BIOLOGICAL NITROGEN FIXATION OF SOYBEAN IN ACID SOILS OF SUMATRA, INDONESIA

Setiyo Hadi Waluyo

Promotors: **Dr. Willem M. de Vos**
Hoogleraar in de Microbiologie

Dr. Leendert 't Mannetje
Hoogleraar in de Graslandkunde

Co-promotor: **Dr. Lie Tek An**
Universitair Hoofdocent bij de leerstoelgroep Microbiologie

Setiyo Hadi Waluyo

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Proefschrift

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Propositions

1. The use of lime-pelleted seeds is an efficient tool for soybean production in transmigration areas in Indonesia.

   This thesis

2. Inoculation of soybean is essential in transmigration areas outside Java, but not in Java.

   This thesis

3. Knowledge is important, but how to use our knowledge to solve a problem is even more important.

4. Many investigations are lost for years, if not forever, in the jungle of journals and tangle of tongues.

   W. J. Humprey cited by Fred *et al.* (1932), *in* Root nodule bacteria and leguminous plants. Madison, WI. University of Wisconsin, United State of America

5. Sandwich PhD scholarships consolidate the international position of Wageningen University.

6. Drinking coffee together is essential for the scientific and cultural exchange in the Netherlands.

7. There is no right or wrong way of doing things, but there are different ways.

   International Communication Workshop, Dean's Office for International Students, Wageningen University

8. One should not judge before one understands.

   International Communication Workshop, Dean's Office for International Students, Wageningen University

9. Bicycle is an essential transporter in The Netherlands, but it is not advisable to bike in Jakarta, Indonesia.

10. Weather is a daily hot topic for Dutch. It has also strong effects on human behaviour and on research activity.

Propositions attached to the thesis :
"BIOLOGICAL NITROGEN FIXATION OF SOYBEAN IN ACID SOILS OF SUMATRA, INDONESIA"

Setiyo Hadi Waluyo
Wageningen, The Netherlands, 6 November 2000

Chapter 1
Introduction

I. Soybean in Indonesia

I.1. The origin and status

Soybean (*Glycine max* Merrill) belongs to the family Leguminosae, subfamily Papilionoideae. Little is known about the origin and the early history of this crop. However, it is generally accepted that the species is native to Eastern Asia and has been known to occur in China, Manchuria and Korea. The first domestication of soybean was recorded in North China around the 11th century BC (Piper and Morse, 1923; Allen and Allen, 1981; Hymowitz and Newell, 1981).

In Indonesia, soybean is an old-established crop. Most probably soybean was introduced some hundreds of years ago through trade with China and Indo-China (Van der Giessen, 1932). Originally the plant was called Cadelium or Kadelee by Rumphius in 1747 (Piper and Morse, 1923), and its common Indonesian name now is Kedelai. The crop is found all over Java and Bali (De Vries, 1932; Brotonegoro *et al.*, 1986; Manwan and Sumarno, 1991; Anonymous, 1997).

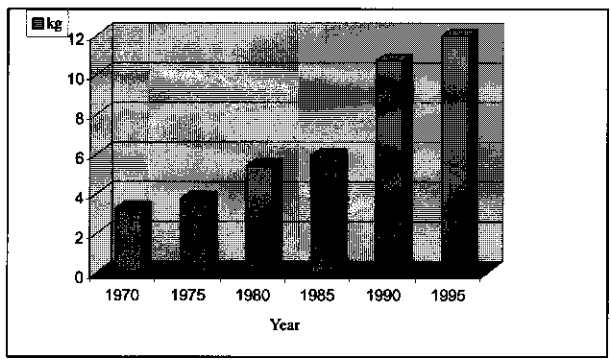

Figure 1. Consumption of soybeans per capita, 1970 to 1995 (Damardjati *et al.*, 1996).

Soybean, one of the major low-cost sources of protein, has already been consumed for centuries, and has become the second main food crop after rice in Indonesia. Notably, soybean consumption per capita has significantly developed during the last decades and more than tripled in the last 25 years (Fig. 1). Mostly, soybean is consumed as processed food products, such as Tempeh (fermented soybean), Tofu (a protein extract), Kecap (soybean sauce), and oil (Damardjati *et al.*, 1996). Consumption of Tempeh and Tofu increased on average 7.9 % per year. In addition, since 1986, the demand for soybean for soybean milk, kecap and oil has increased on average 3.7 % per year (Anonymous, 1998d). Moreover, there is an increasing tendency to use soybean as poultry feed. This all resulted in a total consumption of soybean of approximately 2 300 000 ton in 1997.

I.2. Production

The average yield of soybean in Indonesia is around 1.0 to 1.2 t ha^{-1}, which is quite low compared to the other soybean-producing countries such as Brasil, which produce around 2.0-2.5 t ha^{-1} (Manwan and Sumarno, 1991; Anonymous, 1998d). Recently, by intensifying existing production and the exploitation of new soybean growing areas, the production has been increased from 653 000 t in 1980 to 1 565 000 t in 1994 (CBS, 1995). However, as yet it has been impossible to meet the increasing consumption and hence a large amount of soybean has to be imported (Fig. 2). The import of soybean from 1991 to 1998 was constant, about 700 000 t per year, i.e. ± 50 % of the domestic production (Manwan and Sumarno, 1991; Damardjati *et al.*, 1996; Anonymous, 1998d). In 1995, US$252 million was spent to import 746 330 t of soybean seeds. In addition, in 1996, besides soybean seeds, other soybean-derived products and soybean stover were imported for US $265 million (Anonymous,

1998d). Therefore, to supply the domestic demand, it is vital to increase domestic production. However, there are many constraints for the cultivation of soybean, as discussed below.

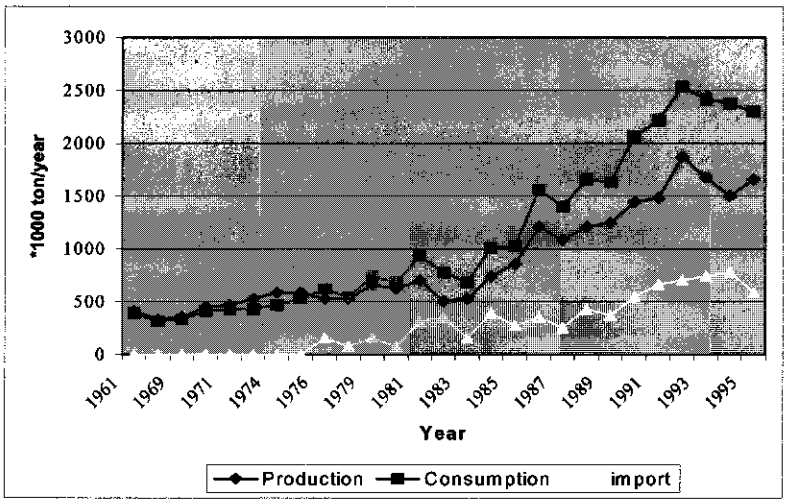

Figure 2. Consumption, production and import of soybean in Indonesia (adopted from Anonymous, 1998d).

The availability of suitable land is the main problem to increase soybean production in Indonesia. With 60 % of the total harvested areas of soybean and 62 % of total production, Java is the main region for soybean production (Anonymous, 1998b, 1998c). The soils on Java are fertile and favourable for soybean production. However, Java is over-populated, and expansion of the soybean area is only possible outside Java, especially in the transmigration areas Sumatra, Kalimantan, Sulawesi and Irian Jaya. Unfortunately, the soils in these areas are usually infertile, acid, and only suitable for soybean production after major improvement of soil fertility. These acid soils cover 48.3 millions hectares, or approximately 30 % of the total land area of Indonesia. These soils are characterised by a pH < 5, an excess of Aluminium (Al) ions, a deficiency in phosphate (P), a poor buffering capacity and are classified as Red

Yellow Podzolic soils (Sudjadi,1984; Adiningsih *et al.*, 1988; Wade *et al.*, 1988; Von Uexkull and Bosshart, 1989; Fig. 3).

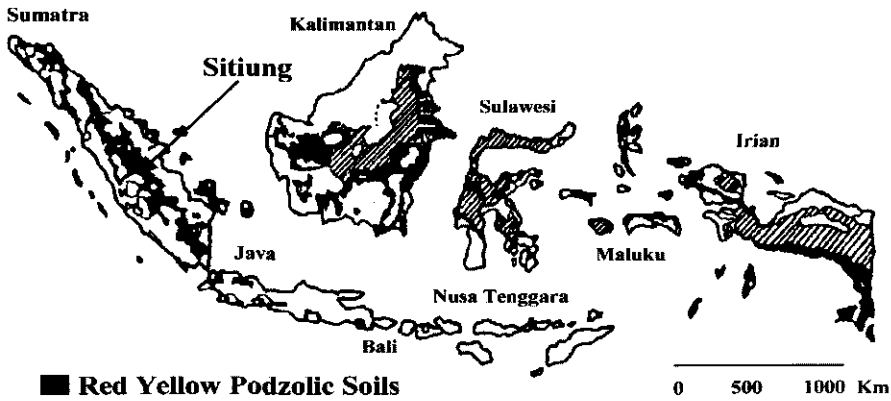

Figure 3. Regional distribution of Red Yellow Podzolic soils in Indonesia (After Driessen and Soepraptohardjo, 1974).

One of the transmigration areas is Sitiung, located in West Sumatra. This area has been populated by transmigrants since 1976, and was transformed into a successful upland rice-soybean production system with the judicious use of lime and fertilisers (high-input technology). However, production of soybean in this area is only possible with the input of large amounts of fertilisers and lime (Wade *et al.*, 1988; see Table 1). Therefore, liming is a compulsory basal treatment of farming in

Table 1. Some properties and characteristics of four representative Sitiung acid soils in West Sumatra, Indonesia (Extracted from Wade *et al.*, 1988).

Parameter	Sitiung I-A	Sitiung IV-D	Sitiung II-E	Sitiung V-C
Clay (%)	74.5	67.0	89.0	49.0
Silt (%)	20.0	13.5	6.5	11.5
Sand (%)	6.5	19.5	4.5	39.5
Organic C (%)	2.0	3.05	1.35	2.45
Exchangeable Calcium (cmol/L)	0.2	0.35	0.1	0.45
Exchangeable Magnesium (cmol/L)	0.05	0.1	0.05	0.25
Exchangeable Potassium (cmol/L)	0.15	0.05	0.05	0.15
Exchangeable Aluminium (cmol/L)	3.95	3.8	3.05	3.05
ECEC (cmol/L)	4.5	4.3	3.25	3.95
Aluminium saturation (%)	87	90	94	80
pH ($H_2O/CaCl_2$)	4.5/4.1	4.0/3.7	4.0/3.8	4.0/3.8

these areas. To stimulate the farmer, 3.0 to 4.0 tons of lime ha^{-1} were provided free of charge. In addition, the government supported the transmigrants by providing free food, seeds, basic farm implements, fertilisers and pesticides in the first year. Heavily subsidised fertilisers were also provided in the following year. It was reported that US$ 19 million was budgeted for free lime to the farmers to increase soybean production between 1983 and 1986. Since the soil is low in nitrogen (N), the farmer still has to use a high rate of N and P fertilisers to obtain maximum soybean yield. This is uneconomic and cannot be adopted by the poor farmers from Java.

II. Biological nitrogen fixation (BNF)

The importance of legume crops in agriculture has been recognised for several thousand years and is due to the presence of N-fixing bacteria, also known as nodulating rhizobia, in legume root nodules, as first demonstrated by Beijerinck (1888). Besides being essential as a source of cheap protein for human nutrition and animal feed, legumes in symbiosis with rhizobia are essential in crop rotation to maintain soil fertility.

Global N fixation has been estimated to amount to around 175 million metric ton per year (Burn and Hardy, 1975; Brockwell *et al.*, 1995). Increased plant protein levels are an obvious consequence of N fixation. Amounts of N fixed ranging between 234 and 643 kg /ha/year have been recorded (Bergersen *et al.*, 1985). BNF can replenish soil N lost by leaching, assimilation and uptake by crops. Table 2 shows how levels of soil N were maintained, when oats and well-nodulated, abundantly N-fixing soybeans were alternated. The crops removed 630 kg N/ha in 3 years (Brockwell *et al.*, 1995). In USA, Burton and Curley (1965) reported that soybean yield was increased from 1 008 t to 2 084 t per ha due to inoculation.

Table 2. Effect on soil N of 3 years of double-cropping with oats and well-nodulated soybeans (Brockwell *et al.*, 1995).

	N removed in biomass of oats and in seed of soybean ($kg\ ha^{-1}$)	Residual soil N	
		Total N ($g\ kg^{-1}$)	Mineral N ($mg\ kg^{-1}$)
Prior to oat crop 1		1.38	30.3
Oat crop 1	107.9	1.24	3.4
Soybean crop 1	174.2	1.32	14.4
Oat crop 2	20.8	1.29	4.3
Soybean crop 2	156.6	-	-
Oat crop 3	33.5	1.18	6.9
Soybean crop 3	137.2	1.34	6.5
Total for 6 crops	630.2		

In Indonesia, yield increases due to BNF have often been reported (De Jongh, 1943; Keleney, 1959; Darmawan, 1987; Brotonegoro *et al.*, 1987). Soybean yield increases of 13 to 215 % were obtained in experiments conducted in Java using N-fixing bacteria (De Jongh, 1943). Subsidised by the Indonesian government, a *Bradyrhizobium* inoculant, termed LEGIN (Legume Inoculant), was produced as part of a soybean intensification programme (INSUS, Intensifikasi Khusus, a special intensification) (Jutono, 1987, 1989). A total of more than 68 000 kg of peat-based *Bradyrhizobium* inoculant was distributed free of charge to farmers participating in the soybean intensification programme in transmigration areas between 1983-1986 (Sebayang and Sihombing, 1987).

Following the INSUS programme, the use of rhizobia inoculants for soybean production became very popular. Sindhoesarojo (1989) estimated that around 125 to 220 ton $year^{-1}$ of soybean-rhizobia inoculants were needed in the years 1989-1993. Besides LEGIN, other soybean inoculants, mainly consisting of *Bradyrhizobium* strains, were also available, e.g. NITRAGIN imported from the USA and RHIZOGIN

from the Bogor Agricultural Institute (IPB). Recently, an inoculant, called RHIZOPLUS, has been introduced for cultivation of soybean on 3000 ha of agricultural land by the Department of Agriculture of Indonesia (Anonymous, 1998a). However, the response of soybean yields to *Bradyrhizobium* inoculants was found to be rather variable. Pasaribu *et al.* (1989) reviewed the effect of the inoculation by *Bradyrhizobium* strains on the yield of local soybean varieties. Based on the seed yield, only 1 out of 11 experimental sites did respond to the inoculation practice. In addition, Sunarlim (1987) reported for a field experiment in West Java that the number of nodules was not increased by the inoculation of imported *Bradyrhizobium* strains. In contrast, Brotonegoro *et al.* (1987) reported that in newly opened land in Tegal, Java, inoculation with *Bradyrhizobium japonicum* significantly increased the yield of soybean. Using the standard inoculant strains *Bradyrhizobium japonicum* USDA 110 and CB 1809 as well as a local isolate (FCB 26, isolated in Lampung, South Sumatra) Simanungkalit *et al.* (1996) found that a positive effect of inoculation was obtained on the number of nodules and yields of soybean (cv. Wilis) in South Sumatra and North Sumatra, but not in Bogor Java. More studies on BNF have been conducted in areas that have never been cultivated with soybean (Adiningsih and Prihatini, 1981; Mahmud and Rumawas, 1983; Setijorini, 1985; Saraswati, 1986; Supriati, 1987; Hendratno *et al.*, 1995; Simanungkalit *et al.*, 1996). Mostly, inoculants are applied to crops grown on acid soils in conjunction with a liming and fertilisation programme of the Indonesia government. In a green-house experiment with acid soil from Sitiung, West Sumatra, inoculation of soybean seeds (cv. Orba) with an inoculant from NifTAL (University of Hawaii, Honolulu, USA) increased the number of nodules on plants grown on limed soils (Adiningsih and Prihatini, 1981). Using a quite similar soil, but with a different soybean cultivar (cv. Clark 63) and an

inoculant SEMIA 5019 from Brasil, Mahmud and Rumawas (1983) reported unsatisfactory results on nodulation. More recently, Hendratno *et al.* (1995) reported that a remarkable positive effect on nodulation and yield was obtained with the inoculation of *Bradyrhizobium japonicum* on soybean grown in acid soils at Palembang, South Sumatra.

The obvious conclusion from all these reports has been that inoculation of soybean is not necessary in areas already cultivated with soybean for a long period but may be essential in new areas that have no history of soybean cultivation (Toxopeus, 1938; Keleney, 1959; Sunarlim, 1987; Pasaribu *et al.*, 1989).

III. Soil acidity

The soils in Sitiung are very acid, and low in nutrients like Ca and P. Moreover, besides having a high level of Al, these soils are also known to have a strong P-fixing capacity. These factors may affect the plant, the bacteria or both, as well as their symbiosis.

III.1. Plant growth

The soybean plant is very sensitive to soil acidity and factors related to acid soils. Besides soil pH, Al toxicity and the lack of P and Ca are the main restricting factors for growth and yield of soybean on acid soils (Kamprath,1978; Sudjadi, 1984; Bell and Edwards, 1987; Wade *et al.*, 1988). Application of lime raises soil pH (Danso, 1977; Mengel and Kamprath, 1978), and reduces Al toxicity (Sartain and Kamprath, 1975; Carvallo *et al.*, 1981a; Munns *et al.*, 1981; Foy, 1984; Murphy *et al.*, 1984; Alva *et al.*, 1987a, 1987b). Liming also increases Ca and P availabilities (Danso, 1977; Andrew, 1978; Alva *et al.*, 1987a, 1987b).

The solubility of Al is pH dependent and in acid soils Al is present in excess. The percentage of Al saturation in acid soils can be more than 60 % whereas the critical level of Al saturation for soybean is already reached at 15 to 20 % (Kamprath, 1984; Wade *et al.*, 1988; Table 1). The main beneficial effect of liming is to reduce the solubility of Al below the critical level for soybean.

In highly weathered acid soils P deficiency is very common. When Al is present in excess, aluminium-phosphate is a common form of P in acid soil, which is unavailable for soybean plant. Increasing the availability of P, either indirectly by liming or directly by P fertilisation is essential for soybean cultivation in acid soils. Therefore, high amounts of P fertiliser are often applied to obtain optimum yield of soybean on acid soils (Cassman *et al.*, 1981c; Sudjadi, 1984; Wade *et al.*, 1988; Hendratno *et al.*, 1995). Increased P availability in acid soils after liming, is one of the reasons for a better growth of soybean plants. Additional application of P may increase yield more e.g. application of 485 kg TSP ha^{-1} yielded more than double that obtained by the application of 2.0 t of lime ha^{-1} in acid soils (Sudjadi, 1984).

Phosphate is an essential plant nutrient, and plays a specific role in promoting root growth and branching. Application of P to soybean increased root surface area per plant and also per gram of root (Hallmark and Barber, 1984; Borkert and Barber, 1985). As the root system is enlarged by P fertilisation, the number of infectible-root sites is increased as well, increasing the chance for rhizobia to infect the root.

III.2. Bradyrhizobium

For the success of BNF, the presence of rhizobial strains, mostly *Bradyrhizobium* spp., with a high N fixing capacity is essential. A high growth rate and ability to colonise the rhizosphere are also essential. It was reported that in

synthetic media, the major limiting growth factors for rhizobia are low pH, Al toxicity, and low P (Keyser and Munns, 1979a, 1979b; Coventry and Evans, 1989; Brockwell *et al.*, 1995). Low pH limits the growth and the survival of rhizobia in acid soils. There is often no multiplication of an introduced *Bradyrhizobium* strain in acid soils. The cell number even declines and this may lead to the failure of nodule formation, while to ensure good nodulation, a large number of cells is needed (Mulder *et al.*, 1966; Danso, 1977; Brockwell *et al.*, 1985). This is the challenge working with N-fixing plants on acid soils. Limitation of BNF on acid soils may be due to the lack of attachment of bacterial cells to roots, or other factors, ranging from direct effects of low pH on rhizobia multiplication to indirect problems associated with the soil nutrient status (Mulder and van Veen, 1961; Dowling and Broughton, 1986; Graham, 1992).

Aluminium is recognised to have a negative effect on growth of rhizobia (Andrew, 1978; Keyser and Munns, 1979a, 1979b; Carvalho *et al.*, 1981a,b, 1982; Franco and Munns, 1982; Hartel and Alexander, 1983; Foy, 1984; Wood and Cooper, 1988; Graham, 1992). It was found that the growth of rhizobia was reduced on either synthetic liquid media or nutrient solution containing Al (Keyser and Munns, 1979a, 1979b; Carvalho, 1981a,b; Hartel and Alexander, 1983). Alleviation of Al by liming acid soils may influence the survival and the growth of introduced rhizobia, and allow nodule formation to proceed naturally (Danso, 1977).

The deficiency of P in acid soils may affect the presence and growth of rhizobia. Therefore, the availability of P, due to liming or P fertilisation, may have a positive effect on rhizobia, nodulation and BNF of the plant (Fig. 4).

In synthetic media, the growth rate of rhizobial strains was reduced by low levels of P. However, the requirement, and the capacity to store and use P are markedly different among rhizobial strains (Cassman *et al.*, 1981a,b; Smart *et al.*, 1984; Beck and Munns, 1984; Singleton *et al.*, 1985). The effectiveness of rhizobial populations in acid soils seems to be limited by the deficiency of P, as well. Singleton *et al.* (1985) observed that there was no relation between P and the effectiveness of *Bradyrhizobium* strains. However, they showed differences among *Bradyrhizobium* strains in soybean response to P. *Bradyrhizobium* strains which are equally effective at moderate P fertilisation, differed substantially when P supply was high in a highly P-fixing Hawaiian ultisol. It was also reported that there was a relation between the tolerance of Bradyrhizobia to low P and in the ability of those bacteria to nodulate soybean (Beck and Munns, 1984).

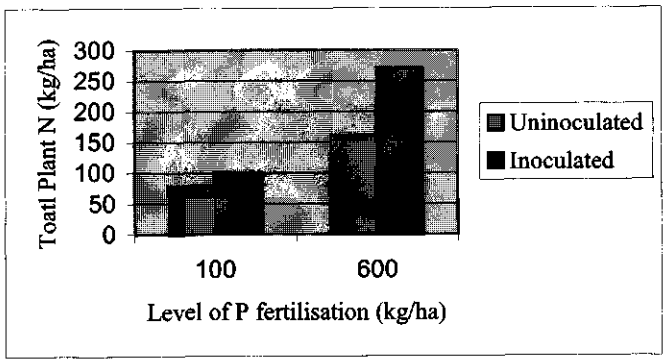

Figure 4. Total N accumulation of inoculated and uninoculated soybeans at two levels of P fertilisation (Anonymous, 1993).

Phosphate is not only essential for growth of the plant and *Bradyrhizobium* but also for the growth and functioning of the nodule (Graham and Rosas, 1979; Cassman *et al.*, 1981c; Beck and Munns, 1984; Leung and Bottomley, 1987; Mullen *et al.*, 1988; Israel, 1993).

III.3. BNF in acid soils

Soil acidity and its related aspects are presumably the most important factors limiting N fixation in the symbiosis between soybean and rhizobia. Acidity itself was suggested to interfere in the initiation of nodulation. Once the nodule is initiated, it will further develop regardless of low pH (Munns, 1968; Lie, 1969).

In acid soils Al toxicity is the limiting factor for BNF. Due to excess of Al, plants grow very poorly, the root systems are poorly developed, they form little fine-branching roots, which may result in a low number of infection sites, and therefore limit the infection process of the *Bradyrhizobium* (Carvalho *et al.*, 1982; Brady *et al.*, 1990; Hecht-Buchholz *et al.*, 1990). Decreasing Al saturation in acid soils by liming, increased number of soybean nodules (Sartain and Kamprath, 1975)

Fertilisation with super-phosphate in acid soils has been shown to improve nodulation of subterranean clover plants and other legumes (Hastings and Drake, 1960). P is essential for the growth and functioning of root nodules (Gates and Muller, 1979; Cassmann *et al.*, 1980; Singleton *et al.*, 1985; Israel, 1987). Gates (1974) showed that the beneficial effect of P on nodulation is probably not entirely due to an increase of plant vigour. Diatloff and Luck (1972) reported that the major effects of P in acid soils are on growth and nodulation of soybean. Singleton *et al.* (1985) found that the dry weight and nitrogenase activity of soybean nodules were significantly increased by P additions. More recently, it was shown by Israel (1993) that P supply has an indirect effect on host-plant growth and more direct effects on the metabolic function of nodules.

