Biological nitrogen fixation by
lucerne (*Medicago sativa* L.) in acid soils

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Biological nitrogen fixation by lucerne (*Medicago sativa* L.) in acid soils

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en
milieuwetenschappen,
op gezag van de rector magnificus,
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des namiddags te vier uur in de aula
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aan Nelleke

aan mijn zussen

aan ons vader en ons moeder

NN08201,1365

STELLINGEN

1. De initiatie van het nodulatieproces bij lucerne (*Medicago sativa* L.) wordt geremd door zowel een lage bodem-pH, als door een geringe beschikbaarheid van calcium in de bodem.

Dit proefschrift.

2. De verbetering van de biologische stikstofbinding in zure gronden door het zaad van lucerne (*Medicago sativa* L.) in een laagje kalk te hullen, is grotendeels toe te schrijven aan de locale neutralisatie van de grond.

Dit proefschrift.

3. De afwezigheid van wortelknollen bij leguminosen is op zich geen indicatie voor het ontbreken van de plantspecifieke *Rhizobium* bacteriën in de grond.

Bushby (1981) Soil Biol.Biochem. 13 (241-245).
Dit proefschrift.

4. De pH waarde verkregen via de agar-contact methode is een reële weergave van de bodem-pH.

Marschner en Römheld (1983) Z.Pflanzenphysiol.Bd. 111 (241-251).
Dit proefschrift.

5. In de (verzurende) nederlandse gronden is biologische stikstofbinding overbodig.

6. Een mens in de maatschappij is als een wortel in grond; beiden creëren hun eigen micro-sfeer.

7. De consensus bij internationale landbouwconferenties een hoge prioriteit te geven aan het oplossen van de problemen in de ontwikkelingslanden, staat in schril contrast tot de fondsen die ter beschikking worden gesteld om vertegenwoordig(st)ers uit deze landen (op zijn minst) aanwezig te laten zijn.

8. Solidariteit houdt meestal op als het eigenbelang in het gedrang komt.

9. Het plan van de Drugs Enforcement Agency (DEA) om de cocateelt in Bolivia met rupsen en chemische middelen te bestrijden is ecologisch, maar vooral sociaal-politiek niet verantwoord.

10. Het heil van de sportbeoefening weegt op tegen de blessures die men er aan overhoudt.

11. De veronderstelling dat de voorwaardsen van een rugbyteam dom zijn, is onterecht.

Dit proefschrift.

Stellingen behorende bij het proefschrift "Biological nitrogen fixation by lucerne (*Medicago sativa* L.) in acid soils". Jan Pijnenborg, Wageningen, 22 juni 1990.

Voorwoord

Voor U ligt het resultaat van drie jaar onderzoek. Ik ben blij dat het karwei nu klaar is, en erkentelijk voor de medewerking van verschillende mensen. Om te beginnen bij de vakgroep Microbiologie, met mijn begeleider Tek An Lie. Hij zorgde voor stimulerende ideeën en stevige discussies. De samenwerking ontwikkelde zich tot een vruchtbare symbiose, die zich uitstrekte tot in Bolivia. Mensen stimuleren behoort ook tot de specialiteiten van Alex Zehnder. Hij heeft mij het artikelen schrijven geleerd. Dankzij Jan van Heerd en Theo Ploeg zijn menig potje en petrischaaltje gevuld met grond. De lucerne plantjes werden met toewijding verzorgd. Anton Houwers wil ik bedanken voor het correctiewerk, Nees Slotboom voor de tekeningen, Bobo Freeke en Ton Feijen (Fotolokatie De Dreyen) voor de foto's en Sjaan Gerritsen en Ria Kooy voor de koffie. Twee studenten hebben hun bijdrage geleverd: Willie Burgman (LUW) en Petri Leinonen (Finland).

In dit interdisciplinaire onderzoek, over het effect van bacteriën op de groei van planten in grond, werd ik verder ook geholpen door Bouke Deinum en Leen Mol (vakgroep Landbouwplantenteelt en Graslandkunde) met de veldproeven, en door Jaap Nelemans en Wim Menkveld (vakgroep Bodemkunde en Plantenvoeding) met het gebruik van de pH micro-electrode in de verschillende bodems.

Het lijkt onvermijdelijk dat het contact met familie en vrienden wordt bepaald door het onderzoek. Om een proefschrift tenslotte tot een goed eind te kunnen brengen moet je er als promovendus helemaal induiken. In de persoonlijke sfeer werd ik daarbij gesteund door Nelleke, die mij er op z'n tijd ook weer eventjes uittrok. Bedankt !

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"*Consider a plant -its life- how a seed faln to ground*
sucketh in moisture for its germinating cells,
and as it sucketh swelleth, til it burst its case
and thrusting its roots downward and spreading them wide
taketh tenure of the soil, and from ev'ry raindrop
on its dribbling passage to replenish the springs
plundereth the freighted salt, while it pricketh upright
with its flagstaff o'erhead for a place under the sun,
anon to disengage buds that in tender leaves
unfolding may inhale provender of the ambient air"

Robert Brigdes (1806-1882)

cited in the book
Root Nodule Bacteria and Leguminous Plants
E B Fred, I L Baldwin and E McCoy, 1932

Chapter 1

Introduction

THE IMPORTANCE OF BIOLOGICAL NITROGEN FIXATION

Nitrogen is usually the fourth most abundant element present in plant tissue, following carbon, oxygen and hydrogen (Wild and Jones, 1988). It is an essential constituent of proteins and nucleic acids. Most plants depend for their growth entirely on combined nitrogen, *i.e.* in the form of ammonium or nitrate. An agronomic practice to replenish the pool of combined nitrogen in soil is the application of nitrogen fertilizer. For industrial production of nitrogenous fertilizers, atmospheric dinitrogen is combined catalytically with hydrogen by the Haber-Bosch process (N_2 + H_2 --> $2NH_3$). This process consumes high amounts of energy.

Atmospheric dinitrogen can be used directly by certain plants *via* their symbiosis with N_2 fixing microorganisms. For agriculture, the most important N_2 fixing system is the symbiosis of leguminous plants with *Rhizobiaceae* (Marschner, 1986). The amount of symbiotically fixed nitrogen per year ranges from 65 to 335 kg of nitrogen per hectare (Subba Rao, 1980).

In developing countries with a rapid increasing population, the intake of protein per capita is insufficient (Subba Rao, 1977). Since the production and application of nitrogenous fertilizers is very limited, biological nitrogen fixation offers the most promising supplement or even the only alternative in these areas of the world (Signurbjörnsson, 1984). Grain legumes are an important source to meet the protein requirements for human nutrition (Döbereiner and Campelo, 1977).

Legumes are also grown as forage crops for animal feed. For this purpose, lucerne or alfalfa (*Medicago sativa* L.) has been cultivated since the mid-sixteenth century ('t Mannetje *et al.*, 1980). Lucerne consistently shows relatively high contents of fixed nitrogen (Vance *et al.*, 1988). By introducing lucerne into pasture, the yield of the mixed sward increases (Olsen and Elkins, 1977), and part of the fixed nitrogen is transferred from the legume to the grass species (Burity *et al.*, 1989).

In affluent parts of the world, agriculture greatly benefits from the application of nitrogenous fertilizers, and the use of legumes as a N-source has slowly been supplanted (Mulder *et al.*, 1977). In the Netherlands for example, the area of land sown with lucerne decreased from 15.000 hectares in 1945 to only 2.000 hectares in 1980 (van der Meer, 1985). Meanwhile, the average amount of fertilizer-N applied per hectare of arable land increased from 32 to 239 kg (*source* Landbouwcijfers). In this period, an intensive dairy and meat production system developed, depending largely on the imports of animal feed from abroad. In the past decade however, the harmfull eutrophying effects of the excess of nutrients from manure applications, mainly N and P, increased alarmingly. This development resulted in the propagation of a more extensive farming practice with lower application levels of manure and fertilizer. Compared to 1980, the amount of fertilizer-N per hectare applied in 1988 was 10 kg less, and the surface area with lucerne doubled (*source* Landbouwcijfers).

The policy of the European Community (EC) to lower the production of Dutch milk, also induced a renewed attention on leguminous crops (Schröder, 1988). A scientific programme of the EC ("Energy in Agriculture") has been the source of financement for the investigation presented in this thesis.

SOIL ACIDITY

A wide range of environmental conditions may affect the legume-*Rhizobium* symbiosis (Vincent, 1988). Amongst them, acidity of the soil is one of the major constraints (Freire, 1984; Russell, 1978). The response of some temperate legume species to different soil-pH levels is plotted in Fig. 1. The plants grew on symbiotically fixed nitrogen. The impaired growth of lucerne in soils with pH below 6 (measured in a soil-water suspension) has been known for a long time (Cunningham, 1928; Joffe, 1920).

In the state of West-Australia, biological nitrogen fixation is of vital interest for agriculture.

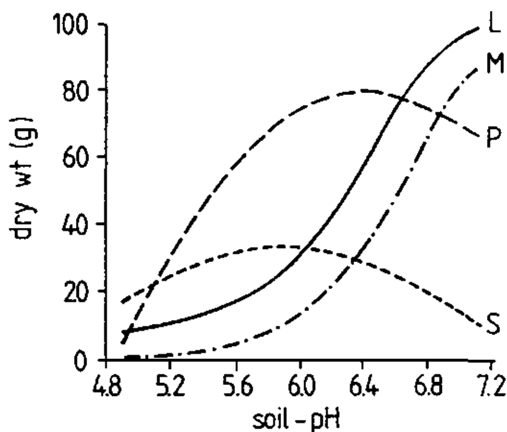

Figure 1. The effect of soil-pH on the yield of some temperate legumes (g dry shoot weight per 0.35 m^2) grown in garden plots. L: Lucerne (Medicago sativa), M: Black Medick (Medicago lupulina), S: Serradella (Ornithopus sativus), P: Pea (Phaseolus), according to Mulder et al., 1966.

Here, nodulation and growth of both lucerne and clover are severely hampered by soil acidity on about 4 million hectares of land (Howieson *et al.*, 1988). In the Netherlands, such poor soils have developed mainly in sandy material of Pleistocene origin. Some 40% of the area, roughly one million hectares, is covered with this material (de Bakker, 1979).

In acid soils the high concentration of proton ions may not *per se* be the only factor limiting plant growth. Soil acidity is generally accompanied by the release of excessive amounts of aluminium and manganese, and deficiencies of calcium and molybdenum (Andrew, 1978; Munns, 1976). At soil-pH values below 5, the free Al and Mn can reach toxic levels (Foy, 1984; Munns, 1976), and below pH 4 the high concentrations of both elements are the most important limiting factors (Marschner, 1986).

Because of the complexity of soil acidity, in which the effects of H^+ ions are confounded with other constraints, many investigators have used defined nutrient solutions instead of soil, to sort out the response of legumes to single stress factors (*e.g.* Kim *et al.*, 1985; Lie, 1969; Munns, 1968). However, a solution is homogeneous, whereas soil is a heterogeneously structured system, allowing considerable spatial variations to occur chemically as well as microbiologically (Marschner, 1986). In particular, the conditions in the vicinity of a plant root (rhizosphere) may differ in many respects from those encountered in the bulk of the soil. The uptake of nutrients is a selective process, leading to depletion or

accumulation of ions. This might result in a root-induced pH change (Dijkshoorn, 1962; Riley and Barber, 1969; Israël and Jackson, 1978). Therefore it is not surprising that the micro-environment around the root plays a decisive role in the nodulation process (Demezas and Bottomley, 1987).

NITROGEN FIXING SYSTEM

The nitrogen fixing system consists of three biological entities (Parker and Chatel, 1982): (i) the host plant (legume), (ii) the nodule bacterium (*Rhizobium*), and (iii) a new organ, the root nodule (symbiosis). Effective nitrogen fixation depends on the intrinsic characteristic of each component, and the influence of environmental factors (Fig. 2; Vincent, 1988). The sensitivity of each component for acidity will be discussed in the following paragraphs.

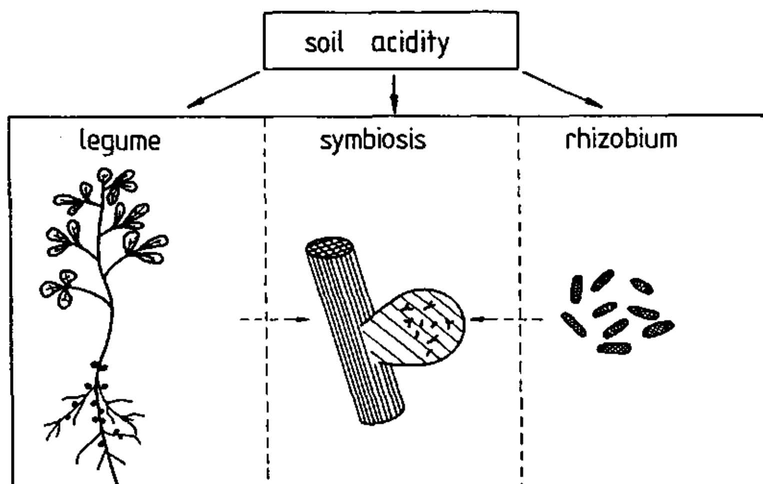

Figure 2. Soil acidity can affect legume, symbiosis, and Rhizobium.

Legume

Comparisons of species within the family of *Leguminosea* show a substantial variation in sensitivity towards acidity; *Medicago* species being the most vulnerable (Fig. 1; Andrew, 1976, 1978;

Mulder *et al.*, 1966; Munns, 1965). The variation in the genus *Medicago* may be utilized to select the less acid sensitive genotypes. *M.truncatula* performs better than *M.sativa* (Andrew, 1976); *M.sativa* performs better than *M.lupulina* (Fig. 1; Mulder *et al.*, 1966). The acid tolerance of the plant can be improved in long-term breeding programmes (Barnes *et al.*, 1984).

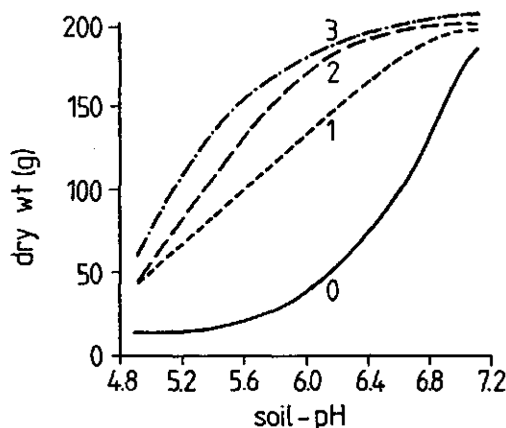

Figure 3. Effect of nitrogen supply on the yield of lucerne (g dry weight per 0.4 m^2) grown on garden plots of different soil-pH. 0: no nitrogen, 1: 40, 2: 80, 3: 120 kg N ha^{-1}, according to Mulder et al., 1966.

To obtain nodules on lucerne roots, soil-pH should at least be around 6 (Fig. 1). When supplied with fertilizer-N, the legume is more tolerant to acidity and the minimum pH for growth of *M.sativa* could be lowered from 6 to around 5 (Fig. 3). Obviously, the symbiosis is more sensitive to acidity than host plant growth. In the range from pH 5 to pH 6 the free Al concentration is low enough. so its toxicity is limited (Hoyt and Nyborg, 1972). In this investigation a sandy soil of pH 5.2 has been used.

Rhizobium

Within the genus *Rhizobium*, which is subdivided into four species, the critical pH for growth differs markedly (Table 1). Again, the least acid tolerant species that infects temperate legumes is the counterpart of lucerne, *R.meliloti*.

In soil, the number of *R.meliloti* (Barber, 1980; Lie, 1974; Lowendorf *et al.*, 1981), as well as *R.trifolii* (Coventry *et al.*, 1985a) are highly correlated with soil-pH. According to the work of Rice *et al.* (1977), the poor growth of lucerne below pH 6 (Fig. 4A) is due to a restricted nodulation

(Fig. 4B), which is caused by insufficient numbers of rhizobia in the soil (Fig. 4C).

Table 1. The minimum pH value for growth of several Rhizobium species

Rhizobium species	Legume*	Critical pH
R.meliloti	Medicago (lucerne)	5.3 (1)**
R.loti	Lupinus (lupin)	3.2 (2)
R.leguminosarum		
biovar. viceae	Pisum (pea)	4.5 (4)
biovar. phaseoli	Phaseolus (bean)	3.8 (3)
biovar. trifolii	Trifolium (clover)	4.5 (4)
R.fredii	Glycine soja (soybean)	-

* Only one representative per cross inoculation group is given.
** source (1) Lowendorf and Alexander, 1983a; (2) Fred et al., 1932; (3) Lowendorf and Alexander, 1983b; (4) Vincent, 1977

Since the conditions in the rhizophere are more favourable for bacteria, rhizobia proliferate mainly in the presence of germinating legume seeds and developing roots (Lowendorf and Alexander, 1983ab; Parker *et al.*, 1977). The presence of the hostplant generally results in increased rhizobial numbers (Barber, 1980; Jensen and Sorensen, 1987) and the harmful effects of soil acidity may partly be counteracted (Richardson and Simpson, 1988). Relatively little quantitative data are available on the growth of rhizobia in rhizosphere-soil of their hostplant (Alexander, 1984), and the correlation between rhizobial survival and the actual rhizophere-pH has not yet been studied.

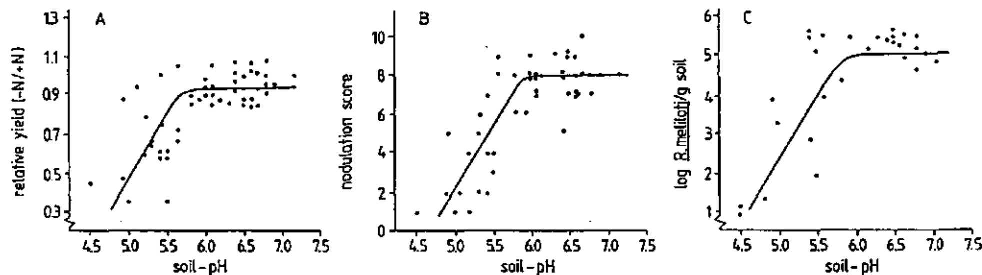

Figure 4. The effect of soil-pH on the relative yield (A) and nodulation score of lucerne (B), and the number of R.meliloti bacteria present in the soil (C), after Rice et al., 1977.

To alleviate the problems of nodulation of lucerne in acid soils, the selection of acid tolerant *R.meliloti* strains seems to be more essential than selection of acid tolerance in the *Medicago* hostplant, since the latter is already more tolerant (Robson, 1988). Due to the selection pressure, the chance to encounter acid tolerant strains is higher in acid soils. This source has been explored by many investigators (*e.g.* Barber, 1980; Date and Halliday, 1979; Lindström and Myllyniemi, 1987; Robson and Loneragan, 1970b). Good results were achieved in Australia with *R.meliloti* strains originating from acid soils in Sardinia (Howieson and Ewing, 1986). However, the rhizobia present in an acid soil do not need to be acid tolerant (Wood and Shepherd, 1987; Richardson and Simpson, 1989); rather they survive by avoiding extreme acidity. The highest proportions of *R.trifolii* were detected in the less acidic regions of the soil profile (Richardson and Simpson, 1988), which coincided with the presence of nodules (Richardson *et al.*, 1988c).

In the laboratory the ability of rhizobia to grow in acidified yeast extract mannitol media has been used as a criterion to test several strains (Date and Halliday, 1979; Keyser and Munns, 1979; Rice, 1982). To maintain a stable pH the bacteria were grown in media supplied with special buffers (Howieson, 1985). The outcome of such tests, however, showed a poor correlation with actual survival rates in soil (Lowendorf and Alexander, 1983a; Lowendorf *et al.*, 1981). A short generation time of the bacteria under acidity stress is a prerequisite for nodulation in acid soils (Coventry and Evans, 1989). However, the nodulating capacity of *R.meliloti* strains that were selected in this way was rather poor (Howieson *et al.*, 1988). Similar results were found with strains of *R.trifolii* (Lindström and Myllyniemi, 1987; Richardson and Simpson, 1989). Obviously, the ability of *Rhizobium* strains to grow in laboratory media of low pH is not sufficient to ensure nodulation in acid soils.

From all this information it is clear that there is no good criterion as yet to select *Rhizobium* strains with a good nodulating performance in acid soils. Recently it has been

suggested that acid tolerance is based on the ability of rhizobial cells to maintain their cytoplasmic pH at a constant level, the so-called pH-homeostasis (Padan *et al.*, 1981). Six *R.meliloti* strains, capable of inducing good nodulation in acid soils (Howieson and Ewing, 1986) were able to keep their cytoplasmic pH around pH 7.2 in a medium with a pH as low as 5.6. Four strains that were less efficient in inducing nodulation in acid soil, could not maintain a relatively large pH gradient (O'Hara *et al.*, 1989). Similarly, Graham *et al.* (1989) reported a reduced ability of acid sensitive mutants of *R.phaseolii* to control their cytoplasmic pH.

Symbiosis

The formation of a root nodule is an interactive multi-step process between plant and bacterium (see reviews by Dart, 1974; 1977; Dazzo, 1980). Compounds exuded by the roots stimulate the rhizobia to multiply and to attach to the surface of root hairs. The curling of these hairs is initiated by a factor which is encoded by nodulation genes in *Rhizobium*. In general, the rhizobia invade the root in infection threads. The cortex tissue reacts by developing a nodule meristem. In these cells the rhizobia differentiate into the bacteroids that are able to fix atmospheric nitrogen.

