* (1988) found that the survival levels of *Rhizobium* introduced into loamy sand could be increased by the addition of bentonite clay, a smectitic clay mineral. The increased survival could be explained by the presence of many pores with equivalent neck diameters < 6 μm in bentonite amended soil as opposed to unamended loamy sand, which were called protective microhabitats. Bacteria situated inside these microhabitats were protected from predation by protozoa (Heijnen and Van Veen, 1991).

The pore size distribution of a soil can be determined in different ways (Lawrence, 1977). Examples are the use of moisture characteristic curves (Heijnen and Van Veen, 1991), or the mercury intrusion method (Wu *et al.*, 1990). Both methods are based on a cylindrical pore model (Lawrence, 1977), but since pores are never truly cylindrical, incorrect pore diameters are likely to be determined. However, both methods are widely used for the description of the pore size distribution in soil. A different way to study pore size distributions is with micro-morphology, involving light-microscopic studies of soil thin sections. An interesting approach was the staining, in thin sections, of bacteria introduced into soil (Postma and Altemüller, 1990; Tippkötter, 1990). If samples are thin enough to be permeable to electrons, transmission electron microscopy (TEM) is also applicable (Foster *et al.*, 1983), allowing for much larger magnifications (2,000 to 1,000,000; Tessier, 1991). A problem arising when using soil thin sections is that soil moisture has to be replaced by resin, thereby introducing the possibility of creating artifacts due to shrinkage of the soil or through damage to, or the solubility of, organics.

Since it was hypothesized in previous studies that the protective action of bentonite clay on rhizobial survival might be due to the formation of pores with diameters < 6 μm (Heijnen and Van Veen, 1991), it was tried to confirm this hypothesis by visualizing these pores. Cryo Scanning Electron Microscopy (CryoSEM, Tessier and Berrier, 1979) was applied, a method previously found to preserve the organization of clay materials in their original hydrated condition (Chenu, 1989). Since kaolinite was also found to influence rhizobial survival (Heijnen and Van Veen, 1991), pores in kaolinite amended loamy sand were also studied.

Materials and methods

Soil and clays:

A loamy sand, kept in experimental field micro-plots for several years, was employed for micro-morphological studies. Some soil characteristics were given by Heijnen *et al.* (in press). X-ray analysis showed that the silt and clay fractions in the loamy sand mainly consisted of quartz and feldspars. Kaolinite and illite were also present, the latter with some smectite interstratification. The loamy sand was amended with kaolinite or bentonite clay, previously used to enhance rhizobial survival (Heijnen and Van Veen, 1991) or was left unamended. The compositions of the cation exchange complex of the bentonite and kaolinite, respectively, are as follows: (in meq/100 g dry clay mineral) Na^+: 55.6 and 1.5; K^+: 1.1 and 0.5; Ca^{2+}: 27.4 and 0.6; Mg^{2+}: 6.9 and 0.8.

Soil treatments:

The aim of our present work was to study the microstructure and to visualize small pores (< 6 μm) in bentonite-, kaolinite- and unamended loamy sand. Bentonite was added as a powder to concentrations of 0.5%, 1% and 5% (mass fractions) to loamy sand. Kaolinite was only added in amounts of 10%, also as a powder. The different soil mixtures were moistened to a water potential of -10 kPa, which resulted in water contents of 32% for the 5% bentonite treatment and approximately 18% for all other remaining treatments (Heijnen and Van Veen, 1991). To determine whether the pore sizes in bentonite amended soil were influenced by the water content, loamy sand amended with 5% bentonite was also studied at a moisture content of 18%.

Cryo scanning electron microscopy (CryoSEM):

A slightly modified sample preparation technique for CryoSEM studies, according to Tessier and Berrier (1979) was applied, using a Hexland CT100 cryotrans system. Soil aggregates of approximately 2-3 mm^3 and containing 18 or 32% water, depending on the treatment, were cryofixed by immersion into melting nitrogen (-210°C). At this temperature ice crystal growth is limited (Robards and Sleytr, 1985), thereby minimizing the chance of the occurrence of freezing artifacts. The sample was then transferred to the preparation chamber (-185°C) where it was fractured. Ice was partially sublimated under vacuum at temperatures of about -90°C, again in order to limit ice crystal growth (Robards and Sleytr, 1985). Sample surfaces were metallized with gold to provide a conductive film. Samples were observed at temperatures < -110°C in the refrigerated column of a Philips SM525 scanning electron microscope. We tried to evaluate the pore sizes and numbers in the different soil treatments. Pores from a 3-dimensional image were traced (2-dimensionally), and maximum pore lengths and minimum pore widths were estimated using a Quantimet 970 image analysis system (Cambridge Instruments).

Results

In the loamy sand (Fig. 1) sand grains ranging from 50-500 μm and large interstitial pores (≈100 μm) were observed. Some sand grains were coated with plasma (fine size fraction), whereas others were bare. The plasma consisted of clay, silt and organic matter. Clay also occurred as bridges between sand grains (Fig. 1). The plasma (Fig. 2) showed some porosity too. In areas of 39 x 24.5 μm^2 (e.g. in Fig. 2), an average of 24 pores was counted with an average maximum length of 5.2 μm, and an average minimum width of 3.1 μm (Table 1).

Loamy sand amended with 10% kaolinite (Fig. 3) had a completely different microstructure. Most of the sand grains were coated with plasma (here: mainly kaolinite), and only few bare grains remained. The large interstitial pores present had about the same size (≈100 μm) as those found in the unamended loamy sand. The

plasma (Fig. 4) was dominated by the plate-like structure of kaolinite. The kaolinite plates were coarse (2 - 10 μm), ranging into the silt-fraction. In the plasma, in areas of 39 x 24.5 μm^2 (e.g. in Fig. 4), an average of 27 pores was counted with an average maximum length of 4.7 μm, and an average minimum width of 1.7 μm (Table 1).

Amendment of loamy sand with 5% bentonite clay (32% moisture content) had a large effect on soil structure (Fig. 5). Sand grains appeared to be embedded in, and covered with, bentonite clay, which was a dominating component of the soil plasma. Large pores (≈100 μm) were less frequent than in unamended and kaolinite-amended loamy sand. The bentonite formed a very porous matrix (Fig. 6), which in some places contained organic remnants (not shown). The pores had a polygonal shape with thin wrinkled walls, resembling a honeycomb structure (Fig. 7). In areas of 39 x 24.5 μm^2 (e.g. in Fig. 7), an average of 78 pores was counted with an average maximum length of 2.7 μm, and an average minimum width of 1.7 μm (Table 1). It was clear that small pores were much (approximately 3 times) more abundant in loamy sand amended with 5% bentonite clay than in unamended loamy sand or kaolinite-amended loamy sand (Table 1).

At a moisture content of only 18% in the 5% bentonite treatment a similar porous structure was visible as was the case with a moisture content of 32% (not shown). However, now the pores in the bentonite plasma were much smaller, with maximum pore lengths ranging up to 1 μm only.

Table 1: Numbers of pores and their average length and width, including the standard deviation (SD), measured from CryoSEM micrographs. Countings were performed on areas of 39.0 x 24.5 μm^2. ls = loamy sand; b = bentonite; k = kaolinite.

Treatment	No. of pores	length ± SD (μm)	width ± SD (μm)
ls	24 ± 1	5.2 ± 0.6	3.1 ± 0.2
ls + 10% k	27 ± 5	4.7 ± 1.1	1.7 ± 0.3
ls + 10% b	78 ± 5	2.7 ± 0.2	1.7 ± 0.2

After amending the loamy sand with 0.5% or 1% bentonite clay it was more difficult to locate the bentonite in the sample. The clay occurred in thinner layers covering the sand grains, leaving more bare sand grains. Small pores with sizes similar to those observed in the 5% bentonite treatment were detected, however they were less frequent since the bentonite percentage was lower. Bentonite clay and

Figs. 1, 2, 3, 4, 5, 6, 7, 8: P = pore; S = sand grain; SP = soil plasma; CB = clay bridge; IC = indigenous clay; K = kaolinite; B = bentonite.

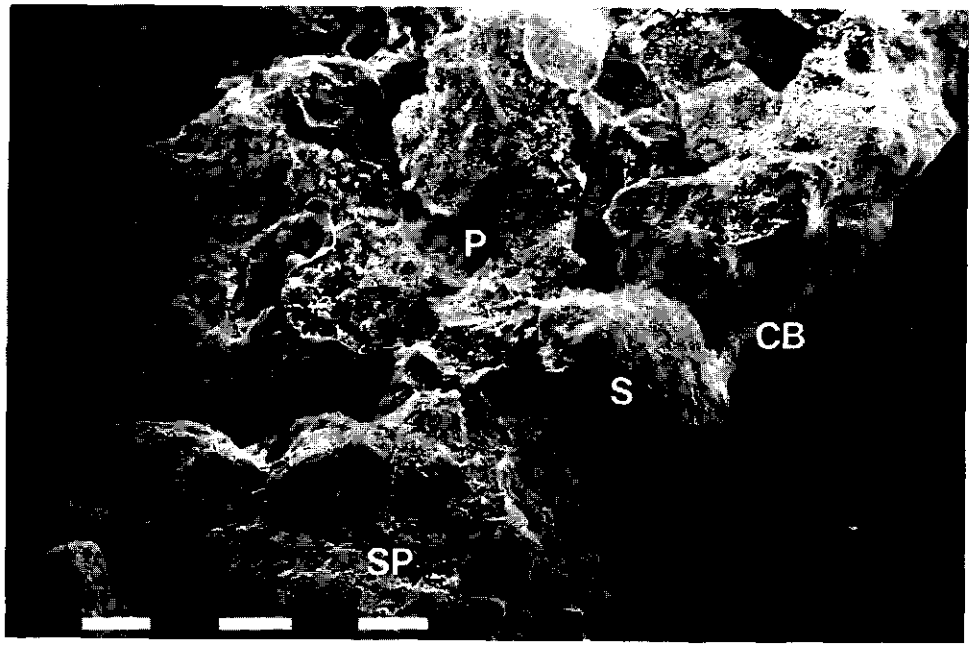

Fig. 1: Unamended loamy sand. White bar = 0.1 mm.

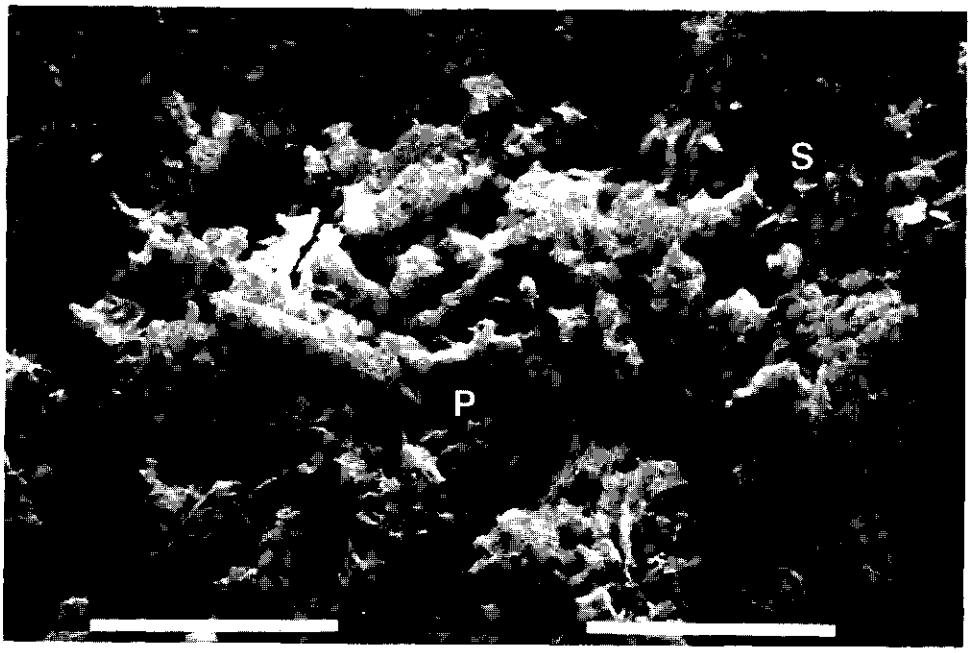

Fig. 2: Soil plasma in unamended loamy sand. White bar = 10 µm.

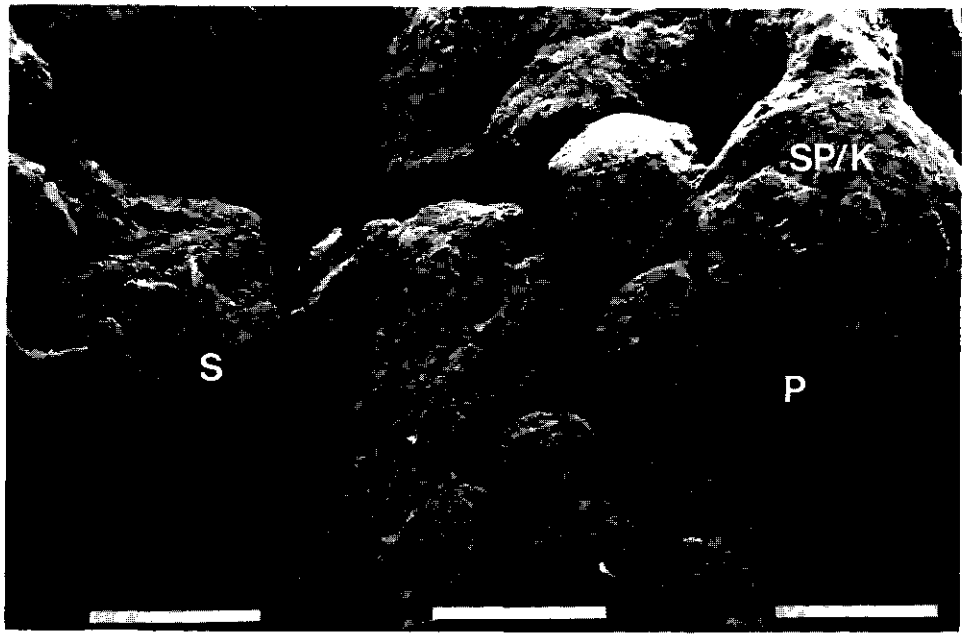

Fig. 3: Loamy sand amended with 10% kaolinite. White bar = 0.1 mm.

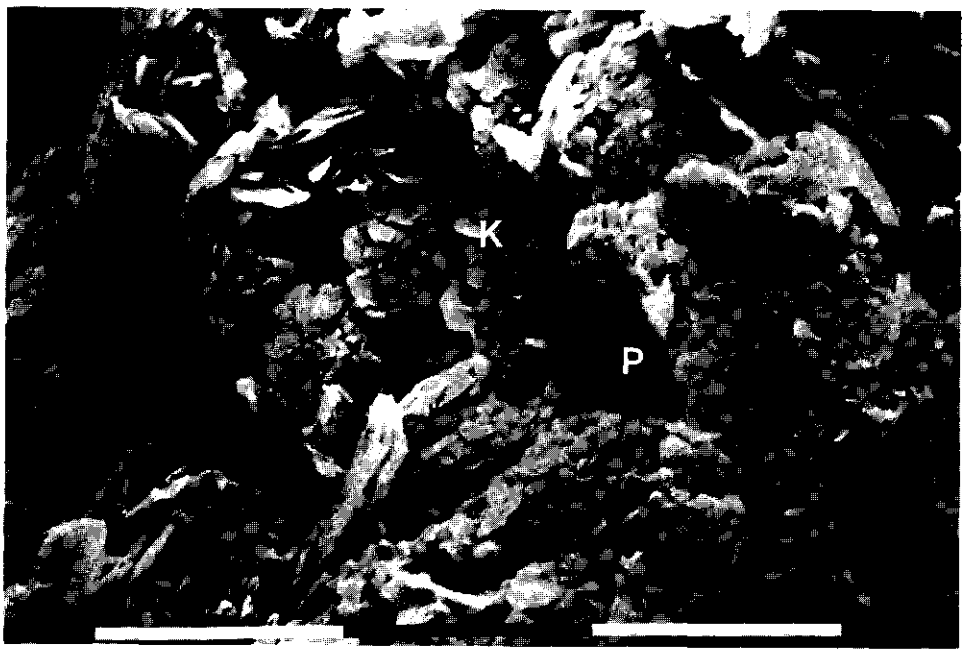

Fig. 4: Soil plasma in loamy sand amended with 10% kaolinite. White bar = 10 µm.