IV. Improvement of soybean cultivation in acid soils

IV.1. Liming the soils

The practice of liming has long been recognised as a way to increase soybean production on acid soils. The added lime neutralises soil acidity, decreases the toxicity of Al and increases the availability of some nutrients such as P. The technique is the simplest and fastest to increase growth and production of soybean in weathered acid soils. In the case of the Sitiung soils, lime applications from 4 to 7.0 t of ha^{-1} costing around US$350 ha^{-1} are needed for soybean production annually. High rates of P fertiliser are often found to be an effective way to obtain a realistic yield of soybean on acid soils (Sudjadi, 1984; Wade *et al.*, 1988). Application of ±15 kg P ha^{-1} yielded soybean similar to that obtained by the application of 2.0 t of lime ha^{-1} in acid soils (Sudjadi, 1984). On the other hand, there are also negative reports of liming heavily acid soils to neutral reaction. Decreased P availability (Kamprath, 1971; Amarasiri and Olsen, 1973) and induced deficiencies of micro-nutrients, particularly Bo, Zn, and Mn (Kamprath, 1971; Pearson, 1975; Munns, 1976; Sanches, 1976; Foy, 1984; Kamprath, 1984; van Uexkull and Bosshart, 1989; Bottomley, 1992) have been reported as a results of heavy liming of acid soils. However, application of high levels of P fertiliser is economically not feasible and cannot be afforded by the farmers.

IV. 2. The use of seed pelleted with $CaCO_3$ and TSP, $Ca(H_2PO_4)_2$

There is a need to develop an appropriate technique allowing optimal utilisation of natural resources, by decreasing the amount of lime and P to an acceptable level in accordance with the means of the farmers. By applying low amounts of lime close to the seed as seed-pellet, instead of broadcasting lime to the soils, the amount of lime can be reduced to less than 100 kg ha^{-1}. Lime-pelleting

legume seeds has been developed as a cheap method in Australia and has been successfully used to improve BNF of legume crops in acid soils. Lime pelleting the seeds increased weight and number of nodulated of subterranean clover plants (Loneragan *et al.*, 1955; Hasting and Drake, 1960; Jones *et al.*, 1967), increased yields of soybean cv. Bragg and plant weight of perennial soybeans, Tinaroo and Desmodium (Diatloff and Luck, 1972; Elkins *et al.*, 1976; Danso, 1988), increased number of nodulated lucerne plants (Mannetje, 1967; Pijnenborg and Lie, 1990).

Lime-pelleted seed gives a protection of rhizobia on the inoculated seeds from the toxicity of acid soils (Hastings and Drake, 1960; Kang *et al.*, 1977). In addition, lime-pelleted seeds can neutralise the micro-environment in the close vicinity to the seedling. This is crucial in establishing BNF. The success of lime pelleting seeds is because the most sensitive-step in BNF to acidity is the infection (Munns, 1968; Lie, 1969), and because the susceptibility of the root-sites for infections are transient (Bhuvaneswary *et al.*, 1980). The germinating seeds and the emerging roots are protected, and nodule formation and N fixation can proceed naturally.

Although liming and P fertilisation can overcome the problems of acid soils, the critical steps that are influenced by such treatments can be variable, depending on the type of soil. Therefore, in this study, the effect of lime addition on growth and nodulation of soybean in Sitiung acid soils are being investigated. Application of a small amount of lime mixed into the top-soil and lime-pelleting of soybean seeds have been investigated in the field, in green house (pot) and in laboratory (rhizotron) experiments. In general, for soybean cultivation, 100 kg P_2O_5 were applied by broadcasting in the field, and this level still has to be increased if a reasonable yield of soybean is expected in acid soils. To reduce this amount, a small amount of P (10 kg TSP ha^{-1}, equivalent to 5 kg P_2O_5) was also included in the pellet.

V. Population structure of soybean rhizobia native to Indonesia

An important aspect for the success of BNF is insight in the structure of indigenous soybean rhizobia populations. This is particularly the case in areas where indigenous ineffective but competitive rhizobia are abundant (Ham *et al.*, 1971). There is a general lack of information about the population structure of indigenous soybean rhizobia native to Indonesian soils. As a consequence, a thorough survey is needed of the occurrence of the bacteria in different locations in Indonesia. Furthermore, it is essential to characterise the isolates using reliable molecular methods. This opens the possibility to select elite indigenous soybean rhizobia under favourable conditions, such as those on Java where they are abundantly present in most soils. In addition, under acid conditions, such as those in Sumatra, which are unfavourable for rhizobia, this survey may be important for the selection of rhizobia adapted to stress conditions. The susceptibility of rhizobia to acidity and related factors is distinctly variable. This permits the isolation of native rhizobia from acid soils by screening acid-tolerant rhizobia on synthetic media (Date and Halliday, 1979; Keyser and Munns, 1979a, 1979b). In general, in acid soils, rhizobia are low in number and are scattered especially in niches of the soils which are not harmful for them (Richardson and Simpson, 1988). This is presumably the reason for the presence of nodules on uninoculated soybean plants grown on acid soils after liming (Adiningsih and Prihatini, 1981; Mahmud and Rumawas, 1983; this study). Continuous cultivation of soybean crops and amelioration of the soils by liming and fertilisation may increase the number of the indigenous strains. For instance, Richardson and Simpson (1988) reported that liming acid soils increased the population density of indigenous *Rhizobium leguminosarum* bv. *trifolii* in pasture either planted or not planted with the legume host. There are some reports that many indigenous rhizobia, which are presumably adapted to local legumes, are not very

effective, or even ineffective, on modern legume cultivars, and after reaching a high level may cause a problem for rhizobia inoculation practices (Thies *et al.*, 1991).

The isolation and examination of soybean rhizobia native to Indonesian soils has already been reported more than 50 years ago (Toxopeus, 1936; 1938). It was also recognised that the rhizobia population varied largely with the location (De Jongh, 1941). Following the increasing demand for rhizobial inoculants, the study of indigenous strains has been accelerated. At the request of the government of Indonesia, LEGIN was produced and introduced to farmers in the transmigration areas outside Java (Jutono, 1987). In the last few decades several rhizobial strains from abroad have been introduced, including USDA 110 and TAL 102 from the USA, CB1809 from Australia, SEMIA-5019 from Brazil, and FA-3 and SA-1 from France. In one of the first systematic surveys, more than 164 strains were isolated from local and imported soybean varieties grown in the region of Yogjakarta (Jutono, 1984). In addition, Rumawas and Rumawas (1989) have isolated several soybean rhizobia from the region of Bogor. More than 150 rhizobial strains isolated from several locations in Java and Sumatra and imported from abroad are presently maintained in the Sukamandi Research Institute (Saono, 1988). However, little is known about the taxonomy of these rhizobial strains. Following the reports of *Bradyrhizobium japonicum* strains isolated from several places in Java (Toxopeus, 1936; de Jongh, 1941; Keleney, 1959; Newton, 1962) only two papers reported the occurrence of other *Bradyrhizobium* spp. (*B. elkanii*) and *Sinorhizobium fredii* in Java and Sumatra (Ozawa *et al.*, 1995; Anonymous, 1998e). This contrasts with the large number of reports describing rhizobia, elsewhere in the world, that nodulate soybean (Keyser *et al.*, 1982; Scholla and Elkan, 1984; Dowdle and Bohlool, 1985; Jarvis *et al.*, 1992; Xu *et al.*, 1995). By using molecular methods the identification and classification of rhizobia have been improved significantly (Young,

1994, 1996; Young and Hauke 1996). It is therefore a great challenge to apply this new technology to characterise the largerly unknown rhizobial communities in Indonesia.

V. Molecular taxonomy of Brady- and Sinorhizobia nodulating soybean

Bacteria nodulating leguminous plants were first described in 1888 (Beijerinck, 1888) and their initial classification as rhizobia was based on their host range specificity. In recent years, other methods, including phenotypic traits, DNA:DNA relatedness and molecular techniques based on Polymerase Chain Reaction (PCR), have been included in rhizobial classification. Presently, there is a great variety of phenotypic and genotypic methods available that permit a different degree of phylogenetic classification varying from the genus, species, subspecies, biovar to the strain level (Fig. 5).

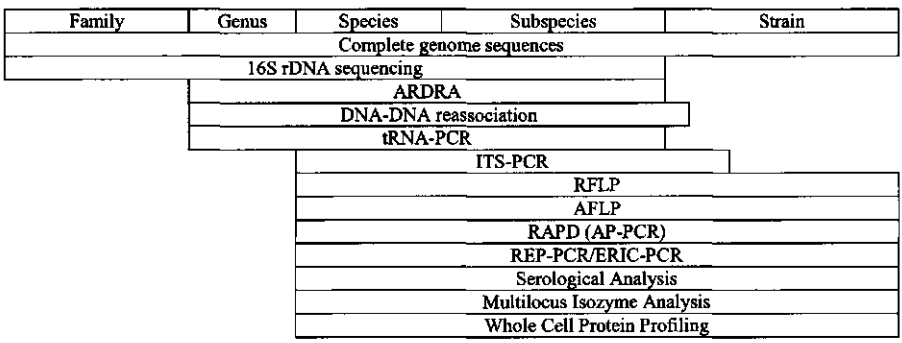

Figure 5.Relative resolution of various fingerprinting and DNA techniques (modified from Rademaker and de Bruijn, 1998). The following abbreviation were used : ARDRA : amplified ribosomal DNA restriction analysis; tRNA : transfer RNA; ITS : intergenic spacer; RFLP : restriction fragment length polymorphism; AFLP : amplification fragment length polymorphism; RAPD : random amplified polymorphic DNA; AP-PCR : arbitrary primer PCR; REP : repetitive extragenic palindromic; ERIC : enterobacterial repetitive intergeneric consensus.

However, the description of new genera and species of root-nodulating rhizobia should fulfill a minimal standard, as has been proposed by Graham *et al.* (1991) and Novikova (1996). The development and implementation of these molecular techniques have accelerated the taxonomic evaluation of rhizobia and the current

classification is mainly based on the nucleotide sequences of the small sub-unit ribosomal RNA (rRNA) and includes *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* spp. (Fig. 6). Several species belonging to the latter three genera have been found to nodulate soybean, and in some cases other plants, as is summarised in Table 3.

Table 3. Recognised species of *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* that nodulate soybean and their nodulation of other plants.

Genus	Species	Host Plants	Reference
Bradyrhizobium	*B. japonicum*	*Glycine* spp.	Jordan, 1982
	B. elkanii	*Glycine* spp.	Kuykendall *et al.*, 1992
	B. liaoningensis	*Glycine* spp.	Xu *et al.*, 1995
Sinorhizobium	*S. fredii*	*Glycine* spp.	Scholla and Elkan, 1984;
		Albizia lebbeck	de Lajudie *et al.*, 1994
		Indigofera tinctoria	
Mesorhizobium	*M. thianshanense*	*Glycine* spp.	Chen *et al.*, 1995; Jarvis
		Glycyrrhiza spp.	*et al.*, 1997
		Sophora spp.	
		Caragana spp.	

A study on soybean rhizobia by Kirchner in 1895 (cited by Fred *et al.*, 1932) described that soybeans did not form nodules in the garden at Hohenheim in Germany, while several other leguminous plants did form nodules. Contrary to that, soybean plants that were grown in soil from Japan successfully produced nodules. Based on this host specificity, it was concluded that the soybean rhizobia are distinct from the other nodulating rhizobia, and were designated as *Rhizobium japonicum* (Fred *et al.*, 1932). Further classification of the legume nodulating rhizobia showed that they could be divided into two groups, the fast- and slow-growing rhizobia (Jordan, 1982). A new genus, *Bradyrhizobium*, was proposed for slow-growing strains specific for many tropical legumes. The fast-growing rhizobia isolated from soybean nodules collected from China (Keyser *et al.*, 1982) were classified as *Sinorhizobium* (Scholla and Elkan, 1984). Recently, a new genus *Mesorhizobium* was proposed to include *M.*

thianshanense, which can be obtained from a variety of legumes such as soybean, *Glycyrrhiza pallidiflora*, *Sophora alopecuroides* and *Caragana polourensis* (Chen *et al.*, 1995; Jarvis, 1997; Table 3).

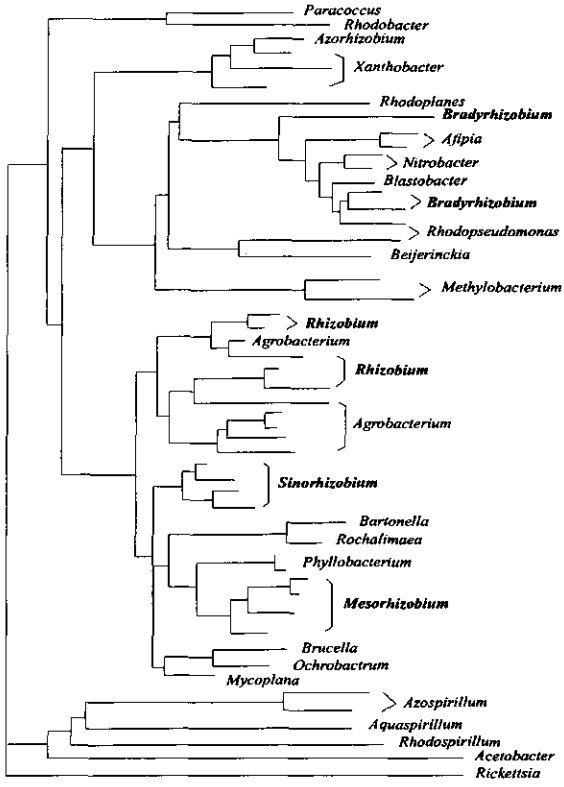

Figure 6. Phylogenetic tree of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* and related bacteria in the α subdivision of the *Proteobacteria* based on aligned sequences of the small-subunit rRNA genes (after Jarvis *et al.*, 1997).

The symbiotic and physiological properties of soybean rhizobia have been found to be more diverse than originally anticipated. The symbiotic relation with the host plants remains very important, since this is the most conspicuous feature of rhizobia and has an important practical value. The discovery of fast-growing soybean rhizobia,

which belong to genus *Sinorhizobium*, underlines the need to consider the symbiotic properties, in particular, since representatives of this genus often fail to nodulate modern soybean cultivars. In addition, there are some rhizobia outside the genus *Bradyrhizobium* and *Sinorhizobium*, now designated *Mesorhizobium* spp., which were reported to form nodules on soybean (Chen *et al.*, 1995; Jarvis, 1997).

The ambiguous results that are often found with host-range nodulation tests have driven the development of determination based on DNA techniques (see Fig. 5). These have been applied to a variety of rhizobia and include serological analysis (e. g. Date and Decker, 1965), DNA-DNA hybridisation (e. g. Hollis *et al.*, 1981; Kuykendall *et al.*, 1992), REP and ERIC PCR, as well as RAPD (e. g. De Bruijn, 1992; Dooley *et al.*, 1993; Judd *et al.*, 1993; Sikora *et al.*, 1997), RFLP (e. g. Laguerre *et al.*, 1994), and sequence analysis of 16S rDNA (e. g. Willems and Collins, 1993; De Lajudie *et al.*, 1994).

Serological techniques, such as immunofluorescence and immunodiffusion, are based on the antigenic uniqueness of microorganisms. There are three groups of recognised antigens for *Rhizobium*, the somatic (cell wall, O), flagellar (H) and capsular (K) antigens (Somasegaran and Hoben, 1995). Serological techniques have been used to identify *Rhizobium japonicum* and *Sinorhizobium fredii* and are widely used for competition studies (Date and Decker, 1965; Sadowsky *et al.*, 1987). Immunological analysis revealed *Bradyrhizobium japonicum* serogroup 123 to be a predominant and very competitive strain in the USA (Ham *et al.*, 1971). Achmad *et al.* (1981) also used this approach to study the diversity of cowpea rhizobia.

Based on DNA-DNA relatedness, Hollis *et al.* (1981) classified slow-growing soybean rhizobia into 2 groups, group I/Ia and group II. Although this method is still in use and essential for describing new bacterial species, at present PCR- based techniques

play a more important role in the characterisation and identification of bacteria, mainly at the strain level.

A specific PCR technique, the Random Amplified Polymorphic DNA (RAPD) analysis, was introduced by Williams *et al* (1990). In RAPD, a PCR-amplification of polymorphic DNA sequences is performed using short arbitrary oligonucleotide primers. Subsequently, the PCR product is separated by gel electrophoresis and analysed either manually or using a computer programme. This approach has the capability to distinguish related strains. Since arbitrary primers are used, RAPD is also known as AP-PCR. Other PCR-techniques are REP- and ERIC-PCR. These are based on the use of primers complementary to repetitive sequences in the genome. These sequences were shown to be highly conserved and widely distributed within bacteria. Using this technique De Bruijn (1992) could distinguish and classify *R. meliloti* strains. It was shown that ERIC and REP sequences are present and highly conserved in rhizobia. This technique had been used successfully to distinguish the genetically and phenotypically nearly identical *Bradyrhizobium* strains of serogroup 123 (Judd *et al.*, 1993).

Bacterial strains can also be characterised by another PCR-based technique, termed Amplified Ribosomal DNA Restriction Analysis (ARDRA). This method is based on the principle that the restriction sites in the RNA operon are conserved and reflect the phylogenetic relationship (Masol-Deya *et al.*, 1995). It involves the use of a pair of universal primer sequences for PCR amplification of either 16S rRNA genetic loci or the intergenic spacer (IGS) of the 16S and 23S rRNA genes. The 16S and 16S-23S PCR products are subjected to restriction endonuclease digestion separated by gel electrophoresis and resulting in a genomic finger-printings that may be used for the identification of bacterial genomes at the species and some times even at the strain

level. Since the bacterial IGS contains non-coding DNA and a tRNA gene, this 16S-23S spacer region gives more restriction length variation than the 16S rDNA in the ARDRA. The ARDRA technique has been used successfully to identify and characterise several rhizobial strains (Laguerre *et al.*, 1994; Selenska-Pobell *et al.*, 1996; Vinuesa *et al.*, 1998). However, the discrimination power of ARDRA is less than that of AP- and REP-PCR, notably since it is based on the diversity of a single genomic locus, while in the latter approaches are targeting the diversity of the whole genome. Subsequent development of molecular techniques allowed for further refinement of the taxonomy of soybean nodulating rhizobia and based on the sequence of the 16S rRNA gene, soybean rhizobia could be grouped into two genera, *Bradyrhizobium* (Kuykendall *et al.*, 1992) and *Sinorhizobium* (De Lajudie *et al.*, 1994). Recently, another genus, *Mesorhizobium*, has been proposed as a new genus for a group of soybean nodulating rhizobia (Chen *et al.*, 1995) (see Table 3 and Fig. 6).

The genus *Bradyrhizobium* consists of two species, *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. This is based on DNA hybridisation probes described by Kuykendal *et al.* (1992) and confirms the grouping proposed by Hollis *et al.* (1981). *Bradyrhizobium japonicum* belongs to group I/Ia, which is very host-specific and forms nodules only on soybean. *Bradyrhizobium elkanii* represents group II, which is very distinct from *Bradyrhizobium japonicum*, and forms nodules on both soybean as well as cowpea plants. Recently, a new group of slow-growing soybean bacteria, *Bradyrhizobium liaoningensis*, was described by Xu *et al.* (1995). Based on DNA homology, strains belonging to this new species are different from *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium elkanii* USDA 76. Sikora *et al.* (1997) also found that indigenous strains of *Bradyrhizobium japonicum* from soybean-producing

areas of the Republic of Croatia were different from the strains that are used as regular inoculants.

The genus *Sinorhizobium* contains a fast-growing strain nodulating soybean, *S. fredii*, which is considered to be specific to soybean gene centre regions, such as China, and other countries in Asia and South East Asia (Keyser *et al.*, 1982; Scholla and Elkan, 1984; Stower and Eaglesham, 1984; Dowdle and Bohlool, 1985; Chamber and Iruthayathas, 1988; Jarvis *et al.*, 1992). This fast-growing strain differs from *Bradyrhizobium* spp. by several genetic, biochemical, physiological and symbiotic properties (Sadowsky *et al.*, 1983; Stower and Eaglesham, 1984). The mean generation time of this species ranges from 1 - 4 hours, whereas *Bradyrhizobium* strains have mean generation times in excess of 6 hours (Jordan, 1982). Fast-growing strains utilise a greater assortment of carboydrates than the slow-growers (Sadowsky *et al.*, 1983). This new species *S. fredii* can be differentiated from *Bradyrhizobium* spp. by the sensitivity to various antibiotics (Dowdle and Bohlool, 1985). It has also broader host-range specificities than *Bradyrhizobium* spp. (Stowers and Eaglesham, 1984). Devine (1985) reported that at least 80 % of 285 introduced soybean plants (*Glycine max*) from Asian countries were nodulated effectively by *S. fredii* strain USDA 205. This strain USDA 205 is rather exotic since it has a broad host-range and can form nodules and fix N with several legumes in addition to soybean. This strain formed effective nodules on soybean, cowpea, pigeon pea but formed ineffective nodules on siratro, phasey bean and mungbean (Scholla and Elkan, 1984). *S. fredii* strain NGR 234 is even more unusual. This strain was isolated from *Lablab purpureus* but formed nodules on at least 37 genera of legumes and the non-legume tree *Parasponia* (Jarvis *et al.*, 1992). Other *S. fredii* strains such as strains USDA 257 and USDA 191 are highly specific for only certain soybean cultivars (Keyser *et al.*, 1982; Hattori and Johnson,

1984; Heron and Pueppke 1984). In addition, there are also many *S. fredii* strains that can effectively nodulate several modern soybean varieties (Hattori and Johnson, 1984; Dowdle and Bohlool, 1985; Lin *et al.*, 1987). Chamber and Iruthayathas (1988) and Young *et al.* (1988) also reported the ability of *Sinorhizobium fredii* to nodulate modern soybean variety Fiskeby V and Clark, from Europe and USA respectively. Rodriquez-Navarro *et al.* (1996) isolated promiscuous fast-growing soybean rhizobia from Vietnam, which are likely to be different from *Sinorhizobium fredii* USDA 257. In addition, it was reported that fast-growing growing soybean nodulating strains have low levels of DNA-DNA homology with *B. japonicum* (Scholla *et al.*, 1984).

Since the methods are rapidly developing and new rhizobial strains are being isolated, the phylogeny and taxonomy of nodulating bacteria is changing and has been reviewed extensively in recent years (Willems and Collins, 1993; Yanagi and Yamasato, 1993; Martinez-Romero and Caballero-Mellado, 1996; Novikova, 1996; Young, 1996; Young and Hauke, 1996).

VI. Outline of the thesis

The aim of this thesis was to develop methods to improve BNF of soybean in acid soils in a new transmigration area in Sitiung, and to replace the common use of large amounts of lime and P fertiliser. Hence the use of seeds pelleted with lime and P was studied (Chapter 2). Pelleting the seeds with lime and P was to ensure that the BNF can proceed optimally. Besides field and pot experiments, a detailed study was made in the laboratory using rhizotrons (Chapter 3). Since P deficiency is common in acid soils, the effect of P, in particular on the initiation of nodule formation, was investigated. For this purpose the rhizotron system, was used in order to observe and count nodule priomordia in the whole root system at the early stages of infection.

A study of the indigenous population of soybean rhizobia was initiated since exploration of indigenous soybean rhizobia may reveal bacterial strains more adapted to local stress conditions. Agronomic treatments may also lead to an increase of indigenous strains, ineffective but very competitive, which may cause a failure of inoculation practices. A comparison was made of the occurrence of soybean rhizobia in old soybean lands (Java) and new lands (Sumatra) (Chapter 4). These rhizobial isolates were characterised phenotypically, based on their symbiotic properties, and genetically using ARDRA. To establish their phylogenetic position, a number of rhizobial isolates from Java and Sumatra were studied in more detail by sequencing the major part of 16S rDNA (Chapter 5). This analysis revealed that the rhizobial population in Indonesian soils is very diverse and include strains belonging to *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii* and, most likely, *Sinorhizobium fredii*. Finally, the agronomic and microbiological aspects of the described work are summarised and discussed in a wider perspective in Chapter 6.

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Chapter 2

Effects of Pelleting the Seed with Phosphate and Lime on the Growth and Nodulation of Soybean in Acid Soils in West Sumatra, Indonesia

Setiyo Hadi Waluyo[1], Tek An Lie[1] and Leendert 't Mannetje[2]

[1] Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT, Wageningen, The Netherlands. Phone: +31 317 483102/482105. Fax.: +31 317 483829. [2] Department of Plant Sciences, Wageningen University, Haarweg 333, 6709 RZ, Wageningen, The Netherlands. Phone: +31 317 483045. Fax.: +31 317 484575.

Keywords : Soybean, acid soils, phosphate, seed pelleting, nodulation, yield.