The early stages of nodule formation are the most acid sensitive (Coventry and Evans, 1989; Coventry *et al.*, 1985b; Dart, 1974; Lie, 1969). The sensitivity is only of short duration, as shown in solution cultures by a temporal exposure of pea plants to a low pH (Mulder *et al.*, 1966; Lie, 1969). In the case of lucerne, this "acid sensitive step" lasts only 12 hours, which coincided with the time of root hair curling (Munns, 1968).

It has frequently been reported that the detrimental effect of low pH on nodulation is diminished by calcium in the rooting medium (Andrew, 1976; 1978; Andrew and Johnson, 1977; Loneragan, 1959; Loneragan and Dowling, 1958; Munns, 1977; 1978; Spencer, 1950). The concomitant influence of calcium and

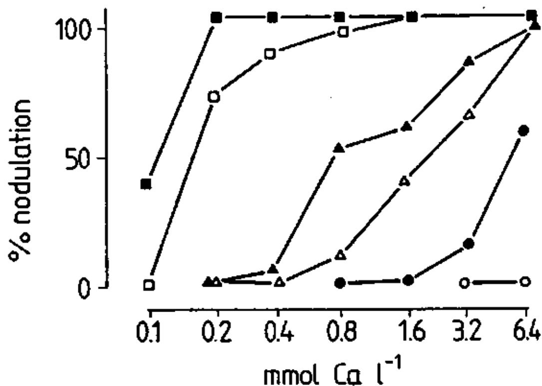

Figure 5. The interaction of calcium concentration and pH on the nodulation of hydroponically grown lucerne plants. ○ pH4.6, ● pH4.8, △ pH5.0, ▲ pH5.2, □ pH5.4, ■ pH5.6, according to Munns, 1970.

pH on the nodulation of lucerne is shown in Fig. 5. Calcium is known to be very effective in detoxifying high concentrations of heavy metals, and to reduce the negative effects of aluminium (Marschner, 1986). However, the involvement of calcium in the first stages of the nodulation process is probably more important for its positive effects. Calcium was found to stimulate the expression of *Rhizobium* nodulation genes by root exudates (Richardson *et al.*, 1988ab). During the initial phase of the attachment to the root hair, calcium is needed to anchor a specific binding protein (rhicadhesin) to the surface of the rhizobial cell surface (Smit *et al.*, 1988ab).

AGRONOMIC MEASURES TO IMPROVE BIOLOGICAL NITROGEN FIXATION IN ACID SOILS

Two practical measures are taken for a better biological nitrogen fixation in acid soils: (i) supplying the legume seed with the appropiate *Rhizobium* strain (inoculation) and (ii) increasing soil-pH by liming.

Inoculation

In the early history, farmers transferred large amounts of soil from a field with a well nodulated crop to another field where the same legume was to be sown. It was thought that the old soil could teach the young soil how to produce a good crop (Fred *et al.*, 1932). The discovery and isolation of root nodule bacteria by Hellriegel in 1886 allowed the direct

inoculation of the seed with pure *Rhizobium* cultures. The bacteria were suspended in water or skim milk and the seeds were moistened with this suspension (Thornton, 1929a). In this way, the rhizobia were already at the right place to infect the emerging root. Nowadays, the seeds are inoculated by immobilizing the rhizobia on the seed coated with gum arabic or methyl cellulose as adhesive agent (Brockwell, 1962; Elegba and Rennie, 1981). To improve bacterial survival, the rhizobia were mixed with peat before immobilization (Brockwell, 1977; Burton, 1976). More recently, synthetic carrier materials such as gels of polyacrylamide (Dommergues *et al.*, 1979; Jung *et al.*, 1982) or alginate (Bashan, 1986), as well as oil (Kremer and Peterson, 1982; 1983) have been tested for their capacity to protect rhizobia from drought and heat stress. Because of practical and economic reasons, however, these materials are not suitable as alternative for peat as carrier material. Therefore, peat-based inoculants are still most commonly used (Graham-Weiss, 1987; Somasegaran, 1985).

Inoculation trials with *Trifolium* and *Medicago* in acid soils were only successfull when high numbers of rhizobia were applied (Adams and Lowther, 1970; Mulder and van Veen, 1960; Munns, 1965; Rice, 1975, 1982; Rice and Olsen, 1983; Robson and Loneragan, 1970a). However, the nodulation and yield of the crop cannot always be restored solely by inoculation. In field experiments with lucerne at pH 5.5 inoculation was successfull, but not at pH 5.0 (Rice, 1982). When soil acidity is too severe, liming is of course inevitable.

Liming and lime-pelleting

Liming has two positive effects. Firstly, it increases the pH and decreases the solubility of the harmfull elements aluminium and manganese. Secondly, it augments the amount of the essential nutrients calcium and molybdenum (Lanyon and Griffith, 1988). These two beneficial effects result in enhanced proliferation of *Rhizobium* (Coventry *et al.*, 1985a; Mulder and van Veen, 1960; Robson and Loneragan, 1970b;

Vincent and Waters, 1954). For lucerne the benefit of lime to ameliorate growth in acid soils has been known for a long time (Thornton, 1929b). Interestingly, tropical legumes have, if at all, a lower lime requirement than temperate legumes (Munns and Fox, 1977; Norris, 1967).

By incorporating the seeds of clover into a layer of lime (lime-pelleting), the necessary amount of lime could drastically be reduced. Since the pioneering work of Loneragan *et al.* (1955), many others have reported the benefit of lime-pelleting (Brockwell, 1962; Cordero and Blair, 1978; Gaur and Lowther, 1981; Hastings and Drake, 1960; Lowther and Johnston e, 1979; Wade *et al.*, 1972). Experiments with broadcast lime applications and lime-pelleting have shown that in moderately acid soils, lime-pelleting is as beneficial as liming (Jones *et al.*, 1967; Lobb, 1958; Lowther, 1974; 1975; 't Mannetje, 1969). Lime-pelleting enhances nodule formation specifically in the uppermost regions of the soil profile, thus making nitrogen fixation more susceptible to moisture deficits (Davey and Simpson, 1989). Contrarily, broadcast amendment enhances root nodulation also in the deeper zones (Davey *et al.*, 1989; Richardson *et al.*, 1988c).

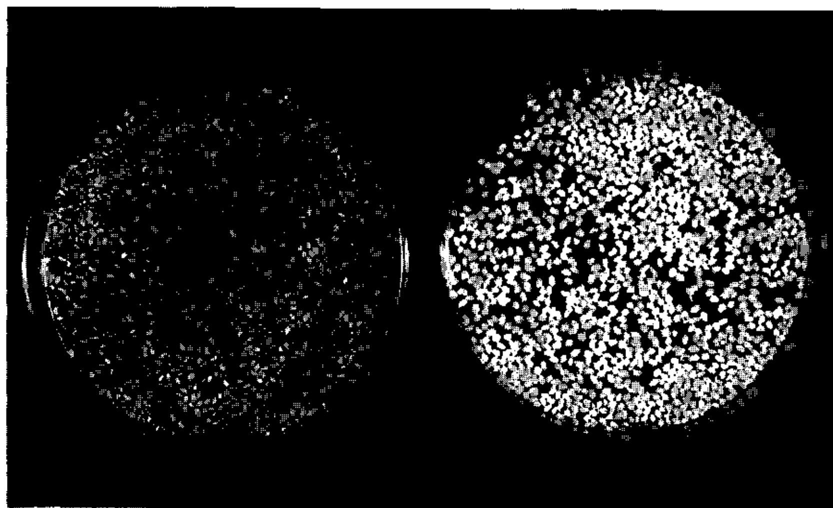

Plate 1. Seeds of lucerne (Medicago sativa L. cv Resis), untreated (left) and lime-pelleted (right).

At different locations in the Netherlands with acid sandy soils, the effect of lime-pelleting has recently been compared to broadcast liming (Deinum and Eleveld, 1986). Lucerne seeds were lime-pelleted with a net amount of 30 kg of $CaCO_3$ per hectare (Plate 1); liming of the soil was done with 1000 kg of $CaCO_3$ per hectare. Both liming and lime-pelleting had a positive effect. The most pronounced effect was found for the location "Wageningen Hoog". Without any lime addition (pH-H_2O 5.2), only 19% of the seedlings were nodulated after two months (Table 2). Broadcast liming increased nodulation to 37% in the same period and lime-pelleting resulted in 89% nodulated seedlings. The dry matter yield in the first growing season increased equally for both lime applications (Table 2).

Table 2. The effect of liming and lime-pelleting on nodulation (60 days after sowing) and shoot dry weight (of the first growing season) of Medicago sativa L. in a sandy soil of pH 5.2 (location Wageningen Hoog), according to Deinum and Eleveld, 1986

	Control*	Liming	Lime-pelleting
Number of nodulated seedlings (%)	19	37	89
Dry shoot weight (1000 kg ha^{-1})	4.8	8.4	9.0

* Seeds were inoculated with R.meliloti

OUTLINE OF THIS THESIS

The work reported in this thesis focusses on the nodulation and subsequent nitrogen fixation by lucerne in acid soils. The acid sandy soil of Wageningen Hoog has been used as a reference throughout. The effects of inoculation and lime-pelleting were first studied in the field. Subsequently, two types of growth systems were designed to follow the early formation of nodules under climatically-controlled conditions in the laboratory (Chapter 2). These systems, pots and rhizotrons (small root boxes), were used for a more detailed study on the effects of lime-pelleting.

The influence of lime on the survival of inoculated *R.meliloti* bacteria in the micro-environment around the seed was measured, as well as the nodulation response of seedlings to inoculation with increasing rhizobial numbers (Chapter 3).

By adding lime to acid soil, the nodulation is affected in two ways, (i) by the increase of pH, and (ii) by the input of calcium. To elucidate the first aspect, a method had to be developed for the local quantification of soil-pH (Chapter 4). With this method, the increase in soil-pH around lime-pellets and the pH dynamics in the rhizosphere of the seedling have been measured (Chapter 5).

Finally, the effect of calcium on the *in situ* process was studied in rhizotrons. Using agar blocks containing a specific calcium chelator (EGTA), calcium was locally removed from the root environment (Chapter 6).

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Chapter 2

Effect of lime-pelleting on the nodulation of lucerne (*Medicago sativa* L.) in an acid soil: A comparative study carried out in the field, in pots and rhizotrons

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ABSTRACT

The nodulation of lucerne was studied in soil (pH-H_2O 5.2) with seeds either inoculated with *Rhizobium meliloti* (R), or inoculated and pelleted with lime (RP). For comparison, experiments were done in the field and in two types of micro-cosmos: pots and rhizotrons. In the field experiments, lime-pelleting improved the establishment of seedlings and augmented the nitrogen yield of the first harvest. These positive responses in plant growth were the consequence of a better nodulation on the upper 10 mm of the seedling tap root. The number of seedlings carrying crown nodules increased from 18% (R) to 56% (RP) at 26 days after sowing.

In both, pots and rhizotrons, lime-pelleting also increased crown nodulation: in pots from 32% (R) to 60% (RP), and in rhizotrons from 5% (R) to 90% (RP). Rhizotrons, made of plastic petri dishes, allowed for continuously following of early root development and nodule formation. Crown nodulation could already be measured after 14 days. Based on these experiments, it was concluded (i) that crown nodulation is an adequate parameter to quantify the benefit of lime-pelleting, and (ii) that rhizotrons, because of the more pronounced effects and shorter incubation time, are more suitable to study the nodulation response in the soil caused by the addition of rhizobia and lime.

INTRODUCTION

Growth of lucerne (*Medicago sativa* L.) is impaired by soil acidity. Already in 1920, Joffe studied this phenomenon in a pot experiment with soils, acidified to different levels. From pH 7.0 to pH 3.5, a gradual decrease in shoot-weight was measured. Below pH 6.0, the number of nodules also decreased. These results have been confirmed with field-grown lucerne (Cunningham, 1928). The beneficial effect of liming was already shown one year later (Thornton, 1929). Acidity of the soil has a stronger effect on growth when lucerne depends for

nitrogen supply on the symbiosis with Rhizobium. When enough mineral nitrogen is present, this plant can grow in soils at much lower pH-values (Andrew, 1976; Mulder *et al.*, 1966; Munns, 1965).

To counteract the acidity, Loneragan *et al.* (1955) coated the seeds of *Trifolium subterraneum* with small amounts of lime, and as a result, obtained better growth. The beneficial effect of lime-pelleting in acid soils for clovers (Cordero and Blair, 1978; Hastings and Drake, 1960; Jones *et al.*, 1967; Lowther, 1974, 1975), was also found for lucerne ('t Mannetje, 1967; Rice and Olsen, 1983). Adams and Lowther (1970) reported that lime-pelleting may also negatively influence plant growth. These authors suggested that lime on the seed coat reduced the survival of rhizobia. Unfortunately, no bacterial counts were made to substantiate this hypothesis.

Recently, lime-pelleted lucerne seeds were succesfully used in the Netherlands (Deinum and Eleveld, 1986). At three different locations with acid sandy soils, the technique of lime-pelleting (30 kg of $CaCO_3$/ha) was compared to broadcast liming (1000 kg of $CaCO_3$/ha). Lime-pelleted seedlings nodulated significantly better, and the dry matter yield of the first growing season was about the same as in the limed soils.

Since field studies are very labour-intensive and allow only a limited amount of experimental variations, we investigated the possibilities of reducing the field system to a smaller and better controlable laboratory system. One of the three acid soil locations mentioned above, Wageningen Hoog, was chosen as a reference and the results obtained at this location, were compared to the outcome of inoculation and lime-pellet experiments in pots and rhizotrons. In the following, we report the result of this comparison plus supplementary observations, which were obtained in these two micro-cosmos.

MATERIAL AND METHODS

Soil

The sandy soil of field station Wageningen Hoog has the following composition: pH-H_2O (measured in a 1:2.5 soil in water suspension), 5.2; organic matter, 2.2%; cation exchange capacity (CEC), 3 meq per 100 g dry soil, consisting of the following elements (meq per 100 g): Na, 0.2; K, 0.2; Ca, 1.2; Mg, 0.2 and Al, 0.6. The soil did not contain native *Rhizobium meliloti*.

Soil of the upper 10 cm of the profile was collected with tools, first sterilized with 96% ethanol. The soil was allowed to dry at room temperature. For experiments in the laboratory, dry soil was mixed with a sterile solution of 0.34 g KH_2PO_4 plus 0.25 g $MgSO_4.7H_2O$ per litre of demineralized water, to obtain a moisture content of 12%.

When neutralized soil was needed, 10 kg of air-dried soil was mixed with 1.4 L of sterile demineralized water, containing 19.0 g of $Ca(OH)_2$. This mixture was incubated for 3 weeks at 30°C in the dark to equilibrate. The final pH-H_2O of the resulting air-dried soil was 7.2.

Plant

In all experiments, seeds of lucerne (*Medicago sativa* L. cv Resis) were used. The seeds were obtained from van der Have, Kapelle, The Netherlands.

Rhizobium

Rhizobium meliloti strain K-24 was grown at 30°C for 4 days in yeast extract mannitol medium (YEM), containing per litre of demineralized water: yeast extract, 1.0 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.25 g; $CaCl_2.2H_2O$, 0.1 g and $CaCO_3$, 1.0 g (Vincent, 1970).

Inoculation and lime-pelleting

For the field and pot experiments, the seeds were inoculated with a peat-based inoculum as follows: a sterile peat-loam mixture (composition: van Schreven, 1970) was mixed with an outgrown culture of rhizobia in YEM medium to obtain a moisture content of 60% (w/w). This mixture was incubated for a week at 30°C. An equal amount (weight) of the peat-bacteria mixture was added to an aqueous solution of 2% methyl cellulose (Tylose, Fluka A.G., Buchs, Switzerland). Four parts (per weight) of seeds were mixed with one part of the peat-bacteria-methyl cellulose mixture. Methyl cellulose was used to fix the rhizobial cells to the seed coat. Final pelleting was done by dusting the wet seed with dry $CaCO_3$ (100 g per 200 g of seed), according to the method described by 't Mannetje (1967). The seeds were shaken, until evenly coated lime-pellets were obtained.

In rhizotrons, the soil was inoculated directly at the positions where the seeds would be sown, by using a syringe to add 12 µL of a washed *R.meliloti* suspension in 0.85% NaCl, containing about 10^9 cells per mL. Instead of lime-pelleting, an equivalent amount (10 µmol per seed) of $CaCO_3$ suspension (12 µL) was added to the place of inoculum.

Prior to sowing, the number of viable rhizobia per inoculated seed was determined, using the Miles and Misra drop-count method (Vincent, 1970). Samples of 100 seeds were suspended in 50 mL solution of 0.1% $Na_4P_2O_7.10H_2O$, and shaken on a rotary shaker at 200 rpm for 20 minutes. A 1:10 dilution series was made in sterile 0.85% NaCl solution. In rhizotrons, the number of cells was determined by diluting the washed rhizobial suspension. From each dilution, four drops (12 µL) were placed on a petri dish containing YEM medium with 1.0% agar. Each dish received 12 drops. The number of colony forming units was counted at 12 x magnification after incubation at 30°C for 3 days.

Field

Differently treated lucerne seeds (Table 1), were sown at Wageningen Hoog location on August 20, 1987. Seeds from each treatment were sown in four randomly chosen plots. To avoid contamination with rhizobia, the sowing machine was sterilized by washing it carefully with 96% ethanol, and sowing the control and P plots first. The sown plots (5 x 2.4 meters) were divided in two equal subplots. One half was left undisturbed for determination of dry matter production. The other half was used for regular sampling of the seedlings; 100 cm plant row (50-100 plants) was carefully excavated, and the number of plants was recorded, as well as their nodulation status.

Table 1. Seed treatments

Control:	Inoculated with heat-sterilized inoculum, not pelleted
P:	Inoculated with heat-sterilized inoculum, pelleted with $CaCO_3$
R:	Rhizobium: inoculated, not pelleted
RP:	Rhizobium + Pellet: inoculated and pelleted with $CaCO_3$

In the growing season of 1988, the crop was harvested three times. Fresh shoot weight was measured immediately after cutting; the dry matter content was determined by drying a representative subsample at 80°C during 24 hours. The nitrogen-content was determined by the indophenol-blue method (Novozamsky *et al.*, 1974), after wet digestion of the plant material in a H_2SO_4-Se-salicylic acid mixture with addition of H_2O_2 (Novozamsky *et al.*, 1983). Analysis of variance was applied to the harvest data using the GENSTAT program (Lawes Agricultural Trust, Rothamsted experimental station).

Pots

Glass jars, 10 cm in diameter and 10 cm in height, were heat-sterilized (120°C) and subsequently filled with 600 g of the soil, moistened as described above. Plant holes of 5 mm depth were made, and each pot was sown with 24 seeds. The plants

were grown in a climate room under a 16 h light (200 lux)/8 h dark cycle at 20°C and a relative humidity of 70%.

Rhizotrons

Rhizotrons were made of plastic petri dishes (9 cm ϕ), by removing the top 2 cm (see Plate 2). Lucerne seeds were surface-sterilized in 70% ethanol (one minute), followed by 20 minutes treatment in 6% H_2O_2 solution, supplied with a drop of detergent (Teepol, Lamers and Pleuger, 's-Hertogenbosch, The Netherlands). Subsequently, the seeds were pre-germinated during one day at 30°C on water-agar (1.0%), which was acidified with 0.1*N* HCl to pH 5.2, to obtain a pH similar to the soil. The empty rhizotrons were kept overnight at 70°C to eliminate possible contaminations with rhizobia. The rhizotrons were filled with 50 g of moist soil. Each unit was planted with 7 uniform seedlings (root length 3 to 5 mm) at 5 mm depth and a distance of 10 mm from each other. The rhizotrons were placed in the climate room at an angle of 60° to force the roots to grow towards the lid. The rhizotrons were covered with aluminium foil to shield the roots from light.

Loss of moisture was reduced by covering both pots and rhizotrons with a layer of sterile washed gravel. During the first 4 days, the systems were in addition sealed with parafilm. Thereafter, moisture content was controlled by keeping the weight of pots and rhizotrons constant. Sterile demineralized water was added, when needed.

RESULTS AND DISCUSSION

To quantify the effect of lime-pelleting on nodulation, a differentiation was made between nodules formed on the upper 10 mm of the taproot, or on lateral roots in this section within 3 mm distance of the taproot, and nodules formed outside this region. The first nodules are defined as crown nodules, and the others as distal (non-crown) nodules.

Field experiment

The dynamics of nodulation, expressed as the ratio between the number of nodulated and the total number of plants (% total nodulation or % crown nodulation), is plotted in Fig. 1. In this soil, devoid of native *R.meliloti*, nodulation before 47 days after sowing (d.a.s.) only occured in the inoculated (R and RP) plots. In an early stage, at 12 and 15 d.a.s., nodulation was confined to the crown region of the root. Also at later stages, crown nodulation contributed to the major part in total nodulation. As a result of lime, crown nodulation increased from 18±8% for *R.meliloti* (R) treated seedlings to 56±17% for inoculated and pelleted (RP) seedlings at 26 d.a.s. (Fig. 1). Obviously, lime-pelleting improved the conditions for nodulation in the micro-environment around the seed, since the seed treatment tended to increase specifically the number of crown-nodulated plants.