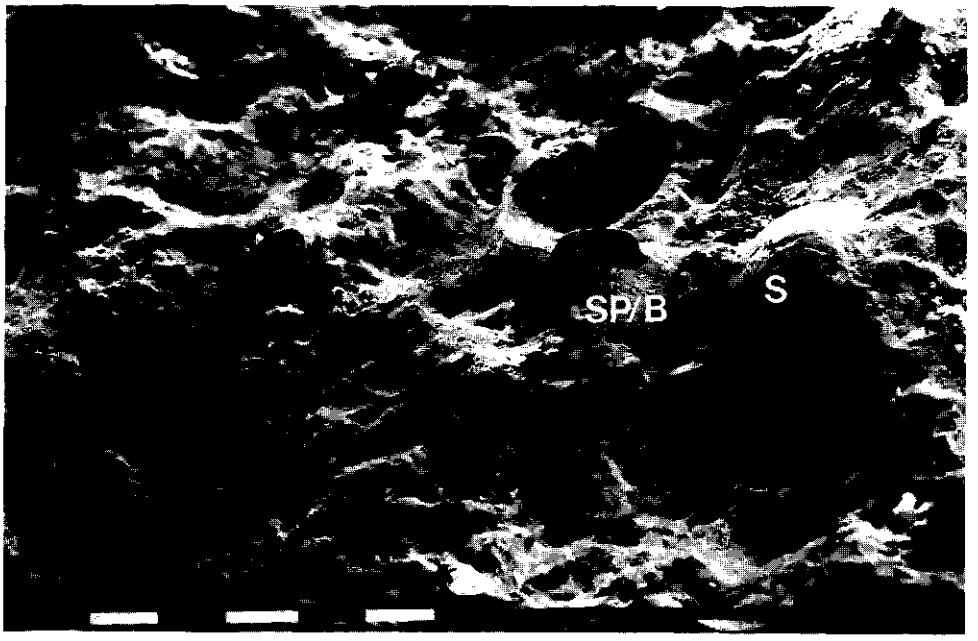

Fig. 5: Loamy sand amended with 5% bentonite, 32% moisture content. White bar = 0.1 mm.

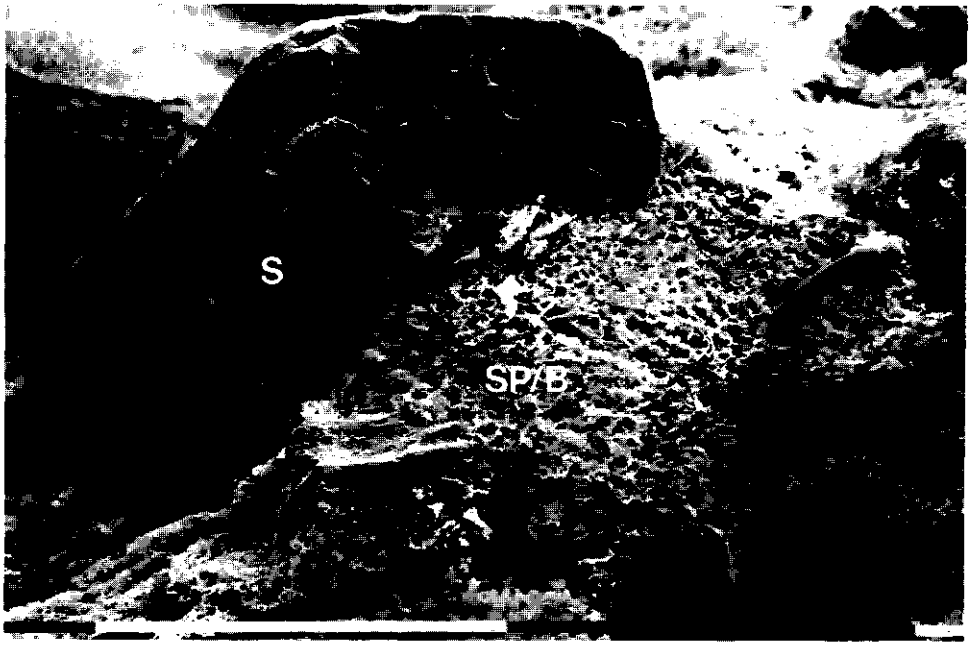

Fig. 6: Loamy sand amended with 5% bentonite, 32% moisture content. White bar = 0.1 mm.

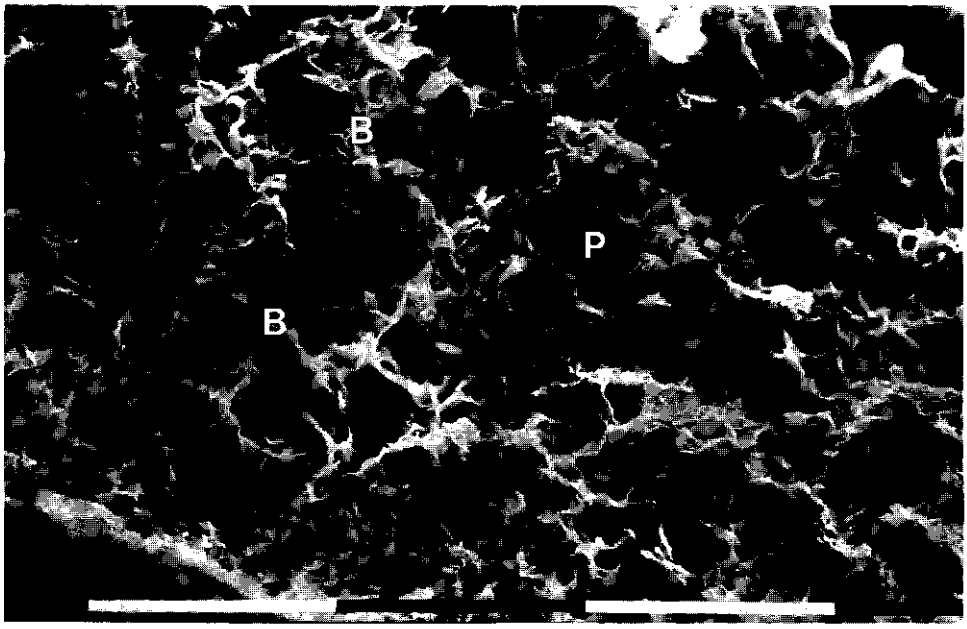

Fig. 7: Soil plasma in loamy sand amended with 5% bentonite, 32% moisture content. White bar = 10 μm.

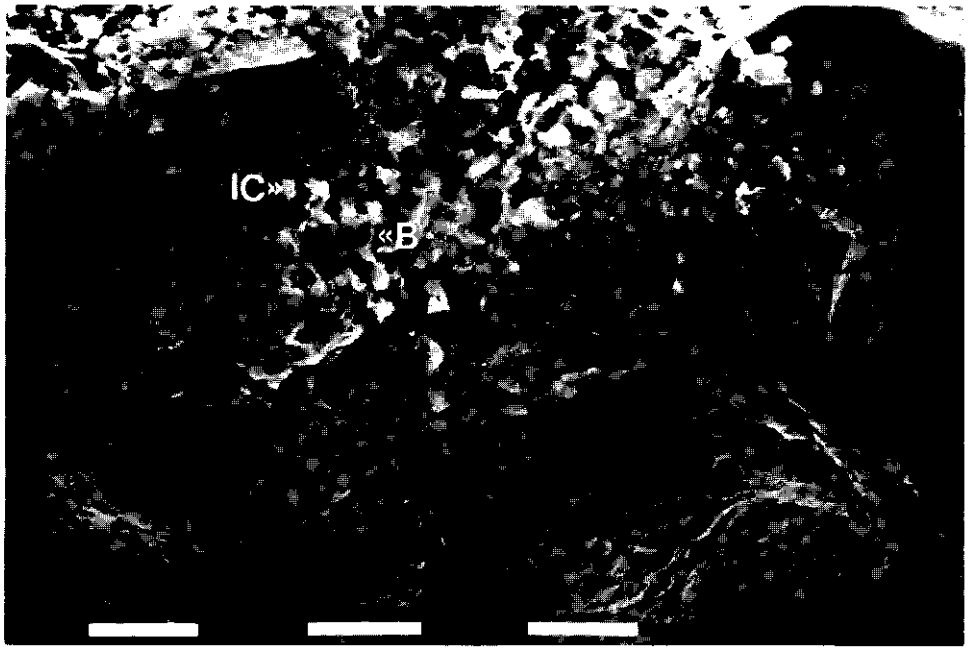

Fig. 8: Soil plasma in loamy sand amended with 1% bentonite. White bar = 10 μm.

indigenous soil clay often occurred mixed together (Fig. 8). The indigenous clay particles could be recognized by their blocky appearance (as was also observed in unamended loamy sand), and were completely surrounded by bentonite. Since bentonite became mixed with indigenous clay when only low concentrations of bentonite were present, it was assumed that this would also happen when higher amounts of bentonite were added to the loamy sand.

Discussion

In previous work (Heijnen *et al.*, 1988, Heijnen and Van Veen, 1991) the survival of *Rhizobium*, introduced into loamy sand, was shown to be improved by the addition of bentonite clay. It was hypothesized that bentonite clay protected introduced rhizobia against predation by protozoa through the creation of many new pores large enough to allow bacteria to enter, but too small to be colonized by predating protozoa. These so-called protective microhabitats were defined as pores with neck diameters < 6 μm (Heijnen and Van Veen, 1991). Here we demonstrated that bentonite-amended soil was characterized by the presence of an extremely porous clay matrix. Large numbers of pores were present, with an average maximum length of approximately 3 μm and an average minimum width of approximately 2 μm (Table 1). In kaolinite-amended or unamended loamy sand pores with comparable dimensions were also present, but they were much less numerous (Table 1) than in bentonite-amended soil. Therefore, the hypothesis that bentonite was responsible for the creation of many small pores (< 6 μm), which could serve as protective microhabitats for introduced bacteria, was confirmed.

Kaolinite amendments to loamy sand also increased rhizobial survival compared to unamended loamy sand, but to a lesser extent than was the case for bentonite amendments (Heijnen and Van Veen, 1991). Our present study showed that the numbers of small pores in kaolinite-amended loamy sand were similar to those found in unamended loamy sand (Table 1). However, the pores were slightly smaller (Table 1), offering more protection against predating protozoa, which may explain the increased survival levels of bacteria introduced into kaolinite amended as compared to unamended loamy sand (Heijnen and Van Veen, 1991). The inability of kaolinite to create many small pores could be explained by its coarse plate-like structure; large particles cannot be stacked in such a way that many small pores (protective microhabitats) are created.

Bentonite clay was found to mix well with indigenous soil clay. Also organic matter, surrounded by bentonite, was observed. Especially this latter aspect may be important for bacterial survival, since it means that a food source is situated near the protective microhabitats.

We could not observe bacteria in the different soil treatments studied, in spite of the fact that at least 10^9 cells were expected to be present in 1 g natural soil. This could be explained by the low colonization percentage (0.1%) of the pore space in soil by bacteria (Adu and Oades, 1978), reducing the chance of detection. Furthermore, if a bacterium was covered with clay or organic matter, it would be difficult to recognize and would probably not be detected at all. This agrees with the fact that on the rare occasions when bacteria were observed in soil, they were usually situated on bare sand grains (C. Chenu, pers. commun., 1991). Therefore, the phenomenon that a large proportion of the soil is not colonized by bacteria, in combination with clay additions and/or organic matter covering the bacteria, could be used to explain the fact that no bacteria were observed in bentonite-, kaolinite- or unamended loamy sand. However, visualization of bacteria in clay with CryoSEM is possible, but only when high bacterial population densities (2×10^{11} cells/g) are present (Schmit and Robert, 1984).

Smectitic clay minerals with polygonally shaped pores and thin wrinkled walls, as shown in Fig. 7, were also found by Tessier (1990). However, the pore sizes of 1-2 μm described by Tessier (1990) were smaller than those which were measured from Fig. 7 (average maximum pore length of approximately 3 μm). Freezing artifacts due to the presence of water were most likely not the reason for the occurrence of the large pores (3 μm as compared to 1-2 μm), since the clay studied by Tessier (1990) was relatively wetter than was the case for our samples (-3.2 kPa and -10 kPa, respectively). Furthermore, no particular orientation of the pores was observed, which also suggests that freezing artifacts were not a major problem in our studies. However, the pores shown in Fig. 7, and probably also the pores shown by Tessier (1990), might be enlarged slightly due to a decrease in the interlayer spacing (from 5-10 nm to approximately 1.5 nm; Tessier, 1990) inside the clay tactoids during freezing. (Tactoids are particles resulting from the face to face stacking of clay plates. Through the interleaving of layers, particles with a large lateral extension as compared to their thickness are formed). However, this decrease in interlayer spacing, and thus the increase in pore size, is on a much smaller size scale (nm) than that of the pores under observation (μm). Differences in pore sizes comparing our present work and work by Tessier (1990) are probably related to the method of sample preparation. Tessier (1990) equilibrated homogenized clay slurries at given water potentials. This preparation method allowed for a good dispersion of the clay particles, resulting in small tactoids and, hence, small pores. In the present work, the bentonite was added to the soil as a powder, and after mixing, the sample was brought up to a moisture content corresponding to a water potential of -10 kPa. The rapid wetting, without an initial dispersion of the clay, will probably cause formation of larger tactoids, and hence the formation of larger pores.

The observed increase in pore lengths from approximately 1 μm to 3 μm in the 5% bentonite treatment, when increasing the moisture content from 18% to 32%,

could be explained by the swelling of the clay. Wilding and Tessier (1988) showed that, contrary to classical concepts, shrink-swell phenomena can only partly be explained by hydration-dehydration cycles of double layers. Only for Na-saturated, low electrolyte smectitic clays systems, the diffuse double-layer model is likely to be functional. In clays with exchangeable Ca and Mg, as was the case for the bentonite clay used here, shrinking and swelling also involves changes in the amount of interparticle pore water, causing size increases or decreases of pores which behave as bellows of an accordion (Wilding and Tessier, 1988). The extent of swelling is determined by the flexibility of the pore walls, which in turn depends on the clay mineralogy (smectitic clays provide high flexibility) and the composition of the exchange complex. Flexible pore walls will stretch at high moisture contents, resulting in large pores. In loamy sand amended with 5% bentonite clay, pore sizes indeed increased at higher water contents (compare -80 kPa and -10 kPa).

Swelling and shrinkage of the soil might affect the survival of introduced bacteria in soil due to a fluctuation in pore size. At a water potential higher than -10 kPa, it is unlikely that pore sizes in the bentonite matrix would exceed 6 μm, due to the geometry of the pore structure (Tessier, 1990). Therefore, at higher moisture contents than corresponding to -10 kPa, protection against protozoa would probably still occur. However, at water potentials lower than -10 kPa (drier soils), pore sizes would decrease and could reach dimensions smaller than those of bacterial cells. A similar decrease in pore size has been suggested to be detrimental to bacteria in pure clay (Schmit and Robert, 1984). It remains to be studied how the survival of introduced bacteria in soil is affected when pores, colonized with introduced bacteria, are reduced in size due to shrinkage of the soil.

Conclusions

The method described here enabled us to study relationships between soil structure and microbial processes on the size scale at which these processes are likely to occur in soil. With the use of CryoSEM the hypothesis, that the protection of introduced bacteria in soil by bentonite clay was caused by an increase in the number of small pores, was confirmed. Pores with an average maximum length of approximately 3 μm and an average minimum width of approximately 2 μm were numerous in bentonite-amended loamy sand, offering the introduced bacteria protection from predating protozoa and resulting in high bacterial survival levels.

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CHAPTER 7

METABOLIC ACTIVITY AND POPULATION DYNAMICS OF BACTERIA INTRODUCED INTO UNAMENDED AND BENTONITE AMENDED LOAMY SAND

Summary

Respiration measurements showed that the cumulative amount of CO_2 respired by rhizobia introduced into sterile bentonite amended loamy sand was significantly higher than in unamended loamy sand. The maintenance respiration of rhizobial cells was not influenced by the presence of bentonite clay. Carbon was used more efficiently during growth in bentonite amended than in unamended loamy sand. The presence of bentonite clay increased the growth rate of rhizobia introduced into sterile soil. Survival studies performed in non-sterile bentonite amended loamy sand showed that using high (10^{10} cells/g dry soil) as compared to lower (10^{4}-10^{7} cells/g dry soil) inoculum densities, increased the final survival levels of introduced rhizobia. In unamended loamy sand, the application of 10^{10} or 10^{7} cells/g dry soil resulted in similar final survival levels. Pore shape and the continuity of the water-filled pore system were suggested to largely determine the colonization rate of protective microhabitats.

Introduction

For effective use of bacteria introduced into the environment for various purposes (Van Elsas and Heijnen, 1990), sufficiently high numbers of active organisms may be required for prolonged periods of time. Key-factors which determine the survival of bacteria in soil are soil structure and texture (Van Elsas *et al.*, 1986), predation (Acea *et al.*, 1988), temperature and moisture content (Beringer and Bale, 1988). Additions of bentonite clay to soil have been shown to greatly enhance survival of introduced rhizobia (Heijnen *et al.*, 1988; Heijnen and Van Veen, 1991) mainly by offering protection against predation by protozoa. This increased survival was hypothesized to result from the presence of many bentonite created protective

microhabitats. Protective microhabitats were defined as pores large enough to allow bacteria to enter but too small to be colonized by protozoa or other bacteriovores and were defined as having necks with diameters of approximately 3-6 μm or less (Heijnen and Van Veen, 1991).