Abstract

Lime-pelleting seeds with the equivalent of 50 kg lime ha^{-1} increased nodulation, growth and yield both in unlimed and limed soils. Considerable increases in nodulation, growth and yield were obtained when a small amount of P fertiliser (10 kg TSP ha^{-1}) was incorporated in the lime-pellet.

The beneficial effects of both lime-pelleting and [lime+TSP]-pelleting were more pronounced on nodulation than on growth and yield, and greater in unlimed soils than in limed soils. Large effects were obtained in nodulation, growth and yield of soybean in field experiments by pelleting seeds with lime or with lime+TSP. However, the pelleted soybean plants grown in unlimed soils remained small and yields were negligible.

To sustain growth and production of soybean in these acid soils, adequate quantities of lime and of P fertiliser would be necessary. In the present study, a

combination of broadcast lime at 2.0 t ha^{-1} with [lime+TSP]-pelleting of inoculated seeds was found superior to the application of 7.0 t ha^{-1} of lime with inoculated seeds only.

Al toxicity and P deficiency were the main problems in these acid soils. For the low-input production of soybeans considerations should be directed to the correction of these factors.

Abbreviations : BNF - Biological Nitrogen Fixation; CV- Cultivar; ECEC - Effective Cation Exchange Capacity; LR - Lime Requirement; P - phosphate; RAS - Percentage of Aluminium saturation; TSP - Triple Super Phosphate.

Introduction

Sitiung is one of the new agricultural areas opened up for new settlement (transmigration) in West Sumatra, Indonesia. The area has infertile acid soils (pH=4.03), and is populated by poor farmers. Recently more attention has been given to the Sitiung areas for the national Indonesian program of attaining self-sufficiency in soybean (Sudjadi, 1984; Wade *et al.*, 1988).

Exploration of soybean BNF has the potential for considerable improvement in yield, and for the development of sustainable agriculture. This is particularly important in areas with economic constraints and where low-input management is practised. However, BNF and growth of soybean on weathered acid soils is limited by acidity and related factors such as Al toxicity, Ca, P and some micro-nutrient deficiencies (Munns, 1977; Alva *et al.*, 1987; Coventry and Evans, 1989).

Liming and P fertilisation increase soybean yield and BNF on acid soils (Sartain and Kamprath, 1975; Abruna, 1979). Those techniques are costly and cannot be afforded by the poor local farmers. Therefore, low-input technologies need to be developed in an attempt to encourage local farmers to increase soybean production. Lime-pelleting seeds have been successfully developed to improve BNF and the

establishment of some temperate legumes in acid soils in Australia (Mannetje, 1967; Diatloff and Luck, 1972). However, this technique is considered less effective on heavily weathered acid soils (Cregan *et al.*, 1989).

Soybean is a plant with high-P requirement, whereas P is deficient in heavily weathered acid soils. In the present study, [lime-TSP]-pelleting of soybean seed, and lime application on soils from the Sitiung area, were investigated.

Materials and methods

This paper reports the results of field, pot, and rhizotron experiments. Two field experiments were conducted in Sitiung in 1990 and 1992 and two pot experiments in 1990 and 1991 in the greenhouse at CAIR-NAEA (Centre for the Application of Isotopes and Radiation, National Atomic Energy Agency), Jakarta.

Rhizotron studies were conducted in 1990-1992 at the Department of Microbiology, Wageningen. The pot and rhizotron experiments were performed to study the treatments used in the field experiments at laboratory scale. Rhizotron experiments allow a more detailed study of various aspects of BNF.

Table 1. Properties of the Sitiung soils

Clay (%)	74.5*
Organic C (%)	2.0*
Available P (ppm)	<5.0*
pH (H2O)	4.03
pH (KCl)	3.65
Cations (meq/100 g soil)	
Ca	0.4
Mg	0.2
K	0.14
Al + H	6.32
Al	5.56
Na	0.4
Al saturation (%)	88
P	<5.0ppm*

* Adapted from Sudjadi (1984).

Field experiments

The chemical properties of the soils in Sitiung are shown in Table 1. Urea (25 kg N ha^{-1}), TSP (100 kg TSP ha^{-1}), and KCl (100 kg KCl ha^{-1}) were applied as basic nutrients. Lime requirement (**LR**) for maximum growth of soybean plants was calculated as 6.75 tons lime per hectare, using the formula of Wade *et al.* (1988):

$$LR = 1.5[\{Al-(RAS \times ECEC/100)\}].$$

[Al= exchangeable aluminium; RAS= percentage of aluminium saturation; ECEC= effective cation exchange capacity].

The amount of lime used for pelleting soybean seeds was equivalent to 60 kg ha^{-1}. The lime used in this experiment, was agricultural lime (**Kapur pertanian** in Indonesian). Soybean seed of the cv. Tidar was sown at the rate of 50 kg ha^{-1}. The seeds for each treatment were treated with an appropriate *Bradyrhizobium* inoculant*. The treated seeds were planted in holes with a depth of 5 cm (3 seeds $hole^{-1}$) on a grid of 0.30 x 0.20 m^2. Plot size was 3.0 x 4.0 m^2. The total numbers of plots were 48 and the total area was 576 m^2. The treatments were applied in an incomplete factorial with 6 replications (Table 2).

Table 2. Treatments used in Fields experiments. (+) indicates presence and (-) indicates absence of treatment

Experiment I				Experiment II				
Lime ($t\ ha^{-1}$)	Inoculation			Lime ($t\ ha^{-1}$)	Control	Inoculation		
	Legin	Legin+ Ca-pellet	Nitrogen ($100kg\ Nha^{-1}$)			Legin	Legin+ Ca-pellet	Legin+ CaP-pellet
0	-	+	+	0	+	+	+	+
				2.0	-	+	+	+
3.5	+	+	+	3.5	+	+	+	+
7.0	+	-	+	7.0	+	+	-	-

*Legin, an peat-base rhizobium inoculant, is an official rhizobium inoculant for soybean cultivation in transmigration areas and produced by Gadjah Mada University, Yogjakarta, Indonesia.

Field experiment II was similar to field experiment I, except that the coating material for the seed pellets was a mixture of TSP (10 kg ha^{-1}) with lime (50 kg ha^{-1}). Planting distance was 0.2 x 0.15 m^2. There were 56 plots of 3.9 x 3 m^2 in a total area of 655.2 m^2. The treatments were applied in an incomplete factorial with 4 replications (Table 2).

Pot experiments

Pot experiment I was an imitation of field experiment I without N treaments. Soil was collected from the area of field experiment I. LR was calculated by assuming that the mass of 1.0 ha of top-soil equal to 2 x 10^6 kg. Air-dried soil was ground and screened by a 0.5 cm sieve. Plastic pots (diam.: 25 cm and height: 30 cm) were filled with 2.5 kg of air-dried soil. Soybean seeds were sown in a hole (2 seeds $hole^{-1}$). There were 3 holes pot^{-1}. The plants were thinned to 2 plants pot^{-1} after emergence. Tap water was added to bring the soils approximately to field capacity. This moisture level was maintained throughout the experiment by regularly weighing and watering the pots. The soybean plants were harvested at the stage of 50 % flowering. Materials and methods for pot experiment II were similar to those of pot experiment I, except that TSP-pellet and [Lime+TSP]-pellet treatments were included.

Rhizotron experiments

A rhizotron was made from a plastic-petri dish 9.0 cm in diameter, cut of the top of 0.5 cm to allow the soybean plant to grow outside the rhizotron (Fig. 1).

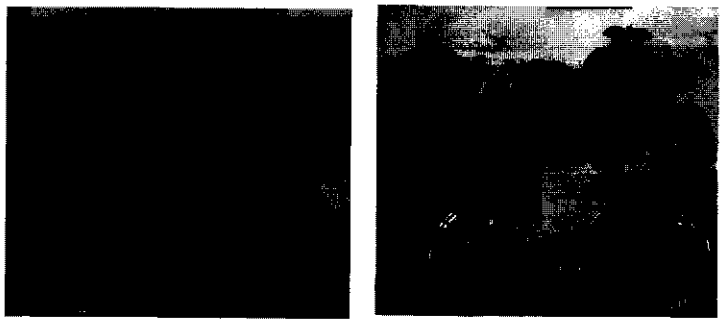

Figure 1. Left : A rhizotron. Empty (left). Filled with soil (right). The lid (bottom).
Right : Rhizotron with soybean plants.

The rhizotron experiment was carried out to investigate the effects of lime and [lime+TSP] pelleting soybean seed on nodulation, growth and development of roots of soybean in acid soils. The air-dried soil was moistened to field capacity before being put into the rhizotrons (70 g rhizotron^{-1}). In this experiment, all treatments, lime-pellet, [lime+TSP]-pellet and *Bradyrhizobium* inoculant (broth) were applied directly to 0.5 cm below the root tips. As pelleting material $CaCO_3$ was used and firstly dissolved in sterile water (0.075 gr 0.5 ml^{-1} seedling^{-1}, equivalent to 50 kg lime ha^{-1}). For [lime+TSP]-pellet treatments, besides lime, 0.5 ml of a solution containing 0.0125 gr TSP seedling^{-1} (equivalent to 10 kg TSP ha) were applied. Soybean seeds were sterilised by immersing them sequentially in ethanol 70 % for 10 minutes, then in 6 % of hydrogen peroxide containing 1 drop of Tween 20 for 10 minutes. The sterile soybean seeds were rinsed with sterile water at least 3 times. They were then germinated on water-agar 0.7 % for 24-48 hours. Seedlings were transplanted into the rhizotrons and incubated for 24 hours.

The plants were harvested 20 days after treatments were applied. Number and fresh weight of nodules were determined.

Results

Growth, nodulation and yield of soybean plants grown in Sitiung were increased by lime (Table 3).

The beneficial effects of *Bradyrhizobium japonicum* inoculation were intensified significantly by coating the inoculated soybean seeds with a small amount of lime (60 kg ha^{-1}), which increased BNF and growth both in field and pot experiments (Fig. 2; Table 3 and Table 4). Similar results were found in the rhizotron experiments (Tables 5). The enhancement of nodulation was pronounced by incorporating a small amount of P (10 kg TSP ha^{-1}) to the lime-pellet (Table 3 and Table 4). In field experiments, on soils which received 2.0 t ha^{-1} of lime, the number of nodules obtained from inoculated soybean seeds pelleted with lime+TSP was almost twice that obtained from inoculated seed only.

Figure 2. Growth and nodulation of soybean (*Glycine max* cv. Tidar) harvested from a field experiment at Sitiung. 01. Control (only 5 % plant grew on this plot). 14. *Rhizobium* + Lime-pellet (60 kg lime ha^{-1}). 17. Lime (3.5 t ha^{-1}) + *Rhizobium* + Lime-pellet (60 kg lime ha^{-1}).

Table 3. Growth and nodulation of soybean cv.Tidar at Sitiung with different treatments of lime and lime- pelleting (Field Experiment).

Lime (t ha^{-1})	Inoculation	Experiment I				Experiment II							
		Nodulation plant^{-1}		Shoots plant^{-1}		Nodulation plant^{-1}		Shoots plant^{-1}		Yield (kg ha^{-1})		P Uptake (kg ha^{-1})	N fix* (mg plant^{-1})
		Number	Dry Weight (mg)	Dry Weight (gr)	N total (mg)	Number	Dry Weight (mg)	Dry Weight (gr)	N total (mg)	Grain	P**		
0	Control (noinoculation)	-	-	-	-	0e[@]	0.0e[@]	0.7d[@]	14.4e[@]	87e[@]	0.6	0.21	0
	Legin	9e[@]	23.0bc[@]	1.2cd[@]	32.5	-	-	-	-	-	-	-	-
	Nitrogen	0f	7.0c	2.4bc	62.3	-	-	-	-	-	-	-	-
	Legin + Lime-pellet	17bc	43.0ab	1.6bcd	46.9	15bcd	51.1de	0.8cd	21.1de	75e	0.5	0.12	0.7
	Legin + [Lime+TSP]-pellet	-	-	-	-	21ab	79.4bcd	1.1bcd	27.8cde	319cde	1.9	0.16	1.3
2.0** (Broadcast and Mixed)	Legin	-	-	-	-	13cd	75.6cd	1.8abc	52.8abcd	471bcd	2.8	2.45	3.8
	Legin + Lime-pellet	-	-	-	-	18abcd	74.4cd	1.6abcd	48.9abcde	498bcd	2.7	2.29	3.4
	Legin + [Lime+TSP]-pellet	-	-	-	-	23a	111.7a	2.3a	76.7a	870a	5.0	3.32	6.2
3.5 (Broadcast and Mixed)	Control (no inoculation)	-	-	-	-	1e	11.7e	1.9ab	57.8abc	213de	1.1	0.79	4.3
	Legin	12ab	44.0ab	2.7b	71.0	19abc	96.1abc	2.1ab	72.2a	328cde	2.0	1.65	5.8
	Nitrogen	4de	20.0bc	4.7a	131.6		-	-	-	-	-	-	-
	Legin + Lime-pellet	13ab	61.0a	3.1b	92.1	16abcd	112.2a	1.9ab	62.8abc	535abcd	3	2.66	4.8
	Legin + [Lime+TSP]-pellet	-	-	-	-	22ab	107.7ab	2.0ab	65.6ab	768ab	4.3	2.55	5.1
7.0 (Broadcast and Mixed)	Control(no inoculation)	-	-	-	-	1e	8.3e	1.6abcd	43.9abcde	343cde	1.9	1.53	2.9
	Legin	7cd	20.0bc	2.8b	89.9	13cd	66.7cd	2.1ab	77.8a	569abcd	3.5	3.19	6.3
	Nitrogen	3de	5.0c	5.0a	165.8	-	-	-	-	-	-	-	-

[@] Values in the same column followed by the same letter at the same column are not significantly different by Multiple Duncan's test at P<0.05 (MSTAT-C, 1988) . * N total any treatment - N total zero control (26 mg/18 plants). ** Pyield - Ppellet+seed. Pseed= 0.7 %*** X 50 kg seed/ha = 0.35 kg P/ha (*** Anonymous, 1981). Ppellet= 0.47 X 10 kg TSP/ha= 4.7 kg P_2O_5= 1.35 kg P/ha.

In pot experiment II (Table 4), however, the effects were stronger for number and weight of nodules than for the growth of soybean. Compared to that was seed inoculated only, the number of nodules obtained from inoculated seeds treated with [lime+TSP]-pellet was increased 4 times on soils limed with of 3.5 t ha^{-1}. Nodule weight was increased 10 times. In the Rhizotron experiment, number of nodules obtained from [lime+TSP]-pellet was greater than from lime-pelleting alone (Table 5). Visual observations showed that the rate of nodule development and leghaemoglobin content of nodules were increased by P supply. Nodules in the [lime+TSP] treatment were more effective, big and red, than those from lime only.

Table 4. Growth and nodulation of soybean cv.Tidar at Sitiung inoculated with *B. japonicum* with different treatments of lime and lime- pelleting (Pot experiment).

Lime (t ha^{-1})	Inoculation	Experiment I				Experiment II			
		Nodule $plant^{-1}$		Shoot $plant^{-1}$		Nodule $plant^{-1}$		Shoot $plant^{-1}$	
		Number	Score	Dry Weight (gr)	N total (mg)	Number	Dry Weight (gr)	Dry Weight (gr)	N total (mg)
0.0	Control (no inoculation)	0	0	2.6	6.2	0.0	0.0	2.45	13
	Legin[1]	11	2.5	2.5	7.2	-	-	-	-
	Legin[1] + Lime-pellet	11	4.5	3.8	11.3	20.0	0.03	3.15	12
	Legin + TSP-pellet	-		-	-	2.0	0.05	2.65	11
	Legin + [Lime+TSP]-pellet	-		-	-	37.0	0.12	5.90	23
2.0(Mixed)	Control(no inoculation)	-		-	-	4.0	0.01	C	C
	Legin	-		-	-	19.0	0.03	5.35	22
	Legin + Lime-pellet	-		-	-	30.0	0.06	5.83	23
	Legin + TSP-pellet	-		-	-	13.0	0.06	7.68	33
	Legin + [Lime+TSP]-pellet	-		-	-	54.0	0.14	7.63	30
3.5(Mixed)	Control (no inoculation)	-		-	-	2.0	0.0	5.53	35
	Legin	13	4.5	4.6	14.4	15.0	0.02	6.0	30
	Legin + Lime-pellet	21	5.0	4.7	15.6	36.0	0.08	6.93	29
	Legin + TSP-pellet	-		-	-	14.0	0.02	3.93	18
	Legin + [Lime+TSP]-pellet	-		-	-	60.0	0.24	8.90	44
7.0(Mixed)	Control(no inoculation)	1	2.0	4.4	12.7	0.0	0.0	5.10	29
	Legin	11	2.0	4.7	14.6	6.0	0.06	6.53	33

* Nodulation score : 0 = no N fixed. 5 = Effective BNF. C = Contaminated.

A similar pattern was found in total N and the dry matter yield (Table 3). In the field experiments I, liming with 3.4 and 6.8 t ha^{-1} more than doubled the dry matter production of shoots. Total N yield of shoots was also increased more than two fold by lime. Liming with 3.4 tons of lime ha^{-1} (broadcast) and pelleting the seeds with 60 kg of lime ha^{-1} produced higher N total of shoots than liming the soils with 6.8 t ha^{-1}. Compared to the control treatment, there was an extra 574 mg N uptake by the plants growing on the limed soils. Total N yield obtained from the application of lime at 3.4 t ha^{-1} together with 60 kg of lime ha^{-1} in a seed-pellet yielded around 45 % of that obtained from the soils receiving lime at 6.8 t ha^{-1} and urea at 100 kg ha^{-1}.

Table 5. Effects of $CaCO_3$ and [$CaCO_3$+TSP] applied as solution on soil pH, Al, P, growth of lateral root and nodulation of soybean using Sitiung soil (Rhizotron experiment).

Treatment	soil pH ($CaCl_2$)	Al@ (mgL^{-1})	P@ (mgL^{-1})	Number of lateral root 1st and 2nd order at 7 days after treatment	Nodulation $plant^{-1}$		Fresh Weight Shoot $plant^{-1}$ (gr)
					Number	Fresh Weight (mg $nodule^{-1}$)	
Control	4.1	9.75	0.004	50a* 56b*	2.0c*	1.0c*	0.426b*
$CaCO_3$	7.1	0.04	0.042	43a 82a	11.0b	1.5b	0.790a
$CaCO_3$+TSP	6.8	0.05	0.044	44a 72ab	16.0a	2.8a	0.890a

*Values followed by the same letter at the same column are not significantly different by Multiple Duncan's Test at P<0.05 (MSTAT-C, 1988). @Extracted with 0.01 M $CaCl_2$ and analysed by Continuous-flow Analysis (Novozamsky *et al.*, 1993; Houba *et al.*, 1994).

The pot experiments confirmed these results (Table 4). However, there were differences between the results from field and pot experiments. The effect of lime on soybean growth was greater in the field than in pot experiments. The differences can probably be explained because plants in the field have a larger volume of soil to grow in.

However, the application of a high level of lime gave negative effects on nodulation. It was found that the number and weight of nodules were increased with 3.4 t ha^{-1} of lime, but decreased when the level of lime was raised to 6.8 t ha^{-1}. Similar results were found in the pot experiment (Table 4).

In the field experiment II (Table 3), application of 2.0 t ha^{-1} of lime broadcast and pelleting the soybean seeds with lime at 50 kg ha^{-1} and 10 kg TSP ha^{-1} produced 301 kg ha^{-1} more yield than from soils with 7.0 t ha^{-1} of lime. The results of Pot experiment II confirm these findings (Table 4).

N-fixation was also increased with lime and pelleting. Without lime, the amount of N-fixed was increased from 12 mg with lime-pelleting to 24 mg with [lime+TSP]-pelleting. Assuming that there was no N mineralisation due to liming, the amount of N fixed by pelleting with lime and TSP of plants grown in the soil with 2.0 t ha^{-1} of lime was similar to the amount of N fixed by the inoculated plants grown in the soils with 7.0 t lime ha^{-1} (Table 3).

P yield was increased by lime and [lime+TSP]-pelleting soybean seeds. In this study it was found that liming the soil increased P yield of soybean regardless of seed pelleting treatments (Table 3). The highest P yield was obtained from applications of 2.0 t ha^{-1} of lime broadcast and pelleting the soybean seeds with 50 kg ha^{-1} of lime and 10 kg ha^{-1} TSP. Similar results were also found on the total P uptake by the plants.

Discussion

The significance of the adequacy of P in the early stages of soybean growth for establishment and growth and BNF were shown in this study. The responses to the added P for nodulation, growth and yield of soybean were obvious both in field

and pot experiments. Plant yield and BNF were greatly increased by the addition of a small amount of P fertiliser with the lime used to pellet the soybean seeds (Table 3). This indicates that P is very essential for the early stage of soybean growth, and the deficiency of P more determinant factor than acidity in the Sitiung soils. It is clearly shown on the results obtained from unlimed soils. Soil pH close to seedling both on lime-pellet and [lime+TSP]- pellet might not differ (Table 5). Most likely the small amount of P in the seed-pellet was readily exploited by the developing seedlings and fostered root growth. This is in agreement with the results of Hallmark and Barber (1984), and is clearly shown in the result of Rhizotron experiment. Root growth of the soybean in soil treated with lime+TSP was much better than in the soils with lime.

The availability of P in the early stage plays an important role in nodulation and BNF of soybean. Nodulation were improved in field, pot and rhizotron experiments. However, the effect was more pronounced on the weight than on the number of nodules, as was also shown by Gates and Muller (1979) and Wan Othman *et al.* (1991). The effect of P on nodule activity was more likely through the host-plant, although this has been disputed (DeMooy *et al.*, 1973; Singleton, *et al.*,1985; Israel, 1993). In the Rhizotron experiment, it appeared that there was a strong correlation between the development of the root system and nodule activity. There were no differences in the root systems of soybean plants of lime-pelleted or [lime+TSP]-pelleted seeds 7 days after the start of treatments (Chapter 3, this thesis). However, the differences were evident in the plants that were harvested 20 days after the start of treatments. The root systems of plants from [lime+TSP]-pelleted seeds had greater root surface areas per plant and per gram of root than from lime-pelleted seeds.

There was an indication in the Rhizotron experiment (Table 5) that P ions also had a direct effect on the *Bradyrhizobium* infection process. P plays an important role in the nodulation process (Israel, 1993), and the infection proces of *Bradyrhizobium* is transient and takes place at around 12-48 hours after inoculation (Turgeon and Bauer, 1982). Adequate P in the early stages of nodulation may sustain the survival of the *Bradyrhizobium* on inoculated seeds and support the colonization of rhizosphere by *Bradyrhizobium*. It has been reported that low soil P contributes to the poor survival of some rhizobial strains in soils (Beck and Munns, 1984). Colonisation of the rhizosphere and nodule initiation are growth rate dependent (Dart, 1977). Casmann *et al.* (1981) also found that *Rhizobium* grown on a P-deficient medium was less effective than *Rhizobium* from a medium with adequate P. The fact that the number of nodules was higher with [lime-TSP]-pelleting than with lime-pelleting is in agreement with these reports in the literature. Besides the importance of P, it can be expected that lime in the seed-pelleting will protect the bradyrhizobia by increasing the soil pH and detoxifying Al ions in the micro-rhizosphere (Robson and Loneragan, 1970; Danso, 1977; Kang *et al*, 1977). While P is sufficient, root hairs and lateral root density are increased with lime, which in turn increase the number of potential sites for infection and the number of nodules (Bell and Edward, 1987). Munns (1968) and Lie (1969) have already shown that this process is the most sensitive step of nodulation in a rhizosphere of high acidity and Al toxicity. Recently Hecht-Buchholz *et al.* (1990) and Brady *et al.* (1990) reported that soybean root hairs were deformed by Al in solution culture. They also assumed that inhibition of emerging root hairs by Al toxicity causes failure in nodulation of soybean.

The increased N resulting from BNF by pelleting the seeds alone, however, was not enough to support good growth and yield of the soybean plants. The plants

remained small and the yields were negligible, although the colour was dark green (Fig. 2; Table 3), indicating that this soil was very toxic for soybeans. Besides soil pH, there were other factors limiting growth. Al saturation of the soil was 88 percent and P was less than 5.0 ppm (Table 1), while for optimal growth of soybean Al saturation should not exceed 10-20 %, and the critical levels of P is 13.0 ppm (Wade *et al.*, 1988). It has been reported that lime-pelleting legume seeds is only potentially succesful where soil acidity is mild (Cregan *et al.*, 1989). Munns (1986) also suggested that soybean grown on acidic soils may be limited by other factors than nodulation failure. They found that the inoculated plants were well nodulated, green, and high in N even when growth was severely reduced by the acid soils. The plant symptoms indicated that soybean grown on Sitiung soils were limited by both Al toxicity and P deficiency to the host plant. Therefore, apart from pelleting the seeds, an amount of lime was still required for the production of soybean. It was found that the effects of lime-pelleting soybean seeds on dry matter and N were negligible in unlimed soils, in contrast to lime pelleting seeds in soils treated with lime (Table 3). The yields obtained by pelleting soybean seeds with 50 kg ha^{-1} of lime both in soil with 2.0 t ha^{-1} and 3.5 t ha^{-1} of lime were comparable to the yield obtained from inoculated seeds in soils limed with 7.0 t ha^{-1}.