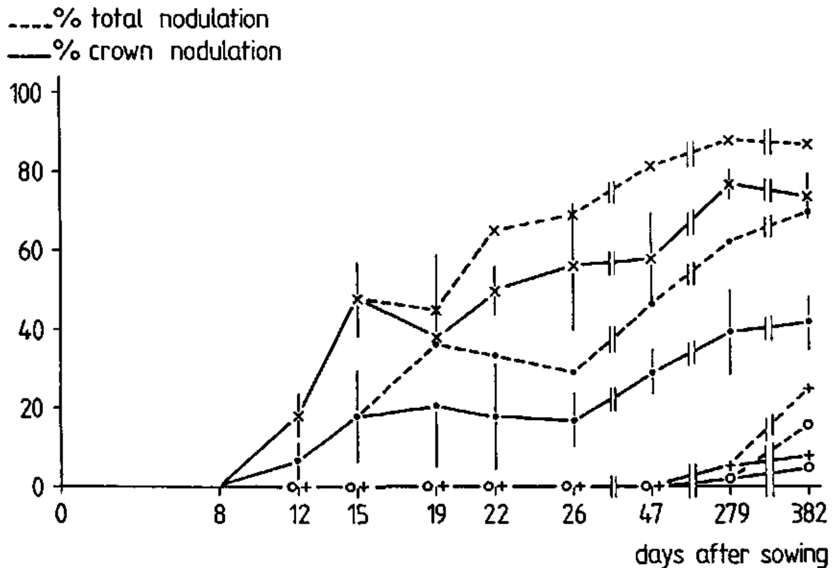


Fig. 1. Influence of seed treatments on the dynamics in the relative number of nodulated plants (% total nodulation, dashed lines) and crown-nodulated plants (% crown nodulation, solid lines; bars indicate SE) in the field at Wageningen Hoog. Treatments: Control (o), P (+), R (•) and RP (x).

During the first 47 days, the difference between total and crown nodulation was quite similar in the presence (RP) and absence (R) of lime (Fig. 1). This difference, which was due to plants carrying only distal nodules, suggests that lime-pelleting does not influence the nodulation on the lower (distal) parts of the root system. These observations agree well with previous reports on the effect of lime-pelleting on clover seeds (Cordero and Blair, 1978; Lowther, 1974). A similar phenomenon was found with soybean in very acid clay soils, when, instead of lime, ash from burned rice straw was added to the plant hole (Duong and Diep, 1986).

In the course of time both total and crown nodulation gradually increased. This was probably the result of the death of non-nodulated seedlings (see ***Seedling establishment***). Due to contamination with *R.meliloti* from adjacent plots, also some non-inoculated (control- and P-treated) plants gradually became nodulated towards the end of the growing season (382 d.a.s.).

At the time of sowing, the number of rhizobia on the lucerne seeds was not affected by lime-pelleting; for RP- and R-treated seeds 1.3±0.3 and 2.2±0.8 x 10^5 cells of *R.meliloti* respectively, were enumerated.

Field experiments, have the advantage that their results can directly be used for agricultural practices. For long term studies, of course, field experiments are obligatory. However, costs and labour involved in such studies are often very high. Furthermore, field experiments can only be done during the growing season, and even then, the climatical conditions may differ extremely from year to year. In this study, *e.g.* water-logging of a part of our field in the winter of 1987, prevented the use of more than two blocks for determination of dry matter yield and nitrogen content. To overcome these limitation and to exactly control the environmental conditions, lucerne was grown in the same soil in either pots or rhizotrons in a climate room. The validity of these micro-cosmos experiments, with regard to nodulation, are discussed below.

Pot experiments

The nodulation response in pots of RP- and R-treated seedlings (inoculated with 5.0 x 10^5 cells of *R.meliloti*) at increasing age (13, 17, 21 and 25 days) is given in Fig. 2A. The initial phase of the experiments in the field (Fig. 1) could be simulated in pots, since lime-pelleting similarly augmented crown nodulation, from 32±3% to 60±3% at the last sampling date.

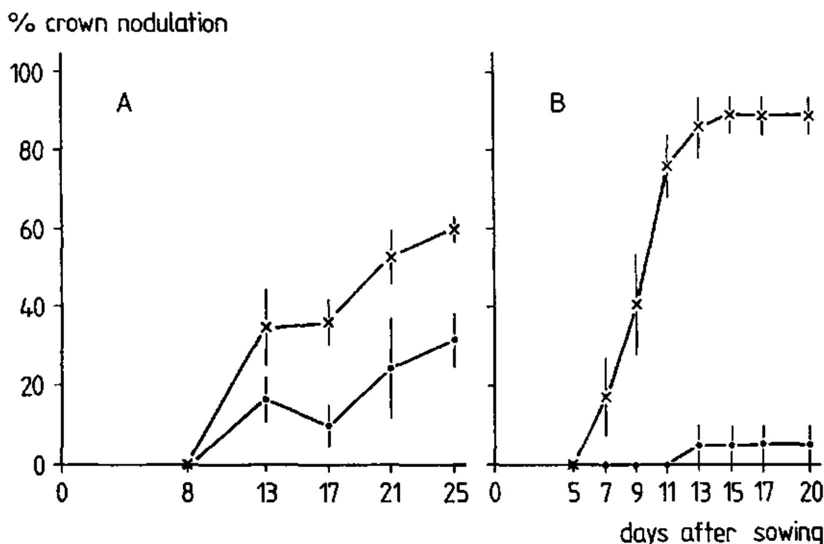


Fig. 2. Development in the relative number of crown-nodulated seedlings (% crown nodulation) in pots (A) and rhizotrons (B), influenced by inoculation (•) and inoculation plus lime-pelleting (x). Data (mean ± SE) for 4 pots (4 x 24 seeds) and 20 rhizotrons (20 x 7 seedlings), respectively.

The consequence of crown nodulation for plant growth in pots was determined by measuring the dry shoot weight at 25 d.a.s. of seedlings, differing in nodulation status (Fig. 3). The crown-nodulated seedlings (status 2), had developed heavier shoots than nodulated seedlings with only distal nodules (status 3). The difference between status 2 and 3 was significant($P<0.01$), when the seeds were lime-pelleted (RP). Since crown nodules were developed first (Fig. 1), they started fixing nitrogen earlier than distal nodules. With the unfertile soil used in this study, the weight of non-nodulated

seedlings (status 1) was, obviously, the lowest.

The effect of lime-pelleting in the soil of pH 5.2 was compared to its effect when the soil was brought to pH 7.2 prior to sowing. The seeds were inoculated with 3.0 x 10^6 cells of *R.meliloti*. The analysis of this experiment (25 d.a.s.) is summarized in Table 2. Nodulation of the roots is shown in Plate 1. Also in this experiment, lime-pelleting more than doubled the number of crown-nodulated seedlings in the acid soil. The roots outside the crown region were sparsely nodulated, as is shown by the very low number of distal nodules (Table 2, Plate 1B). Studies with subterranean clover at low pH, showed that nodules were formed in the less acidic regions of the soil profile (Richardson *et al.*, 1988). Plants compensated for the lower number by increasing the mass of individual nodules (Coventry *et al.*, 1985a; Richardson *et al.*, 1988). Once nodules were formed, acidity did not affect the specific nitrogenase activity (Coventry *et al.*, 1985b; Lie, 1969).

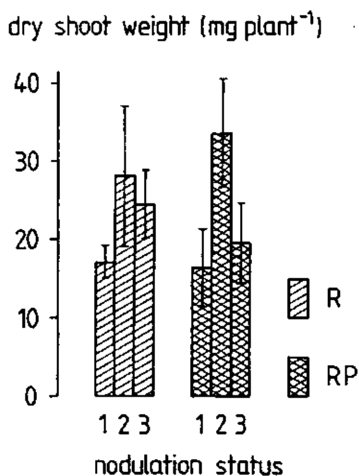


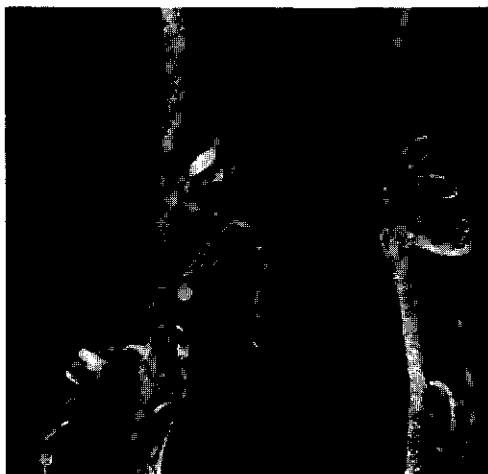
Fig. 3. Shoot dry weight (mean ± SE) of lucerne seedlings (25 d.a.s.) influenced by their nodulation status: (1) not nodulated, (2) crown-nodulated or (3) nodulated with only distal (non-crown) nodules. The seeds were either inoculated (R) or inoculated plus lime-pelleted (RP). Data are averaged for 8 pots (x 24 seeds).

Table 2. The effect of lime-pelleting (+ present, - absent) on the number of crown-nodulated lucerne seedlings 25 d.a.s. (% crown nodulation), and (1) the number of crown nodules per crown-nodulated plant, and (2) the number of distal nodules per plant, at soil-pH 5.2 or 7.2. Data (mean ± SE) averaged for 2 pots, sown with 24 seeds

pH	Lime pelleting	% Crown nodulation	Number of nodules	
			(1)	(2)
5.2	-	37 ± 9	2.2 ± 0.4	0.2 ± 0.3
5.2	+	78 ± 16	2.6 ± 0.1	0.2 ± 0.3
7.2	-	92 ± 11	2.2 ± 0.2	7.7 ± 0.3
7.2	+	100 ± 0	2.2 ± 0.1	7.1 ± 1.3

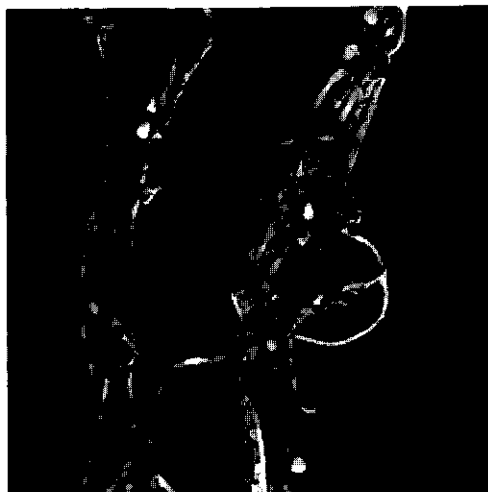
In the neutralized soil, lime-pelleting had, as might be expected, no effect. Roots nodulated abundantly everywhere (Table 2, Plate 1C,1D), similar to the nodulation of subterranean clover in limed acid soils (Coventry *et al.*, 1985a; Richardson *et al.*, 1988).

A



C

B



D

Plate 1. Nodule formation on excavated roots of 25 day-old lucerne seedlings, grown in pots in a soil of pH 5.2 or pH 7.2: crown nodulation (A and C, resp.) and distal nodulation (B and D, resp.).

Rhizotron experiments

In rhizotrons, the nodule development can be followed daily, simply by observing the roots through the transparent plastic lid. The development of crown nodulation is plotted in Fig. 2B. Compared to the nodulation of peat-based inoculated seeds in pots (Fig. 2A), inoculation with a suspension of 1.0×10^7 cells of *R.meliloti*, resulted in hardly any nodulation (5±5%). This is probably the result of the way the inoculum was applied. Peat-based inoculation was found superior to liquid inoculation in comparative trials with soybean (Burton and Curley, 1965). The protective nature of peat as a carrier for rhizobia, is generally accepted (*e.g.* Date, 1970). To the peat used here, a small amount of $CaCO_3$ was added to neutralize its natural acidity (van Schreven, 1970).

In contrast to lime-pelleting, the direct addition of $CaCO_3$ to the soil, increased crown nodulation in rhizotrons to a level as high as 90±5%. Moreover, nodulation developed quicker: in 14 (Fig. 2B) *versus* 25 days (Fig. 2A). This is probably due to the fact that the rhizobia were directly applied to the radicles of selected seedlings. Hence, the infection of the root could take place 1 or 2 days earlier than in the field and in pots.

In rhizotrons, rhizobia and lime can be applied at distinct locations. As could be expected, nodules only developed on roots grown in the spots, previously supplied with both components (see Plate 2). Nodules failed to develop if one component was applied at 5 mm, and the other at 20 mm depth (data not shown). Rhizotrons are

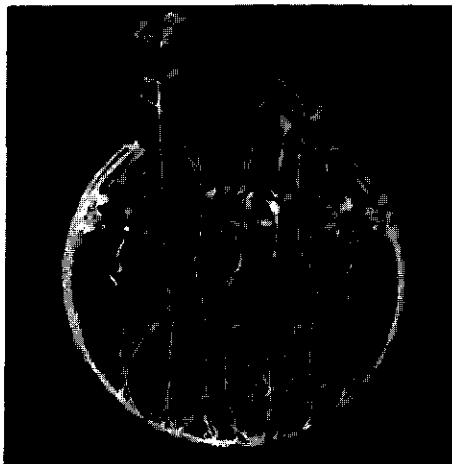


Plate 2. Crown nodulation on roots of 20 day-old lucerne seedlings, growing in rhizotrons. The soil around the seed, sown at 5 mm depth, was locally treated with R. meliloti and $CaCO_3$.

suitable for more detailed studies on the various factors influencing nodulation. In subsequent studies we have utilized rhizotrons to enumerate the *in-situ* survival of inoculated rhizobia after sowing (Pijnenborg *et al.*, 1990a), and to quantify the neutralization caused by lime-pelleting (Pijnenborg *et al.*, 1990b).

Plant growth

Seedling establishment From an agricultural point of view, a good establishment of small seeded crops, like lucerne, is very essential. Plant density is taken as a parameter to quantify the seedling establishment. The treatment effects on the dynamics of plant density are shown in Fig. 4. The sowing-rate used was 33 kg of seed per hectare. This is equivalent to 16.5×10^2 seeds per square meter. Less than 50% of the seeds had germinated 8 days after sowing. At the first harvest of the crop in 1988 (279 d.a.s.), the density of RP-treated plants was almost the same as 8 days after sowing. Much less plants were counted with the other treatments, due to a higher death rate of non-nodulated seedlings (Deinum and Eleveld, 1986). At the final harvest (382 d.a.s.), the density of inoculated plants (RP and R) was significantly ($P<0.01$) higher than the control and P treatments. At that stage, the densities had declined to, respectively, 70, 49, 29 and 26% of the initial number of germinated seeds.

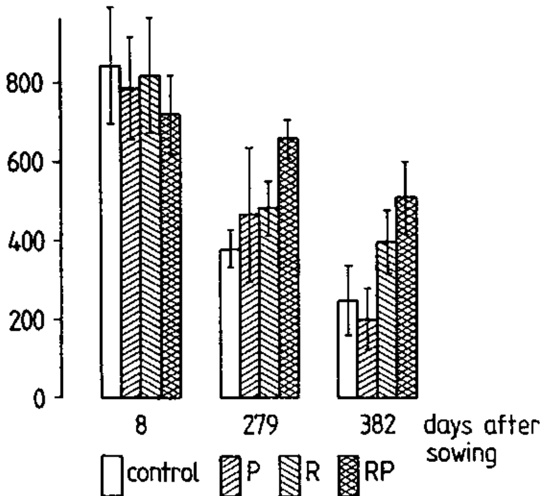


Fig. 4. Effect of the seed treatment (Control, P, R or RP) on the density (mean ± SE) of lucerne plants in the field at Wageningen Hoog at three sampling dates.

Nitrogen fixation In the spring of 1988, the uninoculated plots showed a markedly yellow colour, indicating nitrogen deficiency. This observation is consistent with the analysis of nitrogen in the shoots of the first harvest (Table 3). In this first harvest, the differences in dry matter yield and nitrogen content were most pronounced. The amount of nitrogen increased more than 5 times by inoculating the seed (R). Additional lime-pelleting (RP) rendered an even higher nitrogen yield of the first harvest.

Table 3. The effects of seed treatment on dry matter yield, nitrogen content, and total amount of nitrogen in shoots of lucerne, grown at location Wageningen Hoog in an acid sandy soil, at harvest 1 (279), 2 (329) and 3 (382 d.a.s.). Data are averaged for 2 plots

	Dry matter (1000 kg ha^{-1})			Nitrogen content (% of dry matter)			Shoot-N (kg ha^{-1})		
Harvest:	1	2	3	1	2	3	1	2	3
Control	1.59[a*]	2.37[a]	1.57[a]	1.79[a]	2.25[a]	2.96[a]	28[a]	53[a]	47[a]
P	1.50[a]	1.89[a]	2.01[a]	1.84[a]	3.30[a]	3.33[a]	28[a]	62[a]	67[a]
R	5.99[b]	3.19[b]	3.35[a]	2.55[b]	3.14[a]	3.04[a]	153[b]	100[a]	102[a]
RP	5.56[b]	2.56[a]	2.94[a]	2.94[c]	2.90[a]	3.19[a]	164[b]	74[a]	94

data in one column, followed by the same character, do not differ significantly (LSD 0.05)

At the second and third harvest, treatment differences tended to diminish. This leveling-out effect has been reported previously by others (Deinum and Eleveld, 1986; Rice and Olsen, 1983), and can be attributed to the inevitable rhizobial contamination, resulting finally in nodulation (Fig. 1), and high nitrogen content of the non-inoculated plots (Table 3).

Due to inoculation, the summed total nitrogen yield more than doubled. Despite the higher plant density (Fig 4), lime-pelleting did not give an extra increase over inoculation.

Table 4. Ratio of dry shoot weight of plants from only inoculated, and plants from inoculated plus lime-pelleted seeds (ratio R/RP), at different times after sowing in the field of Wageningen Hoog

Days after sowing	R/RP
8	1.01
12	0.93
26	0.79
47	0.89
279 (harvest 1)	1.48
382 (harvest 2)	1.35

This phenomenon has previously been found at the same location in 1986 (Deinum, personal communication), and can be explained by a compensation effect of the vegetation. In the R plots with lower nodulation (Fig. 1), the plant density was lower as compared to the RP plots (Fig. 4). With time, the remaining (nodulated) plants had more space to grow in the R plots and hence were able to produce heavier shoots (Table 4).

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Chapter 3

Nodulation of lucerne (*Medicago sativa* L.) in an acid soil: Effects of inoculum size and lime-pelleting

J.W.M. PIJNENBORG, T.A. LIE and A.J.B. ZEHNDER

Plant and Soil *in press*

ABSTRACT

The effects of inoculum level and lime-pelleting were studied in an acid soil with respect to the nodulation and growth of lucerne (*Medicago sativa* cv Resis) and the population dynamics of *Rhizobium meliloti*. In small root-boxes (rhizotrons), the *in-situ* survival of inoculated rhizobia was studied in the micro-environment around the seed for a period of 12 days after sowing. During the initial 24 hours, a strong increase in rhizobial numbers was measured, concomitantly with the development of roots. As a result of lime-pelleting, rhizobial numbers were higher only at 3 days after sowing ($P<0.05$). Later, this difference diminished steadily. Addition of lime did not increase the adhesion of the rhizobia to the seedling tap root.

Plant responses to inoculation were studied in pots. To obtain optimal nodulation, the soil had to be neutralized around the seed with lime and at least 10^5 cells of *R.meliloti* were required. With more than 10^5 rhizobia per seed, lime-pelleting increased the number of crown-nodulated seedlings from 24% to 77%. Higher numbers of rhizobia could not equalize the effect of lime. A strong correlation was found between crown nodulation, nitrogen content and dry weight of the shoots.

INTRODUCTION

Under optimal conditions, not more than 10 rhizobia per legume root are initially required for nodule formation (Purchase and Nutman, 1957). However, under adverse soil conditions like acidity, inoculation of the seed with at least 10^5 rhizobial cells is needed (Date, 1970). The critical stage for survival of the rhizobia is the time between sowing and germination of the seed (Brockwell, 1977). After germination, the bacterial number in the vicinity of the developing root (rhizosphere) increases as a result of excretions from the root (van Egeraat, 1975).

Within the genus *Rhizobium*, the microbial symbiont of lucerne *Rhizobium meliloti*, is the most acid sensitive (Vincent, 1977). In Canadian soils, Rice *et al.* (1977) found strongly declining numbers of native *R.meliloti* with increasing soil acidity. These authors concluded that a major factor contributing to the nodulation failure and the stunted growth of lucerne in soils with pH values below 6, is the poor survival of *R.meliloti*.

By applying a pellet of lime around the lucerne seed, the negative effect of acid soils can partially be eliminated. In particular, the number of seedlings carrying crown nodules can be increased (Deinum and Eleveld, 1986; Pijnenborg and Lie, 1990). In the following study, it is investigated whether the addition of a small amount of lime directly influences the infection of *Medicago sativa* with *R.meliloti*, or acts exclusively as a neutralizing agent allowing better proliferation of rhizobia and thus providing a higher chance for infection.

MATERIAL AND METHODS

Plant

In all the described experiments, seeds of lucerne (*Medicago sativa* L. cv Resis) were used. The seeds were obtained from van der Have, Kapelle, The Netherlands. The plants were grown in soil in a climate room at 20 °C, a 16 h light (200 lux)/8 h dark cycle and a relative humidity of 70%.

Soil

The soil had the following characteristics: pH (measured in a 1:2.5 soil in water suspension): 5.2; organic matter 2.2%; cation exchange capacity (CEC): 3 meq per 100 g of soil, containing the following elements (meq per 100 g): Na 0.2, K 0.2, Ca 1.2, Mg 0.2 and Al 0.6. The soil did not contain native *R.meliloti* bacteria. Air-dried soil was mixed with a sterile

solution of 0.34 g KH_2PO_4 plus 0.25 g $MgSO_4.7H_2O$ per litre of demineralized water, to obtain a moisture content of 12%.