According to Stotzky *et al.* (1991) the presence or absence of clay minerals, among other factors, influences the activity of microorganisms in soil. An interesting question emerging is whether a bacterial population introduced into bentonite amended soil and presumably situated in protective microhabitats is metabolically active, and if so, to what extent. Metabolic activity may be determined in many different ways, e.g. by the evolution of carbon dioxide, the uptake of oxygen, the ATP-content, or heat output (Nannipieri, 1984). Here we focused on the determination of the metabolic activity of rhizobia introduced into sterile soil by measuring the amount of CO_2 respired in time, using different inoculum densities ranging between approximately 10^3 and 10^{10} cells/g dry soil.

It was not possible to distinguish rhizobial respiration from respiration by indigenous microorganisms in non-sterile soil. We therefore focused on assessing the population dynamics of rhizobia introduced into soil at the different inoculum densities, in order to obtain information on the process of colonization of protective microhabitats. The colonization rate of small pores by introduced bacteria in soil is low, as evidenced by Postma and Van Veen (1990), who found that in non-sterile loamy sand only 0.001% of the pores with neck diameters between 0.8 and 3 μm were colonized by introduced rhizobia. We aimed at determining whether colonization processes differed between soils with and without bentonite amendments, using inoculum densities ranging between 10^4 and 10^{10} cells/g dry soil. Factors, possibly influencing colonization of protective microhabitats by introduced bacteria are discussed.

Materials and methods

Bacterial strain

A transposon Tn5 mutant of *Rhizobium leguminosarum* biovar *trifolii* strain R62, resistant to kanamycin and rifampicin, was employed as a model organism (Heijnen *et al.*, 1988). Rhizobia were cultured for 2 days at 29°C in 2.5 litre Erlenmeyer flasks containing 1 litre of yeast extract mannitol broth (Heijnen *et al.*, 1988) supplemented with 25 mg/l kanamycin at 300 rpm. This resulted in a cell concentration of approximately 10^9 cells/ml. Cells were harvested by centrifugation (7000 rpm, 15 min.) and suspended in sterile demineralized water, thereby concentrating the cell density to approximately 2×10^{11} cells/ml (high inoculum density). Part of this cell suspension was diluted with sterile demineralized water to obtain cell suspensions containing

approximately 4 x 10^8 cells/ml (medium inoculum density) and 4 x 10^5 cells/ml (low inoculum density), respectively.

Soil and clay mineral

A loamy sand (Heijnen *et al.*, in press) amended with or without 10% bentonite clay was employed. When required, loamy sand and bentonite were sterilized by irradiation with 4 Mrad using a ^{60}Co-source. The pH of the bentonite amended and unamended loamy sand was determined after shaking for 2 hours in 1M KCl.

Soil studies

The metabolic activity of rhizobial cells introduced into loamy sand with and without bentonite amendments was studied in sterilized soil over a period of 30 days. Soil portions corresponding to 40 g dry weight were inoculated with 1 ml of suspensions containing high, medium and low cell concentrations to establish initial cell concentrations of approximately 1 x 10^{10}, 9 x 10^7 and 6 x 10^3 cells/g dry soil, respectively. Different inoculum densities were used to study the metabolic activity of the introduced rhizobial populations which were expected to have different population dynamics. Soil portions were incubated in air-tight 1.5 l jars at 15°C, a temperature occurring frequently in soils in temperate regions. To maintain a humid environment, a glass vial containing sterile demineralized water was placed inside each jar. CO_2 evolving was trapped in 10.0 ml 0.1 N NaOH to measure bacterial respiration. To determine the amount of CO_2 respired in time, the NaOH was regularly replaced. The CO_3^{2-}-ions present in the NaOH solution were precipitated with excess $BaCl_2$. The remaining NaOH was then titrated with 0.1 N HCl after which the total amount of CO_2 respired could be calculated. Portions of sterilized soil, not inoculated with rhizobia, were used as controls. CO_2 evolving from these controls, as a result of contaminations with other micro-organisms, was subtracted from the amount of CO_2 respired from inoculated soil portions. The amount of CO_2 evolving from the controls was always much less than the amount evolving from the inoculated samples. It appeared to be impossible to prevent some contamination with other micro-organisms. Contamination was probably a result of the frequent opening and closing of the jars, necessary for the removal and replacement of the NaOH. The number of rhizobial cells were determined simultaneously with respiration measurements, using plate count techniques.

In a second set of experiments the colonization of protective microhabitats by rhizobia introduced at different initial cell concentrations was studied in bentonite amended and unamended loamy sand. Non-sterilized soil portions corresponding to 10 g dry weight were inoculated with rhizobia using inocula with high, medium and low cell concentrations. We aimed at obtaining approximately the same inoculation levels as used in the respiration experiment. However, in this experiment initial population densities in inoculated soil were slightly higher, approximately 2 x 10^{10}, 4 x

10^7 and 5 x 10^4 cells/g dry soil, respectively. Population dynamics of introduced rhizobia were studied over a period of 80 days. Soil portions were incubated in humid chambers at 15°C in the dark.

For both sets of experiments the loamy sand and the bentonite amended loamy sand were brought up to a water potential of -10 kPa, resulting in a moisture content of 17% for the loamy sand and 45% for the loamy sand amended with 10% bentonite. The bulk density was adjusted to 1.2 g/cm^3 (dry weight).

Enumeration of bacteria

Numbers of culturable rhizobial cells in soil were determined using selective plate count techniques. Soil portions corresponding to 10 g dry weight were transferred into 250 ml Erlenmeyer flasks containing 95 ml of a 0.1% $Na_4P_2O_7$ solution and 10 g gravel (2-4 mm dia). The flasks were shaken for 10 min (200 rpm) at room temperature. The $Na_4P_2O_7$ was used to disperse the bacterial cells, and the gravel to mechanically facilitate this dispersion. Subsequently, serial 10-fold dilutions were made and plated on yeast extract mannitol agar (Heijnen *et al.*, 1988) supplemented with 50 mg/l kanamycin, 20 mg/l rifampicin, 100 mg/l cycloheximide and 50 mg/l benomyl. Cycloheximide and benomyl were applied to inhibit fungal growth. Plates were incubated at 29°C for 3-5 days, and the numbers of colony forming units were determined. Immunofluorescence counts (Postma *et al.*, 1988) were included in the second experiment (80 day incubation period) to determine the percentage of viable but non-culturable cells in the rhizobial population (Colwell *et al.*, 1985).

Statistical analysis

Numbers of colony forming units were determined in time from destructively harvested duplicate soil portions. An analysis of variance was done on the ^{10}log number of cells to test for bentonite effects. Cumulative amounts of CO_2 respired were determined from triplicate soil portions. Because the variation between triplicate soil portions increased with increasing amounts of CO_2, analyses were performed on ln-transformed respiration data. Because the data were the result of repeated measurements on the same soil portions, analysis of variance was performed on each sampling date. The respiration per cell was estimated as the mean respiration divided by the mean number of cells, thereby suggesting that the expected value of a division is the expected value of the nominator divided by the expected value of the denominator ($\epsilon(x/y) \approx \epsilon(x)/\epsilon(y)$. This was a reasonable approximation for these experiments, since the variations of the respiration and the number of cells were small compared to their means. The given SD values were calculated according to Mood *et al.* (1974). When differences are reported as significant, this was at a level of $P < 0.05$.

Results

Population dynamics of introduced rhizobia were dependent of the inoculum density (Fig. 1). In sterile soil inoculated with the low inoculum density of 6 x 10^3 cells/g dry soil a significant increase in cell concentrations of 3-4 log units was observed (Fig. 1) during the first 10 days of the experiment. A significantly faster growth of the population size in the bentonite amended loamy sand was observed. By day 30, both in bentonite and unamended loamy sand, population densities had reached levels of around 10^8 cells/g dry soil. For the medium sized inoculum (Fig. 1), population densities either remained fairly stable (loamy sand) or showed a slight increase (loamy sand amended with 10% bentonite clay). Final differences in population densities were significant, but small. When a high inoculum density was employed (Fig. 1), numbers of colony forming units declined slightly during the 30 day incubation period. In the bentonite amended soil significantly more cells survived than in the unamended loamy sand.

Cells introduced into unamended as well as into bentonite amended loamy sand were found to be metabolically active (Fig. 2). The initial CO_2-flush occurring in the first 24 hours of incubation was not included in these figures, since this was expected to be the result of mixing the bacteria with the soil, and thereby changing the bacterial environment from liquid to solid. This initial CO_2-flush could thus be regarded as an artefact in view of the present objectives. Adu and Oades (1978) also found that when measuring CO_2 release there was a period of adjustment of microorganisms to the new environment after introduction into soil. The addition of 10% bentonite clay to loamy sand resulted in an overall significantly higher amount of CO_2 respired. As from day 10 the cumulative amount of CO_2 respired after applying the large inoculum was significantly higher than the amount respired after applying the medium or low inoculum densities. No significant differences in the cumulative amount of CO_2 respired occurred comparing the treatments with medium and low inoculum densities.

When the rhizobial population size was not increasing (large inoculum), the cumulative amount of C respired during the first 10 days was 106.3 µg/g dry soil for bentonite amended loamy sand and 88.7 µg/g dry soil for unamended loamy sand (Fig. 2). Cell densities were slightly higher in the bentonite amended than in the unamended loamy sand during this same time period (Fig. 1). It could be calculated by dividing the cumulative amount of carbon released during the first 10 days of incubation (Fig. 2) by the number of cells surviving in this same period of time (Fig. 1), that the amount of carbon respired per bacterium was 0.015 ± 0.003 pg for both the unamended and bentonite amended loamy sand. With a population increasing in size (low and medium inoculum levels), the amount of carbon respired for the production of a new cell during the first 10 days of incubation could be calculated in

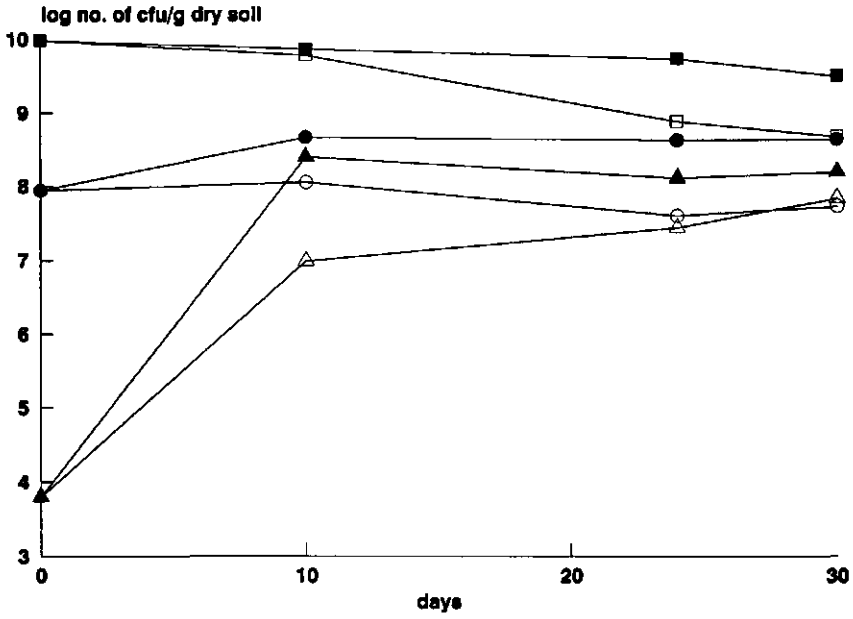

Fig.1: Population dynamics of *R. leguminosarum* biovar *trifolii* introduced into sterile loamy sand at a low inoculum density (△), a medium inoculum density (○) and a high inoculum density (□) and into sterile loamy sand amended with 10% bentonite clay at a low inoculum density (▲), a medium inoculum density (●) and a high inoculum density (■). cfu = colony forming units.

a similar way (cumulative amount of CO_2 divided by the number of new-grown cells). It was assumed that no cell death occurred during these first 10 days. For the production of each new cell, after applying medium inoculum densities, this resulted in 1.97 ± 0.30 pg C respired in the loamy sand, which was significantly higher than 0.16 ± 0.09 pg C respired in loamy sand amended with 10% bentonite. Similar calculations resulted in 2.45 ± 1.67 pg C respired in loamy sand and 0.30 ± 0.03 pg C respired in loamy sand amended with 10% bentonite when applying low inoculum densities. Even though the latter difference caused by the presence of bentonite was large and in the same range as found with the medium inoculum density, it was not statistically significant.

Population dynamics of rhizobia introduced into non-sterilized loamy sand at high inoculation densities, showed a very strong decline (Table 1). In bentonite amended soil inoculated with high numbers of rhizobia, a much smaller decline of the population density was observed. After 80 days the difference in population densities between the unamended- and bentonite amended loamy sand was at least 2 log units, and highly significant. With medium inoculation densities a similar trend was

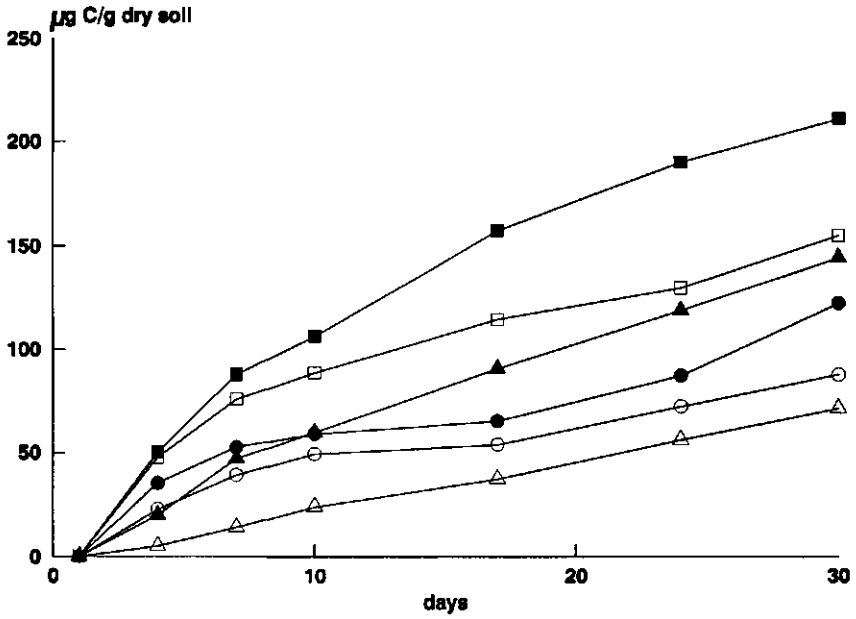

Fig.2: Cumulative amounts of carbon respired from sterile soil inoculated with *R. leguminosarum* biovar *trifolii* expressed as µg C/g dry soil. Sterile loamy sand inoculated with low (△), medium (○) and high (□) inoculum densities. Sterile loamy sand amended with 10% bentonite clay inoculated with low (▲), medium (●) and high (■) inoculum densities. Low inoculum density: 6 x 10^3 cells/g dry soil; medium inoculum density: 9 x 10^7 cells/g dry soil; high inoculum densities: 10^{10} cells/g dry soil.

found (Table 1), but less pronounced and with a final significant difference in numbers of culturable cells between bentonite amended and unamended loamy sand of 1.2 log units. The population dynamics of *Rhizobium* after inoculation of about 5 x 10^4 cells/g dry soil showed a decline till below the detection limit in loamy sand (Table 1). With bentonite amendments the number of cells by day 80 remained stable during the incubation period. Also with the low inoculum density, significantly more cells (0.9 log units on day 56) survived in bentonite amended loamy sand than in unamended soil. In bentonite amended soil, higher inoculation densities resulted in higher survival levels. In unamended soil, this was only the case when comparing low and medium inoculum densities; high inoculation levels did not give an additional increase in final survival levels.

Immunofluorescence detection techniques and selective plating techniques both resulted in similar counts for the numbers of surviving rhizobial cells (results not shown), indicating that non-culturable cells were not (or only in negligible amounts) present.

The pH-KCl of the bentonite amended and unamended loamy sand was found to be 6.4 and 5.7, respectively.