Besides lime, the availablity of P is essential as well in the Sitiung soils. It is clearly shown by the tremendously increased BNF, growth and yields as results of an addition of a small amount of P in the lime-pellet. It was reported earlier by Sudjadi (1984) that applying 200 kg TSP ha^{-1} increased soybean yield from 200 to 700 kg ha^{-1} in unlimed Sitiung soils. It is most likely that P was more available in limed soils than in unlimed soils. The added lime increased availability of P naturally present in soils and that applied in the form of fertiliser. It was observed (not measured) in our

experiment, that an application of 3.4 t ha^{-1} of lime increased the extent and distribution of the root system. Addition of lime also eliminates the toxicity of Al ions, improve root proliferation, increases interaction between root and soil surface, and thereby enhanced P uptake (Sumner and Farina, 1986; Mengel and Kirkby, 1987). Diatloff and Luck (1972) also found that in acid soils with a high level of exchangeable Al, in addition to seed inoculation, a high rate of lime was required for satisfactory legume growth and N fixation. However, it is important to note that the excessive use of lime may cause nodulation failure too.

An interesting result was obtained when TSP was used alone as a seed-pellet, without lime. Pelleting the peat-base inoculated seeds with P (TSP) had a harmful effect on the germination (data not presented). This was most probably caused by salt-injury from the TSP fertiliser. It has been reported that the addition of TSP in a band decreased the pH of the soil surrounding the band by up to 2 units (Forth and Ellis, 1988), and sowing the inoculated seeds in contact with acid superphosphate fertilisers had been found injurious to the bacteria and to germination or the seeds (Diatloff and Luck, 1972).

It is clear that cultural practices aimed at alleviating soil constraints to soybean N fixation and growth in acid soils has to be planned carefully, and must be fine-tuned when attempting to provide soil environments conducive to sustained maximum symbiosis, establishment and growth. P nutrient is a major factor in Sitiung soils. This finding is similar to the previous reports (Sudjadi, 1984; Wade *et al.*, 1988). However the management of P fertilisation is not so simple as reported by Wade (1988). In this study, the availability of P in the early stage is very essential for BNF, growth and yield of soybean. More detailed investigations are essential because it is

difficult to distinguish whether the effect of P is on nodulation *perse* or through the improved host-plant growth.

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Chapter 3

Effect of Phosphate on Nodule Primordia of Soybean (*Glycine max* Merrill) in Acid Soils in Rhizotron Experiments

Setiyo Hadi Waluyo[1], Tek An Lie[1] and Leendert 't Mannetje[2]

[1] *Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen Uinversity, Hesselink van Suchtelenweg 4, 6703 CT, Wageningen, The Netherlands. Phone: +31 317 483102/482105. Fax. : +31 317 483829.* [2] *Department of Plant Sciences, Wageningen University, Haarweg 333, 6709 RZ, Wageningen, The Netherlands. Phone: +31 317 483045. Fax. : +31 317 84575.*

Keywords : Soybean, Phosphate, root growth, nodule primordia, nodulation, acid soils.

Abstract

Addition of a small amount of P (1.35 kg P ha^{-1} as TSP) with lime-pelleted soybean seeds significantly increased nodulation and yields in acid soils, at Sitiung, West Sumatra, Indonesia. To clarify whether P had a direct or indirect effect on the nodulation process, a series of rhizotron experiments, with special attention given to formation of nodule primordia, was conducted.

It was shown that Ca and P were essential nutrients for root-growth, nodule formation and growth of soybean in the acid soils of Sitiung. Ca increased root-growth, number of nodule primordia, nodules and growth of the soybean plant. This positive effect of Ca was increased considerably by the application of P.

Ca and P have a synergistic effect on BNF of soybean in acid soils. Ca is important for the establishment of nodules, whilst P is essential for the development and function of the formed nodules.

P increased number of nodule primordia, thus it has also an important role in the initiation of nodule formation.

From this study, it can be concluded that Ca and P are the most limiting nutrients for BNF of soybean in the acid soils, at Sitiung, West Sumatra, Indonesia.

Abbreviations : BNF- Biological Nitrogen Fixation; cv- Cultivar; TSP- Triple Superphosphate; P- Phosphate; C_a- Calcium; $CaCO_3$- Calcium Carbonate; K_2CO_3- Potasium Carbonate; DAT- Days after treatment. st- first; nd- second; rd- third.

Introduction

P is essential for Biological Nitrogen Fixation (BNF) of legumes. Cassman *et al.* (1981b) found that N-fixing soybean plants required more P than N-supplied plants. In heavily weathered acid soils, P is generally deficient and will limit the potential input of BNF. Therefore, application of P is necessary to improve BNF in acid soils. The possibilities to increase the availability of P in acid soils are by P fertilisation or indirectly by liming ($CaCO_3$ addition), releasing P from the soil. The P supplied may play important roles on establishment, growth and function of nodules (DeMooy and Pesek, 1966; Gates, 1974; Cassmann *et al.*, 1981b; Gates and Muller, 1979; Israel, 1987; Singleton *et al.*, 1985), on growth of rhizobial strains (Cassmann *et al.*,1981a; Beck and Munns, 1984; Leung and Bottomley, 1987), and on host-plants (Munns *et al.* , 1981). Wan Othman *et al.* (1991) reported that nodulation of cowpea (*Vigna unguiculata* L. Walp) was impaired by a very low P status of the soil. It has been reported that growth rate of most *Rhizobium* strains is reduced by low levels of P (Beck and Munns, 1984). Munns *et al.* (1981) have shown that limitation of

growth and BNF of soybean (*Glycine max* Merrill) in highly acid soils is due to the host plant rather than to the failure of nodule formation.

The effects of P on rhizobia have been studied for a long time (Truesdell, 1917 cited by Keyser and Munns, 1979). However, the mechanism of the P effect on BNF is not yet clear, since it is difficult to distinguish between the effects directly on BNF or indirectly via the plant. Cassman *et al.* (1993) and Chapter 2 (this thesis) have shown that application of P increased number of nodules of soybean plants grown in heavily weathered acid soils. In Chapter 2 (this thesis) it was shown that positive effects of P on growth and BNF of soybean in acid soils were obtained, provided that P is applied at the correct place and time. The availability of P in close vicinity to seedlings of soybean seed was suggested (Chapter 2, this thesis) to have a positive effect on the survival of introduced rhizobia, to stimulate root growth and then to promote infection to proceed naturally on acid soils.

In this study, the effects of P on nodule primordia of soybean inoculated with *Bradyrhizobium japonicum*, USDA 110 was investigated in more detail in rhizotron experiments. The rhizotron system was developed for the study of N fixation of seedlings in soil. Results of field studies can be interpreted by using the rhizotron system as an intermediate step between the laboratory and the field (Pijnenborg and Lie, 1990)

Material and Methods

A rhizotron consists essentially of a plastic petri dish, cut at one side, filled with soil, and kept at an angle of ca. 60 degrees, so that the roots are growing towards the lid of the petri dish. The transparent lid allows observation of the root system continuously, and by opening the lid it is possible to inoculate or to apply treatment at a certain location and certain time. Using this system the effect of several factors of

soil acidity affecting N fixation by lucerne was studied (Pijnenborg and Lie, 1990). Many plants can be grown in a limited space, in a relatively short time (less than 3 weeks), and what is more important: the results obtained in rhizotrons are in good agreement with comparable field experiments.

In this study, 5 experiments were conducted using soil samples from Sitiung, West Sumatra. Soybean (cv. Tidar) was used in experiments I, II, IV and V. In experiment III, in addition to cv. Tidar, new soybean mutant-lines 214, 23D and 231A (provided by Agriculture Division, Center for the Application of Isotopes and Radiation, National Atomic Energy Agency, Indonesia) were used. In experiment 1 and 2 only one seedling was used per rhizotron, but in experiment 3, 4 and 5 two seedling were used. The seeds were germinated on 1 % water-agar and were transplanted after 24 hours into rhizotrons filled with acid soil from Sitiung. *Bradyrhizobium japonicum* USDA 110 grown (7 days at 30 °C) in Yeast Extract Mannitol (YMB, Somasegaran and Hoben, 1995) was used as an inoculant. This inoculant was applied directly on root-tip at the day of treatment (DAT) (1 day after transplanting).

Experiment I

The terms **liming** were used for the application of $CaCO_3$ to the soils and **lime-pellet** when the seeds were coated with a layer of $CaCO_3$. In the following experiments instead of lime-pellet, a solution of $CaCO_3$ was applied directly on the root tip. This is comparable to lime-pelleting, but the amount can be applied more exactly. Five levels of $CaCO_3$, the equivalents of 0, 0.8, 1.6, 3.3, and 6.7 t ha^{-1}were applied. The visible part of the root was inspected at 5 DAT. Number of nodules was counted at harvest 20 DAT.

Experiment II

A study was made to compare the effects of **liming** the soil and **lime-pelleting** the seeds on the formation of roots and nodules of soybean. $CaCO_3$ levels similar to Experiment I were used as liming, and $CaCO_3$ with levels of 0, 5, 10, 15, 20 and 25 kg ha^{-1} were used for lime-pelleting. Length of the visible part of the root was measured at 5 DAT. Number of nodules and weight of shoots were determined at harvest 20 DAT.

Experiment III

To study the effect of P on formation of soybean nodule, six treatments: H_2O, $CaCO_3$, K_2HPO_4, TSP, [$CaCO_3$+K_2HPO_4] and [$CaCO_3$+TSP] applied to the root-tips of seedlings, were carried out. Number and weight of nodules were determined at harvest 20 DAT.

Experiment IV

This experiment comprised 3 treatments, H_2O (control), and solutions of $CaCO_3$ and [$CaCO_3$+TSP], to study the effects of Ca and P on root growth, formation of nodule primordia and nodules. The plants were harvested at 5, 10 and 20 DAT. At 5 and 10 DAT, the entire root was excised, number and length of first, second and third order roots were measured and counted. To determine nodule primordia, the root harvested at 5 DAT was fixed with glycerol (15 min) and cleared by immersing the roots in sodium hypochlorite 6 % active chlorine solution (15 min). After clearing, the fixed root was stained with 0.01 % methylene blue (Johnson *et al.*, 1996). Primordia of roots and nodules inside root-tissue (Fig. 1) were observed under a microscope

(magnification 60x) and counted from the entire root system. At harvest 20 DAT the number and weight of nodules were determined.

Experiment V

This experiment was done to study the effect of neutralising acidity on formation of nodule primordia. To cancel the effect of Ca, K_2CO_3 was used instead of $CaCO_3$ to neutralise acidity. Four treatments: H_2O, $CaCO_3$, K_2CO_3 and [K_2CO_3+TSP] were applied. Number and length of first, second and third order roots were counted and measured from entire root harvested at 10 DAT. Then, this root was fixed and coloured in a similar way such as mentioned in experiment IV. Primordia of roots and nodules (Fig. 1) were observed and were counted from all over the roots under the microscope (magnification 60x). Number and weight of nodules were determined at 20 DAT.

Figure 1. Root (Left) and nodule (Right) primordia of soybean excised from the plant in a rhizotron experiment and examined under a microscope with magnification of 125X.

Results

Roots

Ca, P and neutral soil pH were found important to root growth. Applying $CaCO_3$ increased root length and nodule number. The effects were clear and statistically significant at $\alpha = 0.05$ (Table 1). This is in good agreement with results from experiments carried out in pots and in the field. A comparison between applying $CaCO_3$ to the soil (liming) and direct application of $CaCO_3$ to the seedlings (lime-pelleting) clearly demonstrated the efficiency of the latter method (Table 2). Table 2A showed that root length was increased proportionally by liming. The optimal effect was found at 3.3 and at 6.7 t ha^{-1} for main and lateral roots, respectively. Number of nodules and weight of shoot were increased at 6.7 t ha^{-1}. The effect of lime -pellet on root growth is not clear (Table 2B). No effect was obtained on length of main root by applying $CaCO_3$ to the root-tip of the seedling. Although the length of the main root was highest at the equivalent of 25 kg $CaCO_3$ /ha applied, the differences were not significant. The effect of lime-pellet on lateral roots was shown, the best results were already observed at 10 kg ha^{-1}.

Table 1. The effect of liming the soil on root growth and nodulation of soybean growing in acid soils, in rhizotron experiments.

Lime ($CaCO_3$, t ha^{-1})	Main root length (cm) measured at 5 DAT	Number of nodules $plant^{-1}$ harvested at 20 DAT
0	1.16d*	3d*
0.8	2.46c	7c
1.6	4.20b	10ab
3.3	5.16a	8bc
6.7	4.56ab	11a

*Values followed by the same letter in the same column are statistically not significant according to Duncan's multiple test $\alpha = 5$ %, and with 9 replications (MSTAT-C, 1988).
DAT = days after treatment.

Table 2. Comparison of the effect of $CaCO_3$ as a liming agent (A. mixed with soil) and equivalent to lime-pelleting (B. applied near root-tip) on root growth, nodulation and growth of soybean in acid soilsin rhizotrons experiment.

A. $CaCO_3$mixed with the soils (liming)				
$CaCO_3$ (t ha^{-1})	Root length (cm $plant^{-1}$)@		Number of nodules $plant^{-1}$@@	Dry Weight Shoot (mg $plant^{-1}$)@@
	Main	Lateral		
0.0	1.96c*	0.111b*	3.2b	73
0.8	4.80b	0.169b	5.0b	84
1.6	6.14b	0.166b	4.2b	77
3.3	8.70a	0.175b	5.4b	88
6.7	9.12a	0.299a	8.2a	106

B. $CaCO_3$ applied directly on root-tip (imitation of lime-pellet)				
$CaCO_3$ (kg ha^{-1})	Root length (cm $plant^{-1}$)@		Number of nodules $plant^{-1}$@@	Dry Weight Shoot (mg $plant^{-1}$)@@
	Main	Lateral		
0	3.78ab*	0.108c*	0b**	81
5	4.94ab	0.127c	5.4a	155
10	2.96b	0.209ab	5.4a	154
15	5.92a	0.150bc	7.4a	182
20	3.96ab	0.250a	6.8a	174
25	6.26a	0.192a	7.4a	181

* and ** values followed by the same letter in the same column are statistically not significant according to Duncan's multiple test at α = 5% and α = 10%, and with 5 replications (MSTAT-C, 1988). @ measured at 5 DAT (on a visible part of the root). @@ at harvest 20 DAT.

The responses of root growth to Ca, P and neutral soil pH was clearly shown on the plants harvested at 10 DAT (Table 4). Applying $CaCO_3$ increased number and length of roots. Compared to the control, only the number of 2 nd order roots was increased significantly by Ca. The increase in number of 1 st and 3 rd order root s was not significantly different. The effect of Ca on root growth was more pronounced than neutralising soil acidity (Table 6).

Apparently, P has only had a positive effect on root growth in the presence of Ca (Table 4). Number of 1^{st}, 2^{nd} and 3^{rd} order roots of were significantly (at α=5) increased by 1.3, 1.8 and 11 times over the control. A similar respopnse was found for root length. The response of root and nodule growth to Ca and TSP was ultimately shown on the shoot growth of the plants at harvest, 20 DAT (Fig. 2).

Nodules

The effects of Ca and P on number and weight of nodules were significant (Table 3; 4; 5). Using 4 soybean varieties, it was found that there were no specific effects among the soybean varieties (Table 3). Compared to the control, number of nodules on cv.Tidar was increased more by $CaCO_3$ (4.4-fold) than by TSP (2.6-fold). The importance of Ca on nodulation is also shown in Table 6. Number of nodules obtained by applying $CaCO_3$ was higher than those obtained by [K_2CO_3 + TSP], and no effect was obtained by applying K_2CO_3 alone. Application of either KH_2PO_4 or TSP in addition to $CaCO_3$ treatment had no effect on number of nodules (Table 3). There was no significant difference in the number of nodules obtained by applying [$CaCO_3$+KH_2PO_4] or [$CaCO_3$+TSP]. The effect of [$CaCO_3$+TSP] was not statistically different from that of $CaCO_3$ alone. In contrast, considerable increases in weight of nodules were obtained. No differences were found between the effect of [$CaCO_3$ +KH_2PO_4] and [$CaCO_3$+TSP] on weight of nodules, but these values were significantly higher than that obtained by $CaCO_3$ alone (Table 3). Table 4 also shows that the total weight of nodules was increased significantly from 19 with alone $CaCO_3$ to 60 mg with [$CaCO_3$+TSP]. The importance of P on growth and function of nodules was confirmed by the results in Table 5. There was no increase in weight of nodules obtained by applying K_2CO_3 alone.

Table 3. Nodulation of soybean cvs. Tidar, 214, 23D and 231A in acid soils with different treatments in rhizotron experiments.

	Nodule							
	Number plant^{-1}				Weight plant^{-1} (mg)			
	Tidar	214	23D	231A	Tidar	214	23D	231A
Control	3.5d[@]	4.3c[@]	2.3c[@]	0.0c[@]	7d[@] (2.0)	9 e[@] (2.0)	6 e[@] (2.6)	0e[@] (0.0)
$CaCO_3$*	15.3b	21.4a	18.0a	17.8a	29 c (1.9)	50 c (2.3)	59c (3.2)	50c(2.8)
KH_2PO_4**	5.4d	6.6bc	0.8c	0.0c	12 d (2.2)	31 d (4.7)	2 e (2.4)	0e(0.0)
TSP***	9.2c	8.0b	6.5b	9.5b	26c (2.9)	40cd (5.0)	29d (4.5)	36d(3.8)
$CaCO_3$ +KH_2PO_4	19.9a	20.4a	18.7a	18.4a	61a (3.1)	82 a (4.0)	95a (5.1)	76a(4.1)
$CaCO_3$ + TSP	17.6ab	21.8a	21.1a	20.7a	43b(2.5)	66 b (3.0)	71b (3.3)	62b(3.0)

[@] Values followed by the same letter at the same column are not significantly differentiated by Multiple Duncan's test at α = 5%, with 8 replications (MSTAT-C, 1988). Values in bracket are weight of individual nodule.

*$CaCO_3$ was applied 0.175 gram/seed (50 kg ha^{-1}) containing of Ca = 0.0276 gram.

** KH_2PO_4 was toxic (too acid) for seedlings, some seedlings did not grow.

***TSP was applied 0.0125 gram/seed (10 kg ha^{-1}) containing of Ca = 0.0022 gram.

As shown on Table 3 and Table 5, the weight of individual nodules (size of nodule) was also increased by P. Nodules obtained from plants treated with TSP, [$CaCO_3$+TSP], [$CaCO_3$+KH_2PO_4] were heavier than those in the control and $CaCO_3$ treatments (Table 3).

Discussion

The importance of P on legume BNF has long been recognised. However, there is still controversy about the role of P on infection and nodule development since it is difficult to isolate this effect from that of P on host plant growth. The role of P in initiation, development and function of the nodules of soybean plants in the acid soils of Sitiung, has been clearly shown in this study. This is in agreement with reports of Israel (1987, 1993) which indicated that P has specific roles in nodule initiation, growth and functioning in addition to its effects on host plant growth processes. The response of soybean BNF to the availability of soil P has been reported

earlier by Graham and Rosas (1979) and Cassman *et al.* (1980). Freire (1976) noted that in an Al-toxic Brazillian soil, P fertilisation was more important than lime in enhancing nodulation and dry matter production of soybean plants. In addition, soybean plants primarily dependent on N fixation require P more than N supplied plants to obtain a comparable yield (Cassman *et al.*, 1981b).

This study demonstrated that the effect of soil acidity and related factors (Ca and P) on nodulation and root growth of soybean can be studied in detail using soybean seedlings in the rhizotron system. Ca, P and neutral soil pH increased total number of nodule primordia per plant and per cm of root length. P increased number of nodule primordia regardless of the increasing soil pH and the application of Ca (Table 4 and Table 5). However, a more significant increase was found when $CaCO_3$ was added as well. The treatment [$CaCO_3$ + TSP] increased the number of nodule primordia by a factor 3.7 (Table 4) and [K_2CO_3 + TSP] by a factor 3.0 (Table 5) compared to the control. This is in agreement with previous studies with *Stylosanthes humilis* H.B.K (Gates, 1974), and soybean (Mullen *et al.*, 1988), based on visible nodules. In the present study, number of nodule primordia was counted at an early stage when they were still inside root-tissue. Brockwell *et al.* (1985) suggested that optimum nodulation and N fixation are functions of early colonisation of the plant rhizospheres by *Bradyrhizobium japonicum*. It was reported that the early steps in the nodulation process are the most sensitive to acidity for BNF (Munns, 1968; Lie, 1969), and the availability of potential infection sites on roots is transient (Bhuvaneswari, *et al.*, 1980; Turgeon and Bauer, 1982).

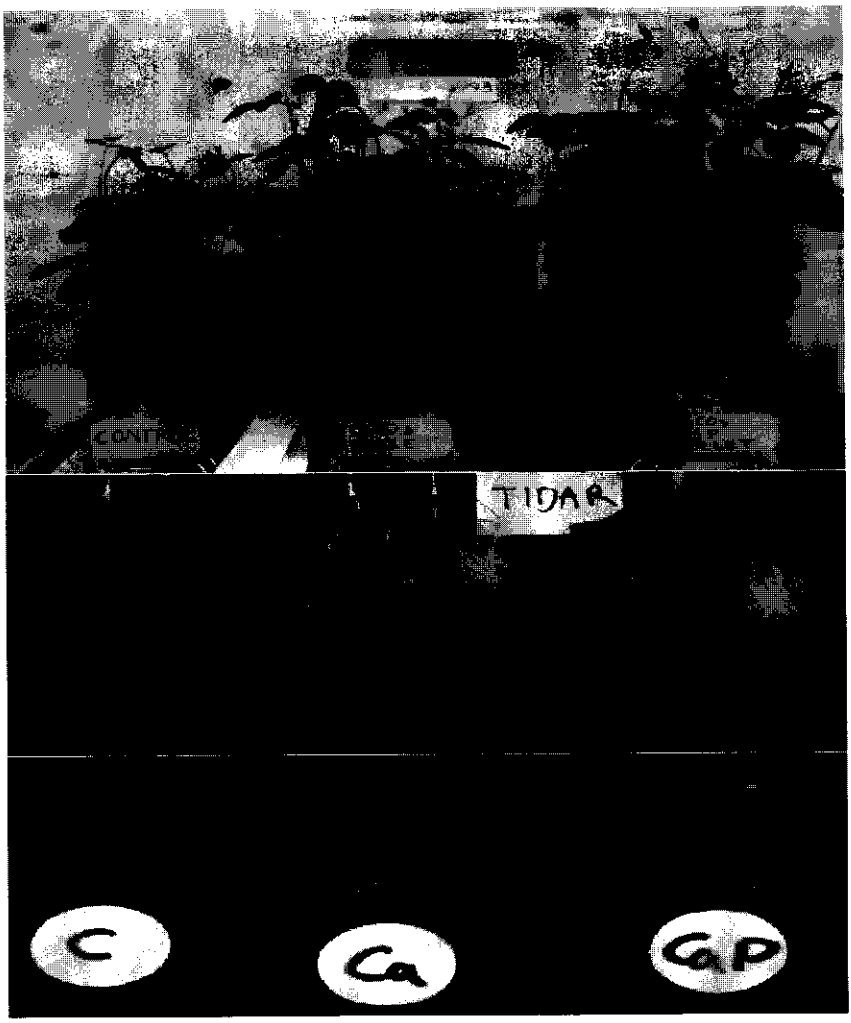

Figure 2. Growth (Top), root (Middle) and nodules (Bottom) of soybean in acid soils, Sitiung, West Sumatra, treated with P (TSP) fertiliser in a rhizotron experiment.

Table 4. Root growth, formation of nodule primordia and nodules of soybean (cv Tidar) affected by $CaCO_3$ and $CaCO_3$+TSP in acid soils in rhizotron experiments.