Rhizobium

R.meliloti strain K-24 was grown at 30°C for 4 days in yeast extract mannitol medium (YEM), containing per litre of demineralized water: yeast extract, 1.0 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.25 g; $CaCl_2.2H_2O$, 0.1 g and $CaCO_3$, 1.0 g (Vincent, 1970).

A spontaneous streptomycin (S) resistant mutant ($K\text{-}24^S$) was obtained, following the procedure of Schwinghamer and Dudman (1973). Strain $K\text{-}24^S$ is resistant to 250 µg of streptomycin per mL of YEM medium. In preliminary studies, the applicability of $K\text{-}24^S$ was evaluated. The strain did not differ in nodulating capacity compared to the parent strain. The S-resistance was found to be a very stable characteristic; tests carried out regularly during two years did not show a significant growth reduction on YEM medium containing 250 µg of S per mL. This is in agreement with earlier work of Brockwell *et al.* (1977), showing stability of the spontaneous S-resistance in *R.trifolii*.

Experimental design

Lucerne seeds were either inoculated with *R.meliloti* (R), or inoculated and pelleted with lime (RP). The investigation dealt with the effects of lime-pelleting on (i) the *in-situ* survival of inoculated rhizobia, and (ii) the nodulation and growth of the hostplant. Survival studies were carried out in rhizotrons. At 1, 3, 6, 9 and 12 days after sowing (d.a.s.) the number of *R.meliloti* was determined (Experiment 1). The adhesion of the bacteria to the seedling tap root was quantified at 6 and 9 d.a.s. (Experiment 2). Plant responses were measured in pots after 25 days of growth. The seeds were either peat-based inoculated (Experiment 3) or liquid inoculated (Experiment 4).

Inoculation and lime-pelleting

To obtain a peat-based inoculum, a sterile peat-loam mixture (van Schreven, 1970) was mixed with an outgrown culture of *R.meliloti* in YEM medium to obtain a moisture content of 60% (w/w). This mixture was incubated for a week at 30°C. An equal amount (weight) of the peat-bacteria mixture was added to an aqueous solution of 2% methyl cellulose (Tylose, Fluka A.G., Buchs, Switzerland). Four parts (per weight) of seeds were mixed with one part of the peat-bacteria-methyl cellulose mixture. Methyl cellulose was used to fix the rhizobial cells to the seed coat. Final pelleting was done according to the method described by 't Mannetje (1967) by dusting the wet seed in a ratio of 2 to 1 with dry $CaCO_3$ until evenly coated lime-pellets were obtained.

Liquid inoculation was carried out with a *R.meliloti* suspension that was washed twice in 0.85% NaCl. Using a syringe, aliquots of 12 µL were applied immediately after sowing to each seed. Instead of lime-pelleting, an equivalent amount (10 µmol) of $CaCO_3$ in aqueous suspension (12 µL) was applied to the plant hole, prior to sowing.

The number of viable rhizobial cells per inoculated seed was determined, using the Miles and Misra drop-count method (Vincent, 1970). Samples of 100 peat-based inoculated seeds were suspended in 50 mL solution of 0.1% $Na_4P_2O_7.10H_2O$, and shaken on a rotary shaker at 200 rpm for 20 minutes. A 1:10 dilution series was made in sterile 0.85% NaCl solution. When liquid inoculation was used, the washed rhizobial suspension was diluted. From each dilution, four drops (12 µL) were placed on a petri dish containing YEM medium with 1.0% agar. Each dish received 12 drops. The number of colony forming units (c.f.u.) was quantified after 3 days of incubation at 30°C using a microscope (12x magnification).

Rhizotron experiments

Rhizotrons were prepared from plastic petri dishes (ϕ 9cm) by removing the top 2 cm of both the dish and the lid (Pijnenborg and Lie, 1990). The rhizotrons were filled with 50 g of moistened soil and sown with three lucerne seeds at 5 mm depth and 20 mm distance (Fig. 1). In the rhizotrons and pots (see below), the soil was covered with a layer of sterile gravel to prevent cross contamination. The moisture content of the soil was checked daily by weight, and adjusted with sterile, demineralised water if necessary.

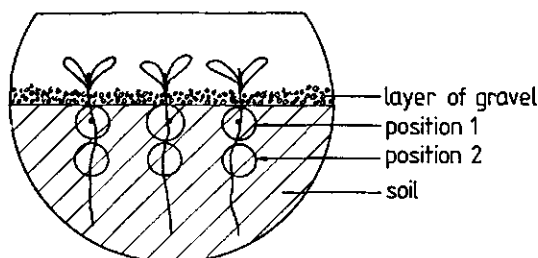


Fig. 1. A rhizotron with the positions where soil samples were taken to determine the number of inoculated R.meliloti bacteria.

For enumeration of *R.meliloti* K-24^{S} (Exp. 1), the soil including the seed and tap root was taken out using a cork-borer (ϕ 10 mm) around or just below the seed (position 1 and 2, resp., Fig. 1). In this way, punched samples of 1.4 g of moist soil were obtained. The samples were diluted as described under ***Inoculation and lime-pelleting*** for the enumeration of inoculated seeds. A selective growth medium was used, containing 100 μg of cycloheximide (to inhibit fungal growth), 1.5 μg of crystal violet (to inhibit spore-forming bacteria) and 50 μg of streptomycin per mL of YEM medium. Three independent samples were counted for each treatment. Non-inoculated soil served as a control. Recovery tests were done by inoculating a known number of K-24^{S} bacteria into the soil. After 3 hours incubation at 4°C, 98% of the introduced bacteria could be recovered.

For the adhesion experiment (Exp. 2), the seeds were surface-sterilized as described previously (Pijnenborg and Lie, 1990), and allowed to germinate at 30°C on 1.0% agar in demineralized water for one day. Uniform seedlings with a root length of 3 to 5 mm, were selected for further growth in soil.

Each rhizotron was sown with seven seedlings at 5 mm depth and placed in the climate room at a slope of 60° to force the roots to grow towards the lid. 10 average-sized plants were carefully taken out and the soil adhering to the root was removed 8 consecutive times by shaking the seedlings for 10 minutes at 50 rpm in 25 mL of sterile 0.85% NaCl solution. The roots were stained by soaking them for 20 minutes in 0.02% methylene blue (Vasse and Truchet, 1984). Finally, the material was washed in an aqueous solution containing 200 μg of cycloheximide, 3 μg of crystal violet and 100 μg of streptomycin per mL. The adhesion of *R.meliloti* was determined according to the procedure of Caetano and Favelukes (1986), by embedding the roots in selective YEM medium at 40°C containing 1.0% of melted agar. After incubation at 30°C for two days, the number of micro-colonies formed on the surface of the taproot was counted at 50x magnification.

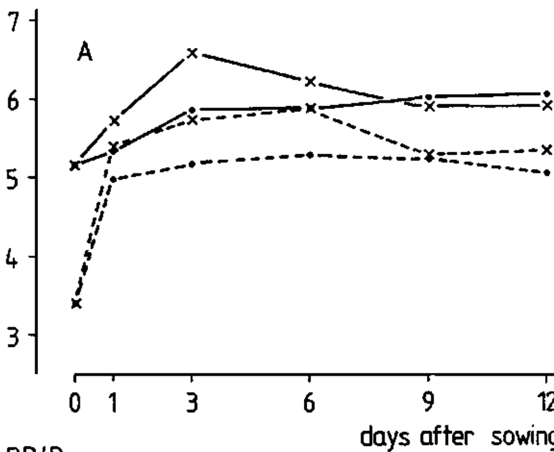
Pot experiments

Pots (10 cm ϕ, 10 cm high) were filled with 600 g of moist soil, and 24 plant holes of 5 mm depth were made. Seeds in one pot were treated identically; each treatment was replicated in four pots. When harvested at 25 d.a.s. the following parameters were measured: (i) crown nodulation, *i.e.* the number of plants carrying nodules on the upper 10 mm of the tap root, or on laterals in this section within 3 mm distance from the tap root, (ii) the weight of the shoots after drying them at 80 °C for 24 hours, and (iii) the nitrogen content of the shoots, which was determined by the indophenol-blue method (Novozamsky *et al.*, 1974), after wet digestion of the dried plant material in a H_2SO_4-Se-salicylic acid mixture with addition of H_2O_2 (Novozamsky *et al.*, 1983).

RESULTS

Survival of inoculated R.meliloti

To study the effect of lime-pelleting on the *in-situ* survival of *R.meliloti*, the number of rhizobia was recorded from 0 (time of inoculation) to 12 days after sowing, *i.e.* the period prior to nodule formation (Exp. 1). The development of the bacteria in the micro-environment around the seeds in rhizotrons (position 1, Fig. 1), is presented in Fig. 2A. The seeds were peat-based inoculated with $2.6x10^3$ or $1.4x10^5$ cells per seed. The relative increase as a result of lime-pelleting is given in Fig. 2B.



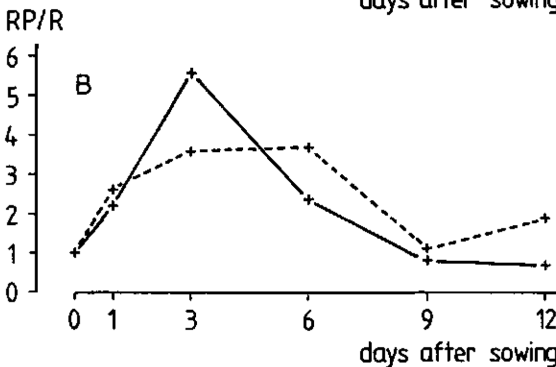


Fig. 2. Experiment 1.
A: Survival of inoculated R.meliloti (log of the number of colony forming units per 1.4 g of punched soil) in the micro-environment (ϕ 10 mm) around lucerne seeds that were inoculated with $2.6x10^3$ (---), or $1.4x10^5$ (——) cells, in the presence (× RP) or absence (• R) of a lime-pellet. Data are the averaged values of three measurements.
B: The number of R.meliloti in RP- relative to R-treated seeds (ratio RP/R), at different times after sowing. The seeds were inoculated with $2.6x10^3$ (---) or $1.4x10^5$ (——) cells per seed.

Within the first 24 hours a strong increase in cell numbers could be measured, which coincided with the development of 1 to 5 mm long radicles. Only at day 3 were more rhizobia found in the presence of lime for both inoculum levels ($P<0.05$). The cell numbers at 3 d.a.s. were 3.8×10^6 (RP) *versus* 6.8×10^5 (R) and 5.4×10^5 (RP) *versus* 1.5×10^5 (R) cells per soil sample for the high and low inoculum level, respectively. At later stages, the growth stimulating effect of pelleting disappeared (Fig. 2B). However, the difference between the two inoculum levels could still be seen after 9 and 12 days after sowing (Fig. 2A).

The number of rhizobia was not only determined in the seed environment (position 1, Fig. 1), but also in the soil around the seedling tap root (position 2, Fig. 1). The results are presented in Table 1. During the first 6 days, the inoculated bacteria were only found in the vicinity of the seed. At later stages, rhizobia moved slowly downwards.

Table 1. Experiment 1. Effect of the treatments R (inoculation) and RP (inoculation plus lime-pelleting) on the number of R.meliloti (c.f.u. per 1.4 g of soil) in the seed environment (0-10 mm depth, position 1 in Fig. 1), and around the tap root of lucerne seedlings (10-20 mm depth, position 2 in Fig. 1) at different times after sowing. Data (mean ± SE) are averaged values of 3 independent measurements

Treatment:	R		RP	
Position:	1	2	1	2
Day				
0	$1.5\pm0.8\times10^5$	-	$1.3\pm0.9\times10^5$	-
1	$2.2\pm1.9\times10^5$	N.D.*	$4.8\pm1.7\times10^5$	N.D.
3	$6.8\pm2.7\times10^5$	N.D.	$3.8\pm0.5\times10^6$	N.D.
6	$7.1\pm6.3\times10^5$	N.D.	$1.7\pm0.4\times10^6$	N.D.
9	$1.1\pm0.7\times10^6$	$1.9\pm3.2\times10^3$	$8.5\pm4.8\times10^5$	$7.9\pm8.9\times10^3$
12	$1.2\pm0.6\times10^6$	$1.7\pm0.4\times10^4$	$8.5\pm4.9\times10^5$	$2.5\pm1.1\times10^4$

* N.D. not detectable; less than 230 cells per soil sample

A more detailed experiment (Exp. 2) was carried out to determine the effect of lime on the number of rhizobia adhering to the seedling tap root. Three and six days after sowing seeds with 500 cells of *R.meliloti* each, the seedlings were washed and embedded in selective medium. The number of micro-colonies per cm of root is given in Fig. 3. At both sampling dates, the adhesion pattern was virtually the same.

Development of micro-colonies was almost entirely confined to the upper cm of the tap root. The presence of $CaCO_3$ did not induce significantly more adhesion. At three days after sowing, 35 (RP, Fig. 3B) *versus* 26 (R, Fig. 3A) micro-colonies were counted on the upper centimeter of tap root. Despite the uniform size of the selected seedlings, a remarkably high standard deviation was found. On the seedlings that did not receive $K\text{-}24^S$, some growth of bacteria from the non-sterile soil could also be detected. However, this non-specific growth was suppressed satisfactorily by the antibiotics added; only 2 micro-colonies were detected on the roots of 3-day old seedlings (Fig. 3A, 3B).

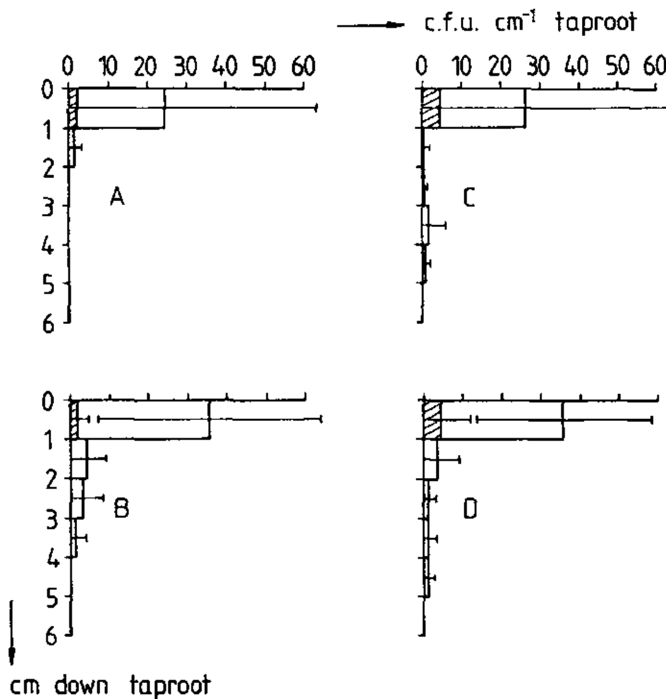


Fig. 3. Experiment 2. Adhesion of R.meliloti to the tap root of lucerne seedlings (number of micro-colonies per cm of root) in an acid soil at 3 or 6 days after sowing. The seeds were supplied with 500 rhizobia (R), and $CaCO_3$ was given additionally (RP). A: R 3 d.a.s., B: RP 3 d.a.s., C: R 6 d.a.s., D: RP 6 d.a.s.. The shaded columns represent the non-inoculated controls. Data are the average of 10 seedlings; bars indicate SE.

Nodulation and growth of lucerne seedlings

Responses in nodulation and plant growth to lime-pelleting at different inoculation levels, were studied in pots. The effect on the number of plants with crown nodules is given in Fig. 4. The seeds were inoculated with a peat-based inoculum (Exp. 3) or directly with a suspension of rhizobia (Exp. 4). Nodulation differed only slightly between the two ways of inoculation. Liquid inoculation scored lower than peat-based inoculation when, in the absence of lime, less than 10^4 cells per seed were applied. This is in agreement with earlier findings of Burton and Curley (1965) and the generally accepted protective nature of peat (Date, 1970).

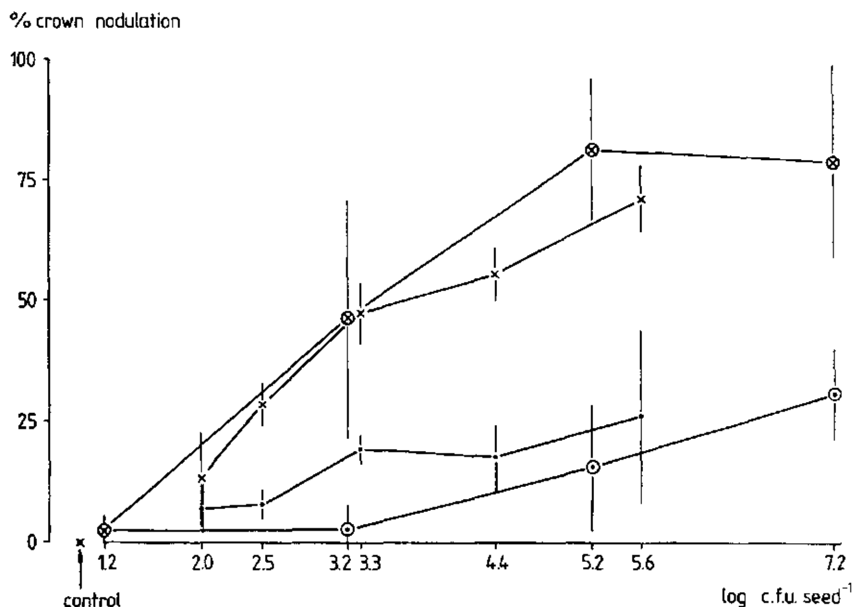


Fig 4. Effect of inoculum size (log c.f.u. of R.meliloti per seed) and lime-pelleting (present, absent) on the relative number of lucerne seedlings carrying crown nodules at 25 d.a.s.. Seeds were either peat-based inoculated (Experiment 3) or liquid inoculated (Experiment 4, encircled symbols).

In the absence of lime, the number of seedlings that was crown-nodulated only increased slightly (from 3% to 15%) by increasing the number of liquid applied rhizobia from 14 to

1.4×10^5 cells per seed (Fig. 4). However, when lime was given, crown nodulation increased from 3% to 81%, respectively. Obviously, in the presence of 10 µmol of $CaCO_3$, inoculation with 10^5 cells per seed is saturating, since addition of more rhizobia did not result in a better crown nodulation (Fig. 4). At optimal numbers (>10^5 cells per seed), lime-pelleting ameliorated the nodulation from 24% to 77% (data averaged for peat-based and liquid inoculation).

In comparison with strain K-24, the acid tolerant *R.meliloti* strain NRG 185 (selected by Rice, 1982), did not perform any better in this soil (data not shown).

Table 2. Experiment 3. The effect of inoculum size and the presence of a lime-pellet on the number of crown-nodulated lucerne plants, and the dry weight and nitrogen content of shoots. Data (mean ± SE) are the averaged values of 4 pots (x 24 seeds)

Inoculum size (cfu/seed)	Lime pellet (+/-)	Crown nodulation (% of plants)	Dry weight of shoots (mg/pot)	Nitrogen content of shoots (% of d.w.)
0	-	0	255 ± 75	1.15
	+	0	273 ± 49	1.03
1.1×10^2	-	6.7 ± 5.2	193 ± 86	1.25
	+	13.2 ± 10.0	228 ± 68	1.30
3.2×10^2	-	7.8 ± 2.9	266 ± 55	1.50
	+	28.5 ± 4.3	324 ± 54	1.97
2.1×10^3	-	18.9 ± 2.9	316 ± 37	1.83
	+	47.5 ± 6.5	388 ± 59	2.46
2.5×10^4	-	17.6 ± 6.9	344 ± 77	1.84
	+	55.6 ± 5.8	424 ± 65	2.52
4.0×10^5	-	26.4 ± 18.3	314 ± 44	2.31
	+	71.0 ± 7.1	589 ± 28	3.22

Lime-pelleting induced better nodulation, and as a result the plants produced heavier shoots with more nitrogen (Table 2). A high correlation was found between the % crown nodulation, and the dry weight and nitrogen content of the shoots (r^2 0.84 and 0.93, resp.).

DISCUSSION

According to several papers, the beneficial effect of lime-pelleting in acid soils on the nodulation of clover and lucerne plants should be attributable to a better survival of the inoculated rhizobia (Brockwell, 1977; Burton, 1976; Lowther and Johnstone, 1982; Robson and Loneragan, 1970ab; Wade *et al.*, 1972). In order to evaluate this hypothesis, the *in-situ* survival of inoculated *R.meliloti* bacteria was determined in the environment of lucerne seeds in rhizotrons. It was found that, consistent with the early suggestion of Thornton (1929), the population of root-nodule bacteria surrounding the root, was but little affected by the original number of bacteria added to the seed. The strong increase in the initial 24 hours (Fig. 2A) is due to a rhizosphere-effect (Parker *et al.*, 1977), caused by exudation of compounds that stimulate bacterial growth (van Egeraat, 1975). In these enumeration studies, the influence of a lime-pellet, if at all, appeared to be of minor importance. Significantly more rhizobia were only counted at 3 d.a.s.. At that stage, the soil around RP-treated seeds contained 3.7 to 5.5 times more rhizobia than around R-treated seeds (Fig. 2B). However, this was not accompanied by a better adhesion of the rhizobia to the seedling tap root (Fig. 3A, 3B).