Table 1: Log no. of colony forming units of *R. leguminosarum* biovar *trifolii* per gram dry soil introduced into loamy sand (ls) and loamy sand amended with 10% bentonite (b) at low (5 x 10^4 cells/g dry soil), medium (4 x 10^7 cells/g dry soil) and high (2 x 10^{10} cells/g dry soil) inoculum densities. ND = not detectable. *: based on one value only. LSD = 0.21.

inoculum	low inoculum		medium inoculum		high inoculum	
soil	ls	10%b	ls	10%b	ls	10%b
day 0	4.74	4.74	7.64	7.64	10.17	10.17
day 1	4.62	4.68	7.73	7.69	10.06	9.99
day 7	4.56	4.99	7.26	7.82	9.45	10.04
day 15	4.12	5.00	7.00	7.58	8.16*	9.65
day 30	4.16	4.59	6.69	7.60	7.66	9.47
day 42	3.76	4.62	6.05	7.41	7.11	9.13
day 56	3.59	4.48	5.84	7.04	6.73	8.74
day 80	ND	4.53	5.37	6.58	5.13	7.47

Discussion

Increasing population densities of rhizobia introduced into sterilized, bentonite amended loamy sand clearly demonstrated that cells were metabolically active under these conditions. In the bentonite amended loamy sand the initial growth rate was even higher than in the unamended loamy sand (low inoculum densities, Fig.1). Thus, cells present in bentonite protective microhabitats were still able to reach and utilize sufficient amounts of substrate. Postma *et al.* (1990) found similar population dynamics of *Rhizobium* introduced into sterile loamy sand or silt loam, also with a stronger initial increase in the soil with the highest clay content. It is difficult to give a reason for the slightly higher population densities found in bentonite amended as opposed to unamended loamy sand. Slightly more cells were recovered from the bentonite amended soil, even though in previous studies the population dynamics were not significantly influenced by the presence of bentonite clay (Heijnen *et al.*, 1988). A possible explanation for higher survival levels in the presence of bentonite might be that less competition occurred between rhizobia. This could be explained as follows: In both treatments the water potential was brought up to a level of -10 kPa, resulting in moisture contents of 17% (loamy sand) and 45% (loamy sand + 10% bentonite). Assuming that bacteria only colonized water filled pores and were distributed randomly, the volume of soil to be colonized and thus the area containing available substrate would be larger in bentonite amended than in the unamended

soil. This could lead to a smaller competitive pressure in the bentonite amended soil as compared to the unamended soil.

Total amounts of CO_2 evolved from sterilized soil inoculated with rhizobia were in the same range, but slightly lower, as found by Thompson *et al.* (1990), who performed similar respiration experiments with *Arthrobacter* and *Flavobacterium* inoculated into sterile soil. Our lower respiration rate could be explained by the use of a different bacterial strain and a lower incubation temperature of 15°C as opposed to 25°C (Thompson *et al.*, 1990).

The use of medium or low inoculum densities resulted in comparable amounts of total CO_2 respired. This could be explained by the fact that already after 10 days, the numbers of cells in both inoculum treatments were similar. The final cell concentration of approximately 10^7-10^8 cells per gram dry soil determined the total amount of CO_2 respired. With the high inoculum density 10-100 times more cells were respiring, which could be given as an explanation for the significantly higher amount of CO_2 respired from soil having received inocula with medium or low cell concentrations.

When looking at the rhizobial population as a whole, it was shown that the presence of bentonite clay in loamy sand stimulated metabolic activity expressed as the cumulative amount of carbon respired in 30 days (Fig. 2). Stotzky (1967) found that metabolic activity, expressed as the rate of conversion of added glycine to NH_4^+ and from NH_4^+ to NO_2^- and NO_3^- was enhanced by amendment of soil with montmorillonite. However, the same author also found that the amount of CO_2 evolving after the addition of glucose to soil was the same whether montmorillonite was present or not (Stotzky, 1972).

Stotzky and Rem (1966) and Stotzky (1986) suggested that increased respiration in the presence of montmorillonite was primarily the result of the buffering capacity of the clay maintaining the pH of the environment at levels adequate for sustained growth. In the experiments described by Stotzky and Rem (1966), the pH in liquid cultures increased from 3.5 to 6.5 through the addition of montmorillonite. Below pH 5 respiration of a wide range of bacterial species was low, above this pH respiration was high. Due to the high cation exchange capacity of montmorillonite, many basic cations could be exchanged for the H-ions produced during metabolic activity, and thus stimulated bacterial respiration (Stotzky and Rem, 1966). It might be that in bentonite-amended soil a similar buffering of the pH in the direct surrounding of rhizobia stimulated prolonged activity. However, the addition of bentonite to loamy sand only increased the pH from 5.7 to 6.4, which already lies within the range of pH values described by Stotzky and Rem (1966) to result in high respiration levels anyway.

For the calculations determining the amount of carbon respired per cell, it was assumed that cell death did not occur. Previously it was found that in sterile demineralized water without added substrate, rhizobial numbers remained unchanged at a level of 10^8 cells/ml for a period of 35 days (Heijnen *et al.*, 1991). Thus, the

calculations described here, assuming rhizobial populations without substantial cell death over a period of 10 days, may be valid. The observation that the carbon respiration of a single rhizobial cell (0.015 pg) in a non-growing population was the same in the absence and presence of bentonite clay, suggests that maintenance metabolism was unaffected by this smectitic clay mineral. For the production of a new cell however, there was a large difference between the amount of carbon respired in bentonite amended and unamended loamy sand (e.g. 0.16 pg and 1.97 pg using medium inoculum densities, respectively). This suggests that a growing rhizobial population used the carbon in bentonite amended loamy sand much more efficiently, than was the case in unamended loamy sand. If we assume a rhizobial cell to have a volume of 1 μm^3 (Postma and van Veen, 1990), 1 cm^3 of bacteria to correspond to 0.22-0.8 g dry weight (van Veen and Paul, 1979), and a bacterium to contain 40% of carbon (van Veen and Paul, 1979), it could be calculated that 1 rhizobial cell contains 0.09-0.32 pg carbon. Thus, for example after applying medium inoculum densities, carbon was used with an efficiency of 36-67% in bentonite amended loamy sand, as opposed to an efficiency of 4-14% in unamended loamy sand. Martin *et al.* (1976) also found that the addition of Ca-montmorillonite to liquid cultures resulted in a more efficient use of glucose C for cell synthesis. Also, Van Veen *et al.* (1985) found the efficiency of the utilization of glucose as a substrate to be slightly higher in a clay soil as compared to a loamy sand.

Survival data (Table 1) found with medium inoculum densities in non-sterile soil were comparable to those found previously (Heijnen *et al.*, 1988; Heijnen and van Veen, 1991). Bentonite clay was found to protect bacteria from predation by protozoa (Heijnen *et al.*, 1988) through the creation of protective microhabitats (Heijnen and van Veen, 1991). The survival of introduced bacteria in soil will largely be determined by the number of introduced cells which were able to successfully colonize protective microhabitats. The colonization rate in bentonite amended loamy sand could be calculated to be higher than in unamended loamy sand, since 16-200 times more cells survived (Table 1, medium and high inocula), with only an approximately 4 times larger volume of protective microhabitats present (calculated from moisture characteristic curves and from Cryo Scanning Electron Microscopic studies; Heijnen and Van Veen (1991) and Heijnen *et al.* (submitted), respectively). To explain this phenomenon, factors determining colonization processes are discussed.

Colonization of protective microhabitats might be influenced by e.g. (a) the moisture content of the soil (continuous water filled pore system), (b) adhesion of introduced cells to soil particles before reaching small pores, (c) the pore shape (narrow pore necks will block the entrance), (d) the occurrence of isolated pores and (e) the numbers of introduced cells in relation to the numbers of non-colonized protective microhabitats (determining the chance of encountering available protective microhabitats). Since the colonization percentage of total pore space and small pores in natural soil by indigenous bacteria are both less than 1% (Adu and Oades (1978)

and Postma and Van Veen (1990), respectively) a sufficient volume of protective pore space should still be available for colonization by introduced bacteria. It was therefore expected that the introduction of higher numbers of cells would result in higher colonization- and survival rates, because with more introduced cells present, the chance of encountering available protective microhabitats would increase. Postma *et al.* (1990) already showed that increasing inoculum densities (10^6-10^8 cells/g soil) resulted in increasing survival levels. In this paper it was demonstrated that the survival level of rhizobia introduced into loamy sand amended with 10% bentonite increased after applying high as compared to medium and low inoculum densities. However, a 300-fold increase in inoculum size only resulted in an 8-fold increase in survival level (calculated from table 1, comparing the final cell numbers for the medium and high inoculum levels in the 10% bentonite treatment). This suggests that not only the chance of encountering available pores, but possibly also the above mentioned factors (a)-(d), determined the colonization rate of protective microhabitats. This was demonstrated even more clearly in the unamended loamy sand, where the use of high inoculum densities, as compared to medium inoculum densities, did not increase final survival levels. Which factor, i.e. adsorption to soil particles, discontinuous water-filled pore system, pore shape, isolated pores or others was most important in determining colonization of pores, is difficult to say. Adsorption of bacteria to soil particles is a very common phenomenon, with higher adsorption rates occurring in clay soils as compared to sandy soils (Breitenbeck *et al.*, 1988). Cells, adsorbed to soil particles before having reached protective microhabitats, would be prevented from colonizing them. If adsorption to soil particles hampered the colonization rate of protective microhabitats, this colonization rate would be expected to be lower in bentonite amended than in unamended loamy sand due to the stronger adsorption in clay soils (Breitenbeck *et al.*, 1988). This does not agree with the fact that the colonization rate was actually 4-50 times higher (16-200 times more surviving cells divided by a 4 times larger volume of protective microhabitats) in the bentonite amended than in the unamended loamy sand. This suggested that adsorption to soil particles before entering protective microhabitats played, at the most, only a minor part. Therefore, only pore shape, a discontinuous water-filled pore system or isolated pores appeared to determine the colonization of protective microhabitats. Completely isolated pores are unlikely to be important in soil, since Glasbey *et al.* (1991) found, through the simulation of a 1 mm^3 cube of one soil aggregate, that all but 0.1% of the pore space was connected to the exterior. Thus, only pore shape (narrow pore necks) or large pores resulting in a discontinuous water filled pore system, were effective in stopping introduced bacteria from penetrating into protective microhabitats. The effectiveness of these two factors was clearly demonstrated by the low colonization rates of small pores found by Postma and Van Veen (1990). The higher colonization rate of protective microhabitats found in bentonite amended soil therefore suggests a more homogenous and continuous

water filled pore system with less narrow pore necks in loamy sand amended with 10% bentonite clay than in unamended loamy sand.

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CHAPTER 8

IMPROVEMENTS TO THE USE OF BENTONITE CLAY AS A PROTECTIVE AGENT, INCREASING SURVIVAL LEVELS OF BACTERIA INTRODUCED INTO SOIL

Summary

Bentonite clay was used to improve the survival of *Rhizobium leguminosarum* biovar *trifolii* cells introduced into soil. Adding 5% bentonite clay to a loamy sand before inoculation markedly improved bacterial survival through the creation of large amounts of protective microhabitats. However, amending loamy sand with 5% bentonite clay also had a strong influence on soil physical properties. We therefore studied different introduction methods, with the aim of applying less bentonite, but maintaining high bacterial survival levels similar to those found with 5% bentonite. Using starved cells instead of a freshly grown culture slightly increased the survival levels in a loamy sand in the absence of bentonite. In the presence of bentonite, no positive effects on survival due to the use of starved cells could be found. Soil-adapted rhizobia survived better in unamended and bentonite-amended loamy sand than freshly cultured rhizobia. However, this was only the case in soil containing up to 1% bentonite. The previously found high survival levels using 5% bentonite could not be reached. Mixing a fresh rhizobial culture or freeze-dried cells with bentonite clay before introduction into soil was very successful in enhancing bacterial survival levels. Only 1% of bentonite was necessary to reach survival levels comparable to those found previously, after mixing 5% bentonite with loamy sand before inoculation. It was concluded that when bacteria and bentonite were mixed prior to inoculation, the clay offered protection at the site of introduction, resulting in a more efficient use of bentonite for enhancing bacterial survival.

Introduction

The introduction of bacteria into soil is a subject which, in recent years, has gained an increasing amount of interest. Selected bacteria may be applied to soil for the improvement of the nutrient supply to crops (nitrogen fixation, increased phosphorous solubility), the

decomposition of xenobiotic compounds, biological control of soil-borne plant pathogens, biological control of frost injury or for plant growth promotion (Van Elsas and Heijnen, 1990). Successful applications of introduced bacteria require that sufficiently high numbers of cells survive over longer periods of time. However, a progressive decline in bacterial cell numbers after introduction into soil has often been observed, for example with *Rhizobium* (Postma *et al.*, 1988; Heijnen *et al.*, 1988), *Pseudomonas*, *Bacillus* (Van Elsas *et al.*, 1986) *Arthrobacter* and *Flavobacterium* spp. (Thompson *et al.*, 1990), all considered to be typical soil-inhabiting bacteria.

Heijnen *et al.* (1988) and Heijnen and Van Veen (1991) found that the addition of 5 or 10% bentonite clay to loamy sand protected introduced rhizobia from predation by protozoa. Thus, the addition of bentonite resulted in increased survival levels of the introduced rhizobial strain over periods of at least 60 days. The protective effects of bentonite additions to soil on the survival of bacteria was found to be the result of the creation of protective microhabitats (Heijnen and Van Veen, 1991). Protective microhabitats were defined as pores < 6 μm, accessible to the introduced bacteria, but inaccessible to protozoa predating on them.

Although the addition of bentonite in concentrations of 5 or 10% is possible in microcosm experiments, in a field situation this would not be feasible because of the large amounts of clay involved. Also, the strong swelling capacity of bentonite (Mason and Berry, 1967) and its effects on the soil moisture characteristics (Schahabi, 1967) do not allow for the application of large amounts of bentonite in agriculture. These negative aspects of bentonite, as opposed to the positive aspect of increasing rhizobial survival in soil, made it necessary to reduce the amount of bentonite used for the protection of introduced bacteria. Therefore, we examined a number of different methods for introducing bacteria into soil, with the aim of obtaining high bacterial survival levels combined with low bentonite concentrations.

Experiments were carried out comparing population dynamics of starved cells as opposed to freshly grown cells after introduction into soil. Here, we combined the possibility that starved cells might have a better chance of survival after introduction into soil (Thompson *et al.*, 1990) with the protective effects of bentonite clay. Also, a gentle adaptation of the bacterial cells to the soil environment, using sterile soil for pre-incubations before the introduction into natural soil, was tested as a method of improving the survival of introduced bacteria. Finally we performed experiments in which bentonite and rhizobia were mixed together, both as powders or suspensions, before introducing them into soil. The rhizobial powder was obtained by freeze-drying a fresh culture, a method previously applied successfully for *Rhizobium* inoculants (Thompson, 1980).

Materials and methods

Soil and clay mineral

A loamy sand, kept in a 6 x 6 m experimental field micro-plot for several years, was used. The particle size distribution was as follows: (in %) <2 μm: 3.8; 2-16 μm: 2.1; 16-50 μm: 6.7; 50-105 μm: 20.3; >105 μm: 67.1. Some other soil characteristics were: pH-KCl: 5.4; organic matter content: 3.1%; $CaCO_3$: 0.1%;. Several weeks before the start of an experiment soil was collected, dried slightly to enable sieving through a 4-mm mesh sieve, and stored in plastic bags at 4°C. One week before commencing an experiment the storage temperature was increased to 15°C, which was also the temperature applied during the incubation experiments. Some characteristics of the bentonite, i.e. the Na and K saturation measured by flame emission spectrophotometry in acidified 0.1 M $BaCl_2$ extracts of the clay sample and the Ca and Mg saturation measured in the same extracts using flame atomic absorption spectrophotometry (Gillman, 1979) were as follows: (in meq/100 g dry soil) Na^+: 55.6; K^+: 1.1; Ca^{2+}: 27.4; Mg^{2+}: 6.9. When appropriate, loamy sand and bentonite were sterilized by irradiation with 4 Mrad using a ^{60}Co-source.

Bacterial strain

A transposon Tn5 mutant of *Rhizobium leguminosarum* biovar *trifolii*, strain R62, resistant to kanamycin (Km) and rifampicin (Rp) was employed (Heijnen *et al.*, 1988). The bacterium was cultured in yeast extract mannitol broth (Heijnen *et al.*, 1988), supplemented with 25 mg/l Km, for 2 days at 29°C on a rotary shaker (150 rpm). Prior to use, cells were harvested by centrifugation (7000 x g, 15 min). Depending on the introduction method, the pellet was either suspended in sterile demineralized water, suspended in 0.01M phosphate-buffered saline (PBS; pH = 7.2) or was freeze-dried. Cells suspended in PBS were incubated on a rotary shaker (150 rpm) for two weeks at 20°C, harvested by centrifugation (7000 x g, 15 min) and suspended in sterile demineralized water. This resulted in a suspension containing starved rhizobial cells. Freeze-dried cells were obtained by mixing the bacterial pellet with milk powder (ELK, DMV Campina, Eindhoven, the Netherlands) followed by fast freezing at -80°C, after which the cells were freeze-dried overnight. 0.01 g milk powder was used when freeze-drying 1 ml cell suspension. The cell-milk powder mixture was ground to a powder using a mortar and pestle. Approximately 6% of the cells survived the freeze-drying procedure.