	5 DAT[@]			10 DAT[@]						20 DAT[@@]		
	Root length 1st order (cm) plant^{-1}	Nodule primordia plant^{-1}		Root plant^{-1}						Nodule plant^{-1}		
		Number	Number/ cm of root length	1st order		2nd order		3rd order				
				Number	Length (cm)	Number	Length (cm)	Number	Length (cm)	Number	Fresh Weight (mg)	
											Nodule^{-1}	Total
H_2O	88a*	7b**	0.08	45b*	108b*	148b**	73b*	4b*	1b**	1c***	4	4c***
$CaCO_3$	71b	11b	0.15	55ab	137ab	245a	173ab	28ab	7ab	9b	2	19b
$CaCO_3$+TSP	89a	26a	0.29	60a	165a	265a	211a	42a	17a	15a	4	60a

DAT= days after treatment. *, ** and ***Values followed by the same letter at the same column are not significantly differentiated by Multiple Duncan's test at α =10 %, α =5 % and α =1 %. [@]Number of replication = 3. [@@]Number of replication = 4 (MSTAT-C, 1988).

Table 5. Effect of neutralising soil acidity by $CaCO_3$ or K_2CO_3 on formation of nodule primordia and on nodule of soybean.

	5 DAT	10 DAT		20 DAT		
	pH (H_2O)	Nodule primordia plant^{-1}		Nodule plant^{-1}		
		Number	Number / cm 1st order root length	Number	Fresh Weight (mg)	
					Nodule^{-1}	Total
Control	4.9	16b *	0.14	3.0d **	1.3	4d **
$CaCO_3$	7.3	34ab	0.25	8.0b	1.6	13 c
K_2CO_3	6.9	31ab	0.25	3.0d	1.7	5d
K_2CO_3+TSP	5.8	48a	0.33	5.0c	3.6	18b

* and ** Values followed by the same letter at the same column are not significantly different by Multiple Duncan's Test at $\alpha = 0.05$ with 5 and 16 replications, respectively (MSTAT-C, 1988). DAT = days after treatment.

Table 6. Effect of neutralising soil acidity on number and length of first, second and third order roots of soybean seedlings in acid soils, in rhizotron experiment.

Treatments	pH (H_2O) at 5 DAT	10 DAT								
		Roots 1st order plant^{-1}			Roots 2nd order plant^{-1}			Root 3rd order plant^{-1}		
		Number	Length (cm)		Number	Length (cm)		Number	Length (cm)	
			total	root^{-1}		total	root^{-1}		total	root^{-1}
Control	4.9	42.0b*	110.0b*	27.0a*	177c*	89b*	50b*	2.4ab*	0.2a*	0.06a*
$CaCO_3$	7.3	58.0a	145.0a	25.0a	229ab	154a	68a	8.2ab	1.15a	0.12a
K_2CO_3	6.9	53.0ab	135.0ab	26.0a	183bc	101b	55ab	1.2b	0.17a	0.09a
K_2CO_3+TSP	5.8	59.0a	144.0a	24.0a	201bc	104b	52ab	1.6b	0.30a	0.21a

*Values followed by the same letter at the same column are not significantly different by Duncan's Multiple Test at $\alpha = 0.05$. Number of replication = 5 (MSTAT-C, 1988). DAT = days after treatment.

Changes in root morphology, and presumably increasing infection sites, may be involved in the number of nodule primordia. Ca was found essential for root branching. However, the availability of P in addition to Ca gave a better root system. Application of Ca+P gave an abundance of root-branching which may provide many potential infection sites available to rhizobia (Fig. 2 middle). Deformation of root hair growth due to Al toxicity, and thus a reduction in the potential number of sites for infections has been considered as one of the reasons for nodulation failure (Alva *et al.*, 1987; Brady *et al.*, 1990). Blamey *et al.* (1983) reported that the toxic effect of Al on soybean root was ameliorated by the addition of P through the reduction of monomeric Al compounds in solution. Availability of P can also increase root branching by precipitation and detoxification of the Al present excessively in the Sitiung soils.

Besides increasing number of nodule primordia, P also had a great influence on growth and function of nodules as shown before by DeMooy and Pesek (1966) and Gates and Muller (1979). This was clearly illustrated on the increase of nodule weight due to application of P (Table 3). The size and weight of individual nodules, and also total nodules, were significantly increased. The stimulating effect of P on nodule growth and function ultimately resulted in the improved growth of shoots of soybean plants, and presumably also in yield.

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Chapter 4

Isolation and Characterisation of Soybean Rhizobial Strains from Java and Sumatra, Indonesia

Setiyo Hadi Waluyo[1], Tek An Lie[1], Leendert 't Mannetje[2], and Willem M. de Vos[1]

[1]*Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands. Phone: +31 317 482105/483102. Fax. : +31 317 483829.* [2]*Department of Plant Sciences, Wageningen University, Haarweg 333, 6709 RZ Wageningen, The Netherlands. Phone: +31 317 483045/483040. Fax. : + 31 317 484575.*

Keywords : ARDRA, Bradyrhizobium japonicum, Indonesia, Java, Soybean, Sumatra.

Abstract

Rhizobial strains capable of nodulating soybean were isolated from soil samples obtained from traditional soybean area's on Java and various new soybean growing area's on Sumatra, Indonesia. All of the 29 soil samples from Java produced nodules on the soybean plant and appeared to effectively fix nitrogen. However, only 42 of the 63 tested soil samples from Sumatra nodulated soybean and from these only 23 appeared to fix nitrogen. A total of 51 different rhizobial strains were isolated from the nodules obtained from the Java (27) and Sumatra (24) soil samples. Based on their nodulation capacity on both soybean and the native legume mungbean, these rhizobial strains could be divided into a group of 16 strains specific for soybean only and another group of 35 promiscuous strains that nodulated both leguminous plants. Based on Amplified Ribosimal DNA Restriction Analysis (ARDRA) of PCR-amplified 16S rDNA and 16S-23S rDNA spacer fragments, the rhizobial strains isolated from Java were found to be different from those from Sumatra. Six isolates from Java and only

one isolate from Sumatra could be classified as *Bradyrhizobium japonicum* and these were found to belong to the same cluster as the reference strain *B. japonicum* USDA 110. All these *B. japonicum* strains were highly specific for soybean. One isolate from Java showed a rather unique position. The remaining strains from Java (20), which are symbiotically promiscuous strains, are clustered in another group. This group and another group containing most Sumatra isolates are distinct from *B. japonicum* USDA 110 and therefore it is tempting to speculate that these represent indigenous soybean rhizobial bacteria.

Abbreviation : ARDRA : - Amplified Ribosomal DNA Restriction Analysis; PCR – Polymerase Chain Reaction; YEMB – Yeast Extract Mannitol Broth; S-ST – Sumatra Sitiung; S-BT – Sumatra Bukit Tinggi; J-YG – Java Yogyakarta; J-NG – Java Ngawi; J-WG – Java Wangon; J-WK – Java Wangkal; J-PLR – Java Palur; J-KH – Java Kraksaan; J-DLG – Java Delanggu; J-SRG – Java Sragen; J-MDN – Java Madiun; J-KLT – Java Klaten; J-MJ – Java Mojosari; J-BSK – Java Besuk; J-TGS – Java Tongas; J-MLD – Java Mlandingan; J-PSR – Java Pasuruan; J-TM – Java Tasik Malaya; J-PN – Java Pusaka Negara; J-JKT - Java Jakarta; J-BGR – Java Bogor and J-CTM – Java Citayam.

Introduction

Soybean (*Glycine max* Merrill) is an important food crop in Indonesia, where it has already been cultivated for several centuries, notably on Java that has fertile soils. To meet the increasing food demands of the last decades, soybean is also grown on other Indonesian islands, such as Sumatra, where soil conditions are less favourable for its growth due to low nutrient content and high acidity.

Soybean is a legume that can fix nitrogen when it is cultured on soils containing the appropriate nodulating rhizobial bacteria belonging to the genera *Bradyrhizobium* or *Sinorhizobium* (Somasegaran and Hoben, 1995). The importance of rhizobial strains for soybean cultivation in Indonesia was already shown a long

that all soybean plants inoculated with the soil samples from Java appeared healthy and carried nodules that all fixed nitrogen. However, the root nodules obtained with Sumatra soil samples were more variable in number than those from Java and varied from many and effective ones to a few small and white ones. It appeared that only 42 of the 63 soil samples from Sumatra produced nodules on the soybean roots. Moreover, only 23 of these soil samples were found to fix nitrogen.

Table 2. Nodulation efficiency and nitrogen fixation capacity of soybean plants inoculated with soil samples from Java and Sumatra.

Source	Number of	Nodulation (%)		N Fixation (%)	
	Soil samples	-	+	-	+
Java	29	0(0)	29(100)	0(0)	29(100)
Sumatra	63	21(33)	42(67)	40(63)	23(37)

+ = nodulated or fixing N, - = not nodulated or not fixing N.

Fifty-one rhizobial strains that nodulated soybean were isolated from effective and ineffective crushed nodules obtained with soils from Java (27) and Sumatra (24). The nodulation efficiency of the isolated rhizobial strains was analysed on soybean plants and compared to that of reference strains, including the *Bradyrhizobium* strain CB756 specific for cowpea (Table 3). In general, rhizobial strains obtained from Java were as effective as the soybean-nodulating reference strains *B. japonicum* USDA 110 and CB 1809. However, the number and weight of nodules, as well as the weight of shoot from the plants inoculated with the rhizobial strains from Java, were significantly higher than those from Sumatra. The differences were most significant in the weight of shoots ($\alpha = 0.01$, Table 3). Remarkably, it was observed that the first two leaves from all the soybean plants inoculated with rhizobial strains from Sumatra were yellow and in most of the cases were lost very quickly, indicating poor N fixing capacity.

The capacity of the isolated rhizobial strains to additionally nodulate mungbean was tested (Fig. 1). Mungbean was chosen because this is a leguminous cowpea plant known to be native to Indonesia (Summerfield and Lawn, 1987). This analysis revealed that most of the strains from both Java and Sumatra showed promiscuous nodulation properties that were not observed with the well-known inoculant strains *B. japonicum* USDA 110 and CB 1809.

Table 3. Efficiency of rhizobial strains isolated from Java and Sumatra compared to reference strains. Number and weight of nodules formed on soybean as well as shoot weight were determined.

Strain		Nodule plant^{-1}		Shoot Weight (g plant^{-1})
		Number	Weight (g)	
None	Control	0	0	0.57±0.32
Bradyrhizobium spp.[@]	CB756	0	0	0.30±0.03
B. japonicum[@]	USDA 110	43±10	0.288±0.07	3.36±0.80
B. japonicum[@]	CB1809	29±5	0.250±0.06	3.70±0.28
Java[@@]		46±10a*	0.263± 0.07a**	3.75± 0.62a***
Sumatra[@@]		37±14 b	0.225±0.07 b	2.43±0.80 b
		CV[#]= 33%	CV=29%	CV=25%

[@] For these reference strains means were calculated from 3 replicates. [@@] A total of 24 randomly choosen bacterial strains were used for each set of soil samples. *, **, *** Values followed by a different letter in same column are statistically different, revealed by T-test with confidence levels at α = 0.10, 0.05 and 0.01 respectively. [#] CV = Coefficient Variation (MSTAT-C, 1988)

Amplified Ribosomal (16S and 16S-23S) DNA Restriction Analysis (ARDRA)

Genomic DNA of the isolated rhizobial strains (27 from Java and 24 from Sumatra) and that of reference strain *B. japonicum* USDA 110 was isolated and used as template for PCR amplification of the 16S rDNA and the 16S-23S rDNAs spacer regions. The amplified 16S rDNA's all showed the expected size of approximately 1.6 kb and upon digestion with 4 different restriction enzymes (*Cfo*I, *Dde* I, *Hae*III

All strains isolated from Java, except for J-YG49, could be grouped into two clusters, one including *B. japonicum* USDA 110. Again the Java isolate J-TGS50 showed a unique position. Similarly, all strains isolated from Sumatra, except for strains S-ST224, S-ST325 and S-ST123, could be grouped in one very large cluster, while two strains (S-BT221 and S-BT322) formed a cluster that was distantly related to that of *B. japonicum* USDA 110.

Discussion

Rhizobial strains capable of nodulating soybean were isolated from soil samples obtained from traditional soybean area's on Java and various new soybean growing area's on Sumatra, Indonesia. Java was found to be occupied by effective soybean rhizobial strains since all of the tested soil samples (29) from Java produced nodules on the soybean plant that effectively fixed N. This is likely to be a consequence of extensive cultivation of soybean on Java for over several centuries (De Vries, 1932) as well as the introduction of many *Bradyrhizobium* strains from abroad (Newton, 1962). In contrast, in Sumatra where soybean cultivation has been introduced quite recently, several locations were found to be devoid of soybean rhizobial strains. Only 42 of 63 tested soil samples were found to nodulate soybean, and from these only 23 appeared to fix nitrogen.

A total of 51 different rhizobial strains (27 from Java and 24 from Sumatra) were isolated from nodules. Based on their nodulation capacity on both soybean and mungbean, these rhizobial strains could be classified as promiscuous strains (35) or strains that show a narrow host-range (16) and only nodulate soybean (Fig. 1). Saono (1988) suggested that the native soybean-rhizobia population in Java appears to be dominated by promiscuous strains, and this is supported here with quantitative data.

All of the soybean plants that were inoculated by rhizobial isolates from Java (except J-MJ44) were growing vigorously in a N-free medium. In contrast, several ineffective bradyrhizobial strains were found in Sumatra. It is likely that the abundance of these strains would have a negative impact on the inoculation by effective *Bradyrhizobium* spp. inoculants.

ARDRA of PCR-amplified 16S rDNA and 16S-23S rDNA spacer fragments was used to differentiate soybean-nodulating rhizobial strains. Based on ARDRA of 16S rDNA, nearly all the soybean-nodulating bacteria could be grouped into two separate large clusters comprising either the Java or the Sumatra isolates. ARDRA of 16S-23S rDNA spacer fragments has reported to be useful for a further sub-classification of bacteria (Jensen *et al.*, 1993; Masol-Deya *et al.*, 1995; Gurtler and Stanisich, 1996; Scheinert *et al.*, 1996). This is due to the fact that the 16S-23S rDNA spacer region of all prokaryotes exhibit a high degree of sequence and size variation at the level of the genus and species. The sequence variation of the 16S-23S rDNA spacer region of the soybean-nodulating bacteria confirmed the clustering of most Sumatra isolates and allowed a further classification of the Java isolates into two large distinct clusters. One of these clusters was found to include strains that were closely related to *B. japonicum* USDA 110, a reference strain which is highly specific for soybean. Based on their grouping and symbiotic properties, we assume that these Java isolates and one isolate from Sumatra (S-ST123) are *B. japonicum* strains. Although clustered at one group, the isolate S-ST123 showed distinct in its symbiotic property to *B. japonicum* USDA 110. It formed many but ineffective soybean-nodules. One unique isolate from Java, J-TGS50, showed an unique position in comparison with the clustered isolates from Java and Sumatra. This and the group containing most Sumatra isolates which were also shown distinct from *B. japonicum* USDA 110, are

all and nodulated both soybean and the indigeneous mungbean. Hence these strains may represent the indigeneous bacterial population capable of nodulating soybean.

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Chapter 5

Phylogenetic Analysis of Soybean Brady- and Sinorhizobia Isolated from Java and Sumatra, Indonesia

Setiyo Hadi Waluyo[1,2], Tek An Lie[1] and Willem M. de Vos[1]

[1]*Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands. Phone: +31 317 482105/483102. Fax. : +31 317 483829.* [2]*From January 2001, Agriculture Division, Center for the Application of Isotopes and Radiation, National Atomic Energy Agency, Jln. Cinere Pasar Jumat, Kotak Pos 7002 JKSKL, Jakarta 12070, Indonesia. Phone: 021 7691607. E-mail: shwaluyo@hotmail.com*

Keywords: Bradyrhizobium elkanii, Bradyrhizobium japonicum and Sinorhizobium fredii, 16S rDNA, Java, Sumatra.

Abstract

The major part of the 16S rDNA genes of 21 soybean brady- and sinorhizobia isolated from Java and Sumatra were amplified, cloned and characterised. Based on comparison of the complete nucleotide sequence of the amplified DNA (approximately 1500 bp) the isolates could be divided into three groups consisting of *Bradyrhizobium elkanii* (12), *Bradyrizobium japonicum* (8) and *Sinorhizobium fredii* (1). The *B. elkanii* strains appeared to be widespread in acid soils, Sitiung, West Sumatra, and only two isolates were obtained from Java. Most isolates from Java (4), two isolates either from Sitiung or from Bukit Tinggi (both located in Sumatra) were identified as *B. japonicum* strains. Finally, a single isolate from Java was identified as *S. fredii*.

Abbreviation: ARDRA – Amplified Ribosomal DNA Restriction Analysis; YEM – Yeast Extract Mannitol; J-KH – Java Kraksaan; J-YG – Java Yogyakarta; J-WG – Java Wangon; J-DLG – Java Delanggu; J-SRG – Java Sragen; J-TM – Java Tasik Malaya; J-TGS – Java Tongas; S-BT – Sumatra Bukit Tinggi and S-ST – Sumatra Sitiung.

Introduction

Rhizobia constitute a heterogenous group of bacteria that belong to the alpha subclass of the Proteobacteria and are able to form root nodules fixing nitrogen on leguminous plants. DNA sequence analysis of 16S rDNA regions has substantiated the diversity of rhizobia, and currently these nodulating bacteria can be divided into 5 genera, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Novikova, 1996; Young, 1996; Jarvis *et al.*, 1997; Ludwig *et al.*, 1998; Vinuesa *et al.*, 1998)

The taxonomy of the genera *Bradyrhizobium* and *Sinorhizobium* is of particular interest since they contain strains that are known to nodulate soybean, which is an important food crop. The genus *Bradyrhizobium* includes the species *B. japonicum*, *B. elkanii* and *B. liaoningensis* that show a rather narrow host-range and slow growth (Kuykendall *et al.*, 1992; Xu *et al.*, 1995; Minamisawa *et al.*, 1997). Recently, rhizobial strains isolated from tree and shrub legumes and unable to nodulate soybean were genetically characterised and identified as *Bradyrhizobium* spp. (Vinuesa *et al.*, 1998). Moreover, based on partial sequence analysis of 16S rDNA, it was reported that peanut bradyrhizobial strains were also closely related to *B. japonicum* (Zhang *et al.*, 1999). The genus *Sinorhizobium* contains various fast-growing species but only strains of *S. fredii* are capable of nodulating soybean (Keyser *et al.*, 1982; Scholla and Elkan, 1984; Chen *et al.*, 1988; de Lajudie *et al.*, 1994). In contrast to the Bradyrhizobia, *S. fredii* shows a broad host-range. Finally, another species with a wide host-range, *R. tianshanenses* was found to nodulate

soybean (Chen *et al.*, 1995). Since this species shows an intermediate growth-rate it has been moved to another genus which is denominated *Mesorhizobium* (Jarvis *et al.*, 1997).

While most rhizobial strains nodulating soybean have been isolated from China or Japan, which are known as the primary gene centers for soybean, there is only limited insight in the diversity of rhizobial strains from Indonesia, which is supposed to be a second gene-centre of soybean plants (Hymowitz and Newell, 1980). Based on symbiotic properties and ARDRA, significant differences between soybean-nodulating rhizobial strains isolated from Java and from Sumatra were found (Chapter 4, this thesis). Most isolates from Java are closely related to *B. japonicum* USDA 110 and more effective on soybean than those isolated from Sumatra. However, several effective isolates could be recovered from soils derived from Sumatra. In this study, therefore, a selected number of isolates from Java and Sumatra were characterised in more detail by the amplification, cloning and sequence analysis of the major part of their 16S rDNA genes.

Material and Methods

Growth, DNA isolation and PCR amplification

Soybean-nodulating rhizobial strains from soil samples derived from Java (7 isolates) and Sumatra (14 isolates) were grown in yeast-extract-mannitol broth (YEM). Growth rates were determined in YEM broth by plating on YEM agar at 30 °C as described previously (Somasegaran and Hoben, 1995). Genomic DNA was isolated from YEM broth grown culture as described earlier (Chapter 4, this thesis). 16S rDNA was amplified by PCR by using approximately 50 ng DNA template and 10 pg the primers 8f [5' CACGGA TCC AGA GTT TGA T(C/T)(A/C) TGG CTC

AG] and 1510r [5' GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT] (Lane, 1991; Laguerre *et al.*, 1994). The temperature profile was as follows: initial denaturation at 94 °C for 5 minutes; 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, and extension at 68 °C for 1 minutes; and final extension at 68 °C for 7 minutes. The PCR products were purified with the Qiaquick PCR purification kit following instructions of the manufacture (Qiagen, Hilden, Germany). Purified PCR products were quantified by electrophoresis on a 1.2 % agarose gel with known amounts of λDNA digested with *Hin*dIII as a standard (GibcoBRL Life Technology, Breda, The Netherlands).

Cloning of the 16S rDNA PCR products and plasmid DNA isolation

The PCR-amplified 16S rDNA fragments were cloned in *Escherichia coli* JM109 by using the pGEM^{R}-T Vector system following a procedure provided by the supplier (Promega, Leiden, The Netherlands). For plasmid DNA isolation, one colony of an ampicillin-resistant transformant was used to inoculate to Luria-Bertani broth medium containing ampicillin (100 μg/ml), and incubated at 37 °C for 24 hours (Manniatis *et al.*, 1982). Plasmid DNA was isolated by using a column Wizard^{TM} Plus Minipreps DNA Purification System (Promega, Leiden, The Netherlands). Purified plasmid DNA was quantified by electrophoresis on a 1.2 % agarose gel with known amount of λDNA digested with *Hin*dIII as a standard (GibcoBRL Life Technology, Breda, The Netherlands).

Sequence analysis

The purified plasmid DNA (250 ng) was used as template for sequencing reaction. Infrared-labelled primers were used for reaction, for the forward reaction primer SP6 IRD800 (5' -GAT TTA GGT GAC ACT ATA G-3') and for the reverse reaction primer T7 IRD800 (5'- TAA TAC GAC TCA CTA TAG GG-3')(MWG Biotech, Ebersberg, Germany). PCR sequencing reaction were performed with reagents provided by the supplier (Amersham Pharmacia Biotech, Freiburg, Germany) and the following temperature profile: 93 °C for 3 minutes; 30 cycles of 93 °C for 30 seconds, 45 °C for 30 seconds, 70 °C for 15 seconds; and storage at 4 °C. The products were separated and analysed on a Li-Cor DNA sequencer 4000L (LiCor, Lincoln, Nebraska, USA). Before loading the samples (1.8 µl), the gel was pre-run for 30 minutes at 1000V. After loading samples, electrophoresis was carried out at 1000 V constant voltage while the gel was heated at 50 °C. The sequence of approximately 1000-1200 nucleotides both in the forward and reverse direction was obtained, corrected manually, and combined into a single contig of 1200 – 1500 of unambiguous sequence by using SeqMan II DNA Star Software (DNASTAR Inc., Madison, Wisconsin, USA).

Identification of the soybean rhizobia on the basis of 16S rDNA homology

The obtained 16S rDNA sequence data were analysed for their homology with the blastn program from The GenBank Network (*http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/*). A rooted phylogenetic tree (neighbour joining) was calculated by using the programmes and database from ARB, an environment for 16S/18S/23S ribosomal RNA sequence data, and *E. coli* position 150

–1114 (Felsenstein correction). The ARB Package is a combination of alignment and dendrogram tools, allowing alignment to a comprehensive 16S rDNA database and detailed phylogenetic analysis (Strunk and Ludwig, 1995).

Results and Discussion

Recently, rhizobial strains nodulating soybean were isolated from Java and Sumatra and characterised based on their nodulation phenotype and ARDRA of 16S and 16S-23S rDNA sequence diversity that allowed classification into 4 groups (Chapter 4, this thesis). The isolates from Sumatra and Java that were found to be distinct from the reference strain *B. japonicum* USDA 110, were considered to be indigenous strains. One isolate from Java, strain J-TGS50, was found to be rather exceptional and showed a unique position in the ARDRA. In the present study, a copy of the 16S rDNA genes of 21 selected, soybean-nodulating rhizobial strains from those Indonesian isolates was PCR amplified, cloned and characterised by nucleotide sequence analysis. The obtained 16S rDNA sequences were compared to those of reference strains obtained from the GenBank and ARB databases (Table 1). The high homology of the rDNA sequences indicated that all the isolates, except for J-TGS50, could be assigned to genus *Bradyrhizobium* and are related to either *B. elkanii* or *B. japonicum* (Table 1). Strain TGS50, the unique isolate from Java, was found to belong to genus *Sinorhizobium* and its 16S rDNA sequence showed 97.7 % homology to that of *S. fredii*. A rooted phylogenetic tree was constructed which showed that all rhizobial isolates from Java and Sumatra except for strain J-TGS50 could be grouped into 2 species, *B. elkanii* and *B. japonicum* (Fig.1).

B. elkanii was found to be dominant in acid soils from Sitiung, West-Sumatra. Ten out of fourteen studied isolates from this location could be assigned to *B. elkanii*. Apart from isolate S-ST518 they form a homogeneous group, and notably strains S-ST17, S-ST45, S-ST117, S-ST215 and S-ST414 show high similarity to the reference strain *B. elkanii* USDA 76 (Fig.1). However in spite of their phylogenetic relation, the strains showed considerable variability in their symbiotic properties (Chapter 4, this thesis).