To achieve a good crown nodulation, which is obviously very important for the nitrogen supply and growth of lucerne in this soil (Table 2), the seeds need a small amount of lime. The beneficial effect of lime-pelleting could not be equaled by inoculating the seeds with numbers as high as 10^7 cells of *R.meliloti* (Fig. 4). A similar interaction between inoculum size and lime-pelleting has been reported for clover varieties (Gaur and Lowther, 1982; Lowther, 1974; 1975). In contrast, Robson and Loneragan (1970a) concluded that for optimal nodulation of *Medicago truncatula*, the effect of lime could to a large extent be substituted by increasing the number of rhizobia. A critical examination of their results reveals however, that this was only warranted to a limited extent,

namely in soils less acid than pH 5.1 (measured in 1:5 soil in 0.01*M* $CaCl_2$). This value corresponds with about pH 5.7 when measured in a 1:5 soil in water suspension (Munns, 1965).

The degree of soil acidity obviously determines the success of attempts to ameliorate the nodulation of lucerne in moderately acid soils with increasing inoculum levels. Munns (1965) was unable to increase the nodulation of the legume by inoculating with 10^5 instead of 10^2 rhizobia per seed in a soil of pH 5.2, whereas in a soil of pH 5.5 (1:5 soil in water) the number of nodulated plants doubled. Similarly, the number of nodulated lucerne plants could substantially be raised by enlarging the inoculum size in soils of pH 5.4 (Rice, 1975) and pH 5.8 (Rice and Olsen, 1983) (pH values determined in 1:2.5 soil in water).

In nodulation experiments in acid nutrient solutions, the length of time required at alleviated pH to obtain nodulation, the "acid sensitive step", could be shortened by applying more rhizobia to lucerne plants (Munns, 1968). Recent work of Richardson *et al.* (1988) indicate that the bacteria must be in a dividing phase of growth to induce the expression of nodulation genes. Under acid soil conditions, the multiplication of rhizobia is adversely affected. A large initial population should then ensure that a part of them is in a responsive phase. Also the probability of the bacteria to encounter infectible root hairs is higher (Coventry and Evans, 1989). The multiplication of the rhizobia in the soil studied, however, seemed not to be a limiting factor; at least 10^5 cells of *R.meliloti* developed around the seed within one day (Fig. 2A). These numbers, if present at the time of sowing, should be sufficient when the seeds were lime-pelleted (Fig. 4).

The persistence of *R.meliloti* bacteria in acid soils has been the subject of several studies (Howieson and Ewing 1986; Howieson *et al.*, 1988, Robson and Loneragan, 1970b). Non-inoculated seeds were sown at increasing distance from the point of inoculation. The proportion of nodulated plants was used as a measure for the colonization and lateral migration

of the rhizobia. Our results indicate that, although rhizobia are present, formation of root nodules is inhibited by soil acidity. Therefore, as also suggested by Bushby (1981), the described bioassay to quantify rhizobial colonization may give an underestimation of the actual number of rhizobia present in the soil.

In conclusion, the benefit of lime-pelleting cannot be explained in terms of an overall better survival of the inoculated rhizobia (Fig. 2A, 2B), nor can it be equaled by a higher rhizobial number (Fig. 4). Hence, the effect of lime on the nodulation of lucerne in acid soil is attributable to other factors, namely local neutralization of soil acidity and supply of calcium. These factors (pH, Ca) will be dealt with in more detail in separate papers (Pijnenborg *et al.*, 1990 a,b).

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Chapter 4

Simplified measurement of soil-pH using an agar-contact technique

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Plant and Soil *in press*

ABSTRACT

A method for the indirect measurement of soil-pH is described. This method allows the spatial arrangement of soil and rhizosphere to be conserved. The soil is brought into contact with a layer of agar, containing bromocresol purple. A nylon gauze is placed between soil and agar. For quantitative pH measurements, a micro-electrode is inserted into the agar after three hours of contact between soil and agar.

The validity of the method was checked by comparing its results with those obtained by standard procedures. At different pH-levels (pH 5.0 to 7.0) in either a sandy or a clay soil, a high correlation (r^2 = 0.98) was found between the two methods. However, in the case of the clay soil, the agar-pH was significantly lower than the standard-pH. In the sandy soil, in the range pH 5.0 to 6.0, the results of both methods agreed very well. The agar method was used to measure the pH dynamics in the rhizosphere of lucerne seedlings, grown in rhizotrons.

INTRODUCTION

The pH in soil is generally not uniform (Davey and Conyers, 1988). Among others, root activities contribute to this heterogeneity. pH changes induced by roots of soybean have been demonstrated by Riley and Barber (1969) by separating the rhizosphere soil from the bulk soil. Schaller and Fischer (1985) made *in situ* pH measurements in unsaturated soil using antimony micro-electrodes. With a set of electrodes fixed in special root boxes, rhizosphere effects were directly quantified. Earlier studies of the same authors however, showed that the outcome of the pH determinations was strongly influenced by the water content of the soil (Schaller and Fischer, 1981). Weisenseel *et al.* (1979) mixed agar with a pH indicator, bromocresol purple, to demonstrate pH changes along roots of barley seedlings. Based on this idea, Marschner and Römheld (1983) developed a simple method to measure pH changes

in soil. The soil was covered with a thin layer of the agar. Qualitative differences in pH could be visualised by a color change of the pH-indicator added. Quantitative measurements were done with the same set-up by inserting a micro-electrode into or through the agar layer (Häussling *et al.*, 1984).

As far as we are aware, no comparative study was made between the agar contact method, and the standard method for measuring soil-pH (Peech, 1965). In this paper we describe an agar method which represents a modification of the method of Marschner and Römheld (1983). The modified method was validated by comparing the soil-pH values with the values, measured according to the commonly used procedure.

MATERIALS AND METHODS

Soil

The experiments were carried out with two soils: a coarse sandy soil of glacial origin (De Bakker, 1979), from Wageningen Hoog, and a clay soil of fluviatile origin of the foreland of the river Rhine at Wageningen. The characteristics of both soils are given in Table 1.

Table 1. Some characteristics of two soils tested at different pH levels

	Sandy soil	Clay soil
pH-H_2O (1:2.5 soil:demi* water)	5.2	6.0
pH-KCl (1:2.5 dry soil:1N KCl)	4.7	5.1
organic matter (%, w/w)	2.2	2.4
cation exchange capacity (meq/100 g)	3	15

* demi = demineralized by ion exchange

To obtain different pH levels, the sandy soil was brought to a moisture content of 12% and mixed with different amounts of K_2CO_3. The soil portions with different pH, were stored at 20°C during two weeks in the dark before use. The pH of the clay soil has been decreased or increased on field plots by the application of sulphur or $Ca(OH)_2$ as described by Jonkers *et al.* (1980).

Experimental design

The soils tested were moistened to 60% of the water holding capacity by mixing with demineralized water. Plastic petri dishes (9 cm ϕ) were filled with 120-150 grams of soil. The soil was slightly pressed to obtain a smooth surface.

The lids of the dishes were filled with 20 mL of agar solution (Marschner and Römheld, 1983), containing 7.5 g agar (Difco Bacto agar, Brunschwig Chemie, Amsterdam), 0.06 g bromocresol purple (Merck) and 0.17 g $CaSO_4$ per litre of demineralized water. After solidification, the agar surface was covered with a 53-mesh nylon gauze (Nijzink, Wageningen) to prevent the soil particles sticking to the agar (Fig. 1). Bromocresol purple is yellow at pH 5.2 and becomes purple at pH 6.8 (Clark, 1928). To measure the pH, a micro-electrode with a tip diameter of 1.2 mm (Type MI-410, Microelectrodes Inc., Londonderry, U.S.A.) was inserted into the agar. Prior to this measurement the nylon gauze was removed.

Fig. 1. The agar-contact system

Validation of the agar method

In a first set of experiments the time of contact required to reach pH equilibrium was determined. Drops of 12 μL containing different concentrations of K_2CO_3 (0, 0.5, 2.0, 4.0 μmol per drop), were placed onto the sandy soil. The agar was brought in contact with the soil 15 minutes later. Besides varying the length of the contact time, the influence of different initial pH's of the agar was also studied. The pH of the agar was brought to pH 5.2, 5.65 or 6.0 with 0.1*N* KOH.

In the subsequent experiments, soil-pH was measured using either the agar method or the standard method, in a 1:2.5 suspension of soil in demineralized water, after shaking for 60 minutes (Peech, 1965). The measurements with the agar method were done four times independently; the standard procedure was carried out in duplicate with aliquots of 20 grams of soil.

Application of the agar method

The described method was applied to study pH changes in the rhizosphere of lucerne (*Medicago sativa* L. cv Resis) growing in the sandy soil. Rhizotrons were made of the plastic petri dishes by removing the top 2 cm, as described in detail elsewhere (Pijnenborg and Lie, 1990). The dishes were filled with 100 grams of 12% moist soil. The seeds were pre-germinated on water-agar (1.0%) during one day, and planted (one per rhizotron) at 5 mm depth in the soil. The rhizotrons were placed in a climate room (20°C, a 16 h light (200 lux)/8h dark cycle) at an angle of 60°. In this way, the roots were forced to grow towards the lid. The rhizophere-pH was determined at day 12 after planting, using the agar method. Subsequently, the root with the adhering rhizoplane soil was taken out of the rhizotron, and embedded in indicator agar of pH 5.2. After three hours of incubation, the micro-electrode was inserted into the agar for pH measurement.

RESULTS AND DISCUSSION

Contact time

The influence of length of contact on the measurement of local soil-pH, using the sandy soil, is presented in Fig. 2. At one hour of incubation, differences in initial pH still significantly affected the measured values. Two to three hours were required to achieve equilibrium (Fig. 2).

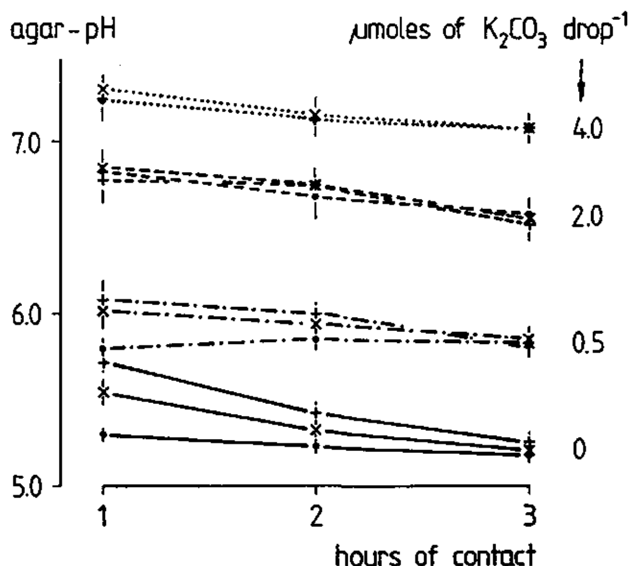


Fig. 2. The local soil-pH values, induced by addition of 0, 0.5, 2.0 or 4.0 µmol of K_2CO_3 in 12 µL drops of water, measured by inserting a microelectrode after 1, 2 or 3 hours of contact into agar of different initial pH (5.2 (•), 5.65 (x) or 6.0 (+)).

Correlation between pH values obtained by the agar method and the standard method

In the following experiments, the validity of the agar method was studied by comparing the results with those obtained by the method used in standard soil analysis (Peech, 1965). At first, the pH in 8 soils, differing in soil type and origin, were measured (Table 2). Both methods gave almost identical results with peat soils, consisting mainly of organic matter,

with sandy soils and with loamy soil of a low pH. The pH values measured with the agar method in loamy soil of a high pH and with two clay soils were up to a half pH unit lower than those obtained with the standard method. This discrepancy between the two methods may be explained by the presence of clay particles. In a sandy soil the fraction of clay particles is low (0-8%, w/w). The clay content is higher in loamy soils (8-25%), and the highest in clay soils (more than 25%, Kuipers, 1984). The clay content is an important factor determining the cation exchange capacity (CEC) of a soil (Buol *et al.*, 1980). The charge on clay particles originates from the isomorphous substitution in the crystal lattice of a cation of lower valence for a cation of higher valence (Tisdale *et al.*, 1985). Obviously, the discrepancy between the two methods tended to increase with increasing clay fraction of the soil (Table 2).

Table 2. Measurement of pH in different soils: (i) in a 1:2.5 suspension of soil in demineralized water (Standard-pH), and (ii) in a layer of agar, after 3 h of contact with the soil (Agar-pH)

Soil type	Origin[a]	Standard-pH[b]	Agar-pH[c]
Peat soil	Hoogeveen	5.24 ± 0.02	5.25 ± 0.03
	Borger Compagnie	6.98 ± 0.04	6.95 ± 0.05
Sandy soil	Bennekom	4.63 ± 0.07	4.74 ± 0.04
	Vredepeel	5.79 ± 0.05	5.58 ± 0.07
Loamy soil	Middachten	4.82 ± 0.03	4.80 ± 0.05
	De Eest	7.92 ± 0.03	7.47 ± 0.11
Clay soil	Wageningen	5.35 ± 0.06	5.12 ± 0.04
	Herveld	7.58 ± 0.04	6.95 ± 0.06

[a] These are all Dutch locations. [b] Mean (± SE) of four replicates.
[c] Mean (± SE) of two replicates.

To obtain more evidence for the possible suitability of the agar method to measure the *in situ* pH without sacrificing the original soil structure and spatial arrangement, more detailed studies were done with two types of soil: a sandy soil and a clay soil (Table 1). The regression lines for the two methods of pH measurement are given in Fig. 3.

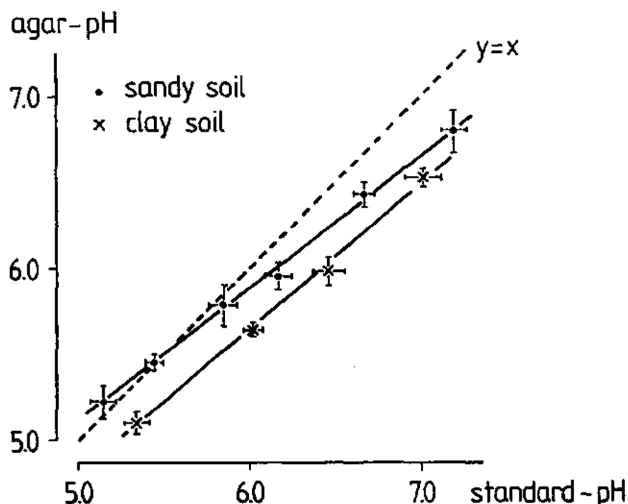


Fig. 3. The regression line for a sandy (•) and a clay soil (x), between the pH-values measured in a 1:2.5 suspension of soil in demineralized water (standard-pH), and values obtained by inserting a micro-electrode into an agar layer, after 3 h of contact (agar-pH). The data are averaged for two and four independent measurements, respectively.

For both soils, a high correlation coefficient ($r^2 = 0.98$) between the methods was found. The values of agar-pH were generally lower than those obtained with the standard method. This is partly due to buffering by the agar solution containing indicator. In the pH range 5 to 7, a maximum buffering capacity of 0.04 meq per litre was measured at pH 6.4 for a solution containing 0.75% agar (50°C). Besides agar, bromocresol purple with its pK of 6.3, also acted as buffer around pH 6.4. But its capacity was lower than that of agar.

For the sandy soil the correlation between the two methods fit the equation $Y = 1.305 + 0.763 * X$. At pH 5.5 this line crosses the theoretical line $Y = X$. As can be seen from Fig. 3, the agar method is suitable for the direct measurement of the soil-pH between pH 5 and 6. The values obtained with the agar method in this range are within the 99% confidence limits of the actual soil-pH, as measured by standard procedures.

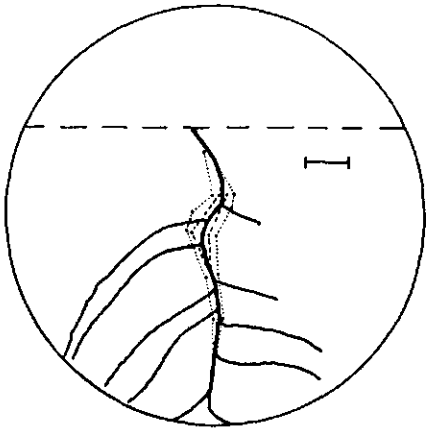
pH measurements in clay soils with the agar method gave consistently values which were too low. As discussed above, this is probably due to the presence a high amount of clay particles. Nevertheless, if a standard curve is made as in Fig. 3, the agar method can be used with these soils as well. With the soil used in our case, the actual pH can be calculated as follows: soil-pH = 1.182 * agar-pH - 0.668. This relation is derived from the equation $Y = 0.565 + 0.846 * X$, describing the linear correlation for the measurements with clay soils in Fig. 3.

Rhizosphere-pH of soil-grown lucerne seedlings

The non-destructive way of measuring the pH with the agar method, makes this method suitable for use in rhizotrons to follow temporal and spatial changes in pH in the rhizosphere. After three hours of contact between the agar and the soil with a root in the rhizotron, the pH changes induced by the plant in the root zone could be measured by inserting the micro-electrode into the agar (Fig. 4). The alkalinization along the tap root could thus be analysed (Pijnenborg *et al.*, 1990).

To show that the root system of the plant is indeed able to change the hydrogen ion activity locally, the same 12-day old seedling, of which the soil-pH was already measured (Fig. 4A), was embedded in 20 mL indicator agar. After three hours of incubation the change in pH was quantified (Fig. 4B) and a clear change of colour could be seen (Fig. 4C). Compared to the root embedded in agar (Fig. 4B), the zone of alkalinization in the rhizotron soil (Fig. 4A) is much smaller. There are various possibilities to explain this difference. The most obvious are: quicker diffusion of ions in the agar solution, higher buffering capacity per volume of soil, and differences in the ionic composition of agar and soil.

Attempts were made in these root systems to compare the agar method with the standard method. Relatively large soil samples (*ca.* 2 mg) had to be taken from the rhizotron to obtain reliable standard-pH values. Their size (spatial extention) prevented, however, an adequate resolution of the pH gradient close to the tap root in soil.



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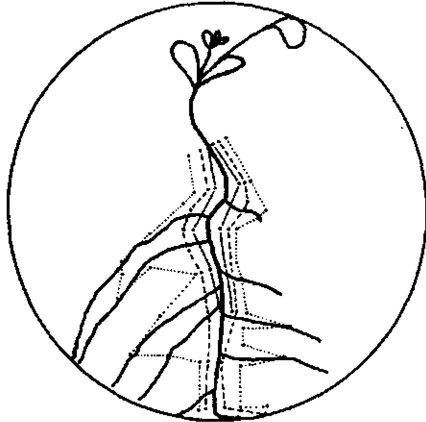

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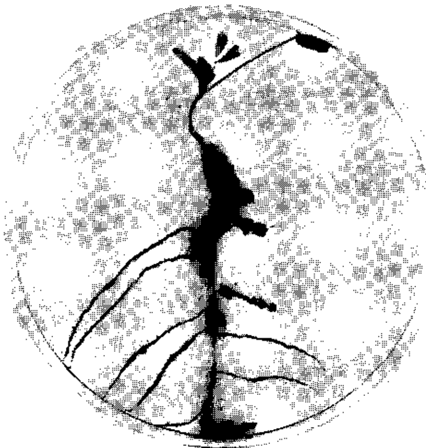

C

Fig. 4. The pH pattern around the root of a lucerne seedling grown during 12 days in a rhizotron with sandy soil of pH-H_2O 5.2. The pH was quantified by inserting a micro-electrode into a layer of agar containing bromocresol purple, 3 hours after contact with the root in the soil (A), and by taking out and embedding the same root for 3 hours in indicator agar of pH 5.2 (B). Iso-pH lines pH 6.0 (——), pH 5.7 (- - - -) and pH 5.4 (··········) and a scale bar (10 mm) are plotted. The photograph (C) corresponds with the embedded root (B).

CONCLUSIONS

The agar method described in this paper is an improvement of the Marschner and Römheld (1983) method. The nylon gauze between soil and agar prevented a contamination of the agar with soil and *vice versa*. The micro-electrode could be inserted into clean agar allowing fast measurements. pH determinations with this agar method were highly reproducable. Temporal and spacial pH variations can be followed with this method in a non-destructive way, provided the soil portion under investigation can be exposed with a minimum disturbance to an agar film. The use of indicators in the agar allows rapid localization of the spots where pH changes have occurred.

The observed discrepancy between the pH measured with the agar method and the standard method in certain soils is certainly a handicap. However, a careful standardization of the method will allow this problem to be overcome.

ACKNOWLEDGEMENTS

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Chapter 5

Nodulation of lucerne (*Medicago sativa* L.) in an acid soil: pH dynamics in the rhizosphere of seedlings growing in rhizotrons

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Plant and Soil *in press*

ABSTRACT

A lime-pellet around seeds of lucerne significantly increased crown nodulation in an acid soil. To investigate whether neutralization or calcium were of importance when lime was supplied, experiments with plants were done either in pots or in rhizotrons. Crown nodulation was used to quantify the effect of these two parameters.

For the neutralization of the soil, KOH (in pots) or K_2CO_3 (in rhizotrons) was added. The crown nodulation of pot-grown plants increased from 31% to 53%. In rhizotrons, the number of crown-nodulated seedlings increased from 9% to 53%. If calcium was supplied additionally (as $CaCl_2$ or $CaSO_4$), 63% crown nodulation was found in pots, and 68% in rhizotrons. These numbers are close to the crown nodulation with lime ($CaCO_3$) alone: 70% in pots and 71% in rhizotrons. In the soil studied, the beneficial effect of lime is largely due to neutralization (80%), and only a minor part (20%) is due to the input of calcium.

Using rhizotrons, the dynamics of the pH in the rhizosphere of lime-treated and untreated seedlings was followed during a period of 12 days. It was found that, even in the absence of lime, the pH along the taproot increased from 5.1 to 5.7. However, this did not result in the formation of root nodules. Nodulation was obtained only by neutralization, which increased the pH during the initial 3 days, the acid sensitive period of the process.