Enumeration of introduced bacteria

Numbers of viable cells (colony forming units, cfu) present in the soil samples were determined by transferring a 10 g soil portion (dry weight) into a 250-ml Erlenmeyer flask, containing 95 ml of a 0.1% sodium pyrophosphate solution and 10 g gravel (2-4 mm diameter) to facilitate dispersal of bacterial cells. The flasks were shaken (200 rpm) for 10 min at room temperature. Subsequently, serial 10-fold dilutions were made and plated on yeast extract mannitol agar (Heijnen *et al.*, 1988) supplemented with 50 mg/l Km, 20 mg/l Rp, 100 mg/l cycloheximide and 50 mg/l benomyl. Cycloheximide and benomyl were added to inhibit fungal growth. To test whether viable but non-culturable cells (Colwell *et*

al., 1985) were present in the samples, immunofluorescence counts using a specific antiserum (Postma *et al.*, 1988) were performed half-way through and at the end of an incubation experiment.

Soil studies

Survival studies were carried out in microcosms containing soil corresponding to 10 g dry weight. Bacteria were introduced at levels of around 10^7-10^8 cells/g dry soil. The different treatments can be distinguished by the state of the rhizobia at the time of introduction into soil. (1) A rhizobial suspension, previously starved in PBS. (2) Soil-adapted rhizobia in loamy sand. (3) Soil-adapted rhizobia in loamy sand amended with bentonite. (4) A fresh rhizobial suspension. (5) A rhizobial powder obtained by freeze-drying a fresh culture. Soil-adapted rhizobia were obtained by inoculating a fresh rhizobial suspension into sterile loamy sand or into sterile loamy sand amended with bentonite at a level of around 10^8-10^9 cells/g dry soil. This soil was then incubated at 15°C for two weeks, and used as an inoculum by mixing it with uninoculated soil (1 g with 9 g, respectively). Bentonite-free inoculants (treatment 2) were used for inoculating bentonite-amended and unamended loamy sand; the bentonite-containing inoculants (treatment 3) were used for inoculating unamended loamy sand.

For treatments (1), (2) and (3) the bentonite was added to the soil as a powder before adding the rhizobia. Treatment (4) was divided into (4a), in which the bentonite powder was mixed with the soil before adding the rhizobia (bentonite and rhizobia added separately) and treatment (4b), in which bentonite and rhizobia were added together as a suspension. This suspension consisted of bentonite, sterile demineralized water and a fresh rhizobial culture. The final concentration of bentonite in the suspension was approximately 20% (w/w). For treatment (5) bentonite powder was mixed with the freeze-dried rhizobial powder and added to the soil (bentonite and bacteria added together). Treatment (4a), adding the bentonite powder and the rhizobia separately, was used in previous work (Heijnen *et al.*, 1988; Heijnen and Van Veen, 1991) and will be used as a reference here.

Final bentonite concentrations in the inoculated soil portions were 0,5%, 1% or 5%. This implied that the inoculant soil in treatment (3) contained 5%, 10% or 50% bentonite clay. Control treatments contained 0% bentonite clay (unamended loamy sand).

For all treatments each soil portion was mixed after adding the bacterial inoculum and adjusted to a dry bulk density of 1.2 g/cm^3. All samples contained sufficient amounts of moisture to correspond to a water potential of -10 kPa, resulting in higher moisture contents in samples with higher bentonite concentrations (0% bentonite: 17% moisture; 0.5% bentonite: 18% moisture; 1% bentonite: 19% moisture; 5% bentonite: 32% moisture).

Inoculated soil portions were incubated at 15°C in the dark. The numbers of bacteria in the soil microcosms were determined after 1 day and after 1, 2, 4, 6 and 8 weeks.

Statistical analysis

Genstat 5 (Lawes Agricultural Trust, Rothamsted Experimental Station) was used for a statistical analysis of the data. On each sampling date duplicate soil portions were harvested destructively to determine the numbers of surviving cells. For each treatment a straight line was fitted to the experimental data on bacterial counts, describing the linear decline of the numbers of bacteria (^{10}log no. of colony forming units) in time. The slopes were used to test whether the decline rate of the population density differed for the various treatments. Differences were considered significant at $P < 0.05$.

Results

In general, the experiments performed here clearly show that survival levels of introduced rhizobia increased when applying more bentonite to soil (Figs. 1, 2; Table 1) for all the different introduction methods tested.

Table 1: Log number of colony forming units of rhizobia introduced into loamy sand with or without bentonite (b) amendments. Bentonite was mixed through the soil as a powder prior to the introduction of rhizobia. Rhizobia were introduced after a pre-incubation in PBS (treatment 1), a pre-incubation in sterile loamy sand (treatment 2) a pre-incubation in sterile loamy sand amended with bentonite (treatment 3) or from a liquid culture (treatment 4a). LSD = 0.15

Introduction method and bentonite concentration	Day 1	Day 7	Day 14	Day 27	Day 42	Day 57
Treatment 1, 0%b	8.15	7.58	7.41	6.90	6.19	5.66
Treatment 1, 0.5%b	8.03	7.67	7.43	6.90	6.16	5.62
Treatment 1, 1%b	7.90	7.68	7.47	6.99	6.23	5.93
Treatment 1, 5%b	7.86	7.91	7.89	7.73	7.49	7.24
Treatment 2, 0%b	7.90	7.64	7.25	6.93	6.43	5.90
Treatment 2, 0.5%b	7.96	7.72	7.44	7.19	6.70	6.27
Treatment 2, 1%b	7.94	8.02	7.89	7.51	6.94	6.77
Treatment 2, 5%b	7.98	7.92	7.71	7.72	7.15	6.70
Treatment 3, 0.5%b	8.04	7.95	7.92	7.67	7.09	6.38
Treatment 3, 1%b	8.08	8.02	7.84	7.60	7.16	6.36
Treatment 3, 5%b	8.10	7.94	8.01	7.95	7.55	6.82
Treatment 4a, 0%b	7.95	7.51	7.24	6.72	6.06	4.80
Treatment 4a, 0.5%b	8.13	7.76	7.52	7.16	6.42	5.48
Treatment 4a, 1%b	8.09	7.93	7.64	7.19	6.65	6.09
Treatment 4a, 5%b	8.02	7.86	7.82	7.74	7.47	7.22

In a first set of experiments, survival data of rhizobia introduced into soil after various pre-incubations of the inocula (treatments 1, 2 and 3), were compared with treatment (4a), using a freshly cultured rhizobial suspension (Table 1). In all these treatments the bentonite powder was added to the soil before the introduction of bacteria. Using cells starved in PBS as an inoculum (treatment 1), compared with the use of fresh rhizobial cells (treatment 4a), only reduced the population density decline rate significantly in the absence of bentonite. Cells pre-incubated in sterile loamy sand with or without bentonite amendments and inoculated into non-sterile soil (treatments 2 and 3) with final concentrations of 0, 0.5 and 1% bentonite, showed significantly lower population density decline rates than freshly cultured cells (treatment 4a). However, this was no longer the case in the presence of 5% bentonite. A remarkable observation was that after pre-incubation of the inoculum in sterile soil, the increase in survival caused by increased bentonite concentrations was much smaller than when freshly cultured cells were used (Table 1).

In a second set of experiments a comparison was made between inoculation methods in which bentonite and bacteria were added separately or mixed together (treatments 4a, 4b and 5). Population densities of rhizobia added to loamy sand together with a bentonite suspension (treatment 4b) showed significantly lower decline rates (Fig. 1) than when powdered bentonite was added to the soil prior to the introduction of the bacteria (treatment 4a). Lower concentrations of bentonite applied as a suspension could induce population dynamics comparable to those found with higher concentrations of bentonite applied as a powder (for example 5% bentonite as a powder and 1% bentonite as a suspension, Fig. 1).

Treatments using a freeze-dried rhizobial culture mixed with powdered bentonite (treatment 5) showed an initial increase in population densities (Fig. 2). This was probably due to the presence of milk powder in the inoculum which apparently served as substrate for the rhizobia. As from day 14, no significant differences in the population dynamics were found between treatments 4b (*Rhizobium*-bentonite suspension) and 5 (*Rhizobium*-bentonite powder), comparing soil portions containing 0.5%, 1% and 5% bentonite. This was also the case for the controls, in which fresh or freeze-dried rhizobia were added to unamended loamy sand (no bentonite). As from day 14 population dynamics were therefore only influenced by bentonite amendments, and not by the presence of milk powder in the inoculum.

A final comparison was made between treatments (1), (2) and (3), performed in the first set of experiments (Table 1) and treatments (4b) and (5), performed in the second set of experiments (Figs. 1, 2). A direct comparison between these two sets of experiments was not possible, since the inoculum densities applied to treatments (4b) and (5) were lower than those applied to treatments (1), (2) and (3) (Figs. 1, 2; Table 1). This alone might already result in different population dynamics between the treatments (Postma *et al.*, 1990). However, treatment (4a), in which bentonite powder and rhizobia were added separately) was included in both sets of survival studies (Fig. 1, Table 1) and could be used as a means by which to compare survival data from treatments (4b) and (5) with

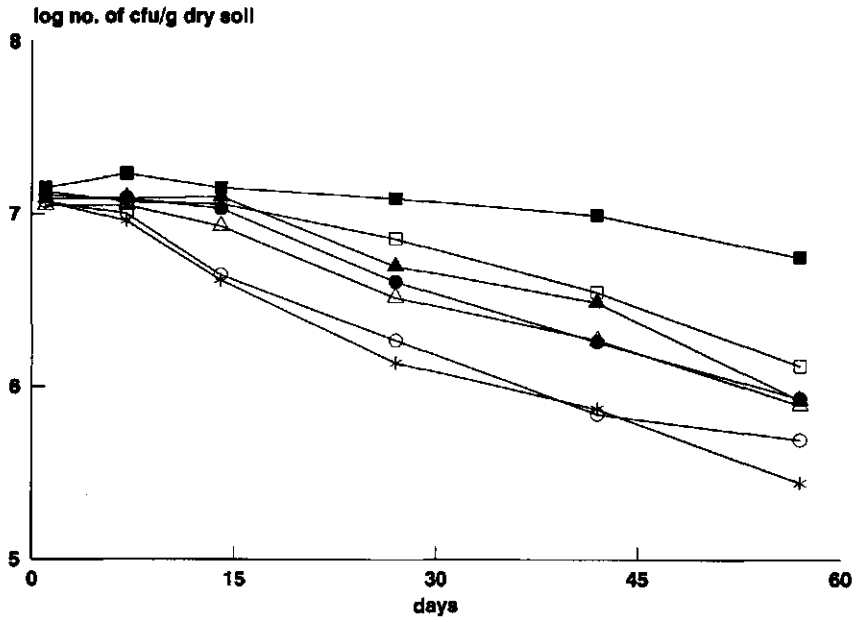

Fig. 1: Population dynamics of *R. leguminosarum* biovar *trifolii* introduced into loamy sand, amended with or without bentonite clay, at a water potential of -10 kPa. The bentonite was mixed with loamy sand as a powder in amounts of 5% (□), 1% (△), 0.5% (○) and 0% (∗) prior to the introduction of *Rhizobium* (treatment 4a). Alternatively, the bentonite was first mixed with sterile demineralized water and a fresh rhizobial culture forming a suspension, and then added to the loamy sand (treatment 4b), resulting in bentonite concentrations of 5% (■), 1% (▲), and 0.5% (●). LSD = 0.17.

survival data from treatments (1), (2) and (3). Therefore the survival data found with treatment (4a), presented in Table 1, were subtracted from the data, also found with treatment (4a), presented in Fig. 1. This amount was assumed to be caused by the difference in inoculum densities, and was subsequently subtracted from the data found for treatment (4b) and (5) (Figs. 1, 2). These corrected data were then used for comparing the decline rates of rhizobial population densities in treatments (4b) and (5) with the decline rates in treatments (1), (2) and (3). For treatment (5) only the data as from day 14 were included due to the increase in cell numbers observed. It was concluded that the use of fresh rhizobial cells mixed with a bentonite suspension (treatment 4b) and the use of freeze-dried cells mixed with bentonite powder (treatment 5) offered the best improvement in the survival of bacteria introduced into soil over the range of bentonite concentrations studied here.

Immunofluorescence counts (results not shown), performed for the various treatments half-way through and at the end of each experiment, showed that there was no reason to

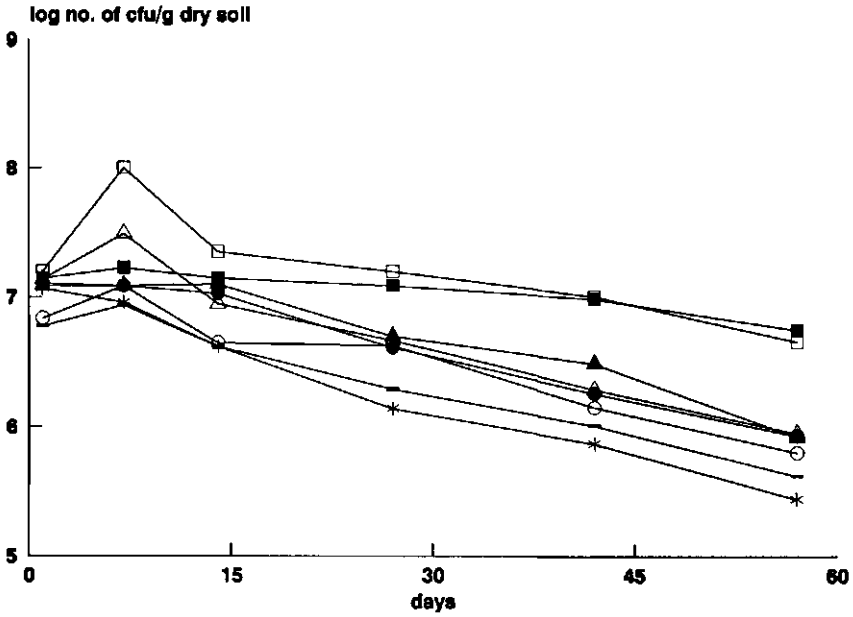

Fig. 2: Population dynamics of *R. leguminosarum* biovar *trifolii* introduced into loamy sand, amended with or without bentonite clay, at a water potential of -10 kPa. The bentonite was first mixed with sterile demineralized water and a fresh rhizobial culture forming a suspension, and then added to the loamy sand (treatment 4b), resulting in bentonite concentrations of 5% (■), 1% (▲), and 0.5% (●). Alternatively, the rhizobial inoculum was freeze-dried, mixed with the powdered bentonite, and introduced into loamy sand (treatment 5), resulting in bentonite concentrations of 5% (□), 1% (△) and 0.5% (○). In control, treatments, fresh (∗) or freeze-dried (–) rhizobial cells were added to unamended loamy sand. The data obtained applying treatment 4b are the same as presented in Fig. 1. LSD = 0.19.

assume the presence of viable but non-culturable cells. This was also found previously in survival experiments using the same rhizobial strain (Heijnen and Van Veen, 1991).

Discussion

Bentonite additions to loamy sand greatly improved the survival of introduced rhizobia by offering them protection against predating protozoa (Heijnen *et al.*, 1988). As was shown by Heijnen and Van Veen (1991), the protection against predation was caused by an increase in the volume occupied by protective microhabitats, which are pores large enough to allow bacteria to enter, but too small to be colonized by protozoa (pores with equivalent neck diameters < 6 μm). Our present results showed that the extent of this protection could be influenced by the method of introduction.

Rhizobium cells starved in PBS, compared with freshly cultured cells, showed significantly higher survival levels after introduction into unamended loamy sand. Similar results were found by Thompson *et al.* (1990) using *Flavobacterium* and *Arthrobacter*. However, Wessendorf and Lingens (1989) found lower survival levels with *Pseudomonas fluorescens* cells introduced into sandy loam after growth in a poor, as opposed to a rich medium. Since soil is considered to be a nutrient-limited environment (Alexander, 1977), our results could be explained through an adaption of the cells to low nutrient levels, prior to their introduction into soil. However, the positive influence of this so-called starvation-adaption of the cells in PBS was only observed in the unamended loamy sand and not in bentonite-amended soils. Therefore it was concluded that the presence of bentonite-created protective microhabitats had a greater influence on rhizobial survival than the physiological state of the cells prior to the introduction into soil. This was in accordance with work by Wessendorf and Lingens (1989), who found that soil conditions (for example soil texture) were of greater importance than bacterial culture conditions for the survival of *Pseudomonas fluorescens* introduced into soil.

Cells, pre-incubated in sterile loamy sand and subsequently introduced into non-sterile soil (treatment 2) containing 0%, 0.5% or 1% bentonite clay, survived better than PBS-starved or freshly cultured cells (treatments 1 and 4a). This might be explained by the fact that prior to introduction into non-sterile soil, the cells had become adapted to an environment consisting mainly of solid particles after their liquid culturing medium. Furthermore, the cells were surrounded with, and presumably also adsorbed to, soil particles which could act as a protective environment against adverse biological interactions. These soil particles could hamper predation by protozoa, since the movement of these organisms between soil particles is limited (Kuikman *et al.*, 1990). Combining this with the fact that in our experiments protozoan movement in the soil will be restricted, on the basis of water availability, to pores < 30 µm (-10 kPa), it is possible that the soil portion in which the cells were introduced will remain devoid of protozoa for a considerable length of time, thus resulting in a greater chance for rhizobial survival. However, if soil particles surrounding the introduced bacteria hampered protozoan movement and predation, these same soil particles would also prevent the bacteria from entering the bentonite-created protective microhabitats which were present in the non-sterile soil portions (treatment 2). This was supported by the fact that increasing bentonite percentages (0-5%) had only a small influence on the population dynamics. Possibly only a minor fraction of the introduced bacteria could enter bentonite-created protective microhabitats. For this reason the use of 5% bentonite in treatment 2 (pre-incubation in sterile loamy sand) did not result in the high survival levels as found using 5% bentonite in treatment 4a (bentonite powder, fresh rhizobial suspension). It can be concluded that in these treatments, the impact of soil particles was more important in enhancing rhizobial survival than bentonite protection.