Table 1. Numbers of nucleotide differences and % 16S rDNA homologies in the aligned sequences of well-known soybean-nodulating strains *B. elkanii* (accession number BEU3500), *B. japonicum* USDA 110 (accession number Z35330) and *S. fredii* USDA 205 (accession number D14516).

		B. elkanii USDA76			*B. japonicum* USDA 110				*S. fredii* USDA 205		
Strain	Number of nucleotides	ΔN[1]	%H[2]	Strain	Number of nucleotides	ΔN	% H	Strain	Number of nucleotides	Δ N	%H
S-ST17	1390	6	99.6	J-WG2	1089	7	99.4	J-TGS50	1440	30	97.9
S-ST414	1415	3	99.8	J-DLG10	1265	28	97.8				
J-KH5	1199	11	99.1	J-SRG9	1443	15	99.0				
J-Y49	1445	7	99.5	J-TM3	1426	3	99.8				
S-ST316	1443	9	99.4	S-ST123	1329	23	98.3				
S-ST29	1054	9	99.1	S-ST325	1445	11	99.2				
S-ST33	1442	10	99.3	S-BT221	1447	21	98.5				
S-ST215	1348	3	99.8	S-BT322	1446	16	98.9				
S-ST16	1446	25	98.3								
S-ST518	1425	18	98.7								
S-ST45	1360	6	99.6								
S-ST117	1445	9	99.4								

[1]Number of nucleotide differences. [2] % of 16S rDNA homology.

Only two out of seven strains from Java (strain J-KH5 and J-YG49) were found to be *B. elkanii*. In contrast to the *B. elkanii* isolates from Sumatra, the N fixation capacity of these *B. elkanii* strains from Java was all very high on soybean (Chapter 4, this thesis).

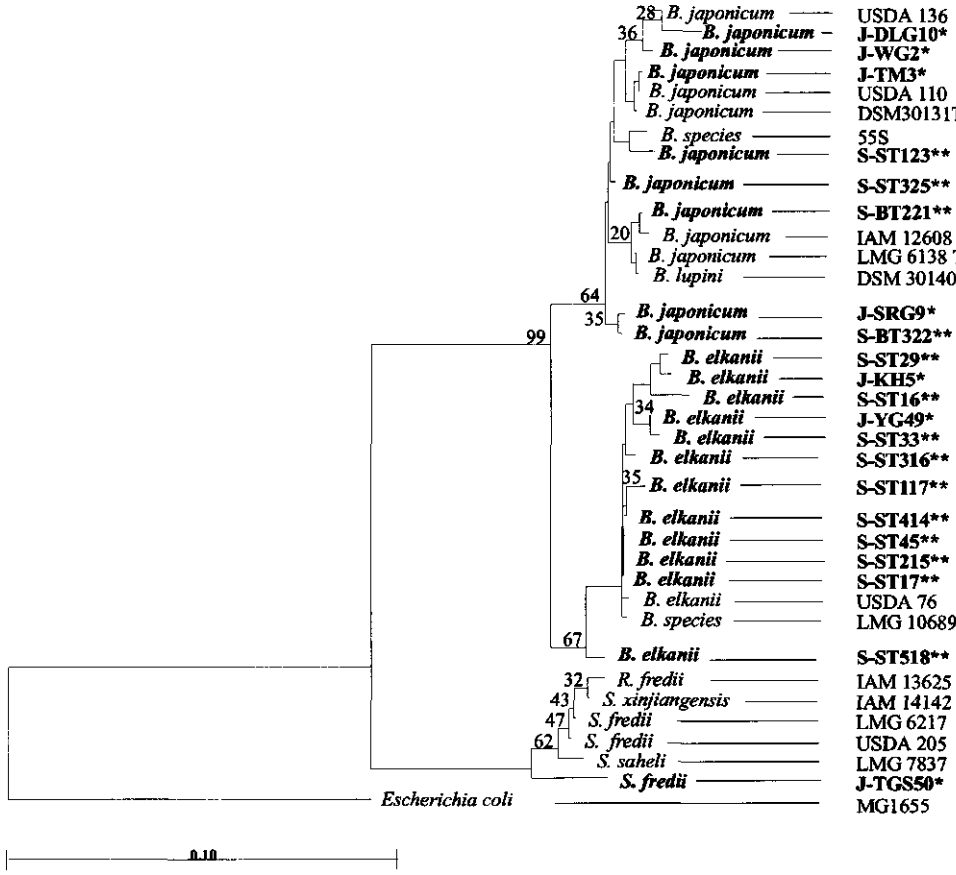

Figure 1. A rooted phylogenetic tree based on 16S rDNA sequences of the Indonesian rhizobial isolates (bold, * from Java and ** from Sumatra) and related other *Bradyrhizobium* and *Sinorhizobium* spp. constructed by the ARB Software with 100 times bootstrap. The bar indicates the phylogenetic distance (0.1 knuc).

B. japonicum was found dominant in Java. Four isolates (J-DLG10; J-WG2; J-TM3; J-SRG9) from seven analysed strains could be classified as *B. japonicum*. This confirms earlier results based on symbiotic properties and ARDRA 16S rDNA and 16S-23S rDNA (Chapter 4, this thesis). One of these isolates, strain J-TM3, was very specific and effective on soybean (Chapter 4, this thesis). This could be due to the cultivation of imported soybean seeds contaminated with soils (Toxopeus, 1938) or the introduction as inoculant by researchers (Newton, 1962). It is not be possible to differentiate between these or other possibilities, but the observation that strain J-TM3 is highly related to *B japonicum* USDA 110, a well known inoculant isolated from Japan, suggests strongly that it is not a native strain (Fig. 1/Table 1). Remarkably, the other strains (J-DLG10; J-WG2 and J-SRG9) have been shown to nodulate mungbean plants (Chapter 4, this thesis). This is in apparent contrast with the common phenotype of *B. japonicum* which is known to nodulate only soybean. Several *B. japonicum* strains (S-ST123; S-ST325; S-BT221; S-BT322) were also found in Sumatra soils. However, while all strains from Java are highly effective, these *B. japonicum* strains from Sumatra are ineffective (Chapter 4, this thesis).

It is interesting to note that a strain belonging to the genus *Sinorhizobium* that include fast growing species, was also found in Indonesia. Hence, the growth properties of this strain, J-TGS50, were determined and compared to that other *S. fredii* strains and *B. japonicum* of USDA 110. Mean generation time (g) of J-TGS50 was almost 6-fold lower than that of *B. japonicum* USDA 110 and even lower than that previously reported for *S. fredii* (Table 2). This was also apparent during growth of colonies on YEM agar plates. While colonies of the strain J-TGS50 with a diameter size between 0.10 – 1.0 mm could be observed already at 3 days after inoculation, no colonies of strain USDA 110 were detectable at that time. Analysis of 16S rDNA

sequences of strain J-TGS50 showed it to be related to *S. saheli* and *S. fredii*. Up till now, only *S. fredii* and not *S. saheli* strains have been reported to nodulate soybean (Keyser *et al.*, 1982; De Lajudie *et al.*, 1994; Young, 1996). However, *S. fredii* strains were found to show a broad host-range and formed nodules on soybean as well as many other legumes, including cowpea, pigeon pea and mungbean (Scholla and Elkan, 1984; Stowers and Eaglesham, 1984; Chamber and Iruthayathas, 1988). This contrasts with the restricted nodulation properties of strain J-TGS50 which did not nodulate mungbean (Chapter 4, this thesis).

Table 2. Mean generation time (hours) of isolate J-TGS50 compared to the other fast growth soybean rhizobia that have been reported elsewhere.

Strain	Generation time (hours)	References
USDA 205	3.6	Keyser *et al.* (1982)
HH303	2.6	Dowdle and Bohlool (1985)
SB357	1.7	Young *et al.* (1988)
SMX11	1.5	Rodriguez-Navarro *et al.* (1996)
J-TGS50	1.5	This study

From the data presented here and the earlier results (Waluyo *et al.*, 2000), it can be concluded that there is no relationship between the nodulation phenotype and rRNA traits. This confirms earlier reports for BTAi1, a phototrophic symbiont of the legume *Aeschynomene* (Young *et al.*, 1991) and for peanut bradyrhizobial strains (Zhang *et al.*, 1999). Therefore, besides nodulation phenotype, other traits, eg. 16S rDNA sequences, should be taken into account for identification and classification of rhizobial strains.

This study demonstrates that species known to nodulate soybean, *B. japonicum*, *B. elkanii* and most likely also *S. fredii* are present in Indonesia. There appeared a great diversity in effectiveness between these brady- and sinorhizobia strains (Chapter 4, this thesis). In the region Java, many effective strains are already

present in the soil, presumably by selection of soybean over a long period of cultivation. This could be the reason for the absence of a response to soybean inoculation practice in Java (Saono, 1988).

Bradyrhizobia strains are present in low numbers in acid soils of Sumatra and are suggested to occupy niches in the acid soils, which are not toxic to these bacteria. It is of interest that there is a large variation in N fixation capacity among the isolates from Sumatra (Chapter 4, this thesis). Hence, combination of 16S rDNA sequence analysis as described here with nodulation phenotype trait analysis (Chapter 4, this thesis) opens the possibility to select for elite, effective strain, adapted to acid soils. These strains may be used as inoculants for other transmigration areas in Indonesia, where poor acid soils are usually predominant.

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Chapter 6

Summary and Concluding Remarks

The aim of this research was to increase soybean production in acid soils, in Sitiung, Sumatra, Indonesia, one of the transmigration areas recommended for soybean production. This was realised via enhancement of Biological Nitrogen Fixation (BNF). However, this is not a simple task since the Sumatra soils are acid and toxic to soybean due to a high exchangeable aluminium (Al) content. Improved cultivars, selected bacterial strains, and appropriate management practices must be developed to realise a high production. The research described in this thesis was directed to the development of cheap and simple agricultural practices to improve BNF in soybean production. In addition, the use of cross-inoculation and molecular techniques, resulting in the isolation and characterisation of Brady- and Sinorhizobia, native to Indonesian soils, is of particular interest since the presence of competitive but inefficient indigenous rhizobial strains may influence the success of the inoculation.

The significance of soybean and BNF in Indonesia

In Indonesia, soybean is the main legume crop and the second main food crop after rice. However, the total production is very low and not sufficient to meet domestic demand. In 1998, 700 000 ton of soybean, representing half of the domestic production was still being imported. In view of the present economic situation, the increase of domestic production of soybean is the only option for Indonesia.

However, the main problem to increase soybean production in Indonesia is a lack of suitable, fertile soils. There is no more land on Java, the main soybean growing area, due to over-population. On the other hand, there is enough vacant land

outside Java on the islands of Sumatra, Kalimantan, Sulawesi and Irian Jaya, but these soils are not suitable for soybean cultivation. The soils are acid, contain high levels of Al, and are deficient in calcium (Ca) and phosphorus (P). A programme by the government of Indonesia, termed INSUS Tanaman Kedelai (INSUS = **In**tensifikasi Khu**sus**), to intensify soybean production has been set up for these areas. The main solution was liming the soils, and lime, costing US$ 19 million, was provided free of charge to farmers between 1983-1986 (Sebayang and Sihombing, 1987). However, this appeared to be a very expensive programme, since approximately the application of 4.0 to 7.0 t ha^{-1} of lime is needed. In addition, due to low N and P levels of the soils the farmer still has to apply N and P fertilisers to obtain a good yield of soybean.

To meet the lack of N, BNF is a promising option to increase soybean production. Soybean (*Glycine max* Merrill), being a legume, can symbiotically fix N from the atmosphere with rhizobial strains, such as *Bradyrhizobium japonicum*, and convert this to ammonia. This symbiosis can provide significant amounts of N to the crop to increase growth and yield, and thus has a significant impact on agriculture.

The significance of pelleting seeds with lime and P fertiliser

To reduce the amount of lime, instead of liming the soils, the use of seed pelleted with lime was investigated. In addition, instead of broadcasting P fertiliser, the inclusion of P as triple superphosphate (TSP) in the pellet was also studied. A combination of a small amount of broadcast lime and pelleting soybean seeds with lime and TSP was found an appropriate management practice to improve soybean BNF and production on strongly acid soils (see Chapter 2). This is important for poor farmers and adds to a sustainable agricultural practice.

Lime pelleted seed has been successfully developed to improve BNF and the establishment of some temperate legumes in acid soils in Australia (Mannetje, 1967; Diatloff and Luck, 1972) and in The Netherlands (Pijnenborg and Lie, 1990). In this research it was shown that seed pelleting with a combination of lime (50 kg ha^{-1}) and P (10 kg TSP ha^{-1}), was very successful for soybean (cv. Tidar) grown on heavily weathered acid soils in Sitiung, West Sumatra (Chapter 2). Results from field and pot experiments showed that pelleting seeds with lime and phosphate could partly substitute heavy liming and high P fertilisation. The seed pelleting was found sufficient to ensure optimal nodulation and N fixation. A considerably increased number and the weight of nodules developed a more efficient BNF, and could enhance N nutrition of soybean in addition to N taken up from the soil. In contrast, most N taken up by the plants grown on the limed acid soils (7.0 t ha^{-1}) was entirely obtained from the soil. This is because the root system was well developed on limed acid soils, leading to an efficient N and other essential nutrients uptake from the soil, in particular those released by mineralisation.

The small amounts of lime and P supplied in the pellets also increased the yield of soybean from 87 kg ha^{-1} to 319 kg ha^{-1} (Chapter 2). This yield is comparable to that obtained either from inoculated seed and liming with 3.5 t ha^{-1} or from un-inoculated seed but liming with 7.0 t ha^{-1}. However, in view of the high sensitivity of soybean to soil acidity, it is still necessary to apply about 2.0 t ha^{-1} of lime to obtain optimal plant growth and yield. Combination of liming and pelleting the seeds produced 301 kg ha^{-1} more yield than from soils with 7.0 t ha^{-1} of lime. The results of pot experiments confirm these finding.

The importance of P nutrition on BNF of soybean in heavily weathered acid soils

Application of P is usually essential to growth and BNF of soybean in strongly acid soils. P is deficient in most highly weathered acid soils (Wade *et al.*, 1988). However, it is interesting to note that the positive effect of P on strongly acid soils is clearly dependent on the way of application. Phosphate must be applied so that it can be easily reached by the roots. This is because of the fact that the soil has a strong capacity to fix P, which is also the reason for a recommendation of using high levels of P fertiliser in the past (Sanchez and Salinas, 1981; Sudjadi, 1984; Wade *et al.*, 1988). However, since TSP is a very soluble fertiliser, its direct contact with seedling must be avoided, the more so since it evokes a strong acidic reaction, which is very toxic to seedlings. To avoid this negative effect, P and Ca must be applied simultaneously. Therefore, P was applied together with lime to pellet the soybean seeds, and this approach was found to be successful for increasing the growth and BNF of soybean grown in the acid soils of Sitiung, West Sumatra (Chapter 2).

There are several ways the applied P close to seedlings promotes the establishment of soybean BNF in acid soils. In general, the improvement of the micro-environment close to the seedling is very essential to establish an infection. The applied lime and P on the seed-pellet neutralises soil acidity, reduces Al toxicity, and increases P and Ca availability. The available P is readily accessed by the emerging roots, increases ramification of the root system and produces a high number of susceptible root-sites (Borkert and Barber, 1985; Brady *et al.*, 1990; Hecht-Bucholz *et al.*, 1990). These are crucial phases of the infection process, and thus for the establishment of BNF (Munns, 1968; Lie, 1969; Bhuvaneswari *et al.*, 1980).

Phosphate thus plays an important role in the initiation of nodule formation and may also be needed by the rhizobial strains to survive and to initiate infection. The importance of P for growth of rhizobial strains has been reported elsewhere (Cassman *et al.*, 1981; Beck and Munns, 1984). It was considered that optimum nodulation and N_2 fixation are functions of early colonisation of the rhizosphere by *Bradyrhizobium japonicum* (Brockwell *et al.*, 1985). To clarify this, more detailed studies using soybean seedlings growing in rhizotrons were performed (Chapter 3). A rhizotron system is supposed to be more reliable to mimic the field conditions than hydrophonic experiments, since soil samples were used to support growth instead of a nutrient solution.

In Chapter 3 it is described that the number of nodule primordia was increased significantly by the addition of P. In this study, the nodule primordia and also root primordia inside the roots were observed and quantified at an early stage, 5 days after inoculation, using a microscope with 60 times magnification. It gives more direct data compared to previously studies, which were based on observation of the emerging nodules (Gates and Muller, 1979). Moreover, by using the rhizotron system, the intact root can be easily released from the soil matrix, and hence nodule primodia can be quantified from whole root systems. Applying P also increased the growth and the capacity for N-fixation of already formed nodules. This is in agreement with earlier reports that P is important for development, growth and function of nodules (Gates, 1974; Gates and Muller, 1979; Cassman *et al.*, 1980; Israel, 1987; Singleton *et al.*, 1985; Wan Othman *et al.*, 1991), and to the host-plant (Munns *et al.*, 1981). Giller and Cadisch (1995) and Thomas (1995) stated that P deficiency is a factor commonly limiting the realisation of the potential of N_2 fixation by legumes.

However, besides pelleting the seeds, liming the soil is still needed to increase soybean production in the infertile strongly acid soils (pH <5.0; Al saturation > 80 %; deficient in Ca and P). The lime compensates the Ca deficiency and improves the soil pH and hence reduces the inhibition of the soybean crop by acid. The liming of acid soils is also used to decrease Al saturation from more than 80% to 15%, the critical level of Al saturation for maximum yield of soybean. Healthy growth of soybean on limed acid soils may also be due to the increased availability of P. As a consequence, growth and BNF of soybean are often better on limed than on un-limed acid soils (Sartain and Kamprath, 1975).

However, from an economic point of view, application of high levels of lime and P fertiliser is not acceptable. Hence, in the present study, a low input agricultural practice was developed to replace it. The results indicate that this approach is successful (Chapter 2). Although lime at 2.0 ton ha^{-1} was still needed, this input is much lower than that reported by Sudjadi (1984) and by Wade *et al.* (1988), and ca. 4.0 ton ha^{-1} of lime could be saved.

The importance of the structure of indigenous rhizobial populations on BNF.

The beneficial effect of rhizobial strains inoculation on the increase of soybean yields has been reported previously (Burton and Curley, 1965; Ham, 1980; Herridge and Brockwell, 1988; Peoples and Crasswell, 1992). In Indonesia, the importance of rhizobial strains to increase soybean production has already been recognised from the work of Toxopeus (1936). To cope with the increasing domestic demand for soybean, some *Bradyrhizobium* strains from abroad were imported to

Indonesia and applied in the traditional soybean areas in Java (Keleney, 1959; Newton, 1962). It has also been suggested frequently that indigenous, and hence adapted, rhizobial strains are present in high numbers in the soil in Java, and their presence may be responsible for the lack of response of the inoculation of elite *Bradyrhizobium* strains. However, there are very limited systematic data on the diversity of indigenous rhizobial strains in Java.

A study of the indigenous rhizobial populations, notably in acid soils, is of interest in view of the possible interference with inoculant *Bradyrhizobium* strains in later years. Of course in the first years of soybean cultivation in the transmigration areas, there is no danger of competition due to the low numbers of indigenous rhizobial cells in the acid soils (Adiningsih and Prihatini, 1981; Mahmud and Rumawas, 1983; Chapter 2). However, a continuous cultivation of acid soils for soybean production will increase native rhizobia in the soils and at a certain level will interfere with the introduced, more effective rhizobial inoculant strains. Indigenous rhizobial strains are suggested to be present in Indonesian soils. This was reported for the acid soils of Sitiung West Sumatra. Nodules were formed on soybean plants grown on un-inoculated but limed soils (Adiningsih and Prihatini, 1981; Mahmud and Rumawas, 1983; Chapter 2). In the present study, it was observed in field experiments that occasionally a few plants in the untreated plots, had a nodule on the lateral roots, in the deeper layers of the soil. Upon transfer of these soils from Sumatra to Wageningen, and their use in rhizotron experiments, the same phenomenon was also observed (Chapter 2). Presumably, these native rhizobia are part of the indigenous flora and scattered in the soils at niches that are not toxic. Richardson and Simpson (1988) provided support for this explanation and reported that *Rhizobium leguminosarum* bv. *trifolii* can persist in very acid soils by avoiding extreme acidity

rather than by tolerance to acidity. Incorporation of lime enhanced colonisation of the soil and produced a homogeneous population throughout the entire soil profile.

Cultivation of soybean on acid soils may increase soybean rhizobia cell numbers in the soils. Mahler and Wollum (1982) reported that 194-fold higher soil populations of *B. japonicum* existed in cultivated fields with a soybean history compared to cultivated fields without a soybean history. Brockwell *et al.* (1987, 1989) also reported increases in the population of *B. japonicum* in soil following soybean cropping. Simanungkalit *et al.* (1995) found that the number of soybean rhizobia in a latosol soil, Bogor, Indonesia, was increased from $\log_{10}$ 1.29 g^{-1} of the total volume of soil to $\log_{10}$ 4.84 g^{-1} soil in the soybean rhizosphere. More recently, it was reported that enrichment of soil bradyrhizobial populations is host-specific, and symbiotic legumes can enrich their soil environment with microsymbionts up to a threshold level (Thies *et al.*, 1995).

Hence, it is important to survey soils prior to agricultural practice for the presence of rhizobial strains and to classify them using molecular approaches. Molecular techniques have been developed for studying rhizobial strains. Effective and competitive strains have been developed by genetic engineering for *R. leguminosarum* (Chen *et al.*, 1991), and for *Sinorhizobium meliloti* RMBPC-2 (Scupham *et al.*, 1996). Using a combination of *gusA* and *celB* marker genes, Sessitsch *et al.* (1996) studied competition between *R. tropici* strains. Significant insight in the potential, activity and taxonomy of rhizobial strains was obtained by a variety of molecular biological approaches, including analysis of their genome (Freiburg *et al.*, 1997). Among others, this has promoted a progressive change in rhizobial strain classification. Due to the economic value of soybean, rhizobial strains nodulating this crop received more attention than other rhizobial strains. Hollis *et al.*

(1981) used DNA-DNA hybridisation to reveal that *R. japonicum* could be divided into three groups, *R. japonicum* group I, group Ia and group II. Subsequently, Jordan (1982) split the genus *Rhizobium* into the genera *Rhizobium* and *Bradyrhizobium*. The discovery of fast-growing rhizobia isolated from soybean root nodules collected in China (Keyser *et al.*, 1982) has changed dramatically the nomenclature of soybean rhizobia. These fast-growing soybean-nodulating rhizobia were designated as *S. fredii* strains by Scholla and Elkan (1984). Kuykendall *et al.* (1992) proposed that *R. japonicum* group II should be classified as *B. elkaniii*, and strain USDA 76 was designated as the type strain. Xu *et al.* (1995) has isolated a new soybean rhizobial strain, which was designated *B. liaoningensis*. Recently, a *Mesorhizobium thianshanense* strain has also been reported to nodulate soybean (Chen *et al.*, 1995). Based on 16S rDNA sequences, a new phylogenetic tree of rhizobia has been proposed by Young (1996) and Jarvis *et al.* (1997), that now include the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*.

In contrast, only limited attention has been given to study rhizobial strains indigenous to Indonesian soils and merely included the isolation, inoculation and effectiveness testing in green house and field experiments. The ambiguous results obtained from the response of inoculation may contribute to this slow development. Since it has been suggested that Indonesia is a secondary gene centre of soybean (Hymowitz and Newell, 1981), there should be many different rhizobial strains present in Indonesia, which could be further exploited. Chapter 4 describes the isolation of soybean-nodulating rhizobial strains from soil samples collected in a traditional soybean area (Java) and from a new soybean area (Sumatra). While, there are many studies demonstrating the diversity among rhizobial strains nodulating soybean, only relatively few studies have assessed the potential symbiotic

significance of the resulting groupings. Therefore, the isolates from Java and from Sumatra were analysed symbiotically by bio-assay on soybean and mungbean (*Vigna radiata* cv. Manyar), and genetically by ARDRA of 16S rDNA and 16S-23S rDNA. The results show that *Bradyrhizobium* strains specific for soybean as well promiscuous soybean rhizobial strains were obtained both from Sumatra's and Java's soil samples.

Differences in the capacity to fix N are important in agriculture for the selection of effective strains. It has been shown that soybean plants inoculated with rhizobial obtained from Sumatra were less healthy than those obtained from Java (Chapter 4). At harvest, most of the first two leaves from soybean plants inoculated with rhizobial strains from Sumatra were yellow in colour, and dropped off. This indicated that the plant was lacking N since in that case, the N from the oldest part would be relocated to the younger part (Mengel and Kirckby, 1987). In contrast, there were no indications of N deficiency of plants inoculated with rhizobial strains from Java. This implies that the soybean rhizobia from Java have a greater capability to fix N than those from Sumatra.