INTRODUCTION

Nodulation of many legumes is sensitive to low pH (Andrew, 1976; Mulder *et al.*, 1966). The obvious remedy in practical agriculture is liming acid soils. There are several reports about the beneficial effect of liming on the nodulation of *Trifolium* and *Medicago* species (Cordero and Blair, 1978; Coventry *et al.*, 1985; Munns, 1965; Munns and Fox, 1977; Richardson *et al.*, 1988; Spencer, 1950). With plants growing

in nutrient solutions, an increasingly higher calcium requirement at lower pH was found for optimal nodulation of both plant species (Loneragan and Dowling, 1958; Munns, 1970). Such an interaction between calcium and pH has also been described for soybean (Albrecht and Davies, 1929). One of the explanations given in literature for this antagonistic effect, is that the uptake of calcium at low pH seems to be inhibited (Andrew and Johnson, 1976; Schmehl *et al.*, 1952).

Recently, the effect of lime-pelleting on nodulation of field grown lucerne in an acid sandy soil was studied in Wageningen (Deinum and Eleveld, 1986; Pijnenborg and Lie, 1990). It was found that lime-pelleting specifically increased the number of seedlings carrying nodules on the upper 10 mm of taproot, the so-called crown nodules. As a result of $CaCO_3$ addition, the crown nodulation more than doubled, and a stronger seedling establishment and more nitrogen fixation were found. The initial phase of the field experiments could be simulated in the laboratory, by growing plants in soil from the field, either in pots or in rhizotrons (Pijnenborg and Lie, 1990). It was not clear from the field and the laboratory experiments whether the positive lime-effect was due to the higher pH in the presence of lime or due to the availability of more calcium. To obtain more information about pH and calcium effects, experiments have been designed to separate the influence of these two parameters. In the following, the results of this study are reported.

MATERIALS AND METHODS

Soil

The soil had the following composition: pH (measured in a 1:2.5 soil in water suspension), 5.2; organic matter, 2.2%; cation exchange capacity (CEC), 3 meq per 100 g dry soil, consisting of the following elements (meq per 100 g): Na, 0.2; K, 0.2; Ca, 1.2; Mg, 0.2 and Al, 0.6. The soil did not contain native *Rhizobium meliloti*.

Soil of the upper 10 cm of the profile was collected with tools, which were first sterilized with 96% ethanol. Air-dried soil was mixed with a sterile solution of 0.34 g KH_2PO_4 plus 0.25 g $MgSO_4.7H_2O$ per litre of demineralized water, to a moisture content of 12%.

Rhizobium

Rhizobium meliloti strain K-24 was grown at 30°C during 4 days in yeast extract mannitol medium (YEM), containing per litre of demineralized water: yeast extract, 1.0 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.25 g; $CaCl_2.2H_2O$, 0.1 g and $CaCO_3$, 1.0 g (Vincent, 1970).

In the first series of experiments, which were done with pots, the bacterial suspension was washed twice in physiological salt solution (145 m*M* NaCl) before use. Since the amount of soil in the root boxes is much smaller and care has to be taken not to change soil-pH, the grown culture for the rhizotron experiments was washed twice in diluted PBS (70 m*M* NaCl solution + 10 m*M* KH_2HPO_4/K_2HPO_4 buffer) of pH 5.2.

Plants

Seeds of lucerne (*Medicago sativa* L. cv Resis), obtained from Van der Have (Kapelle, The Netherlands) were used in all experiments. The plants were grown in pots and rhizotrons, as described in detail by Pijnenborg and Lie (1990).

The pots (10 cm ϕ, 10 cm high) were filled with 600 g of soil, moistened as described above, and 24 plant holes of 5 mm depth were made. 12 µL of aqueous solution of the various chemicals (Table 1) were added locally into the holes using a syringe. As in the field experiment (Pijnenborg and Lie, 1990), the seeds were not surface-sterilized. Each plant hole was sown with a seed and subsequently inoculated with a 12 µL drop containing *ca.* 10^7 cells of *R.meliloti*. The bacteria were enumerated as described by Pijnenborg *et al.* (1990c).

Table 1. Chemicals used to study the nodulation response of lucerne (Medicago sativa cv. Resis) in pots and rhizotrons

Treatment	Pot*	Rhizotron**
pH	KOH	K_2CO_3***
Ca	$CaSO_4$	$CaCl_2$
Ca + pH	$CaSO_4$+KOH	$CaCl_2$+K_2CO_3
Ca + pH (lime-pellet)	$CaCO_3$	$CaCO_3$***
Ca + pH (control)	$CaCO_3$+K_2SO_4	$CaCO_3$+KCl

* Supplied with 20 μeq per seed of the chemical(s) indicated.
** Supplied with 2 μeq per seedling of the chemical(s) indicated.
*** Applied at 1.0, 2.0, 4.0, 8.0 and 12.0 μeq per seedling.

For experiments in rhizotrons, the seeds were surface-sterilized. They were first exposed to 70% ethanol for one minute, and then treated for 20 minutes in 25 mL of 6% H_2O_2, containing a drop of detergent (Teepol, Lamers and Pleuger, 's Hertogenbosch, The Netherlands). The seeds were pre-germinated by incubating them on 1.0% agar in demineralized water for 24 hours (30°C). Rhizotrons were made of plastic petri dishes (9 cm ϕ) by removing the top 2 cm. They were filled with 50 g of moist soil. Chemicals and rhizobia were applied in the same way as in pots. Each rhizotron received 7 pre-germinated seeds with radicles of 3 to 5 mm in length.

The plants were grown in a climate room at 20 °C, with a 16 h light (200 lux)/8 h dark cycle and a relative humidity of 70%. The moisture content of the soil was kept constant with sterile demineralized water. Loss of water was quantified by weighing pots and rhizotrons.

Quantification of nodule formation

All 24 seeds in one pot received the same chemical(s); each treatment was replicated in four pots. Similarly, in one rhizotron, each seedling was treated identically; one replication consisted of four rhizotrons (28 seedlings) and each treatment was carried out in four replications. The number of seedlings with crown nodules, *i.e.* nodules formed on the upper 10 mm of the taproot, or on lateral roots in this section within 3 mm distance of the taproot, was counted either after 14 days (rhizotrons) or after 20 days (pots) of inoculation.

Determination of soil-pH

To determine local changes in soil pH, a modified indicator agar method of Marschner and Römheld (1983) was used (Pijnenborg *et al.*, 1990a). A 3 mm layer of agar was brought into contact with the rhizotron-soil. Soil and agar were separated by a nylon gauze. The solution contained per litre of demineralized water 7.5 g agar (Difco Bacto-agar, Brunschwig Chemie, Amsterdam), 0.06 g bromocresol purple (Merck) and 0.17 g $CaSO_4$.

Qualitative changes in pH could be seen by a change of the indicator color from yellow (pH 5.2) to purple (pH 6.8). For a quantitative determination of the pH change, a micro-electrode (Type MI 410, Microelectrodes Inc., Londonderry, U.S.A.) was inserted into the agar. The pH measurements were independently carried out four times.

RESULTS

Nodulation affected by Ca and/or pH

The effect of calcium on crown nodulation, without altering soil acidity, was first studied in pots. The amount of calcium added per seed (20 μeq of $CaSO_4$) was equivalent to the calcium in lime-pellets used in field experiments (Pijnenborg and Lie, 1990). In comparison to inoculation only, $CaSO_4$ did not significantly alter the number of crown-nodulated seedlings (31% and 26%, resp., Fig. 1A). When KOH was used as a neutralizing agent, the nodulation increased ($P<0.05$) from 31% to 53%. Addition of $CaCO_3$ resulted in 70% crown nodulation, similar to the effect of KOH plus $CaSO_4$ (63%).

In rhizotrons, nodule formation could be followed in the course of time. When the seeds were supplied with *R.meliloti*, only 9% of the seedlings became crown-nodulated (Fig. 1B). Again, addition of calcium ($CaCl_2$) did not affect nodulation (15%). By adding K_2CO_3 the percentage of plants crown-nodulated increased ($P<0.05$) from 9% to 53%. Adding

either $CaCO_3$ or K_2CO_3 plus $CaCl_2$ increased crown nodulation to 71% and 68%, respectively.

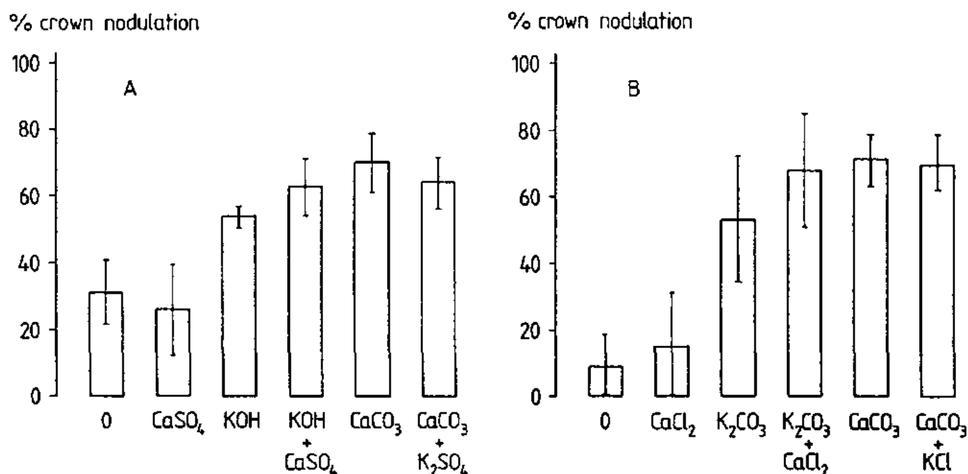
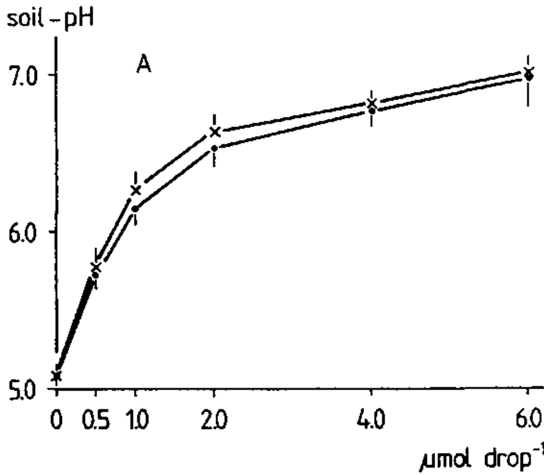


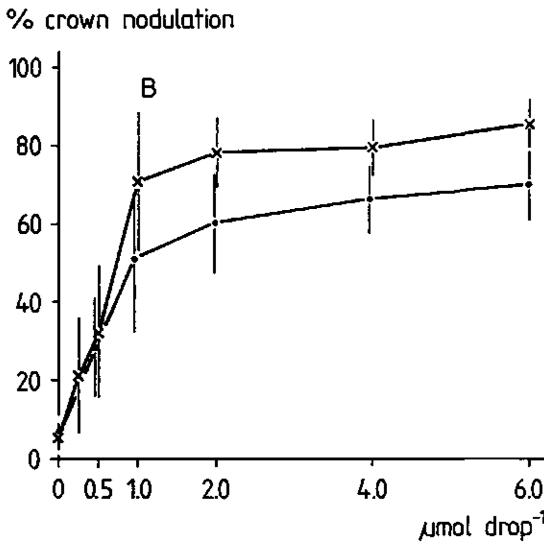
Fig. 1. Effect of chemicals on the relative number of crown-nodulated lucerne seedlings, measured in pots (A) or rhizotrons (B) at 20 and 14 days after inoculation, respectively. 0 = inoculated with R.meliloti only.

Nodulation of plants in rhizotrons was quicker (14 *versus* 20 days) and much more sensitive to neutralization than that of pot-grown plants (9% to 53%, Fig. 1B, *versus* 31% to 53%, Fig. 1A). This difference might be caused by the fact that in rhizotrons the seeds were pre-germinated and *R.meliloti* bacteria were directly applied to the radicles. The infection process in pots was probably delayed, since it took 1 to 2 days before the seeds started to germinate. Because of the quicker nodulation and the higher sensitivity to neutralization, further research was done in rhizotrons.

Dose response of nodulation to neutralization

Experiments were carried out to determine the minimum amount of K_2CO_3 and $CaCO_3$, required for optimal nodulation of lucerne. Apart from plant responses, soil-pH values were also quantified. Virtually the same increase in pH was found after neutralization with either K_2CO_3 or $CaCO_3$ (Fig. 2A).





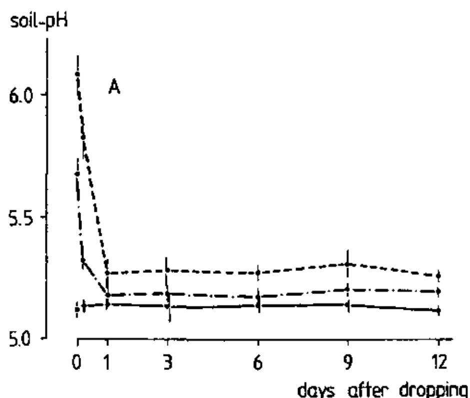
Only 6% of the non-neutralized seedlings formed crown nodules (Fig. 2B). Optimal nodulation was already reached at 1.0 µmol of $CaCO_3$ or K_2CO_3. Higher amounts of these chemicals did not increase nodulation. At optimal concentrations of $CaCO_3$ and K_2CO_3, (averaged for 1.0, 2.0, 4.0 and 6.0 µmol), 79 ± 10% and 62 ± 12% of the seedlings were crown-nodulated, respectively. At 6 µmol of carbonate, the nodulation with $CaCO_3$ was higher ($P<0.05$) than with K_2CO_3.

Fig. 2. Effect of $CaCO_3$ (×) and K_2CO_3 (•) on local soil-pH (A), and the relative number of crown-nodulated lucerne seedlings (B) in rhizotrons. The pH was measured after 15 minutes of treatment, the nodulation after 14 days of growth.

pH-dynamics

Soil-pH was followed for a period to 12 days after local application of K_2CO_3 and $CaCO_3$. Unplanted rhizotrons were included as controls, to be able to distinguish between pure physical-chemical processes and changes due to activities of plants.

The development in soil-pH, measured in K_2CO_3-treated soil without plants, is presented in Fig. 3A. The untreated soil remained constant at pH 5.1. One day after applying 0.5 and 1.0 µmol of the carbonate, the initial soil-pH of 5.7 and 6.1 had decreased to a level of 5.2 and 5.3, respectively, and remained constant thereafter. In the planted rhizotrons, soil-pH was measured around the root crown, 5 mm down the seedling-taproot. Results of planted, K_2CO_3-treated rhizotrons are given in Fig. 3B. During the first day the pH dropped just as in unplanted rhizotrons. Three days after planting, a slight increase could be measured.



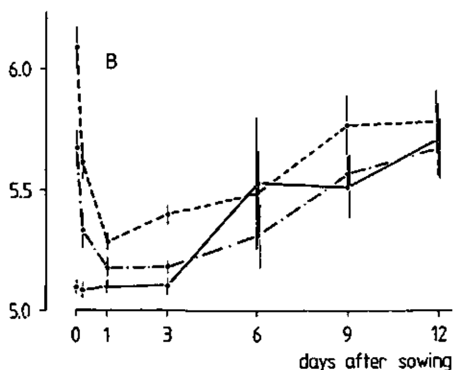
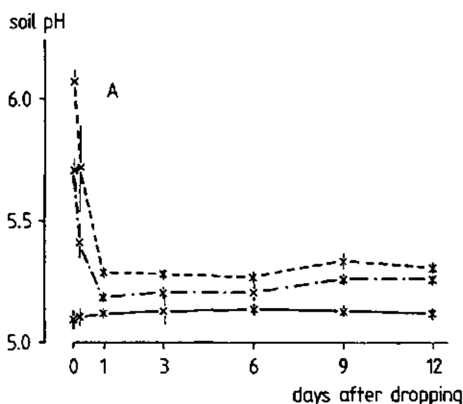


Fig 3. pH dynamics as a result of K_2CO_3 addition (0 (——), 0.5 (—·) or 1.0 (---) µmol per drop of 12 µL) measured in unplanted (A) and planted (B) rhizotrons.



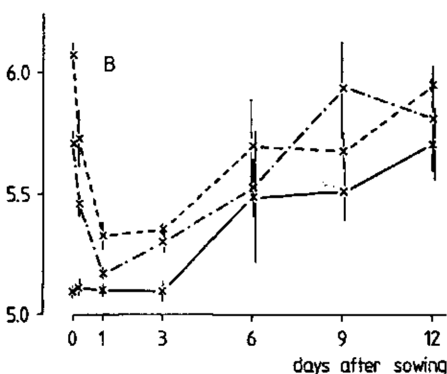


Fig 4. pH dynamics as a result of $CaCO_3$ addition (0 (——), 0.5 (·—·) or 1.0 (---) µmol per drop of 12 µL) measured in unplanted (A) and planted (B) rhizotrons.

At day 6, 9 and 12, soil-pH increased independently of the initially applied amount of K_2CO_3; namely to 5.5, 5.6 and 5.7 (±0.15; data averaged for 0, 0.5 and 1.0 µmol).

Results with lime in comparable experiments, were very similar to the K_2CO_3 treatments (Fig. 4A and 4B). Only slightly higher pH values were found in the planted rhizotrons at day 6, 9 and 12: 5.6, 5.7 and 5.8 (±0.15), respectively. These data are averaged over 0, 0.5 and 1.0 µmol of $CaCO_3$.

DISCUSSION

Importance of neutralization

The increase of nodulation in an acid soil due to pelleting seeds of lucerne with lime, could be attributed to two separate factors, namely neutralization of acidity and supply of calcium. It was possible to achieve a nodulation response comparable to $CaCO_3$ in pots and rhizotrons, by combining the addition of KOH or K_2CO_3 with the addition of $CaSO_4$ or $CaCl_2$, respectively. Quantification of the actual increase in soil-pH is a requirement to make a decisive statement as to which magnitude each of the two factors contributed. This is possible by using rhizotrons. Addition of $CaCO_3$ or K_2CO_3 resulted in the same soil-pH (Fig. 2A), but a lower number of seedlings nodulated when K_2CO_3 was supplied (Fig. 2B). In this soil, the neutralization contributed 79% of the nodulation increase due to lime and the remnant, only 21%, is attributable to the supply of calcium.

The relative contribution of calcium and pH to the nodulation of subterranean clover (*Trifolium subterraneum* L.) in acid soils has been the subject of previous studies. Soil properties, if specified at all, were different from the soil studied in this paper. This makes a comparison of the results difficult. Spencer (1950) found only abundant nodulation, when the original soil (pH-H_2O 5.0) was neutralized with magnesium hydroxide and additionally supplied with calcium sulfate. Very few nodules developed when the soil was only neutralized, or

only supplied with calcium. Coventry *et al.* (1985), working on soils of pH-H_2O 5.0 to 5.2, suggested calcium to be the main contributor to the positive response of nodulation to lime. These authors based their argument on the fact that only a small amount of calcium (0.6 meq per 100 g) was present in the soil. In contrast, recent work of Richardson *et al.* (1988) indicated that the improved nodulation was primarily due to the rise in soil-pH. It is obvious from these reports, that the native calcium content in the soil might be decisive for the outcome of such nodulation experiments. In a subsequent paper (Pijnenborg *et al.*, 1990b), the native calcium in the studied soil (1.2 meq per 100 g) was removed with a specific chelator. In that way, the role of the calcium cation in the nodulation of soil-grown lucerne could be studied in more detail.

Rhizosphere-pH

The dynamics of pH, measured around the root crown of lucerne seedlings, treated with K_2CO_3 or $CaCO_3$ (Fig. 3B and 4B), can be divided into two stages. In the first stage, addition of the carbonates increased the alkalinity locally. Subsequently, the surrounding bulk of acid soil diminished the pH gradient. Within one day, soil-pH in the neutralized spots dropped to 5.2 and 5.3 for 0.5 and 1.0 µmol of carbonate, respectively (Fig. 3A and 4A). These values, slightly higher than in the untreated soil (pH 5.1), might allow root nodules to develop. Munns (1965) showed that lucerne, when supplied with 10^5 of *R.meliloti* bacteria per seed, nodulated well at pH 5.5, moderately at pH 5.3 and poor at pH 5.2 (pH values measured in 1:5 soil in water suspension).

During the second stage, from day 3 to 12 after sowing, local soil-pH was markedly influenced by root activities. Obviously independent of the initial neutralization, the average rhizosphere-pH increased from pH 5.1 to around pH 5.7. Blanchar and Lipton (1986) found an even more pronounced increase when they measured the rhizophere-pH of 10 day-old

lucerne seedlings in a soil of pH 5.5. The soil at 0 to 0.5 mm distance from the older tap roots was more basic (pH 6.8), whereas the soil around the younger lateral roots was more acidic (pH 4.2). They assumed that this was a physiological characteristic of *Medicago sativa*, since they argued that the older parts of the root system absorbe more anions than cations. Such an alkalinization of the rhizosphere was first found by Dijkshoorn (1962) in nitrate-fed plants. To maintain electro-neutrality, OH-ions have to be excreted (*e.g.* Israël and Jackson, 1978). The acidification by phosphate deficient rape plants (*Brassica napus* L.) should be due to an excess of cations (Hedley *et al.*, 1982). Recent work of Hoffland *et al.* (1989a,b) indicated however, that this process may not be related to the nutrient uptake pattern of the plant, but rather due to the production of organic acids in zones just behind the root tips. As a result, rape plants were able to solubilize phosphorus from rock phosphate.