Bacteria, pre-incubated in sterile loamy sand amended with bentonite (treatment 3), encountered a similar protection by sterile soil particles as described above. However, the fact that now the rhizobia could have entered protective microhabitats in the inoculant, did

not seem to further increase survival levels. It was surprising to find that 5% bentonite in treatment (3) did not have a stronger influence on survival levels, especially since the rhizobia could have entered bentonite-created protective microhabitats. We did however have difficulties in mixing the inoculant with the non-sterile soil portions. Also, the use of 50% bentonite in the inoculant needed to obtain 5% bentonite in the final soil portions could have been too high, and might have been unfavourable for bacterial survival. Combining these two aspects, the bacteria might still have been surrounded by the high bentonite concentrations after introduction into non-sterile soil, resulting in reduced survival rates.

The improved survival rate in soil of cells mixed with a bentonite suspension and of freeze-dried cells mixed with bentonite powder prior to the introduction (treatments 4b and 5) as compared to cells added to soil previously amended with bentonite powder (treatment 4a), showed that it was possible to employ bentonite more efficiently. A good example of this increased efficiency was the similarity in population dynamics between loamy sand amended with 5% bentonite, applied as a powder, and loamy sand amended with 1% bentonite, applied as a suspension (Fig. 1). The increased efficiency might be explained as follows. When bentonite was mixed homogeneously through the soil, the chance that introduced bacteria entered protective microhabitats would depend on the number of these microhabitats. With less bentonite present, the chance of reaching and entering a protective microhabitat would be smaller and the chance of protection against predation by protozoa would, consequently, be smaller too (Figs. 1, 2). Therefore, if less bentonite is applied, it is necessary to make sure that bacteria are brought to these bentonite-created protective microhabitats. One way of accomplishing this was by mixing the bacteria with the protective agent before the actual introduction into soil.

However, a problem arising when applying treatment 5 is that the freeze-drying of bacteria may cause difficulties concerning inoculant survival. This was found using a Tn5 mutant of a *Pseudomonas fluorescens* strain, often employed in soil studies (Van Elsas *et al.*, 1988, 1989). More than 99.9% of the *Pseudomonas* cells died during the freeze-drying procedure, and it was not possible to obtain enough cells to get inoculum densities similar to those used with *Rhizobium.* When high inoculum levels are required, freeze-dried cell suspensions will only be applicable if the bacterial species can survive the freeze-drying procedure. If, however, low inoculum densities are acceptable, freeze-drying of the cultures in combination with the use of bentonite appears to be a useful method for increasing the survival of introduced bacteria, particularly since freeze-drying of a bacterial suspension is a good way of preserving an inoculum (Thompson, 1980).

It could be concluded from these experiments that optimal survival of introduced rhizobia could be obtained using only 1% of bentonite clay, providing the rhizobial culture and the clay were mixed thoroughly before introduction into soil. Using this introduction method, even 0.5% bentonite could improve rhizobial survival as compared to a control without bentonite amendments. When rhizobia and bentonite were mixed before introduction, the rhizobial cells would be located there where the protective microhabitats were

being created. The introduced rhizobia would then be able to make optimal use of the new bentonite-created microhabitats in soil. The significance of using lower bentonite concentrations is that the negative influence on some soil physical characteristics of the use of bentonite clay might be reduced considerably by using 1% instead of 5% bentonite. For example, with lower bentonite concentration, swelling of the soil is less likely to occur. An other positive aspect of adding low amounts of bentonite might even be the slight increase in the water holding capacity of loamy sand in the layer where bacteria were introduced, reducing the chances of crop yield losses during dry periods in summer. However, also the use of 1% bentonite amendments to soil in agriculture will probably be limited, due to the large amounts required. It still remains to be seen whether crop production is influenced directly by the use of bentonite clay and whether introduced bacteria will be able to colonize plant roots in bentonite amended soil.

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CHAPTER 9

ROOT COLONIZATION BY *PSEUDOMONAS* INTRODUCED INTO BENTONITE AMENDED SOIL

Summary

The influence of bentonite clay amendments to loamy sand on the colonization of wheat roots by introduced *Pseudomonas fluorescens* cells was studied. In a first experiment, root colonization was determined over a 6 week plant growth period. In a second experiment the colonization was studied after a one week plant growth period, either directly after introducing the bacteria, or after a 6 week pre-incubation of the soil. A rhizosphere effect, i.e. the occurrence of higher cell concentrations in rhizosphere soil than in bulk soil, was observed in the presence and in the absence of bentonite clay. The size of the rhizosphere effect was usually smaller in the presence of bentonite. This suggested that bacteria were physically hindered, making it more difficult to invade the immediate root environment. However, due to the fact that bentonite enhanced survival to such an extent, overall survival in the rhizosphere in bentonite-amended soil was still higher than in unamended loamy sand. No bentonite effects on the rhizoplane populations were observed. In an additional experiment *P. fluorescens* was found to be sensitive to moisture fluctuations. This therefore probably seriously reduced survival levels of the introduced *P. fluorescens* cells in plant growth experiments involving moisture fluctuations.

Introduction

In the past, bacteria have frequently been introduced into the environment for various different purposes (Van Elsas and Heijnen, 1990). A main problem reducing the effectiveness of introduced bacteria is probably the fact that poor and ineffective survival levels frequently occur soon after introduction into soil (Crozat *et al.*, 1987; Thompson *et al.*, 1990). The use of carrier materials or soil amendments has been studied extensively with the aim of improving bacterial survival, however with varying results (Van Elsas and Heijnen, 1990). Heijnen *et al.* (1988) showed that the survival

of introduced bacteria in the bulk soil of a loamy sand could be enhanced by amending the soil with bentonite clay, a smectitic clay mineral. This increased survival was hypothesized to result from the presence of many bentonite created protective micro-habitats (Heijnen and Van Veen, 1991). These protective microhabitats (pores with neck diameters of 3-6 μm or less) are apparently large enough to allow bacteria to enter, but are probably too small to be colonized by grazing protozoa (Heijnen and Van Veen, 1991).

If bacteria introduced into soil are to be used for crop protection, these bacteria will generally have to be able to colonize plant roots in order to be effective (e.g. the suppression of Take-all of wheat by fluorescent pseudomonads; Weller and Cook, 1983). Consequently, if bentonite clay amendments are applied to soil with the aim of increasing survival levels, bacteria introduced into bentonite-created protective microhabitats will have to be able to migrate from these sites in order to colonize plant roots. Bacteria will then either have to move through the bulk soil towards the plant roots, or they will have to colonize plant roots when these roots grow past their protective microhabitat.

In this paper experiments are described in which root colonization by *Pseudomonas fluorescens* introduced into bentonite created protective microhabitats is investigated. In a first experiment however, the population dynamics of *P. fluorescens* introduced into non-planted bentonite amended soil was compared with that of *Rhizobium*, a bacterium previously used in survival studies involving bentonite (Heijnen *et al.*, 1988; Heijnen and Van Veen, 1991; Heijnen *et al.*, in press), but not applicable in nodulation experiments since too many indigenous rhizobia were present in the soil, and proper controls were not available. Colonization of wheat roots by *Pseudomonas* was then studied in time over a period of six weeks. In order to describe the extent of root colonization, the numbers of *Pseudomonas* cells in rhizosphere soil (soil closely adhering to roots) and in the rhizoplane (cells strongly associated with roots) were determined. Also, wheat root colonization by freshly inoculated *Pseudomonas* cells was compared with root colonization by cells inoculated into soil 6 weeks prior to planting. Since moisture fluctuations as a result of water uptake by plants appeared to have a great influence on population dynamics of *Pseudomonas*, survival in unplanted soil, with or without moisture fluctuations, was also studied.

Materials and methods

Bacterial strains

Pseudomonas fluorescens strain R2f (chr::Tn5) was used for survival and root colonization experiments. The wild type strain was isolated from a grassland rhizosphere soil (Van Elsas *et al.*, 1990). Transposon Tn5 was introduced into *P.*

fluorescens by a standard filter mating procedure described by Simon *et al.* (1983), resulting in a strain resistant to kanamycin (Km) and streptomycin (Sm). *P. fluorescens* was cultured overnight in Luria broth (tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; demineralized water, 1l; pH 7.2) supplemented with 50 mg/l Km at 29°C on a rotary shaker (150 rpm). Prior to use cells were harvested by centrifugation (7000 x g, 15 min) and suspended in sterile demineralized water. Stock cultures were maintained at -80°C in the presence of 15-20% glycerol.

Rhizobium leguminosarum biovar *trifolii* strain R62 (chr::Tn5) was used for a survival experiment in bulk soil. Characteristics and culturing conditions for *R. leguminosarum* biovar *trifolii* are described in Heijnen *et al.* (1988).

Soil and bentonite

A loamy sand, kept in an experimental field micro-plot for several years, was used. When appropriate, the loamy sand was amended with bentonite clay in amounts of 0.5%, 1% or 5% (w/w). Soil and bentonite characteristics were described by Heijnen *et al.* (in press). Bentonite was added to the loamy sand either as a powder before introducing *P. fluorescens*, or as a suspension together with *P. fluorescens*. The latter method was expected to result in better survival levels, since this was previously found for rhizobia introduced into unplanted soil (Heijnen *et al.*, in press).

After inoculation, all soil portions had a moisture content corresponding to a water potential of -10 kPa, i.e.: 5% bentonite: 32% moisture content; 1% bentonite: 19% moisture content; 0.5% bentonite: 18% moisture content; unamended loamy sand: 17% moisture content. In all experiments bulk densities were adjusted to 1.2 g/cm^3

Plants and plant growth conditions

Wheat seedlings (*Triticum aestivum* cv. Sicco) were allowed to germinate for approximately 4 days on moist, sterile filter paper and were then used in root colonization experiments. Plants were grown in a climate chamber with a 16h day (20°C), an 8h night (16°C) and a relative humidity of 65%. The light intensity was 25,000 lx. When plants were growing in the soil for 6 weeks, 1 g Sporumix (Merckx *et al.*, 1987) was added per kg dry soil in order to support plant growth.

Soil and root colonization studies

Survival of *P. fluorescens* was studied in unplanted, bentonite-amended or unamended soil over a period of 60 days. Population dynamics of *P. fluorescens* was compared with that of *R. leguminosarum* biovar *trifolii*, a strain previously found to be protected against protozoan predation by bentonite amendments (Heijnen *et al.*, 1988, 1992; Heijnen and Van Veen, 1991). Replicate soil portions (10 g, dry weight) were inoculated with *P. fluorescens* or *R. leguminosarum* biovar *trifolii* at levels of

approximately 10^8 cells/g dry soil, and were incubated in a humid chamber at 15°C in the dark. At regular time intervals soil portions were destructively harvested in order to determine the numbers of surviving *P. fluorescens* or *R. leguminosarum* biovar *trifolii* cells.

To determine whether pseudomonads introduced into protective microhabitats were still capable of colonizing plant roots, two experiments were performed. In the first, root colonization by introduced *P. fluorescens* cells was studied over a period of 6 weeks. Plastic pots (total volume 1400 cm^3) were filled with 1360 g inoculated (approximately 10^8 cells/g dry soil) bentonite-amended or unamended soil (dry weight). All pots were planted with 2 wheat seedlings. The soil surface was covered with gravel to protect it from extensive drying and to minimize algal growth. Water was added daily to readjust the soil moisture content to levels corresponding to a water potential of -10 kPa. Randomly chosen replicate pots were harvested 1, 3, 5, and 6 weeks after planting. Numbers of *P. fluorescens* cells surviving in the bulk- and rhizosphere soil and in the rhizoplane were determined.

In the second experiment, root colonization by *P. fluorescens* was studied over a 1 week period in plastic containers (total volume 150 cm^3) which were filled with 100g inoculated (3 x 10^7 cells/g dry soil) bentonite-amended or unamended soil (dry weight). Less soil was used than in the first root colonization experiment, since plants were only allowed to grow for 1 week instead of 6 weeks. One half of the soil portions was planted with 4 wheat seedlings per container. A higher plant density as compared to the first root colonization experiment was used in order to obtain enough root material for sampling during the 1 week growth period. The soil surface was covered with gravel. Containers were placed in a humid chamber allowing for light penetration. The humid chamber was incubated in the plant growth climate chamber for 1 week, after which the plants were harvested. The second half of the soil portions was left unplanted for 6 weeks and incubated in a humid chamber at 15°C in the dark. After this initial incubation period the soil portions were planted with 4 wheat seedlings per container, incubated in a similar way as the first half of soil portions, and harvested after 1 week. On both harvesting occasions, the numbers of *P. fluorescens* cells surviving in bulk soil, rhizosphere soil and rhizoplane were determined. Since the plant growth period lasted for only 1 week and evaporation was minimized due to the humid conditions, it was not necessary to water the plants.

Population dynamics in rhizosphere and bulk soil suggested that fluctuations in the soil moisture content might influence the survival of introduced *P. fluorescens* cells. In a final experiment, soil portions corresponding to 30g dry weight were inoculated with *P. fluorescens* at levels of approximately 10^7 cells/g dry soil. Only treatments containing unamended loamy sand and loamy sand amended with 5% bentonite (added as a powder only) were included. All soil portions were adjusted to a water potential of -10 kPa. Water was allowed to evaporate from one half of the soil portions, and the moisture content was adjusted to the original level (corresponding

to a water potential of -10 kPa) 3 times a week. The other half of the soil portions was incubated in a humid chamber, and no water losses were observed. In order to compare environmental circumstances with those of the root colonization experiments, the soil surface was covered with gravel, and soil portions were incubated in the plant growth climate chamber. Numbers of surviving *P. fluorescens* cells were determined over a period of 4 weeks.

Enumeration of bacteria

Numbers of culturable *P. fluorescens* cells in bulk soil were determined by transferring approximately 10 g of soil into a 250-ml Erlenmeyer flask, containing 95 ml of sterile 0.1% sodium pyrophosphate solution (NaPP) and 10 g gravel (2-4 mm diameter). The flasks were shaken for 10 min at room temperature (200 rpm). Serial 10-fold dilutions in NaPP were made and plated on King's B agar (proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4.7H_2O$, 1.5 g; glycerol, 10g; agar, 15g; demineralized water, 1 l; pH 7.2) supplemented with 100 mg/l Km, 100 mg/l Sm, 100 mg/l cycloheximide and 25 mg/l benomyl (the latter two antibiotics act as inhibitors of fungal growth). After incubation of the plates for 1-2 days at 29°C *P. fluorescens* colonies could be recognized through the production of a fluorescent yellow pigment. Immunofluorescence detection techniques (Postma *et al.*, 1988; Trevors *et al.*, 1990) were applied for determining the total numbers of *P. fluorescens* cells (including viable but non-culturable cells; Colwell *et al.*, 1985) in the bulk soil.

Prior to determining the numbers of culturable *P. fluorescens* cells in rhizosphere soil, wheat roots and adhering rhizosphere soil were separated from the bulk soil by careful, manual shaking. Wheat roots and adhering rhizosphere soil (approximately 10 g) were then transferred into a 250-ml Erlenmeyer flask, containing 95 ml 0.1% NaPP and 10 g gravel (2-4 mm diameter). Shaking, dilution and plating procedures were similar to those described for the bulk soil.

For the determination of the numbers of viable *P. fluorescens* cells in the rhizoplane of the wheat roots, the root/soil suspension left over from the rhizosphere sampling was sieved (0.5 mm). The roots were rinsed with demineralized water, and excess water was removed with filter paper. The roots were cut up and mixed thoroughly. A root sample of approximately 0.5 g was taken and macerated in 10 ml sterile NaPP using a Potter S glass homogenizer. Further dilution and plating procedures were similar to those described for the bulk soil.

Statistical analysis

On all sampling days, duplicate soil portions or pots were destructively harvested. Genstat 5, Lawes Agricultural Trust (Rothamsted Experimental Station) was used for an analysis of variance on the data. Differences were considered to be significant at $P < 0.05$.