Genetically, soybean bradyrhizobia from Sumatra are in general different from isolates from Java. Several isolates from Java were closely related to *B. japonicum* USDA 110, a reference strain for soybean. Soybean bradyrhizobia from abroad may have been indirectly imported by soil-contaminated soybean seeds or directly introduced by previous researchers. In contrast, isolates from Sumatra are distinctly different from the reference strain USDA 110, and likely to be the indigenous bradyrhizobia.

Some selected rhizobial isolates, representative of the population from a new soybean growing area (Sumatra) and a traditional area (Java), were identified by

complete 16S rDNA sequence analysis (Chapter 5). Based on comparison of the 16S rDNA sequences, two genera of soybean rhizobia, *Bradyrhizobium* and *Sinorhizobium* were found in Indonesian soils. Both *B. japonicum* and *B. elkanii* were obtained from Java and Sumatra, while a *S. fredii*-like strain was found only in a soil sample from Java.

It is interesting to note that *B. elkanii* is widespread in the acid soils of Sitiung, West Sumatra. It seems that *B. elkanii* is more resistant to the encountered acidity than *B. japonicum*. This suggestion is supported by an earlier report describing the isolation of some *B. elkanii* strains from acid soils from Indonesia (Ozawa *et al.*, 1995). The *S. fredii*–like isolate from Java does not belong to the fast-growing soybean brady- and sinorhizobia reported earlier, and may represent a new *Sinorhizobium* species since this isolate could not form nodules on mungbean and the homology of its 16S rDNA with that of *S. fredii* USDA 205, the closest reference strain in the phylogenetic tree, is only 97.9 %.

In conclusion, BNF is a very important natural process in agricultural practices. However, improvement of sustainable agricultural production will need an efficient BNF as a main source of N for crops. To do so, improved crops, efficient brady- and sinorhizobia, and appropriate management practices must be developed.

It is tempting to suggest that introduction of legume crops into other new regions may have an impact on structure of native rhizobial population. Continuous cultivation of soybean crops is expected to promote the selection of rhizobial strains with narrow host ranges and higher specificity for some host plants. Knowledge of the indigenous rhizobial population is therefore important for the success of BNF. The presence of indigenous strains of *Bradyrhizobium japonicum* and *Bradyrhizobium*

elkanii, and a strain resembling *Sinorhizobium fredii*, in Indonesian soils is demonstrated, and offer the selection of elite *Brady-* and *Sinorhizobium* strains.

This study has shown that a combination of broadcast lime at 2.0 ton ha^{-1} with the pelleting by lime and TSP of inoculated seeds with an effective *B. japonicum* or *B. elkanii* can enhance BNF with the ultimate goal to increase production of soybean in the acid soils of Sitiung, West Sumatra.

A low-input management practice is an appropriate agriculture technique to improve growth and yield of soybean in heavily weathered acid soils. Whereas it is not expensive and affordable by poor local farmers, the technique is also very important for the sustainability of agriculture in general.

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Samenvatting

Het doel van dit onderzoek is de soja productie te verhogen op de zure gronden, in Sitiung, Sumatra, Indonesië, door verbetering van de biologische stikstof fixatie (BSF). Dit is echter niet zo eenvoudig, want deze zeer zure gronden zijn toxisch voor soja en ook vanwege het hoge aluminium gehalte. Verbeterde planten rassen, bacterie stammen en geschikt landbouw beleid moeten ontwikkeld worden om een hoge productie te bereiken. Het beschreven onderzoek moet leiden tot de ontwikkeling van goedkope en eenvoudige methoden om de BSF in soja te verbeteren. Daarnaast wordt een studie gemaakt van de Brady- en Sinorhizobia stammen, die van natuur voort komen in Indonesisch gronden, waarbij gebruik wordt gemaakt van plant-infectie proeven en moleculaire technieken. Zij zijn van bijzondere interesse omdat de aanwezigheid van competitieve maar inefficiënte inheemse stammen het succes van enten kunnen beïnvloeden.

Het belang van soja en BSF voor Indonesië

Soja is een veelgegeten peulvrucht en na rijst het belangrijkste voedsel in Indonesië. De productie is echter erg laag en onvoldoende om aan de nationale vraag te voldoen. In 1998 werd de helft van de benodigde soja (700.000 ton) ingevoerd. In het licht van de huidige economische situatie, is intensivering van de binnenlandse productie de enige mogelijkheid om aan de vraag te voldoen.

Hier ligt de uitdaging om de soja productie in Indonesië te verhogen. Een groot probleem om dit te bereiken is de afwezigheid van geschikte, vruchtbare grond. Er is steeds minder grond beschikbaar op Java, een van de belangrijkste landbouw gebieden, dat overbevolkt is. Buiten Java is voldoende ruimte op de eilanden Sumatra, Kalimantan, Sulawesi en Irian Jaya, maar deze gebieden zijn niet geschikt voor verbouw van soja. De gronden zijn te zuur, hebben een hoog aluminium gehalte en missen de benodigde calcium en fosfaat. Een programma van de regering van Indonesie, INSUS Tanaman Kedelai (INSUS = Intensifikasi Khusus), is opgezet voor de intensivering van de soja teelt in deze gebieden. De belangrijkste oplossing was toevoegen van kalk aan de grond, 4,0 tot 7,0 ton per hectare. De kalk, ter waarde van 19 miljoen dollar, werd gratis verstrekt aan de boeren tussen 1983-1986. Daarnaast moesten de boeren nog stikstof en fosfaat bijmesten om een goede opbrengst te

krijgen. BSF is een goede optie om aan dit stikstof tekort te voldoen. De peulvrucht soja (*Glycine max* Merrill), kan in symbiose met *Bradyrhizobium japonicum* stikstofgas uit de lucht fixeren en dit omzetten in ammonia. Deze symbiose, kan de plant van significante hoeveelheden stikstof voorzien waardoor groei en opbrengst toenemen, wat van grote invloed is op de landbouw.

Het invloed van kalk en fosfaat coating van zaden

Om de hoeveelheid kalk te reduceren werd onderzocht of in plaats van kalk toe te dienen aan de grond, het zaad in een laagje kalk te omhullen. Tevens werd bekeken of het insluiten van fosfaat (als tri-superfosfaat, TPS) in de kalkcoating een mogelijkheid biedt in plaats van het strooien van fosfaat op de bodem. Een combinatie van een kleine hoeveelheid 'strooi' kalk en coating van zaad met kalk en TPS bleek een goede methode om BSF te verbeteren en soja productie te verhogen op zeer zure grond. Dit is erg belangrijk voor arme boeren en om de landbouw in stand te houden.

Kalk omhuld zaad is ontwikkeld om BSF te verbeteren en om kweek van niet tropische peulvruchten mogelijk te maken op zure bodems in Australië en in Nederland. In hoofdstuk 2 wordt beschreven dat [kalk (50 kg ha^{-1}) en fosfaat (10 kg TPS ha^{-1})]- coating zeer succesvol was voor soja (cv. Tidar) die groeide op uitgeloogde grond in Situng, West Sumatra. Resultaten van veld- en potproeven toonden dat coating van zaden met kalk en fosfaat deels de zware bemesting met deze stoffen konden vervangen. De zaad omhulling was genoeg om optimale wortelknol vorming en stikstof binding te bewerkstelligen. Het aantal wortelknollen nam aanzienlijk toe, ze konden efficiënter stikstof fixeren en leverden de stikstof voor de plant, zodat deze de stikstof niet meer uit de bodem hoefde op te nemen.

Op bekalkte, zure grond (7,0 t ha^{-1}) werd daarentegen de meeste stikstof door planten opgenomen uit de grond. Dit omdat het wortelsysteem goed ontwikkeld was op gekalkte, zure grond wat leidt tot een efficiënte stikstof en andere essentiële voedingsstoffen opname vanuit de grond, vooral die stoffen die vrijkomen door mineralisatie.

De kleine hoeveelheden kalk en fosfaat toegediend in de coating verhoogde de soja opbrengst van 87 kg ha^{-1} naar 319 kg ha^{-1}. Deze opbrengst is vergelijkbaar met

die je krijgt na enten van zaad en kalk toedienen (3,5 t ha^{-1}) of zonder enten en 7,0 t ha^{-1} kalk bemesten. In het licht van de gevoeligheid van soja voor zuurte van de grond, is het nog steeds nodig om ca 2,0 t ha^{-1} kalk te geven voor de optimale groei en opbrengst. Deze combinatie van kalk en coating van het zaad leverde 301 kg ha^{-1} meer opbrengst van soja dan op grond gekalkt met 7,0 t ha^{-1}. De potproeven bevestigen deze resultaten.

Het belang van fosfaat bemesting op BSF van soja op uitgeloogde bodems

Toepassing van fosfaat is meestal essentieel voor groei en BSF van soja op zeer zure grond. Fosfaat is deficiënt in de meeste uitgeloogde bodems. Het is echter interessant te zien dat het positieve effect van fosfaat sterk afhangt van de wijze waarop toediening plaats vond. Fosfaat moet zodanig beschikbaar zijn dat het makkelijk te bereiken is door de wortels. De grond heeft een grote capaciteit fosfaat te binden, wat aanleiding was voor de hoge bemestingshoeveelheden in het verleden. TSP is een goed oplosbaar nutriënt en moet niet in contact komen met de zaailingen. Het geeft een sterk zure reactie, wat toxisch is voor de zaailing. Om dit negatieve effect te vermijden, moeten fosfaat en calcium gelijktijdig toegediend worden. Daarom werd fosfaat gelijk met de kalk coating toegevoegd aan de zaden en dit resulteerde in een toename in de groei en BSF van soja op de zure gronden in Sitiung, West Sumatra (hoofdstuk 2).

Er zijn verschillende manieren waarop de toegediende fosfaat dichtbij de soja zaailingen BSF stimuleert. In het algemeen is verbetering van het microklimaat dichtbij de zaailing essentieel voor de infectie. De kalk en fosfaat in de coating neutraliseren de zure grond, ontgiften de aluminium toxiciteit en maken fosfaat en calcium meer beschikbaar. De aanwezige fosfaat kan makkelijk door de kiemwortel worden bereikt, zorgt voor vertakkingen in het wortelsysteem en produceert een groot aantal vatbare niches op de wortels. Deze zijn cruciaal in het infectieproces en daardoor voor het tot stand komen van de symbiose.

Fosfaat speelt een belangrijke rol in de start van de wortelknol vorming. Het is ook nodig voor de rhizobia om te overleven en te kunnen infecteren. Het idee was dat optimale knolvorming en BSF functies zijn van vroege kolonisatie van de rhizosfeer

door *Bradyrhizobium japonicum*. Om dit te bestuderen werden soja planten in rhizotrons gekweekt (hoofdstuk 3). Een rhizotron opstelling is betrouwbaarder om de veldcondities na te doen dan een hydrocultuur, omdat grond gebruikt wordt i.p.v. vloeibaar medium. In dit onderzoek werd gevonden dat het aantal initialen van wortelknollen significant verhoogd werd door fosfaat.

Toediening van fosfaat zorgde voor betere groei en hogere BSF capaciteit door de reeds gevormde knollen. Dit komt overeen met ander onderzoek, waar beschreven wordt dat fosfaat belangrijk is voor de ontwikkeling, groei en functioneren van de wortelknollen en voor de gastheer plant. Het is een algemene opvatting dat fosfaat tekort een limiterende factor is voor de stikstof binding door peulvruchten.

Naast een coating van de zaden, is het nog steeds nodig de zure grond te bekalken bij de verbouw van soja. Kalk toediening aan zure grond heeft tot doel om aluminium verzadiging terug te brengen van 80% naar 15%, de kritische waarde voor soja. Daarnaast veroorzaakt bekalking van zure grond een betere beschikbaarheid van fosfaat aan de plant.

Gezien de armoede van de boeren en de huidige economie is bemesten met grote hoeveelheden kalk en fosfaat niet aanvaardbaar. Daarom moest een eenvoudig, goedkoop landbouw beleid ontwikkeld worden zoals hier beschreven. Er is nog steeds 2,0 t ha^{-1} kalk nodig, maar dit is veel lager dan in eerdere studies werd gevonden, en levert een besparing op van c.a. 4,0-5,0 ton ha^{-1} kalk.

De betekenis van de structuur van inheemse rhizobial populaties op BSF

Het gunstige effect van enting op de verbetering van soja opbrengst is ook door anderen beschreven. Om aan de grote vraag naar soja in Indonesië te voldoen werden bacterie stammen geïmporteerd, ter verbetering van BSF in de traditionele soja gebieden op Java. Er is vaak verondersteld dat de aanwezigheid van grote aantallen, inheemse geadapteerde rhizobia in de bodem van Java, verantwoordelijk is voor de slechte resultaten van beenting van soja. Dit is echter speculatief, de diversiteit van inheemse rhizobia flora is nog onvoldoende bestudeerd in Indonesië.

Een studie naar de inheemse rhizobia populaties in grond is interessant vanwege de mogelijke verstoring van de enting met brady- en sinorhizobium

stammen in latere jaren. In de eerste jaren van soja teelt bestaat er geen gevaar voor competitie vanwege de lage aantallen inheemse brady- en sinorhizobium cellen in de zure bodem. Na een aantal jaar cultivatie van soja op zure grond zal de inheemse populatie rhizobia toenemen en op zeker ogenblik zullen ze de geïntroduceerde, meer effectieve stammen verstoren. Er zijn aanwijzigingen dat inheemse *rhizobium* stammen reeds aanwezig zijn in de Indonesische bodem. Soms worden wortelknollen gevormd aan sojaplanten op bekalkte, maar niet geënte grond. In het hier beschreven onderzoek werd in veldproeven gevonden dat enkele planten in de onbehandelde percelen toch wortelknollen vormden aan de zijwortels dieper in de grond. Dit werd bevestigd door rhizotron experimenten in Wageningen met grond die gedurende drie jaar werd verzameld op verschillende locaties in Sitiung. Verondersteld wordt dat deze bacteriën deel zijn van de inheemse flora en zich bevinden in niches in de grond die minder toxisch zijn. Toedienen van kalk doet de aantallen soja rhizobia toenemen en de grond wordt meer uniform gekoloniseerd dan in niet bekalkte grond. In de literatuur was aangetoond is dat *Rhiziobium leguminosarum* bv *trifolii* in zeer zure grond kan standhouden door extreme zuurte te vermijden en niet door zuurtolerantie. Inbreng van kalk vestigt kolonisatie in de grond en zorgt voor een homogene populatie door de gehele grond.

Teelt van soja op zure grond kan zorgen voor toename van *rhizobium* aantallen in de grond. In verscheidene andere studies wordt melding gemaakt van hogere grondpopulaties van *B. japonicum* in grond waarop soja werd verbouwd dan op landbouwgrond zonder soja voorgeschiedenis. Recentelijk is gevonden dat verrijking van grond met *bradyrhizobium* populaties gastheer specifiek is en dat peulvruchten hun bodemmilieu met microsymbionten kunnen verrijken tot een bepaalde drempelwaarde.

Het is van belang om voor de teelt de grond te onderzoeken op de aanwezigheid van rhizobia en om ze te classificeren door middel van geavanceerde moleculaire technieken. Deze technieken zijn ontwikkeld om de verschillende rhizobia goed te onderscheiden. Effectieve en competitieve stammen van *R. leguminosarum* en *S. meliloti* zijn ontwikkeld door genetische technieken. Vele nieuwe stammen zijn ontdekt door de nieuwe moleculaire technieken. Dit bevordert voortschrijdende veranderingen in de classificatie van rhizobia. In 1982 werd het genus *Rhizobium* inde genera *Rhizobium* en *Bradyrhizobium* opgedeeld. Vanwege hun economische waarde kregen de soja rhizobial stammen meer aandacht dan de andere

stammen. Door middel van DNA -DNA hybridisatie technieken werd *B. japonicum* opgesplitst in drie groepen, *B. japonicum* groep I, groep Ia en groep II. De ontdekking van snelgroeiende rhizobia uit soja wortelknollen gevonden in China heeft de naamgeving volledig op zijn kop gezet. Deze snelgroeiende knolvormende rhizobia werden omgedoopt in *S. fredii* (was *R. fredii*). *B. japonicum* groep II werd *B. elkanii*. Een nieuw stam werd geïsoleerd, *B. liaoningensis*. Meer recent is melding gemaakt van een knolvormende stam, die *Mesorhizobium thianshanense* werd genoemd. Op basis van 16S rDNA sequenties is een nieuwe fylogenetische boom voorgesteld.

De Indonesische rhizobia stammen hebben zeer weinig aandacht gekregen. Studies beperkten zich tot isolatie, enting- en effectiviteitproeven in kassen en in veldexperimenten. De onduidelijke resultaten verkregen uit entproeven hebben bijgedragen aan deze vertraagde ontwikkeling. Indonesië is een secundaire genenpool van soja, zodat er dus veel verschillende rhizobial stammen aanwezig moeten zijn, dit moet nog nader onderzocht worden. In hoofdstuk 4 wordt de isolatie en karakterisatie beschreven van rhizobia uit grondmonsters uit traditionele soja teelt gebieden (op Java) en van nieuwe landbouwgebieden (op Sumatra). Het gebruik van fenotypische eigenschappen om rhizobia in te delen kan een waardevolle strategie opleveren om een genetische karakterisering op te zetten van ongeidentificeerde soja rhizobia, zoals de inheemse grondpopulaties. Veel studies die diversiteit aantonen onder rhizobia die wortelknollen vormen op soja worden nu uitgevoerd, maar slechts enkele onderzoeken hebben het potentiële symbiotische belang van de bacterie onderzocht. Vandaar dat deze isolaten van Java en Sumatra zowel op hun symbiotische eigenschappen werden getest in een bio-assay op soja en mungbean (*Vigna radiata* cv. Manyar) en genetisch geanalyseerd door middel van ARDRA van 16S rDNA en 16S-23S rDNA. Plant-specifieke en minder plant- specifieke soja *Rhizobium* stammen werden verkregen uit grondmonsters uit beide gebieden. Verschillen in stikstof fixatie capaciteit is een belangrijk criterium om effectieve stammen te selecteren. In dit proefschrift wordt beschreven dat sojaplanten, die zijn geënt met rhizobia geïsoleerd uit grond van Sumatra, minder gezond waren dan die geënt met bacteriën van Java. Bij de eerste groep vielen tijdens de oogst de onderste twee bladeren af, zij waren geel geworden. Dit is een indicatie voor stikstof tekort. Als stikstof limiterend is wordt de stikstof uit de oudere planten delen gebruikt voor de groei van nieuwe delen. Er werden geen indicaties gevonden voor stikstof deficiëntie in planten geënt met

rhizobia van Java. Dit impliceert dat de rhizobia van Java een grotere stikstof bindingscapaciteit hebben dan die van Sumatra.

Genetisch gezien zijn deze isolaten verschillend. Een aantal isolaten van Java waren gerelateerd aan *B. japonicum* USDA 110, wat een referentie stam voor soja is. Soja bradyrhizobium uit het buitenland kunnen ongemerkt geïmporteerd worden via met grond besmette soja zaden of direct door eerdere onderzoekers. De duidelijk van USDA 110 verschillende isolaten van Sumatra worden verondersteld de oorspronkelijke inheemse bradyrhizobia te zijn.

In hoofdstuk 5 worden enkele geïsoleerde stammen beschreven waarvan verwacht werd dat ze representatief zijn voor de soja rhizobia voor de nieuwe landbouwgebieden en voor de traditionele gebieden. Hiervan werd de complete 16S rDNA sequentie bepaald voor de identificatie van deze stammen. Op basis hiervan werden twee genera van rhizobia, *Bradyrhizobium* en *Sinorhiobium*, gevonden. Zowel *B. japonicum* als *B. elkanii* werden aangetoond in Javaanse en Sumatraans bodem, terwijl *S. fredii* alleen op Java voorkwam.

Het is interessant de wijdverspreide aanwezigheid van *B. elkanii* te zien in de zure grond van Sitiung, West Sumatra. Het lijkt erop dat *B. elkanii* beter bestand is tegen zuurte en/of Aluminium dan *B. japonicum*. Reeds eerder werd vermeld dat enkele *B. elkanii* stammen werden geïsoleerd uit zure Indonesische bodems. Schijnbaar behoort de *S. fredii* van Java uit ons onderzoek niet tot de eerder bekende *Sinorhizobium* stammen, wat wijst op een mogelijke nieuwe *Sinorhizobium* stam.

BSF is een heel belangrijk natuurlijk proces voor de landbouw. Verbetering van de permanente agrarische productie heeft echter een efficiënte BSF nodig, als belangrijke stikstofbron voor gewassen. Om dit te bereiken moeten verbeterde planten, brady- en sinorhizobia en landbouw beleid worden ontwikkeld.

Dit onderzoek steunt de veronderstelling dat introductie van peulvruchten in nieuwe gebieden invloed heeft op de structuur van de inheemse *rhizobium*- populatie, en dat continue teelt van soja leidt tot selectie van brady- en sinorhizobia promoot met een hogere specificiteit voor dit gewas. Kennis van de inheemse *rhizobium* populaties is belangrijk om goed gebruik te kunnen maken van BSF. Diversiteit van inheemse *Bradyrhizobium japonicum, Bradyrhizobium elkanii* en *Sinorhizobium fredii* stammen in de Indonesische bodem is aangetoond maar er moet nog veel werk verzet worden. Selectie van de beste brady- en sinorhizobia die aangepast zijn aan de omgeving moet nog gedaan worden.

Dit onderzoek heeft aangetoond dat het gebruik van (kalk + TSP) gecoate zaden voldoende is voor BSF, en het gebruik van kalk op zure gronden bij de teelt van soja sterk kan verminderen, van 7,0 ton tot 2,0 ton ha^{-1}.

Deze eenvoudige, weinig kapitaalintensieve methode is een geschikte landbouwmethode om groei en opbrengst van soja te verbeteren op sterk uitgeloogde bodems. Het is niet duur en te gebruiken door de arme, lokale boeren en de methode is ook belangrijk voor het instand houden van de permanente landbouw in het algemeen.

Ringkasan

Penelitian ini dilakukan untuk mendapatkan alternatif lain dalam rangka meningkatkan produksi tanaman Kedelai. Alternatif tersebut ialah peningkatan fiksasi nitrogen secara hayati (BNF, Biological Nitrogen Fixation) yang diuji coba di daerah transmigrasi, di Sitiung, Sumatra Barat, Indonesia. Banyak kendala yang harus dihadapi, terutama karena tanah di daerah Sitiung bersifat sangat masam dengan kadar Al yang dapat dipertukarkan sangat tinggi dan beracun bagi tanaman Kedelai. Oleh karena itu, untuk mencapai produksi yang normal, kerjasama antara ahli pemulia tanaman, ahli ilmu tanah, ahli ilmu mikrobiologi dan ahli ilmu agronomi sangat diperlukan. Penelitian dalam thesis ini dimaksudkan untuk mengembangkan tekhnologi tepat guna yang murah, mudah dan terjangkau oleh petani. Untuk melengkapi usaha peningkatan BNF ini, beberapa bakteri pembentuk bintil akar pada tanaman Kedelai, Brady- and Sinorhizobia, yang ada pada tanah di Indonesia diisolasi dan dikarakterisasi dengan metoda uji inokulasi (bio-assay) dan dengan biologi molekuler (molecular techniques). Hal ini dilakukan terutama untuk mengantisipasi keberadaan bakteri pengikat nitrogen bebas aseli dari tanah-tanah Indonesia yang sangat kompetitif tetapi mempunyai daya kemampuan untuk mengikat nitrogen bebas sangat rendah (tidak efektif). Bakteri yang tidak efektif ini mungkin bisa mempengaruhi keberhasilan usaha inokulasi tanaman Kedelai. Selain itu juga untuk seleksi sebuah strain unggulan dari brady- dan sinorhizobia yang asli dalam tanah di Indonesia.

Peranan Kedelai dan Fiksasi Nitrogen di Indonesia

Tanaman Kedelai adalah tanaman kacang-kacangan yang utama dan merupakan tanaman makanan utama kedua setelah tanaman padi di Indonesia.

Produksi nasional tanaman ini sangat rendah, sehingga tidak mampu memenuhi kebutuhan yang cenderung meningkat terus. Tujuh ratus ribu ton Kedelai, yaitu sekitar setengah dari produksi nasional masih diimpor dari luar negeri di tahun 1998.