When the seeds were supplied with 1.0 µmol of K_2CO_3 or $CaCO_3$, significantly higher pH values were measured only during the first 3 days (Fig. 3B, 4B) and a good nodulation was found (Fig. 2B). At day 6, untreated seedlings increased soil-pH to a level (pH 5.5) at which lucerne can nodulate well (Munns, 1965). However, only 6% of the seedlings nodulated (Fig. 2B). The failure to nodulate cannot be the result of a too low number of *R.meliloti*, since the rhizobia added to the soil multiplied just as well in the presence or absence of lime-pelleting (Pijnenborg *et al.*, 1990c).

Probably, once the roots start to raise pH, the root tissue is too old to be susceptible for rhizobial infection. Only the younger root cells of a legume, between the zone of root elongation and the zone of developing root hairs, are infectible (Bhuvaneswari *et al.*, 1981). This transient susceptibility was shown previously by Dart and Pate (1959) who found that nodules developed on lower positions on the root, when the inoculation of *Medicago* seedlings was postponed until 5 to 25 days after sowing. As a result of root growth in this period, the infectible zones, 1 to 2 cm behind the root

tips, moved further down.

The measurements of rhizosphere-pH indicate that the initial period, in this soil from day 0 to day 3, is crucial for the later nodule development. A high correlation (r^2 = 0.98) is found between pH values measured directly after neutralization (Fig. 2A) and crown nodulation two weeks later (Fig. 2B). However, the exact duration of the sensitive phase cannot be deduced from the results presented. Previous research has been done in acid nutrient solutions to study the nodulation of peas (Lie, 1969; Mulder *et al.*, 1966) and lucerne plants (Munns, 1968). With lucerne, this "acid sensitive step" lasted only 12 hours.

It can be concluded that the benefit of small amounts of lime for the nodulation of lucerne in the studied soil, is largely due to (local) neutralization (80%), and only for a minor part to the supply of calcium (20%). The success of crown nodulation is determined by the neutralitiy in the environment around the tap root during the period 0 to 3 days after inoculation. The alkalinization induced by the roots at later stages does not affect the nodulation process.

ACKNOWLEDGEMENTS

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Chapter 6

Inhibition of nodulation of lucerne (*Medicago sativa* L.) by calcium depletion in acid soil

J.W.M. PIJNENBORG, T.A. LIE and A.J.B. ZEHNDER

Plant and Soil *in press*

ABSTRACT

The effect of calcium on the nodulation of lucerne was studied using EGTA, a specific calcium-chelator. First, the effects of the chelator were tested on hydroponically grown plants at pH 7.0. Optimal numbers of nodules were obtained in nutrient solution containing 0.2 m*M* $CaCl_2$. When 0.4 m*M* EGTA was given additionally, nodulation was completely inhibited. Nodulation could be restored specifically with $CaCl_2$, but not with $MgCl_2$.

For studies in an acid soil (pH-H_2O 5.2), lucerne seedlings were grown in rhizotrons. 67% of the seedlings became nodulated when the soil around the seed was neutralized locally with 1.0 µmol of K_2CO_3 in drops of 12 µL volume. However, when native calcium was removed with 2 µmol of EGTA, nodulation was reduced to 12%. However, addition of EGTA to soil resulted in a drop of pH from 6.1 to 5.2. A phosphate buffer could also not keep soil-pH sufficiently stable. Such pH-decreases could be avoided by placing agar blocks containing 6 µmol of EGTA for three hours on freshly developed roots. This treatment reduced nodulation from 87% to 32%, with soil-pH lowering only from 6.2 to 6.0. Nodulation could be restored by adding 2 µmol of $CaCl_2$. The depletion of soil-calcium could depress nodule formation only during the first day after inoculation.

INTRODUCTION

Soil acidity and calcium deficiency tend to occur together (Munns, 1977, 1978). As a macro nutrient, calcium is essential for plant growth (Marschner, 1987). Temperate legumes generally require a relative high calcium concentration, especially when they depend on symbiotic nitrogen fixation. More calcium is needed for the formation of nodules than for nitrogen fixation and plant growth (*e.g.* Andrew, 1976; 1978; Lie, 1974; Lowther and Loneragan, 1968; Munns, 1978).

Studies on the nodulation of lucerne grown in nutrient solutions, showed that for optimal nodulation at pH 4.8, six

times more calcium was needed than at pH 5.6 (Munns, 1970; 1977). This effect was ascribed to an inhibition of calcium uptake by hydrogen ions (Andrew and Johnson, 1978; Loneragan and Dowling, 1958; Schmehl *et al*, 1952). Addition of calcium acetate to acid soil has been shown to beneficially affect nodulation of soybean without altering soil-pH (Scanlan, 1928). The benefit of calcium in acid soils for soybean was confirmed by Albrecht and Davis (1929) by applying calcium chloride. The same authors found no improvement in neutral soils. Loneragan (1959) showed that at least 0.5 meq of calcium per 100 g of soil is required for optimal nodulation of clovers.

In previous work (Pijnenborg and Lie, 1990), a strong improvement of the nodulation of lucerne was found in an acid sandy soil, when the seeds were pelleted with lime. Subsequent studies (Pijnenborg *et al*, 1990a) suggested that up to 20% of the improvement could be due to the input of calcium, and 80% to the neutralization of the soil. In the following, the effect of calcium on nodulation of lucerne is studied in more detail. Experiments were done with plants growing in nutrient solution and in soil in rhizotrons (Pijnenborg and Lie, 1990).

MATERIAL AND METHODS

Plant

In all the experiments seeds of lucerne (*Medicago sativa* cv. Resis) were used. The seeds were obtained from van der Have (Kapelle, The Netherlands). The plants were grown in a climate room at 20 °C, a 16 h light (200 lux)/8 h dark period and a relative humidity of 70% in either soil or hydroponic cultures.

Rhizobium

Rhizobium meliloti strain K-24 was grown at 30°C for 4 days in yeast extract mannitol medium (YEM), containing (g per litre of demineralized water): yeast extract, 1.0; mannitol, 10.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.1 and $CaCO_3$, 1.0 (Vincent, 1970). Before inoculation, the cells were washed twice in sterile physiological salt solution (0.85% NaCl).

Soil

The soil was taken from the field station at Wageningen Hoog (Pijnenborg and Lie, 1990). The main characteristics are:
pH-H_2O 5.2; organic matter, 2.2%, cation exchange capacity (CEC), 3 meq per 100 g of dry soil, consisting of the following elements (meq per 100 g): Ca, 1.2; Mg, 0.2; Al, 0.6; Na, 0.2 and K, 0.2. The soil did not contain native *R.meliloti*. Air-dried soil was mixed with a sterile solution of 0.34 g KH_2PO_4 plus 0.25 g $MgSO_4.7H_2O$ per litre of demineralized water to obtain a moisture content of 12%.

Nutrient solution

The composition of the nutrient solution was (mg per litre of demineralized water): K_2HPO_4, 360; KH_2PO_4, 120; $MgSO_4.7H_2O$, 250. Trace elements: $MnSO_4.4H_2O$, 1.0; $ZnSO_4.7H_2O$, 0.25; $CuSO_4.5H_2O$, 0.25; H_3BO_3, 0.50; $Na_2MoO_4.2H_2O$, 0.05; and Fe(III)citrate 30. Calcium was added as $CaCl_2$. The exact concentrations are given for each experiment individually. The pH of the final solution was 7.0.

Hydroponic system

Lucerne seeds were surface-sterilized by first treating them with 70% ethanol for one minute, and subsequently washing them for 20 minutes in a 6% H_2O_2-solution containing a drop of detergent (Teepol, Lamers and Pleuger, 's-Hertogenbosch, The Netherlands). The seeds were allowed to germinate in petri dishes during 5 days at 30°C on 0.9% water-agar in the dark. Autoclaved nutrient solution was added to 350 mL heat-sterilized jars (120°C, overnight). The seed coats were removed and only uniform seedlings with root length of about 40 mm were used. Eleven seedlings were transferred aseptically from the petri dishes to a 2 mm thick plastic plate with holes of 7 mm diameter. The seedlings were held in position by wrapping the hypocotyles in small pieces of foam plastic. The jars were covered with the seedling containing perforated plates. The nutrient solution was aerated with sterile air. The air was sterilized by first passing it through a cottonwool filter (15 cm long, 3 cm ϕ), and then through a glassfibre bacterium filter with a pore size of 0.6 µm (Sartorius, Breukelen, The Netherlands). After 7 days of growth in nutrient solution of 1.0 m*M* calcium, the roots were washed in calcium-free nutrient solution and transferred to nutrient solutions with final calcium concentrations of 0, 0.2 or 1.0 m*M*. The following day, the plants were inoculated with 10^8 cells of *R.meliloti* per jar. Nodule numbers were counted at 8, 14 and 20 days after inoculation. The plants were harvested at day 20. Weight and acetylene reduction activity (ARA) were quantified. ARA is taken as a measure for the nitrogenase activity of the nodules. It was determined by incubating the plants in 250 mL flasks in air containing 10% acetylene (v/v). Samples of 100 µL of the gaseous phase were analysed for the ethylene content in a Beckerd Packard gas chromatograph (model 417), equipped with FID connected to a 3.25 mm x 1 m column containing Porapak R (80-100 mesh).

Rhizotron system

Rhizotrons, made of plastic petri dishes (ϕ 9 cm), were filled with 50 g of soil (Pijnenborg and Lie, 1990). Lucerne seeds were surface sterilized and pre-germinated for only one day. To deplete the native calcium, a 1*M* EGTA (Sigma, Brunschwig Chemie, Amsterdam) stock solution of pH 7.0 was made in 10*M* KOH. The chelator was applied to the soil in two ways; either directly as an aqueous solution, or in agar blocks that were placed onto the soil.

In the first procedure, the soil spots where the pre-germinated seeds would be sown later, were locally neutralized with drops (12 µL) of either a K_2CO_3 solution (1 µmol per drop) or a K_2HPO_4/KH_2PO_4 buffer solution (pH 7, 8 µmol per drop). Subsequently, various amounts of EGTA solution and, where necessary, also $CaCl_2$ solution were added in the same way. Finally, the treated spots of soil were inoculated with 10^7 cells of *R.meliloti*. Seven pre-germinated lucerne seeds were sown in each rhizotron.

The application of EGTA in agar blocks was used earlier by Lee *et al* (1983) to study gravitropic sensitivity in roots. To prepare these agar blocks, 25 mL of water containing 1.0% of melted agar (50°C) was mixed with various amounts of 1.0*M* EGTA solution and brought to pH 8.2 with 0.1*N* KOH. This mixture was poured into petri dishes and, after solidification, small agar cylinders were made with a cork borer (ϕ 10 mm, 10 mm thick). The rhizotrons were placed at an angle of 60° to force the roots to grow towards the lid. On the surface of the soil the roots developed one day later to a length of about 15 mm. The area around the root tips was neutralized with drops of 4 µmol of K_2CO_3 and inoculated as described above. The agar blocks containing EGTA were placed on the treated root sections and incubated for three hours. Subsequently, $CaCl_2$ solution was applied, if necessary. Each treatment consisted of 8 rhizotrons, containing a total of 56 seedlings. Immediately after beginning the treatments, the pH in the treated soil spots was measured using a previously described agar-contact

technique (Pijnenborg *et al*, 1990b). Nodulation of the plants was quantified after 14 days.

RESULTS

Plants grown in nutrient solution

The addition of EGTA to the nutrient solution resulted in a drop of pH (Table 1), which was neutralized with 0.1*N* KOH.

Table 1. pH of the nutrient solution, influenced by Ca and EGTA concentrations (mM)

(Ca)	(EGTA)	pH
0.2	0	7.0
0.2	0.2	6.8
0.2	0.4	6.6
0.2	0.4 + 0.8 mM $CaCl_2$	6.4
0.2	0.4 + 0.8 mM $MgCl_2$	6.6

This drop is due to the complexation reaction, and is specific for calcium, since no such drop was obtained with magnesium (Fig. 1). The pK values of the four carboxyl groups in EGTA are 2.0, 2.7, 8.9 and 9.4 (Blaedel and Meloch, 1963). At pH 7.0, two of the four groups are protonated. These two protons are released when EGTA reacts with calcium.

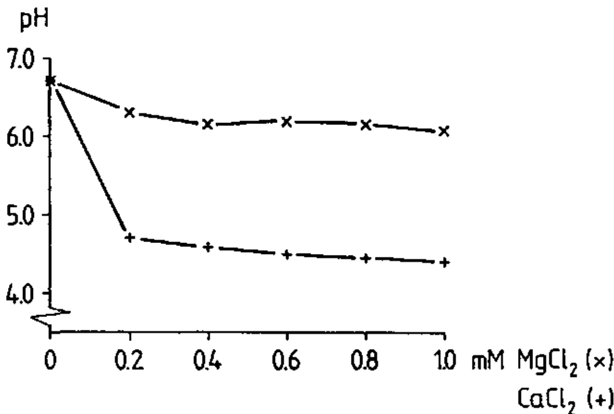

Fig. 1. Effect of addition of $CaCl_2$ (+) or $MgCl_2$ (×) on the pH of an 1.0 mM aqueous EGTA solution.

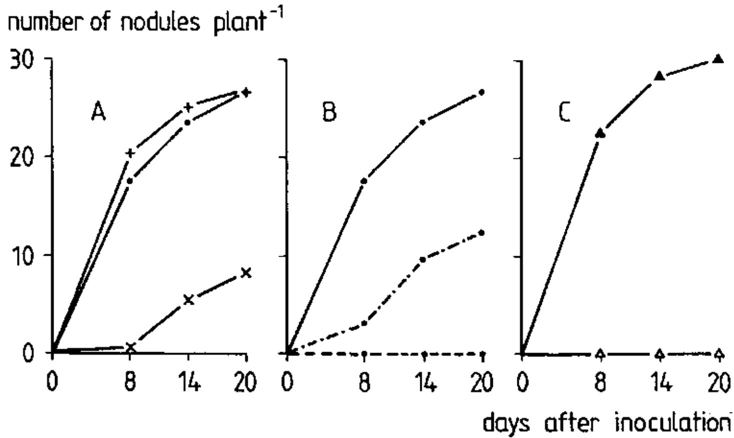

Fig. 2. *The effect of the availability of calcium in the nutrient solution* on the number of nodules per lucerne plant at 8, 14 and 20 days after inoculation (data averaged for 22 plants). A. Effect of the calcium concentration: 0 mM (×), 0.2 mM (•) or 1.0 mM (+). B. Chelation of a 0.2 mM Ca solution with 0 mM (——), 0.2 mM (·—·) or 0.4 mM (---) of EGTA. C. Effect of addition of 0.8 mM $CaCl_2$ (▲) or 0.8 mM $MgCl_2$ (△) to a 0.2 mM Ca solution containing 0.4 mM EGTA (----, Fig. 2B).

Optimal nodule numbers developed in nutrient solutions supplied with 0.2 m*M* calcium (Fig. 2A). The same calcium requirement was found by Munns (1970). Increasing calcium concentrations up to 1.0 m*M* did not result in higher numbers of nodules. When no calcium was given, the number of nodules decreased by 70%. A similar decrease was observed with 0.2 m*M* calcium solution in the presence of 0.2 m*M* EGTA (Fig. 2B).

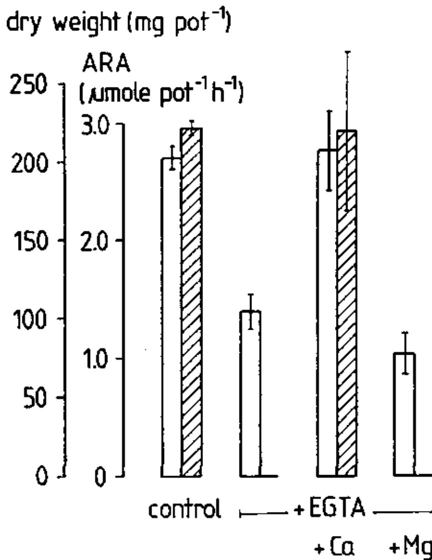

Fig. 3. Response in total dry weight and acetylene reduction activity (ARA; shaded columns) of hydroponically grown lucerne to the availability of calcium, 20 days after inoculation. Treatments: control (nutrient solution of 0.2 mM Ca), 0.2 mM Ca plus 0.4 mM EGTA, supplied with either 0.8 mM $CaCl_2$ (+ Ca) or 0.8 mM $MgCl_2$ (+ Mg). Data are averaged for 2 pots of 11 plants; bars represent SE.

This bioassay is in agreement with the theoretical molar ratio of 1 : 1 for the complexation of calcium with EGTA (Blaedel and Meloche, 1963). At 0.4 m*M* EGTA, root growth was severely reduced and nodule development was totally inhibited. Complete restoration of nodulation was obtained by adding 0.8 m*M* $CaCl_2$ to the 0.2 m*M* calcium solution containing 0.4 m*M* EGTA (Fig. 2C). In contrast, nodules remained absent when an equivalent amount of magnesium was given. Differences in nodule number were paralleled by plant dry weight and nitrogenase activity (ARA). Addition of the chelator completely eliminated ARA and reduced dry weight by 50% (Fig. 3).

Plants grown in soil (rhizotrons)

The effect of EGTA was also examined on lucerne growing in an acid soil in rhizotrons. To obtain crown nodulation, *i.e.* nodulation on the upper 10 mm of the seedling taproot, the soil around the seed had to be neutralized with either K_2CO_3 or a phosphate buffer. Increasing amounts of EGTA were applied in combination with one of these neutralizing agents. As can be deduced from the nodulation response, 2 µmol of EGTA removed all calcium in the soil area which could influence crown nodulation (Fig. 4).

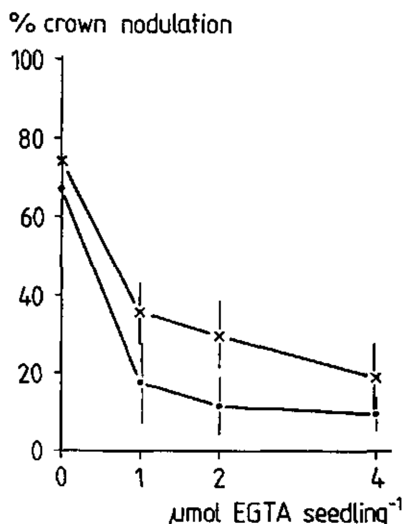

Fig. 4. Reduction of the number of crown-nodulated lucerne seedlings in rhizotrons (% crown nodulation) 14 days after adding increasing amounts of EGTA in 12 µL drops. The soil was locally neutralized (12 µL) with either 1 µmol of K_2CO_3 (•) or 8 µmol of K_2HPO_4/KH_2PO_4 buffer of pH 7 (×).

Addition of 1 μmol of K_2CO_3 increased soil-pH from 5.2 to 6.1, and 67% of the seedlings became crown-nodulated (Fig. 5A). EGTA reduced nodulation to 12% but caused soil-pH to drop to pH 5.2. Addition of extra calcium did not improve nodulation (13%).

To reduce these variations in soil-pH, a phosphate buffer was used instead of K_2CO_3. After neutralization, crown nodulation increased from 15% to 74% (Fig. 5B), and was reduced to 30% with EGTA. Addition of $CaCl_2$ (1, 2, 3 and 4 μmol) resulted in a gradual increase of crown nodulation (to 32%, 50%, 60%, 70%, respectively). Similar to Fig. 5A, the highest response was achieved by combining neutralization with a supply of calcium (83%). In contrast to K_2CO_3 (Fig. 5A), the phosphate buffer allowed restoration of nodulation by applying calcium (Fig. 5B). With phosphate buffer soil-pH could also not be kept completely stable. Chelation caused a pH reduction from 6.3 to 6.1, and calcium addition led to a further drop to 5.7 (Fig. 5B). To avoid these acidification problems, another procedure for local application of EGTA to soil-grown lucerne was used.

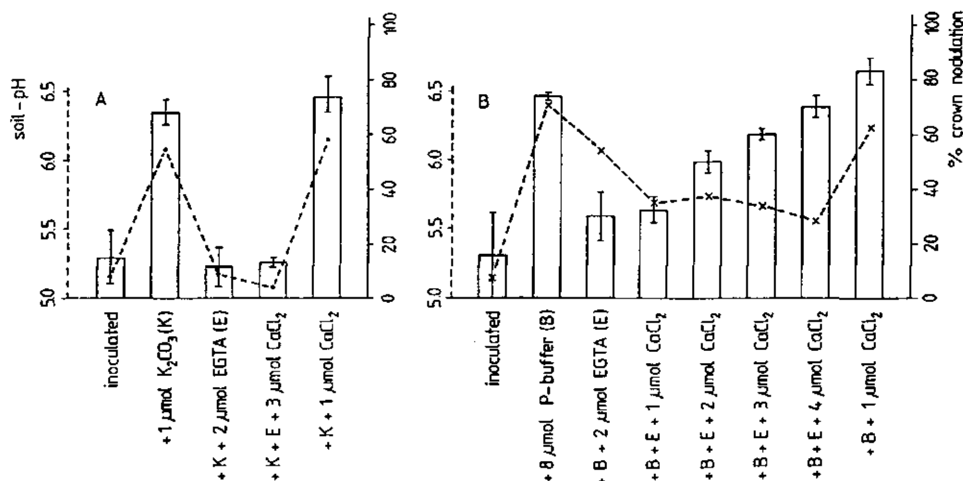

Fig. 5. A: Number of crown-nodulated lucerne seedlings recorded at day 14 (% crown nodulation, histograms) and local soil-pH (dashed line), measured directly after local neutralization (12 μL) with K_2CO_3 and addition (12 μL) of aqueous EGTA and/or $CaCl_2$ solution. B: as A, but the soil was locally neutralized with K_2HPO_4/KH_2PO_4 buffer of pH 7.