Results

In unplanted soils *P. fluorescens* and *R. leguminosarum* biovar *trifolii* both showed a significantly smaller reduction in the survival levels of introduced cells in the presence than in the absence of bentonite clay for all treatments (Table 1). The addition of increasing amounts of bentonite clay to loamy sand generally increased survival levels significantly for both strains. It was also demonstrated that on day 60 both strains encountered a significantly higher protection when bacteria and bentonite were added together as a suspension than when bentonite powder and bacteria were added separately. This resulted in comparable survival levels between treatments in which 1% bentonite was added as a suspension and 5% bentonite added as a powder. In general, no significant differences were found between the survival of *P. fluorescens* and *R. leguminosarum* biovar *trifolii*. *P. fluorescens* appeared to be a suitable organism for studying possible bentonite effects in root colonization experiments.

Table 1: Population dynamics of *P. fluorescens* R2f and *R. leguminosarum* R62, expressed as the log no. of colony forming units/g dry soil over an incubation period of 60 days. Initial cell densities were approximately 10^8 cells/g dry soil. Bentonite (B) was added to loamy sand (Ls) as a powder (p) or as a suspension (s) in amounts of 5, 1 or 0.5% (w/w). LSD = 0.17.

	R2f			R62		
treatment	24h	day 28	day 60	24h	day 28	day 60
5%Bs	8.13	7.10	6.94	7.86	7.80	7.24
1%Bs	7.95	7.62	6.89	7.65	7.34	6.52
0.5%Bs	7.89	7.11	6.23	7.70	7.15	6.36
5%Bp	7.90	7.25	6.63	7.78	7.55	6.46
1%Bp	7.85	6.70	6.15	7.75	6.67	5.86
0.5%Bp	7.90	6.40	5.57	7.78	6.86	5.16
Ls0%B	7.86	6.10	4.95	7.73	6.06	4.97

In the bulk of the planted soil (Fig. 1), cell numbers of *P. fluorescens* introduced into unamended loamy sand showed a strong significant decrease of at least 5 log units in a period of 6 weeks. Addition of increasing bentonite concentrations generally resulted in significantly higher survival levels. Also, the treatments in which cells and bentonite were added together (as a suspension) resulted in significantly higher survival levels than when they were added to soil separately. Final survival levels (week 6) in bulk soil covered a range between approximately 10^3 and 10^8 cells/g dry soil, showing that 5% bentonite (added as a suspension together with the inoculum)

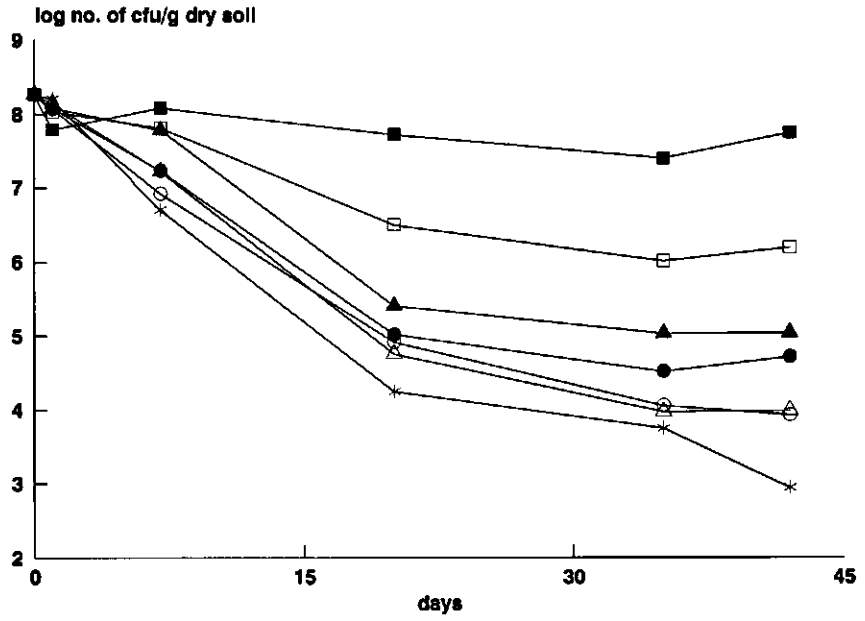

Fig. 1: Population dynamics of *P. fluorescens* in bulk soil of loamy sand (Ls) or loamy sand amended with bentonite (B) over a period of 6 weeks. The soil was planted with wheat seedlings and inoculated with approximately 10^8 cells/g dry soil. The bentonite was added to the soil as a powder (p) before inoculation or as a suspension (s) together with the inoculum, in amounts of 5, 1, or 0.5% (w/w). ■: 5% Bs; ▲ :1% Bs; ● :0.5% Bs; ✳ :Ls; □ :5% Bp; △ :1% Bp; ○ :0.5% Bp. LSD = 0.83.

increased survival with 5 log units compared with unamended loamy sand. In unplanted soil however, a similar comparison (on day 60) showed an increase in the number of surviving cells of only approximately 2 log units (Table 1).

In the rhizosphere (Fig. 2) of the planted soil, population dynamics of introduced *Pseudomonas* cells showed a similar declining trend as in the bulk soil. However, survival levels by week 6 covered a smaller range, between approximately 10^8 and 3 x 10^4 cells/g dry soil. An overall analysis including all soil treatments showed that as from week 3 the rhizosphere contained significantly higher cell concentrations than the bulk soil (rhizosphere effect). The difference between rhizosphere and bulk after 6 weeks was largest for unamended loamy sand (no. of cells in rhizosphere / no. of cells in bulk = 30.9), and decreased when bentonite powder concentrations increased to 5% (no. of cells in rhizosphere / no. of cells in bulk = 3.6). When bentonite was added as a suspension a similar calculation resulted in a rhizosphere effect of only 3.5 using 0.5% bentonite, and decreased further to 1.4 for the 5% bentonite treatment.

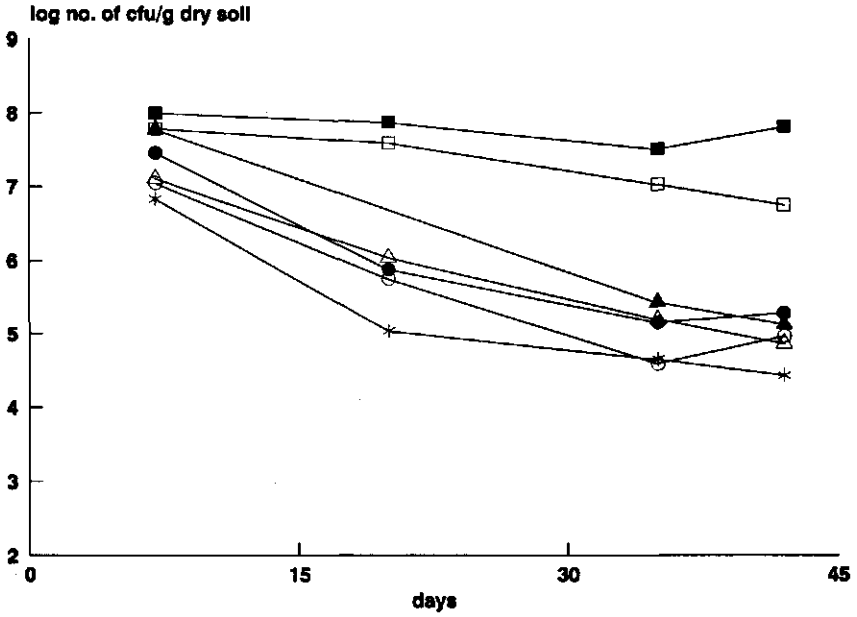

Fig. 2: Population dynamics of *P. fluorescens* in rhizosphere soil of loamy sand (Ls) or loamy sand amended with bentonite (B) over a period of 6 weeks. The soil was planted with wheat seedlings and inoculated with approximately 10^8 cells/g dry soil. The bentonite was added to the soil as a powder (p) before inoculation or as a suspension (s) together with the inoculum, in amounts of 5, 1, or 0.5% (w/w). ■ : 5% Bs; ▲ :1% Bs; ● :0.5% Bs; ✳ :Ls; □ :5% Bp; △ :1% Bp; ○ :0.5% Bp. LSD = 0.83.

In the rhizoplane (Fig. 3), large fluctuations occurred between the numbers of cells in replicate treatments, resulting in a large variation between duplicate samples. A significant decline in the number of cells surviving in the rhizoplane was observed during the 6 week incubation period. By week 6, no significant effects of bentonite additions could be found, even though the 5% bentonite suspension treatment consistently promoted survival to be 1-2 log units higher than in the unamended control.

In the second root colonization experiment (Table 2) the cell concentration in the bulk soil after one week plant growth showed a significant decrease when comparing initially planted soil and soil planted after a 6 week pre-incubation period. Bentonite effects (amounts, powder, suspension) were not as clear as described in the first root colonization experiment. The total numbers in the rhizosphere soil showed a significant decline when comparing 1 and 6 weeks, except for the two 5% bentonite treatments. When including all treatments, an overall significant rhizosphere effect was observed on both sampling occasions. This rhizosphere effect was significantly

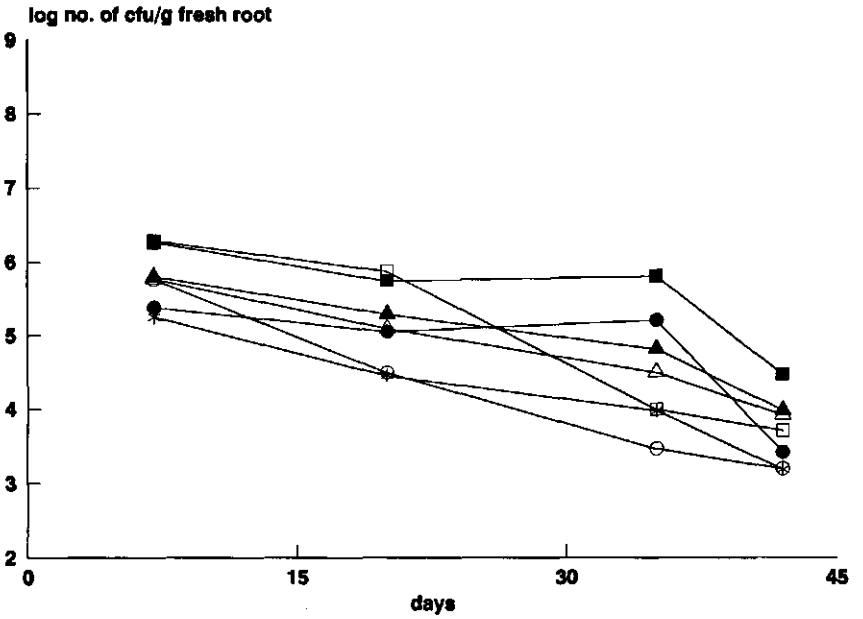

Fig. 3: Population dynamics of *P. fluorescens* in the rhizoplane of wheat planted in loamy sand (Ls) or loamy sand amended with bentonite (B) over a period of 6 weeks. The soil was inoculated with approximately 10^8 cells/g dry soil before planting. The bentonite was added to the soil as a powder (p) before inoculation or as a suspension (s) together with the inoculum, in amounts of 5, 1, or 0.5% (w/w). ■: 5% Bs; ▲ :1% Bs; ● :0.5% Bs; ⁎ :Ls; □ :5% Bp; △ :1% Bp; ○ :0.5% Bp. LSD = 1.75.

larger when the soil was planted after a pre-incubation period of 6 weeks. Without a pre-incubation, the rhizosphere effect after 1 week of plant growth was more pronounced than in the first root colonization experiment (Figs. 1 and 2). After a 6 week pre-incubation, unamended loamy sand again showed a large rhizosphere effect. However, now this effect did not decline significantly with increasing bentonite concentrations (Table 2), and at the highest bentonite concentration, the rhizosphere effect was in the same order of magnitude as the unamended control. An overall analysis, including all the treatments, showed that the numbers of cells in the rhizoplane were significantly higher after a pre-incubation period of 6 weeks before planting, than when the soil was planted directly after inoculation. The use of 5% bentonite, added as a powder, resulted in a remarkably high cell concentration in the rhizoplane after a 6 week pre-incubation period (Table 2).

The decrease in the numbers of introduced *Pseudomonas* cells observed in the bulk soil was larger in the first colonization experiment in which plants were watered, resulting in moisture fluctuations (Fig. 1), than in the second in which no moisture

fluctuations occurred (Table 2). In a final experiment the influence of moisture fluctuations on the survival of *P. fluorescens* introduced at a level of approximately 10^7 cells/g dry soil into unplanted soil was therefore studied. Continuously moist loamy sand amended with 5% bentonite (added as a powder) showed no decline of introduced cell numbers over a period of 28 days. Soil amended with bentonite undergoing a wet-dry cycle showed a significant reduction in the cell numbers to 3 x 10^4 cells/g dry soil. Populations in continuously moist unamended loamy sand declined significantly to 2 x 10^6 cells/g dry soil, and those in unamended loamy sand undergoing wet-dry cycles to 8 x 10^2 cells/g dry soil. Thus, the difference between unamended and bentonite amended loamy sand was approximately 1.7 log units under fluctuating and under constant moisture conditions. However, also under fluctuating moisture conditions, survival in bentonite-amended loamy sand was significantly higher than in unamended loamy sand. Minimum moisture contents of the soil amended with 5% bentonite clay undergoing wet-dry cycles and of the planted soil (Fig. 1) reached values corresponding to water potentials between -300 and -600 kPa for the bentonite amended loamy sand. Minimum water potentials between -100 and -600 kPa were reached for the unamended loamy sand. It was clearly demonstrated for unplanted soil, that *P. fluorescens* was sensitive to water potentials fluctuating between -10 and -600 kPa, which are smaller fluctuations than generally occurring in the field in summer.

Table 2: Log number of colony forming units/g dry soil (cfu's) of *P. fluorescens* in bulk- and rhizosphere soil and in the rhizoplane (cfu's/g fresh root) of wheat roots after 1 week plant growth. The soil was inoculated with 3 x 10^7 cells/g dry soil, and planted with wheat seedlings immediately after inoculation or after a 6 week pre-incubation period. LSD = 0.38 for bulk and rhizosphere soil; LSD = 0.83 for the rhizoplane.

	1 week			6 + 1 weeks		
treatment	bulk	rhizo-sphere	rhizo-plane	bulk	rhizo-sphere	rhizo-plane
5%Bs	6.91	7.97	6.33	6.43	7.97	6.47
1%Bs	7.47	7.40	6.19	6.59	6.64	6.60
0.5%Bs	6.89	6.90	5.90	5.59	6.06	6.80
5%Bp	7.42	8.42	6.51	6.99	8.59	8.02
1%Bp	6.79	7.24	5.95	5.63	6.11	6.76
0.5%Bp	6.83	6.96	5.71	5.16	6.26	6.47
Ls0%B	6.51	6.97	5.65	4.64	6.04	6.38

Immunofluorescence detection techniques were included for determining the numbers of cells in bulk soil. In unplanted soil with a constant moisture content, immunofluorescence counts and plate counts gave comparable results. In bulk soil undergoing moisture fluctuations (Fig. 1; wet-dry cycles in unplanted soil) immunofluorescence counts were up to 2 log units higher than plate counts, suggesting the presence of non-culturable cells in these treatments.

Discussion

Corresponding to previous results, *Rhizobium*, and now also *Pseudomonas*, showed increasing survival levels in the presence of bentonite clay apparently due to the creation of microhabitats protecting introduced bacteria against predation by protozoa (Heijnen *et al.*, 1988; Heijnen and Van Veen, 1991). Thus, the protective effects of bentonite clay were not limited to *Rhizobium*. Like *Rhizobium*, *Pseudomonas* also showed higher survival levels at a certain bentonite concentration when the bentonite was added to soil together with the inoculum (as a suspension), than when bentonite and inoculum were added separately (Heijnen *et al.*, in press). This could be explained, according to Heijnen *et al.* (in press) by the fact that when bacteria and bentonite were mixed prior to inoculation, the clay offered protection at the very site of introduction, resulting in a more efficient use of bentonite for enhancing bacterial survival. Thus, 1% bentonite added as a suspension could offer a comparable amount of protection as 5% bentonite added as a powder.

Rhizobium would appear to be a suitable organism for studying bentonite effects in root colonization experiments, due to its nodulation capacity with legumes. However, *R. leguminosarum* biovar *trifolii* strain R62 could not be used in nodulation experiments, since there was a large indigenous rhizobial population present in the soil which nodulated *Trifolium repens*. Nodules formed by the introduced rhizobial strain hardly added to the total number. However, our present results suggested that *P. fluorescens* strain R2f is a suitable organism for studying root colonization in bentonite-amended soil.

The *P. fluorescens* cells isolated from the rhizosphere and rhizoplane samples are not necessarily the same cells as introduced at the beginning of the experiment. The plant root environment is a relatively nutrient-rich environment, in which inoculant growth may occur. This was actually the case in both root colonization experiments, in which a significant rhizosphere effect was demonstrated. Similar effects were recently described by Breland and Bakken (1991) and Nijhuis *et al.* (submitted).