Mempertimbangkan krisis ekonomi di Indonesia saat ini, maka peningkatan produksi Kedelai secara nasional adalah langkah yang terbaik. Namun demikian, usaha ini menemui banyak kendala. Berkurangnya lahan pertanian yang subur dan produktif disertai dengan pertumbuhan penduduk yang cukup tinggi adalah beberapa masalah utama yang dihadapi. Salah satu dari alternatif yang ada adalah memanfaatkan secara optimal potensi lahan-lahan yang ada diluar pulau Jawa, seperti di pulau Sumatra, Kalimantan, Sulawesi dan Irian Jaya (Papua). Akan tetapi produksi tanaman Kedelai di daerah tersebut juga sangat dibatasi oleh sifat tanahnya yaitu sangat masam, mengandung Al yang berlebihan, kekurangan Ca dan kahat P. Program INSUS tanaman Kedelai pernah dilakukan di daerah-daerah tersebut dengan program pengapuran secara nasional. Kapur setara dengan 19 milyar US Dollar diberikan secara cuma-cuma ke petani selama tahun 1983-1986 (Sebayang and Sihombing, 1987). Program ini tentu sangat mahal dan tidak dapat diadopsi oleh petani, terutama di daerah transmigrasi Sitiung tersebut, di mana sekitar 4,0 sampai 7,0 ton kapur diperlukan untuk tanaman Kedelai. Selain itu, petani harus memberikan pupuk N dan pupuk P untuk mendapatkan hasil yang normal.

Peningkatan nitrogen secara hayati (BNF) adalah pilihan yang baik untuk meningkatkan produksi tanaman Kedelai, terutama untuk memenuhi kekurangan nutrisi N dari tanaman tersebut. Sebagai tanaman kacang-kacangan, Kedelai dengan brady- dan sinorhizobia, secara simbiosis dapat menambat (mengikat) N bebas dari udara dan merubahnya ke ammoniak, yaitu senyawa N yang dapat dipakai oleh tanaman. N hasil penambatan itu dapat menambah kebutuhan nutrisi N pada tanaman

untuk bertambah dan berproduksi serta mempunyai peranan yang penting bagi kegiatan pertanian yang berkesinambungan.

Peran penting dari pelet-biji Kedelai dengan kapur dan pupuk P

Untuk mengurangi jumlah kebutuhan kapur yang besar, percobaan lapangan telah dilakukan di daerah transmigrasi, Sitiung, Sumatra Barat. Dengan menggunakan contoh tanah dari Sitiung, beberapa percobaan Pot dan Rhizotron juga dilakukan, masing-masing di PAIR-BATAN Jakarta dan di laboratorium Mikrobiologi, Universitas Wageningen, Belanda. Dalam percobaan ini dipelajari pengaruh pengapuran (kapur disebarkan pada lapisan atas tanah) dan pelet-biji (kapur bersama pupuk P dalam jumlah yang sangat sedikit dilekatkan ke biji Kedelai dengan bantuan perekat (Lem)) terhadap kemampuan menambat N dari tanaman Kedelai tersebut. Dari hasil-hasil yang diperoleh, dapat disimpulkan bahwa kombinasi pengapuran tanah asam (dengan jumlah kapur lebih sedikit dari tataran normal) dengan pelet-biji adalah sebuah tekhnik tepat guna untuk meningkatkan BNF dan untuk produksi tanaman Kedelai di tanah sangat masam (Bab 2).

Dalam studi ini, perlakuan pelet-biji sendiri dengan kapur (50 kg ha^{-1}) dan pupuk P (10 kg TSP ha^{-1}) telah berhasil meningkatkan penambatan N tanaman Kedelai (cv. Tidar) dan dapat mengurangi jumlah pemakaian kapur dan pupuk P yang besar pada tanah sangat masam seperti di Sitiung, Sumatra Barat (Bab 2). Hasil dari percobaan lapangan dan pot menunjukkan bahwa pembentukan bintil akar dan penambatan N adalah optimal. Jumlah persediaan dan berat nodule dapat ditingkatkan secara nyata. Hal ini membuat penambatan N dari tanaman ini menjadi lebih efisien, dan dapat memperbesar jumlah nutrisi N tersedia untuk tanaman selain yang dapat diambil dari tanah. Sebaliknya, semua kebutuhan nutrisi N pada tanaman yang

tumbuh di tanah yang dikapur dengan 7,0 ton per ha diambil dari tanah. BNF tidak ada (tidak punya bintil akar) dan akar dari tanaman ini tumbuh sangat subur dan dapat mengambil secara besar-besaran unsur N dari tanah, terutama yang dilepaskan ke dalam tanah oleh proses mineralisasi.

Pelet-biji juga telah berhasil meningkatkan hasil tanaman Kedelai dari 87 kg per ha menjadi 319 kg per ha (Bab 2). Hasil ini sebanding dengan hasil yang diperoleh dari tanaman yang telah diinokulasi dengan bakteri penambat N dan ditanam pada tanah dengan pengapuran 3,5 ton per ha atau pada tanaman tanpa diinokulasi oleh bakteri penambat N tetapi ditanam pada tanah yang telah diberi kapur 7,0 ton per ha.

Namun demikian, karena tanahnya sangat masam, untuk mendapatkan pertumbuhan dan hasil yang optimal, pengapuran dengan 2,0 ton kapur per ha masih diperlukan disamping pelet-biji itu sendiri. Gabungan dari pengapuran dan pelet-biji ini dapat menghasilkan 301 kg per ha lebih besar daripada pengapuran dengan 7,0 ton per ha.

Peranan unsur P untuk BNF tanaman Kedelai pada tanah sangat masam

Pemberian unsur P sangat penting untuk pertumbuhan dan BNF tanaman Kedelai di tanah masam. Hal ini disebabkan oleh kekahatan P pada kebanyakan tanah sangat masam (Wade *et al.*, 1988). Tanah masam juga mempunyai daya mengikat P yang sangat besar. Oleh karena itu pemberian P pada tanah masam akan bermanfaat apabila dilakukan secara benar. Rekomendasi pemakaian pupuk P dalam jumlah yang besar dimaksudkan untuk mengatasi masalah tersebut (Sanchez and Salinas, 1981; Sudjadi, 1984; Wade *et al.*, 1988). Untuk efisiensi, pupuk P harus diberikan pada tempat-tempat yang mudah dijangkau oleh pertumbuhan akar tanaman. Akan tetapi

pemakaian pupuk P sendiri, terutama dalam bentuk TSP juga harus hati-hati, kontak langsung dengan biji harus dicegah, karena reaksi pupuk P dengan tanah akan menghasilkan reaksi kimia yang sangat masam, dan merusak pertumbuhan biji. Untuk menghindari efek negatip tersebut, pupuk P dan Ca harus diberikan secara bersamaan. Oleh karena itu, dalam penelitian ini pupuk P dan kapur diberikan secara bersama-sama pada biji Kedelai, dan pendekatan ini telah berhasil untuk meningkatkan pertumbuhan dan BNF tanaman Kedelai pada tanah masam di Sitiung, Sumatra Barat (Bab 2).

Fosfat dianggap mempunyai peranan penting di dalam tahap-tahap awal pembentukan bintil akar. Selain itu, P mungkin juga diperlukan oleh kehidupan rhizobia untuk menginfeksi rambut akar. Untuk mempelajari pengaruh P yang lebih teliti terhadap pembetukan bintil akar, sebuah percobaan rhizotron telah dilakukan (Bab 3). Percobaan rhizotron ini dilakukan untuk mempermudah pelaksanaannya dan sekaligus untuk memdekati kondisi tanah seperti yang terjadi pada percobaan di lapangan. Selanjutnya dengan memakai rhizotron, akar tanaman dengan mudah dipisahkan dari media tanahnya, dan primordia bintil akar yang masih berada didalam jaringan sel akar, sekitar berumur 5 hari setelah diinokulasi, dapat diamati dan dihitung dengan bantuan sebuah mikroskop dengan pembesaran 60 kali. Data yang diperoleh juga lebih realistis daripada data yang telah dilaporkan oleh peneliti terdahulu, yaitu berdasarkan pada jumlah bintil akar yang sudah diluar sel jaringan akar. Dalam penelitian ini, pemberian P telah meningkatkan jumlah primordia bintil akar secara nyata.

Secara umum dapat disimpulkan bahwa pelet-biji dengan kapur dan pupuk P dapat meningkatkan BNF dan pertumbuhan tanaman Kedelai pada tanah masam. Walaupun begitu, untuk tanah yang sangat masam, kapur masih diperlukan untuk

meningkatkan produksi Kedelai. Bahan kapur ini diperlukan untuk memberikan Ca, meningkatkan pH tanah dan juga untuk mengendapkan unsur Al agar tidak meracuni lagi tanaman. Pengapuran tanah masam sering juga meningkatkan ketersediaan unsur hara P. Karena itu, pertumbuhan tanaman Kedelai sering lebih baik pada tanah masam yang dikapur (Sartain and Kamprath, 1975). Akan tetapi dari segi ekonomi pemakaian kapur dan pupuk P dalam jumlah besar tidak dapat diterima oleh petani. Oleh karena itu, dalam studi ini tekhnik yang murah dan tepat guna telah dicoba dan berhasil. Walaupun kapur dengan tataran 2,0 ton per ha masih diperlukan, pendekatan ini masih lebih murah dibandingkan dengan pendekatan lain yang dikemukakan oleh peneliti-peneliti sebelumnya (Sudjadi, 1984; Wade *et al.*, 1988).

Peranan penting dari struktur populasi rhizobia untuk BNF

Pentingnya inokulasi brady- dan sinorhizobia untuk meningkatkan hasil panen Kedelai telah lama diketahui (Burton and Curley, 1965; Ham, 1980; Herridge and Brockwell, 1988; Peoples and Crasswell, 1992). Di Indonesia, hal ini telah dilaporkan oleh Toxopeus pada tahun 1936, bahkan untuk meningkatkan produksi Kedelai di Jawa beberapa strain brady- dan sinorhizobia telah diimpor dari luar negeri (Keleney, 1959; Newton, 1962). Tanah di Jawa juga diduga banyak mengandung brady- dan sinorhizobia asli, dan keberadaan bakteri ini juga diduga merupakan salah satu penyebab kurangnya respon dari tanaman Kedelai terhadap inokulasi strain *Bradyrhizobium* unggulan. Namun demikian, sampai saat ini data sistematik dari keaneka-ragaman rhizobia asli dari tanah-tanah Jawa masih juga tidak tersedia.

Studi tentang populasi rhizobia asli adalah penting, terutama untuk mengantisipasi kemungkinan gangguan (kompetisi) bakteri tersebut pada usaha-usaha

inokulasi dengan strain unggulan *Brady-* and *Sinorhizobium*. Bakteri brady- dan sinorhizobia alami diduga ada pada tanah-tanah masam di Indonesia. Rhizobia alami ini kemungkinan bagian dari flora alami yang menempati tempat-tempat yang tidak berbahaya. Pada awal pertanaman Kedelai di tanah masam, gangguan kompetisi dari brady- dan sinorhizobia alami bisa dikatakan tidak ada karena jumlahnya yang sangat sedikit (Adiningsih and Prihatini, 1981; Mahmud and Rumawas, 1983; Bab 2). Akan tetapi, dengan menanam Kedelai secara terus menerus pada tanah masam akan meningkatkan jumlah rhizobia alami, dan pada tatanan tertentu akan mengganggu inokulasi dari rhizobia yang lebih effektif. Mahler and Wollum (1982) melaporkan bahwa populasi *B. japonicum* adalah 194 kali lebih besar pada tanah-tanah bekas tanaman Kedelai dari pada tanah-tanah yang belum pernah ditanami Kedelai. Brockwell *et al.* (1987, 1989) juga melaporkan terjadinya peningkatan jumlah populasi dari *B. japonicum* pada tanah-tanah yang ditanami Kedelai. Simanungkalit *et al.* (1995) menemukan bahwa jumlah rhizobia untuk Kedelai di tanah Latosol, Bogor, Indonesia, meningkat dari $\log_{10}$ 1,29 g^{-1} menjadi $\log_{10}$ 4,84 g^{-1} tanah dari total volume tanah di daerah perakaran Kedelai. Akhir-akhir ini dilaporkan bahwa peningkatan jumlah rhizobia dipengaruhi oleh tanaman inangnya, dan simbiosis tanaman kacang-kacangan bisa meningkatkan jumlah rhizobia sampai beberapa level (Thies *et al.*, 1995).

Pengapuran juga akan meningkatkan jumlah dan penyebaran brady- dan sinorhizobia secara merata pada lapisan tanah-tanah masam. Seperti yang telah dilaporkan oleh Richardson and Simpson (1988) pada *Rhizobium leguminosarum* bv. *trifolii*. Oleh karena itu, dengan bantuan tekhnik biologi molekuler, survey dan klasifikasi rhizobia di tanah-tanah Indonesia sebelum dibudidayakan untuk usaha pertanian adalah sangat penting. Tekhnologi molekuler untuk rhizobia telah

dikembangkan, seperti rekayasa genetik untuk mendapatkan strain yang efektif dan kompetitif pada *R. leguminosarum* dan pada *S. meliloti* (Chen *et al.*, 1991; Scupham *et al.*, 1996). Dengan menggunakan kombinasi marker gen dari *gusA* dan *celB*, Sessitsch *et al.* (1996) melakukan studi kompetisi antara *R. tropici* strains. Pengetahuan yang dalam tentang potensi, aktifitas dan taksonomi rhizobia juga telah diperoleh dengan serangkaian pendekatan secara biologi molekuler (Freiburg *et al.*, 1997). Diantara pendekatan lainnya, pendekatan secara biologi molekuler ini telah mendorong terjadinya perubahan yang progresif dalam klasifikasi rhizobia.

Dengan pertimbangan nilai ekonomi, dibandingkan dengan rhizobia untuk tanaman kacang-kacangan lainnya, rhizobia untuk tanaman Kedelai (brady- dan sinorhizobia) lebih banyak mendapatkan perhatian. Seperti yang telah dilakukan oleh Hollis *et al.* (1981), dengan metoda DNA-DNA hibridisasi dapat membedakan *R. japonicum* group I, group Ia and group II. Selanjutnya, Jordan (1982) memisahkan genus *Rhizobium* menjadi genera *Rhizobium* and *Bradyrhizobium*. Penemuan bakteri rhizobia tumbuh cepat dari bintil akar Kedelai di China oleh Keyser *et al.* (1982) telah memberikan perubahan yang dramatis pada numenklatur rhizobia untuk tanaman Kedelai, dan Scholla dan Elkan (1984) memberi nama *S. fredii*. Kuykendall *et al.* (1992) mengusulkan bahwa *R. japonicum* group II seharusnya diklasifikasikan sebagai *B. elkaniii*, dan sebagai tipe strainnya adalah USDA 76. Xu *et al.* (1995) telah mengisolasi rhizobia baru untuk tanaman Kedelai, dan dinamakan *B. liaoningensis*. Akhir-akhir ini, *Mesorhizobium thianshanense* strain dilaporkan mampu membentuk bintil akar pada tanaman Kedelai (Chen *et al.*, 1995). Berdasarkan pada pemetaan 16S rDNA, phylogenetic tree yang baru untuk rhizobia telah diusulkan oleh Young (1996) and Jarvis *et al.* (1997), yang sekarang didalamnya termasuk genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*.

Sebaliknya, perhatian pada brady- dan sinorhizobia asli terdapat pada tanah-tanah Indonesia sangat sedikit sekali. Sejak Toxopeus (1936) sampai sekarang, kegiatan hanya terbatas pada isolasi dan uji efektifitasnya. Perkembangan yang lambat ini mungkin disebabkan oleh tidak adanya respon yang nyata dari hasil kegiatan inokulasi rhizobia yang telah banyak dilakukan. Padahal dikenal sebagai negara pusat gen kedua untuk tanaman Kedelai (Hymowitz and Newell, 1981), kemungkinannya besar sekali bermacam-macam rhizobia berada di dalam tanah di Indonesia, dan pembudidayaan lebih lanjut secara optimal masih terbuka. Bab 4 thesis ini menguraikan isolasi rhizobia pembentuk bintil akar pada tanaman Kedelai dari contoh-contoh tanah yang diambil dari daerah penghasil tradisional Kedelai (Jawa) dan dari daerah pengembangan baru (Sumatra). Walaupun telah banyak studi menceritakan tentang keanekaragaman rhizobia tanaman Kedelai, hanya beberapa yang mempelajari potensi BNF dari hasil klasifikasi tersebut. Oleh karena itu isolat-isolat dari Jawa dan dari Sumatra dianalisis secara symbiotik, diujikan pada tanaman Kedelai dan tanaman Kacang hijau, dan secara genetik dengan bantuan beberapa "restriction enzyme", memotong gen ribosome (16S rDNA dan 16S-23S rDNA) yang telah diperbanyak dengan mesin PCR (ARDRA). Hasilnya menunjukkan bahwa *Bradyrhizobium* strains spesifik untuk tanaman Kedelai dan promiscuous rhizobia telah diperoleh dari contoh tanah yang berasal dari Jawa dan Sumatra tersebut.

Perbedaan dalam kapasitasnya untuk mengikat N adalah parameter yang sangat penting dalam menyeleksi strain-strain yang efektif untuk usaha pertanian. Pada Bab 4 telah ditunjukkan bahwa pertumbuhan tanaman Kedelai yang diinokulasi dengan isolat rhizobia dari Sumatra kurang bagus dibandingakan jika diinokulasi dengan isolat rhizobia dari Jawa. Pada saat dipanen, sebagian besar dari dua daun pertama berwarna kuning dan jatuh. Hal ini menunjukkan bahwa tanaman tersebut

kekurangan unsur N untuk pertumbuhannya, sehingga unsur N pada bagian tanaman yang lebih tua direalokasikan kebagian tanaman yang lebih muda (Mengel and Kirckby, 1987). Sebaliknya, tanaman yang diinokulasi dengan isolat rhizobia dari Jawa tidak menunjukkan symtom seperti tersebut di atas. Hal ini menunjukkan bahwa rhizobia untuk tanaman kedelai dari Jawa mempunyai kapasitas pengikat N yang lebih besar dari pada isolat rhizobia dari Sumatra.

Secara genetik, bradyrhizobia untuk tanaman Kedelai dari Sumatra umumnya berbeda dengan yang dari Jawa. Beberapa isolat dari Jawa lebih dekat hubungannya dengan referensi strain untuk tanaman Kedelai, yaitu *B. japonicum* USDA 110. Ada kemungkinan tanah-tanah di Jawa telah terkontaminasi dengan brady- dan sinorhizobia dari luar negeri, baik melalui impor biji Kedelai yang telah terkontaminasi atau introduksi secara langsung oleh peneliti-peneliti sebelumnya. Sebaliknya, isolat-isolat dari Sumatra sangat berbeda dengan referensi strain USDA 110 itu. Dengan mempertimbangkan bahwa Sumatra adalah daerah produksi Kedelai yang relatif sangat baru, maka ada kemungkinan besar bahwa bakteri tersebut adalah brady- dan sinorhizobia asli.

Untuk identifikasi lebih lanjut, beberapa isolat baik dari Jawa dan dari Sumatra dipilih untuk mewakili dari populasi rhizobia dari tanah daerah produksi Kedelai lama dan dari daerah pertanaman Kedelai yang baru. Gen 16S rDNA dari bakteri-bakteri tersebut kemudian dipeta (Bab 5) dan hasilnya dibandingkan dengan data-data yang telah ada di GenBank melalui internet (http://www.ncbi.nlm.nih.gov/cgi-bin/Blast). Hasil yang diperoleh adalah dua genera rhizobia untuk tanaman Kedelai, yaitu *Bradyrhizobium* dan *Sinorhizobium* ada di dalam tanah di Indonesia. *B. japonicum* dan *B. elkanii* diperoleh dari contoh tanah

dari Jawa dan dari Sumatra, sedangkan sebuah isolat seperti *S. fredii* ditemukan hanya dari satu contoh tanah dari Jawa.

Penting untuk dicatat bahwa *B. elkanii* menyebar di tanah masam Sitiung, Sumatra Barat. Kelihatannya *B. elkanii* lebih tahan terhadap keasaman tanah daripada *B. japonicum*. Sedangkan isolat dari Jawa yang menyerupai *S. fredii* itu bukan seperti rhizobia tumbuh cepat untuk tanaman Kedelai yang telah dilaporkan sebelumnya, dan ada kemungkinan merupakan sebuah species baru dari *Sinorhizobium*. Isolat tersebut tidak mampu membentuk bintil akar pada tanaman Kacang hijau, dan tingkat kesamaan dari 16S rDNAnya dengan *S. fredii* USDA 205, sebuah referensi strain yang paling dekat posisinya di dalam "phylogenetic tree", hanya 97,9 %.

Dari penelitian ini bisa ditarik kesimpulan bahwa fiksasi N secara hayati (BNF) adalah proses alam yang sangat penting untuk pertanian. BNF yang efektif adalah sumber utama unsur N untuk tanaman, dan sekaligus untuk meningkatkan produksi pertanian yang berkesinambungan. Walaupun begitu, untuk mencapai sasaran yang diharapkan, breeding tanaman pangan, brady- dan sinorhizobia yang efisien, serta tekhnik yang tepat dan berdaya-guna harus dikembangkan.

Introduksi tanaman kacang-kacangan pada areal yang baru mungkin mempunyai pengaruh pada struktur dari populasi rhizobia asli. Produksi tanaman Kedelai yang terus menerus dapat menyeleksi rhizobia strain dengan tingkat kompatibilitas yang rendah dengan induk semangnya dan sangat khusus untuk beberapa induk semang. Oleh karena itu, pengetahuan tentang populasi rhizobia alami adalah penting untuk keberhasilan dari usaha BNF. Keberadaan *Bradyrhizobium japonicum* dan *Bradyrhizobium elkanii*, dan *Sinorhizobium fredii* asli dari tanah Indonesia telah ditunjukkan dan membuka peluang melakukan seleksi *Brady-* dan *Sinorhizobium* strain unggulan.

Penelitian ini juga menunjukkan bahwa kombinasi dari pemakaian kapur 2,0 ton ha^{-1} disebar, dan inokulasi biji Kedelai dengan *B. japonicum* atau *B. elkanii* dan dilapisi dengan campuran kapur dan TSP dapat menigkatkan BNF, dan dengan sendirinya dapat dipakai untuk mencapai tujuan akhir yaitu meningkatkan produksi Kedelai pada tanah masam di Sitiung, Sumatra Barat.

Tekhnologi murah, tepat dan berdaya-guna adalah salah satu cara yang sesuai untuk menigkatkan pertumbuhan dan hasil dari tanaman Kedelai pada tanah-tanah yang sangat masam. Selain tidak mahal dan dapat diadopsi oleh petani miskin, tekhnik ini juga sangat penting untuk kegiatan pertanian yang kerkesinambungan.

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Curriculum Vitae

Setiyo Hadi Waluyo was born on April 12, 1959 in Probolinggo, East Java, Indonesia. He attended elementary and secondary school in Kraksaan, Probolinggo and high school in Surabaya. He graduated as 'Ingenieur' in Agriculture from the Faculty of Agriculture, Gadjah Mada University, Yogjakarta in 1983. In the same year, he was employed by the Agriculture Division, Centre for the Application of Isotopes and Radiation, National Atomic Energy Agency, Jakarta. In 1986, The Ministry of Education and Science allowed him to work with the *Rhizobium* Group of the Laboratory of Microbiology, Wageningen Agricultural University, The Netherlands, under the supervision of Dr. Ir. Tek An. Lie. He obtained the M.Sc. degree in Soil Fertility at Wageningen University in 1989.
He was involved in a joint EC research project (contract number: C11*CT900551) between the Laboratory of Microbiology, of Wageningen University, The Netherlands, the Research and Development Centre for Biotechnology, Indonesian Institute of Sciences, Bogor, Indonesia and the Centre for the Application of Isotopes and Radiation, National Atomic Energy Agency, Jakarta Indonesia on "***Enhancement of BNF of Soybean in Indonesia, particularly in acid soils of Sumatra***" in 1990 – 1993. In 1994 he started a Ph.D. research programme at the Laboratory of Microbiology, Wageningen University, The Netherlands, under the supervision of Prof. Dr. Alex J. B. Zehnder, Prof. Dr. Willem M. de Vos, Prof. Dr. Leendert 't Mannetje, and Dr. Ir. Tek An Lie. After returning to Indonesia, he will join the Agriculture Division, Research and Development Centre for Isotopes and Radiation Technology, National Nuclear Energy Agency, Jakarta, Indonesia. His address will be the following:

Setiyo Hadi Waluyo

Bidang Pertanian
Pusat Penelitian dan Pengembangan Teknologi Isotop dan Radiasi
Badan Tenaga Nuklir Nasional
Jln. Cinere Pasar Jumat Kotak Pos 7002 JKSKL
Jakarta. 12070
Indonesia
Phone : 62-21-7690709
Fax. : 62-21-7691607
E-mail : shwaluyo@hotmail.com