EGTA applied in agar blocks

One day after sowing the pre-germinated seeds, the lids of the rhizotrons were taken off. The lowest 10 mm of the seedling-taproots were neutralized with K_2CO_3, inoculated with rhizobia and covered with the chelator-containing agar blocks. The plant- and soil-reponses to different chelator quantities in the agar are summarized in Fig. 6. Nodule formation was mainly confined to the treated root sections. Of the seedlings incubated with blocks of plain agar, 87% nodulated. Nodulation decreased to 25% when the agar blocks contained 8 µmol of EGTA. This reduction was accompanied by a minor acidification of the soil, from pH 6.2 to 5.9 (Fig. 6).

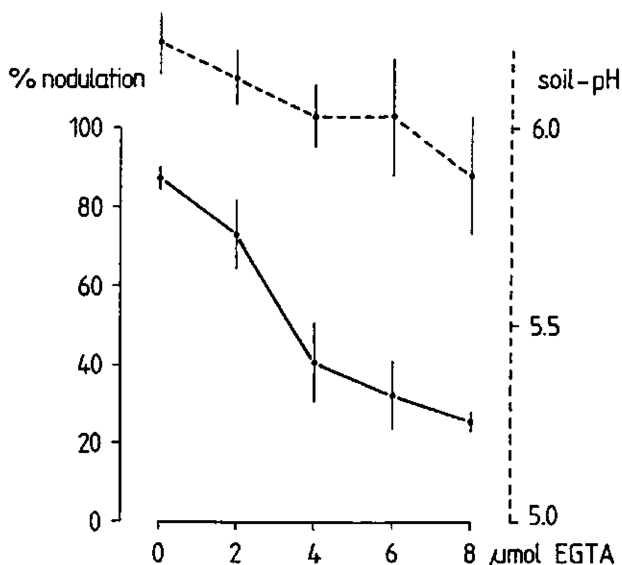

Fig 6. Effect of increasing amounts of EGTA in agar blocks on the number of nodulated lucerne seedlings (% nodulation, solid line) measured at day 14, and soil-pH measured at day 0 (dashed line).

A decrease in nodulation (32%) by EGTA (6 µmol per block) could be restored to 83% by adding 2 µmol of $CaCl_2$ to the soil (Fig. 7); the use of $MgCl_2$ instead of $CaCl_2$ had no effect (35% nodulation). Soil-pH was not affected when the chelated spots of soil were treated with either calcium or magnesium (Table 2).

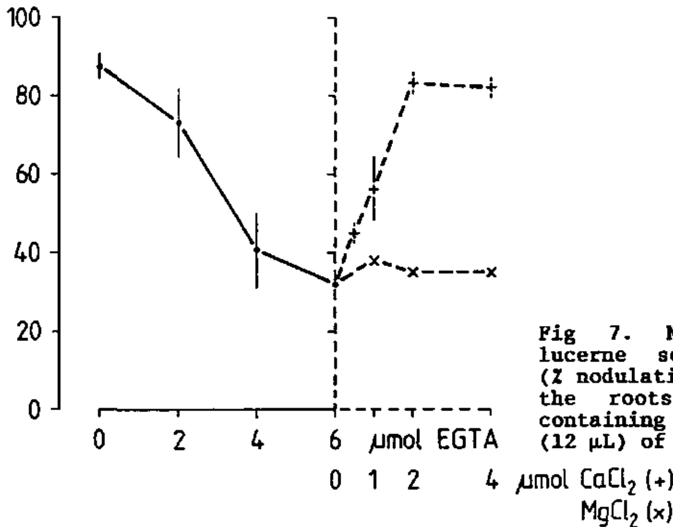

Fig 7. Number of nodulated lucerne seedlings at day 14 (% nodulation) after treating the roots with agar blocks containing EGTA (•) and addition (12 µL) of $CaCl_2$ or $MgCl_2$.

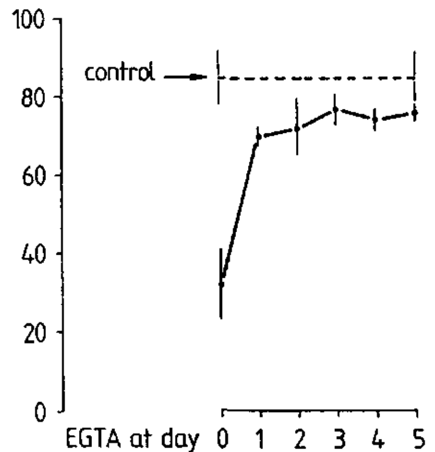

Fig 8. Number of nodulated lucerne seedlings at day 14 (% nodulation) after treating the roots at different times after inoculation with agar blocks containing 6 µmol of EGTA.

Table 2. Nodulation (number of nodulated seedlings) and soil-pH affected by the application of EGTA in agar blocks and subsequent addition of $CaCl_2$ or $MgCl_2$. Data (mean ± SE) averaged for 2 x 28 seedlings and 4 pH measurements, respectively

Treatment	Nodulation (%)	Soil-pH
Control (no EGTA/block)	87 ± 3	6.23 ± 0.08
6 µmol EGTA/block	32 ± 9	6.03 ± 0.12
6 µmol EGTA/block + 2 µmol $CaCl_2$	83 ± 2	5.95 ± 0.06
6 µmol EGTA/block + 2 µmol $MgCl_2$	35 ± 4	6.01 ± 0.10

To study the calcium-sensitivity of the nodulation with the course of time, the roots were treated with agar blocks (6 µmol EGTA) in the period between 0 and 5 days after inoculation. During the first day, the nodulation was strongly depressed; chelation at day 1 to day 5 did not result in an overall significant reduction in the number of nodulated plants (Fig. 8).

DISCUSSION

The data presented in this paper clearly show the requirement of the nodulation of lucerne for calcium, not only in a hydroponic system but also in soil. With EGTA, the particular need for calcium could be quantified, since this compound specifically chelates the calcium ions present in the rooting medium.

Acidification by EGTA

EGTA lowered the pH, not only in nutrient solutions (Table 1), but also in soil (Fig. 5). In solution cultures this acidification could be neutralized simply by titration with KOH. However, when the chelator was directly applied to the soil, it was difficult to maintain the pH above critical levels. Studies in the acid soil used in these experiments, showed that the pH for optimal nodulation should at least be 6.0 in the micro-environment around the lucerne seed (Pijnenborg *et al.*, 1990a). Addition of calcium to soil treated with K_2CO_3 and EGTA lowered the pH to 5.1 (Fig. 5A).

Despite a surplus of calcium, this low pH prevented nodule formation. Even phosphate-buffer, four times the amount of the chelator, could not prevent the pH reduction from 6.3 to 5.7 (Fig. 5B). Higher amounts of phosphate buffer resulted in necrosis of the seedling root. The release of protons with EGTA could partially be overcome by exposing the nodulation sensitive area of the seedling root, *i.e.* just behind the tip of the root (Bhuvaneswari *et al.*, 1981), to chelator containing agar blocks. Such a treatment also allowed to the soil to be supplied with extra calcium, without further acidification (Table 2).

Calcium requirement in the initial stage of the nodulation process

The nodulation of lucerne seedlings in soil was sensitive to calcium depletion only during the first day following inoculation; when the root tissue was treated with EGTA one to five days later, nodule formation was not significantly reduced (Fig. 8). Obviously, at later stages, the nodulation process was not susceptible anymore to the depletion of calcium. These results agree well with work done by Munns (1970) on hydroponically grown lucerne. This author found that the first day, coincidental with the initiation of infection, is the most calcium demanding and also the most acid sensitive stage (Munns, 1968).

Richardson *et al* (1988a,b) studied the expression of nodulation genes of *Rhizobium trifolii*. A stimulating effect of calcium was found on the *nod* gene-induction activity of flavonic compounds exudated by clover roots. In the presence of calcium, the critical pH for expression of the genes was lowered. A limited activity of these *nod* inducing compounds may contribute to the calcium dependent and acid sensitive step in nodule formation.

The adsorption of *Rhizobium meliloti* to lucerne roots requires a high concentration of calcium when the pH of the rooting medium is lowered from 7 to 6 (Caetano *et al.*, 1989).

By treating either the microsymbiont or the root prior to inoculation, it was shown that only the bacterium is affected. Pre-treatment of *R.meliloti* with calcium tended to increase adsorption, whereas pre-exposure to low pH suppressed adsorption in a neutral incubation medium. Similarly, the attachment of *Rhizobium leguminosarum* to pea root hair tips was reduced when the bacteria were grown under low calcium conditions (Smit *et al.*, 1987). During the initial phase of the attachment process a calcium-binding protein is involved, which is commonly present in bacteria of the genus *Rhizobiaceae*. Hence, this adhesin was termed Rhicadhesin (Smit *et al.*, 1988a). Calcium is specifically needed to anchor Rhicadhesin in the rhizobial cell surface (Smit *et al.*, 1988b).

In the period following rhizobial attachment, 3 to 6 days after inoculation, the nodulation of *Pisum sativum* could be inhibited when the roots were supplied with EDTA (Lie and van Egeraat, 1988). These authors attributed this inhibition to a disturbance in growth of infection threads. Microscopic examination of infected *Trifolium* root hair cells showed that the threads were composed of calcium rich material (Sethi and Reporter, 1981). Infection threads show an anatomical analogy with pollen tubes. In these tubes a similar increased calcium concentration was measured (Reiss and Herth, 1979a). The growth of pollen tubes of *Lilium longiflorum* could be stopped with the calcium-ionophore A 23187 (Reiss and Herth, 1979b). A23187 is a mobile carrier and transports calcium ions across membranes (Reed and Lardy, 1972). Nodulation of soybeans could be inhibited with this calcium-ionophore (Blevins *et al.*, 1977).

From the above it is evident that calcium exerts different modes of action in succeeding symbiotic events during the initial stage of the nodulation process. In soil, the natively present amount of calcium can locally be removed by applying EGTA in blocks of agar, without altering the rest of the root system. This specific technique allows quantification of the *in-situ* effects of calcium on nodulation in soil.

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Summary

Growth of lucerne (*Medicago sativa* L.) is poor in soils with values of pH-H_2O below 6. This is often due to nitrogen deficiency, resulting from a hampered performance of the symbiosis with *Rhizobium meliloti*. This thesis deals with the factors affecting biological nitrogen fixation by lucerne in acid soils.

In a field experiment, lucerne seeds were either inoculated with *R.meliloti* only, or inoculated and pelleted with lime, before sowing in a sandy soil of pH 5.2. Lime-pelleting significantly improved the establishment of the seedlings. This was caused by earlier nodulation, as evidenced by the formation of crown nodules, *i.e.* the nodules on the upper 10 mm of the seedling taproot. The number of seedlings carrying crown nodules tripled from 18% to 56%, 26 days after sowing as a result of lime-pelleting. The dry matter yield and nitrogen fixation of the plants at later stages showed a close correlation with crown nodulation.

To minimize the problems inherent to field experiments, *e.g.* costs and labour, besides pots, rhizotrons (small root boxes made of plastic petri dishes) were developed to study the symbiosis under controlable conditions in the laboratory (Chapter 2). Comparative trials showed that crown nodulation in pots was practically similar to that in the field. In rhizotrons, the early root development and nodule formation could continuously be observed. Moreover, lime-pelleting resulted in a greater increase of crown nodulation (5% to 90%) during a shorter incubation period (14 days).

R.meliloti bacteria are notorious for their acid sensitivity. As a consequence the increased crown nodulation by lime-pelleting might be the result of a better proliferation of these bacteria, thus resulting in a higher chance of root infection. To verify this hypothesis, the effect of lime-pelleting on the survival of inoculated *R.meliloti* around the seed was recorded during a period of 12 days following sowing. In the initial 12 hours a strong

increase in rhizobial numbers coincided with the germination of the seed. Little differences in multiplication were measured in the presence or absence of a lime-pellet. To obtain optimal nodulation in this soil, however, lime-pelleting was a prerequisite and at least 10^5 cells of *R.meliloti* per seed were required. In the absence of lime, only a few plants nodulated, even when the number of rhizobia was increased to 10^7 per seed (Chapter 3).

The nodulation effect of lime-pelleting could not be explained by a better rhizobial multiplication. The benefit of $CaCO_3$ can be due to either neutralization of soil acidity and/or to the input of calcium. To study the pH changes in the rhizosphere while leaving the spatial arrangement of the soil intact, the following method was deviced: The soil was covered with a thin layer of agar containing the pH indicator bromocresol purple. To prevent the contamination of agar with soil particles, a nylon gauze was used to keep the soil separated from the agar. Quantitative pH measurements were done by inserting a micro-electrode into the agar after 3 hours of contact with the soil. The validity of the data obtained by this method was investigated by comparing the results with those obtained using standard procedures for pH measurement in a soil-water suspension (Chapter 4).

Using the agar-contact method, it was shown that the benefit of lime on the nodulation of lucerne was largely (80%) due to neutralization of acidity, and to a lesser extent (20%) to the supply of calcium. For nodulation, soil-pH measured around lime-pelleted seeds, should at least be 5.8. Untreated roots were also able to induce a pH-increase of 0.6 units at 6 days after sowing (d.a.s.) or later, but only very few plants nodulated. These results, obtained with soil-grown plants, confirm earlier findings with hydroponically grown plants, that nodulation is sensitive for acidity only during the early phase of the process (0 to 3 d.a.s.) (Chapter 5).

The soil already contained 1.2 meq of calcium per 100 g. Hence, the contribution of calcium in the lime effect was rather small. When the native calcium was removed with a specific calcium chelator (EGTA), crown nodulation could further be reduced to 12%. However, an additional complication was the acidification that accompanied the chelation of soil-calcium, with soil-pH dropping from 6.1 to 5.2. This problem could be overcome by applying small agar blocks containing the chelator. This technique allowed local application and temporal treatment of the root with EGTA. It was shown that the nodulation could be inhibited by calcium depletion only during the first day (Chapter 6). Obviously, the early phase of the nodulation of lucerne in acid soil is transiently susceptible to low calcium availability, similar to the transient sensitivity to low pH.

Samenvatting

Het gewas lucerne (*Medicago sativa* L.) groeit slecht op zure gronden met pH-H_2O waarden lager dan 6. Een van de oorzaken is een tekort aan stikstof in de plant. Dit is een gevolg van een gebrekkige symbiose met *Rhizobium meliloti* bacteriën, waardoor geen lucht-stikstof gebonden wordt. Dit proefschrift handelt over de factoren die een rol spelen bij de biologische stikstof binding van lucerne in zure gronden.

In een veldproef op zandgrond (pH 5.2), werd een vergelijking gemaakt tussen planten waarvan de zaden alleen waren geënt met *R.meliloti*, en zaden die behalve geënt ook nog waren ingehuld in een laagje kalk ($CaCO_3$). De kalkinhulling zorgde (i) voor een betere opkomst van de zaailingen en (ii) voor een verhoogde nodulatie aan de bovenste 10 mm van de hoofdwortel, de zgn. kroonknollen. Dit laatste is een gevolg van een vroege nodulatie. De toename van het aantal planten met kroonknollen (op 26 dagen na het zaaien van 18% naar 56%) is in het vervolg van het onderzoek als maat gebruikt om het effect van kalkinhulling op de nodulatie te kwantificeren. De drogestof opbrengst en stikstofbinding van de planten bij de oogst waren sterk gecorreleerd met deze kroonnodulatie.

Om de problemen die proeven in het veld met zich meebrengen (zoals bijvoorbeeld de kosten en de slechte controle) te verminderen, werden de proeven uitgevoerd in kleine glazen potten en ook in plastic petrischaaltjes (de zogenaamde rhizotrons), gevuld met de grond uit het veld (Hoofdstuk 2). In beide systemen kan de symbiose onder meer gecontroleerde omstandigheden in het laboratorium nader worden bestudeerd. De resultaten in glazen potten waren praktisch gelijk aan die van het veld. Proeven in rhizotrons hadden het voordeel dat de vroege wortel- en wortelknolvorming voortdurend kon worden geobserveerd. Bovendien was het effect van kalk veel duidelijker dan in potten; kroonnodulatie werd verhoogd van 5% naar 90% gedurende een aanmerkelijk kortere groeiperiode, namelijk in 14 dagen.

Rhizobium meliloti bacteriën staan bekend om hun geringe zuurtolerantie. De verbeterde kroonnodulatie als gevolg van de kalkinhulling is daarom mogelijk een gevolg van een betere overleving van de bacteriën waardoor een kans op infectie van de wortel vergroot wordt. Om deze hypothese te verifiëren, werd de invloed van het kalklaagje op de vermeerdering van de geënte *R.meliloti* bacteriën rond het zaad bestudeerd gedurende de periode van 0 tot 12 dagen na het zaaien. In de eerste 12 uur, tijdens de kieming van het zaad, vond een sterke toename plaats. Kalkinhulling had echter geen invloed op de bacteriële groei in de rhizosfeer; slechts kleine verschillen in aantallen werden geconstateerd in de aan- of afwezigheid van kalk. Voor optimale nodulatie in deze grond is kalkinhulling echter wèl een voorwaarde; bovendien moest er dan nog met minimaal 10^5 *R.meliloti* bacteriën per zaad geënt worden. Bij niet ingehulde zaden werd zelfs bij enting met 10^7 bacteriën per zaad geen verbetering van nodulatie gevonden (Hoofdstuk 3).

Aangezien het gunstig effect van $CaCO_3$ op de nodulatie van lucerne in de zure grond niet verklaard kon worden door een grotere vermeerdering van de *Rhizobium* bacteriën in de rhizosfeer, werd verondersteld dat de werking een gevolg was van enerzijds kalk als neutralizerend agens en anderzijds kalk als calcium bron.

Om pH veranderingen in de zaad en wortel omgeving *in-situ* te meten zonder de compositie van de grond te veranderen, werd de volgende methode ontwikkeld (Hoofdstuk 4): Lucerne planten werden gekweekt in rhizotrons en de grond werd bedekt met een dun laagje agar waarin de pH indicator broomcresol purper was toegevoegd. De indicator diende voor een kwalitatieve indicatie; kwantitatieve metingen werden uitgevoerd door een pH micro-electrode in de agar te prikken na 3 uur contact met de bodem. Om de agar vrij te houden van bodemdeeltjes werd tussen de grond en de agar een nylon gaasje aangebracht. De geldigheid van de op deze manier bepaalde resultaten werd getoetst door een vergelijking met de standaard methode om bodem-pH te meten in een grond-water suspensie.

Met de agar-contact methode werd aangetoond dat het gunstig effect van kalkinhulling op de nodulatie van lucerne in de grond voor het grootste deel (80%) toe te schrijven was aan neutralizatie van de grond. De rest (20%) werd veroorzaakt door het inbrengen van calcium (Hoofdstuk 5). Om nodulatie te krijgen moet de bodem-pH tenminste 5.8 bedragen. Dit is het geval met kalkingehulde zaden. Onbehandelde lucerne wortels bleken vanaf dag 6 na het zaaien in staat te zijn om de pH tot dezelfde waarde te kunnen verhogen. De verhoging had echter geen nodulatie tot gevolg. Deze resultaten met in grond geteelde lucerne planten zijn in overeenstemming met eerdere proeven met in watercultuur gekweekte planten, dat het nodulatieproces juist in het begin (0 tot 3 dagen na het zaaien) geremd wordt door een lage pH.

De gebruikte zandgrond bevat reeds een geringe hoeveelheid calcium (1.2 meq per 100 g grond), en de bijdrage van calcium in het kalklaagje is slechts beperkt. Wanneer deze van nature aanwezige calcium werd weggevangen met EGTA, een specifieke calcium chelator, kon de kroonnodulatie worden teruggebracht tot 12% (Hoofdstuk 6). Een complicatie bij de chelering van bodem-calcium was een verzuring, waarbij de bodem-pH werd verlaagd van 6.1 naar 5.2. Dit probleem werd ondervangen door toepassing van blokjes agar met daarin EGTA, waarmee de wortel plaatselijk en tijdelijk kon worden behandeld. Op deze manier kon de nodulatie slechts gedurende de eerste dag na het enten worden geremd. Blijkbaar is zowel de behoefte aan calcium als ook de gevoeligheid voor lage pH beperkt tot de initiële fase van het nodulatie proces.

Curriculum vitae

De schrijver van dit proefschrift werd op 5 augustus 1958 geboren in het brabantse Haaren. Hij doorliep het Maurick College te Vught en begon in 1976 met de Landbouw Hogeschool Wageningen. Zijn bul werd in juni 1983 uitgereikt voor de specialisatie Bodemkunde en Bemestingsleer met als bijvakken Microbiologie en Organische Chemie.

In het najaar van 1983 vertrok de auteur voor een verblijf van 6 maanden naar Latijns Amerika. Na terugkeer werkte hij anderhalf jaar als leraar op de Middelbare Agrarische School in Oss. In april 1986 begon hij het onderzoek bij de vakgroep Microbiologie, waarvan de resultaten zijn verwerkt tot dit proefschrift. Sinds oktober 1989 werkt de promovendus bij het Centro de Investigación Agrícola Tropical in Santa Cruz, Bolivia, als resident expert bij het project Rhizobiología. Dit project houdt zich bezig met de productie van entstof voor leguminozen.