Root colonization involves the capacity of introduced bacteria to migrate from their initial colonization site in soil to the plant root. This could involve several different processes. In the experiment studying root colonization over a period of six weeks (Figs. 1, 2, 3), bacteria could have been transported to the roots by a downward

water flow created when watering the plants, as was also shown by Trevors *et al.* (1990). This means of transport is likely to occur in the presence of a (continuously) water filled pore system, in which the pore necks are wider than the bacteria (Griffin and Quail, 1968). The presence of growing plant roots probably had no influence on vertical transport (Worrall and Roughley, 1991; Trevors *et al*, 1990). In the second root colonization experiment (Table 2) no downward water flow existed to transport the bacteria. For colonization, bacteria would now have to move laterally towards the plant root. This movement could be a result of a water flow in the direction of the plant root (root suction) or of chemotaxis. Chemotaxis could be responsible for the movement of bacteria over several centimetres (Bashan, 1986). An other possibility for bacteria to reach plant roots is when growing roots make contact with bacterial cells in soil. In this case, root colonization would hardly involve any bacterial movement. Obviously, apart from bacterial movement, soil structure will also be a determinant of successful root colonization. Certain sites of introduction, e.g. protective microhabitats in bentonite-amended soil, might be more difficult to migrate from than the mainly unprotected sites in unamended loamy sand.

It is clear from the extent of the rhizosphere effect in unamended loamy sand (Figs. 1, 2) that the introduced bacteria met with few problems when colonizing plant roots. In the presence of bentonite clay the rhizosphere effect appeared to be reduced (Figs. 1, 2). This might be resulting from a physical hinderance caused by bentonite, hampering bacterial invasion of the immediate root environment. This effect was more pronounced when bentonite and bacteria were introduced together, as a suspension. This agrees with the earlier hypothesis that this way of introducing bacteria and bentonite resulted in a very close contact between the two, creating a more effective protection of bentonite against predation (Heijnen *et al.*, in press). Even though the rhizosphere effect was not as large with as compared to without bentonite, the total number of introduced pseudomonads was higher in bentonite-amended rhizosphere than in unamended rhizosphere soil. Taking this latter aspect into account, it was remarkable that cell numbers isolated from the rhizoplane were not significantly affected (even though there was an increasing trend) by the presence of bentonite clay (Fig. 3). However, for determining bacterial numbers in the rhizosphere, a sub-sample of the total root system was used. It might have been better to separate the roots into root-base and root-tip segments, since Liljeroth *et al.* (1991) had found that different bacterial groups occupied different root segments. By taking a sub-sample of the total root system, a possible bentonite effect on the rhizoplane population might therefore not have been recognized in our experiments. From the first root colonization experiment (Figs. 1, 2, 3) it can be concluded that, since colonization of the rhizosphere and rhizoplane is of importance for plant protection, the presence of bentonite is a major factor determining the success of soil-introduced bacteria in plant protection.

The second root colonization experiment strengthens this conclusion. Here a rhizosphere effect was found in the presence of 5% bentonite clay, of a similar extent as found with unamended loamy sand. A significant increase in the cell concentrations in the rhizoplane in the presence of 5% bentonite (added as a powder) compared to the other treatments after a pre-incubation period of 6 weeks was also observed. These findings might appear to be inconsistent with the results of the 6-week root colonization experiment. However it must not be forgotten that growth circumstances were purposely made different between the two experiments, depending on their individual aim. In the second root colonization experiment it was also clearly demonstrated that introduced bacteria, present in the soil for 6 weeks, were still capable of colonizing plant roots. This supported similar data provided by Van Elsas *et al.* (1990). The rhizosphere effect was even larger after pre-incubation, which might result from fast growth on root exudates after a relatively long starved period. Thus, if bacteria are introduced into soil and are capable of surviving over longer periods (e.g. by amending the soil with bentonite clay), they should also be able to colonize the roots of a crop then planted. The necessity of additional inoculations might therefore be considerably reduced. On the other side however, this should also be included in the safety evaluation concerning introduced GEM's (genetically engineered microorganisms) in soil.

The strong decrease in inoculant cell numbers in the bulk and rhizosphere of (unamended) planted soil (Fig. 1) compared to the much smaller decrease in unplanted soil (Table 1) was remarkable. The experiment using unplanted soil with a wet-dry cycle strongly suggested that *P. fluorescens* was sensitive to moisture fluctuations, thereby explaining the strong decrease in the cell concentrations in the planted soil (Fig. 1). Bentonite clay gave a similar protection to introduced pseudomonads under continuously moist and under fluctuating moisture conditions. This conflicts with previous results (Bushby and Marshall, 1977), describing that bentonite protected rhizobia specifically against moisture fluctuations. The use of *P. fluorescens* strain R2f, genetically engineered in such a way that it can be applied for the protection of plants against soil-borne plant pathogens, might be limited since this strain could succumb to moisture fluctuations which are likely to be more extreme in a field situation than those described here.

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SUMMARY

Bacteria have frequently been introduced into the soil environment, e.g. for increasing crop production or for biological control purposes. Many applications require high numbers of surviving organisms in order to be effective. However, survival of bacteria after introduction into soil is generally poor, and numbers of introduced bacteria have been known to decrease from 10^9 to approximately 10^3 cells/g soil in 25 days. Thus, if bacteria are to be used as effective microbial inoculants to, a means to increase survival levels in soil needs to be found.

Survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into loamy sand was found to be greatly enhanced by amendments of bentonite, in amounts of 5 or 10%, to the soil. Bentonite appeared to offer introduced bacteria protection against protozoan predation, resulting in increased bacterial survival levels in bentonite amended soil as compared to unamended soil.

Also in liquid cultures, protozoan activity was strongly hampered by the presence of bentonite, thereby also improving the survival of *Rhizobium*. Bentonite did not release any substances toxic to protozoa in liquid cultures, and presumably this would not occur in the soil environment either. Bentonite toxicity could therefore not explain the increased survival levels of bacteria introduced into bentonite amended soil. It was suggested that, in liquid cultures, bentonite clay increased the minimum level of bacteria for effective predation by protozoa.

Changes in soil structure as a result of bentonite additions could explain the observed increases in bacterial survival levels. A mathematical relationship was found describing the log numbers of introduced rhizobia surviving in soil samples after an incubation period of 57 days using 3 pore size classes. Pores with necks < 3 μm and between 3 and 6 μm positively affected survival levels. These pores apparently were large enough to allow bacteria to enter, but were too small to be accessible to predating protozoa. Pores with necks between 6 and 15 μm had a negative influence on rhizobial survival levels, because bacteria situated inside these relatively large pores could be reached and predated upon by protozoa. Therefore, pores < 6 μm were found so serve as protective microhabitats for bacteria introduced into soil. A larger number of such protective microhabitats in bentonite amended loamy sand than in unamended loamy sand could explain the observed increase in survival levels of bacteria introduced into bentonite amended soil. The colonization potential of protective microhabitats was suggested to be determined largely by pore shape and

the continuity of the water-filled pore system. Increased numbers of protective microhabitats (pores < 6 μm) in bentonite-amended soil as compared to unamended soil were demonstrated visually by micro-morphological studies using Cryo Scanning Electron Microscopy.

The effectiveness of bentonite was strongly determined by the way in which the clay and the inoculum were added to the soil. When a bentonite suspension and bacteria were mixed together prior to inoculation, the clay offered more protection against predation than when bentonite powder and bacteria were added separately. This suggested that when the protective agent was present at the site of introduction, a more efficient use of the clay could be made, resulting in enhanced survival levels.

Apart from survival, bentonite additions to soil also influenced bacterial respiration. The cumulative amount of CO_2 respired by rhizobia introduced into sterile bentonite-amended loamy sand was significantly higher than in unamended loamy sand. Carbon was used more efficiently during growth in bentonite-amended than in unamended loamy sand. The maintenance respiration of rhizobial cells was not influenced by the presence of bentonite clay. The growth rate of rhizobia introduced into sterile soil was increased by the presence of bentonite.

Pseudomonas fluorescens was also found to survive at higher levels in bentonite-amended than in unamended soil, suggesting the bentonite effects were not limited to *Rhizobium* only. *Pseudomonas fluorescens* was used to study root colonization by bacteria introduced into bentonite amended soil. A rhizosphere effect (i.e. the occurrence of higher cell concentrations in rhizosphere soil than in bulk soil) was observed both in the absence and in the presence of bentonite clay, but it was less pronounced in the latter case. This finding suggested that bacteria were physically hindered by bentonite, making it more difficult to invade the immediate root environment. However, protection against predation by bentonite enhanced survival to such an extent, that overall survival in the rhizosphere was still higher in bentonite amended loamy sand than in the unamended soil.

It can be concluded from this thesis that soil structure, and especially the pore size distribution of the soil is a key factor determining the survival chances of bacteria introduced into soil. Application of introduced bacteria for e.g. biological control will probably stand a larger chance of being successful in soils with relatively high numbers of pores < 6 μm. However, it is unlikely that bentonite will ever be applied to soil in amounts of e.g. 5%, because of the large impact of bentonite additions on, for example, the moisture characteristics of the soil. However, the knowledge obtained on the importance of the pore size distribution of a soil, and the fact that the site of introduction of the bacteria will largely determine survival chances, will be of great importance for the future development of successful carrier materials for introducing bacteria into soil.

SAMENVATTING

In het verleden zijn bacteriën veelvuldig in het bodemmilieu geïntroduceerd. Mogelijke toepassingen voor geïntroduceerde bacteriën zijn b.v. het verhogen van de gewasproduktie of de biologische bestrijding van ziekten en plagen. Voor een effectieve toepassing van geïntroduceerde bacteriën is een groot aantal overlevende organismen vaak een vereiste. Een groot probleem is echter de slechte overleving van bacteriën na introduktie in de bodem; er zijn gevallen bekend waarbij er een afname van 10^6 cellen/g grond werd waargenomen in een periode van 25 dagen. Gezien de vele toepassingen voor effectieve microbiële inoculanten in de bodem, is het van groot belang een manier te vinden om de overleving van geïntroduceerde bacteriën in de bodem te verbeteren.

De overleving van *Rhizobium leguminosarum* biovar *trifolii* in zwak lemig zand bleek sterk verbeterd te kunnen worden door een toevoeging van het kleimineraal bentoniet, in hoeveelheden van 5 of 10%, aan de grond. Bentoniet bleek geïntroduceerde bacteriën te kunnen beschermen tegen predatie door protozoën. Hierdoor was de overleving in zwak lemig zand met bentoniet beter dan in zwak lemig zand zonder bentoniet toevoegingen.

Ook in vloeistof cultures bleek de aktiviteit van protozoën sterk geremd te worden door de aanwezigheid van bentoniet. Dit resulteerde eveneens in een verbetering van de overleving van *Rhizobium*. Uit bentoniet kwamen geen, voor protozoën, toxische stoffen vrij. Er werd verondersteld dat dit in het bodemmilieu ook niet zou gebeuren. Bentoniet toxiciteit kon dus geen verklaring zijn voor de verbeterde overleving van bacteriën na introduktie in een zandgrond in aanwezigheid van bentoniet. Er werd verondersteld dat bentoniet ervoor zorg droeg dat het minimum aantal bacteriën, vereist voor een effectieve predatie, verhoogd werd.

Verandering van de bodemstruktuur als gevolg van bentoniet toevoegingen bleek een goede verklaring voor de toename van de overleving van geïntroduceerde bacteriën. In een wiskundige relatie bleek het mogelijk om met behulp van 3 poriegrootteklassen het log aantal overlevende bacteriën na een incubatie periode van 57 dagen te beschrijven. Poriën met equivalent diameters < 3 µm en tussen 3 en 6 µm hadden een positieve invloed op de overleving. Deze poriën waren kennelijk groot genoeg om voor bacteriën toegankelijk te zijn, maar te klein voor prederende protozoën. Poriën met diameters tussen 6 en 15 µm hadden een negatieve invloed op de overleving van *Rhizobium*, omdat bacteriën in deze poriën makkelijk ten prooi

vielen aan protozoën. Poriën < 6 µm konden daarom dienen als beschermde microhabitats voor geïntroduceerde bacteriën in de bodem. Het grotere aantal beschermde microhabitats in een zandgrond met bentoniet toevoegingen kon de waargenomen verbeterde overleving van geïntroduceerde bacteriën, in vergelijking tot een zandgrond zonder bentoniet toevoegingen, verklaren. Het al dan niet koloniseren van een beschermde microhabitat werd verondersteld afhankelijk te zijn van de vorm van de poriën en van de continuïteit van het watergevulde poriën systeem. Grotere aantallen kleine poriën (< 6 µm) in een zandgrond met bentoniet toevoegingen in vergelijking tot de onveranderde zandgrond, werden daadwerkelijk aangetoond m.b.v. Cryo Scanning Elektronen Microscopie.

De effektiviteit van bentoniet toevoegingen aan zandgrond bleek sterk bepaald te worden door de wijze van toevoegen van inoculum en bentoniet. Indien een bentoniet suspensie en het inoculum voorafgaand aan de introduktie gemengd werden, bleek de klei meer bescherming te bieden tegen predatie dan wanneer bentoniet poeder en bacteriën na elkaar werden toegevoegd. Het leek daardoor aannemelijk dat bescherming efficiënter was indien de klei op de plaats van introduktie gesitueerd was.

Naast de overleving, bleken bentoniet toevoegingen aan grond ook de bacteriële respiratie te beïnvloeden. In steriele zandgrond met bentoniettoevoegingen, geïnoculeerd met *Rhizobium*, was de cumulatieve hoeveelheid gerespireerde CO_2 groter dan in afwezigheid van bentoniet. In aanwezigheid van bentoniet werd koolstof bovendien veel efficienter gebruikt tijdens groei dan in afwezigheid van bentoniet. De onderhoudsrespiratie van *Rhizobium* werd niet beïnvloed door de aanwezigheid van bentoniet. De groei snelheid van *Rhizobium* in steriele grond nam na toevoeging van bentoniet toe.

Pseudomonas fluorescens bleek eveneens beter te overleven in zandgrond met bentoniet toevoegingen dan in zandgrond zonder bentoniet toevoegingen. Bentoniet effecten leken daardoor niet beperkt te zijn tot *Rhizobium*. *Pseudomonas fluorescens* werd vervolgens gebruikt in een experiment, waarbij wortelkolonisatie door bacteriën, geïntroduceerd in zandgrond met en zonder bentoniet toevoegingen, bestudeerd werd. Een rhizosfeer effect (het voorkomen van grotere celaantallen in de rhizosfeer dan in de bulkgrond), werd zowel in aan- als afwezigheid van bentoniet aangetoond. In aanwezigheid van bentoniet was het rhizosfeer effect echter kleiner. Een mogelijke verklaring hiervoor zou kunnen zijn dat bacteriën een fysische hinder ondervonden van bentoniet, waardoor het binnendringen in de onmiddellijke wortelomgeving bemoeilijkt werd. Toch was in aanwezigheid van bentoniet de overleving in de rhizosfeer beter dan in afwezigheid van bentoniet, dit als gevolg van de sterk verbeterde overleving van de geïntroduceerde bacteriën.

Uit dit onderzoek kan geconcludeerd worden dat de bodemstruktuur, en in het bijzonder de poriegrootteverdeling, de overlevingskansen van geïntroduceerde bacteriën sterk bepaalt. Toepassingen van geïntroduceerde bacteriën voor b.v.

biologische bestrijding zal een grotere kans van slagen hebben in grondsoorten met een relatief groot aantal poriën < 6 μm. Echter, het is onwaarschijnlijk dat bentoniet ooit in hoeveelheden van b.v. 5% zal worden toegepast, alleen al vanwege de grote invloed op de vochthuishouding van de bodem. De kennis die verkregen is over het belang van de poriegrootteverdeling, gecombineerd met het feit dat de plaats waar de geïntroduceerde bacteriën terecht komen de overlevingskansen grotendeels bepaalt, zijn van groot belang voor de verdere ontwikkeling van bruikbare dragermaterialen voor de introduktie van bacteriën in de bodem.

NAWOORD

Dit proefschrift is het resultaat van 5 jaar onderzoek. Veel mensen hebben mij gedurende die periode wetenschappelijke en morele steun gegeven.

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CURRICULUM VITAE

Caroline Heijnen werd geboren op 25 september 1962 in Den Haag. Na het behalen van het Atheneum diploma aan de Chr. Scholen Gemeenschap "De Vlietschans" in Leiden, begon zij in 1980 met de studie Bodemkunde aan de toenmalige Landbouw Hogeschool. Zij maakte voor het eerst kennis met de bodembiologie tijdens haar stage by het Rothamsted Experimental Station in England. Tijdens haar doctoraal studie werd haar bodembiologische kennis verder uitgebreid door middel van haar hoofdvak, uitgevoerd bij het toenmalige Ital in Wageningen. In januari 1987 studeerde zij met lof af aan de Landbouwuniversiteit. Sinds februari 1987 is zij werkzaam als bodemmicrobioloog/bodemkundige in de onderzoeksgroep bodembiologie op het Ital. Inmiddels is de groep bodembiologie ondergebracht bij het Instituut voor Bodemvruchtbaarheid (IB-DLO, Haren), waarbij zij haar standplaats Wageningen vooralsnog behouden heeft.