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# EFFECT OF PHOSPHATE ON NODULE PRIMORDIA OF SOYBEAN (*Glycine max* Merrill) IN ACID SOILS IN RHIZOTRON EXPERIMENTS

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## ABSTRACT

To clarify whether P had a direct or indirect effect on the nodulation process of soybean grown in acid soils from Sitiung, West Sumatra, Indonesia, a series of rhizotron experiments, with special attention given to formation of nodule primordia, was conducted at Laboratory of Microbiology, Wageningen University in 1998-2000. It was shown that Ca and P were essential nutrients for root growth, nodule formation, and growth of soybean in the acid soils (Oxisols). 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 biological nitrogen fixation (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 also has 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 of Sitiung, West Sumatra, Indonesia.

[***Keywords:*** *Glyicine max*, phosphates, roots, root nodulation, root nodules, acid soils]

## INTRODUCTION

Phosphate is essential for biological nitrogen fixation (BNF) of legumes. Cassman *et al*. (1981a) 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 releasing P from the soil by liming or adding $CaCO_3$ or $CaMg(CO_3)_2$. The P supplied may play important roles on establishment, growth, and function of nodules (DeMooy and Pesek 1966; Gates 1974; Cassmann *et al*. 1981a; Gates and Muller 1979; Israel 1987; Singleton *et al*. 1985), growth of rhizobial strains (Cassmann *et al*. 1981b; Beck and Munns 1984; Leung and Bottomley 1987), and growth of 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 susceptibility of 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 *in* 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) has shown that application of P increased number of nodules of soybean grown in heavily weathered acid soils. In our previous study, 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 was suggested 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.

## MATERIALS AND METHODS

Five rhizotron experiments were conducted at Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands in 1998-2000. Experiment I was conducted to study the effect of liming on root growth and formation of nodules, and experiment II was performed to compare the effect of liming soil and lime pelleting seed on the formation of roots and nodules. The effect of P on the formation of nodules was studied in the experiment III. Experiment IV was conducted to study the effects of Ca and P on root growth, formation of nodule primordia and nodules. Finally, experiment V was conducted to study the effect of neutralising acidity on the formation of nodule primordia.

A rhizotron consists essentially of a plastic petri dish (9.5 cm diameter and 1.2 cm tall), cut at one side 0.5 cm, filled with 70 g soil sample, and the soil surface adjusted at 1 cm below the upper part of the rhizotron. The rhizotron was then kept at an angle of ca. $60^{o}$ so that the roots are growing towards the lid of the petri dish (Fig. 1). 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.

**Fig. 1**. A rhizotron filled with soil and the lid (left, above)]; the entire excised nodulated roots (left, bottom); and rhizotron with soybean plants (right).

Red Yellow Podzolic soil (Oxisols) samples were collected from Sitiung, West Sumatra (Table 1). Air-dried soil sample was ground and screened by a 2 mm sieve and then was moistened to field capacity before being put into the rhizotrons (70 g soil per rhizotron). The terms liming were used for the application of $CaCO_3$ to the soils (mixed) and lime-pellet when the seeds were coated with a layer of $CaCO_3$. Lime requirement (LR) for maximum growth of soybean plants was calculated as 6.75 t lime $ha^{-1}$, using the formula of Wade *et al.* (1988) as follows: LR = 1.5[{Al-(RAS x ECEC/100)}], where Al is aluminium concentration in cmol (+) $kg^{-1}$ soil, ECEC (effective cation exchange capacity) is the sum exchangeable Ca, Mg, and K plus 1 M KCl extractable Al in cmol (+) $kg^{-1}$ soil, and RAS is the required aluminium saturation. For soybean, Wade *et al*. (1988) suggested the RAS value of 15%.

In the following experiments instead of lime-pellet or (lime+TSP)-pellet, a solution of $CaCO_3$ or lime+TSP was applied directly on the root tip. As pelleting material $CaCO_3$ was used and firstly dissolved in sterile water (0.075 g in 0.5 ml per seedling, equivalent to 50 kg lime $ha^{-1}$ for 50 kg soybean seeds). For (lime+TSP)-pellet treatments, besides lime, 0.5 ml of a solution containing 0.0125 g TSP per seedling (equivalent to 10 kg TSP $ha^{-1}$ for 50 kg soybean seeds) were applied. The treatments of $KH_2PO_4$ (equivalent to 0.0125 g TSP), $K_2CO_3$ (equivalent to 0.075 g $CaCO_3$), $CaCO_3$+$KH_2PO_4$, and $K_2CO_3$+TSP were also applied as a solution.

Soybean seeds were sterilised by immersing them sequentially in ethanol 70% for 10 minutes, then in 6% of hydrogen peroxide containing one drop of Tween 20 (a non-ionic detergent polyethylene glycol sorbitan mono laurate solution) for 10 minutes. The

**Table 1. Properties of the Sitiung soils, West Sumatra.**

| Soil analyses | Value |
|---|---|
| Clay (%) | 74.5[1] |
| Organic C (%) | 2.0[1] |
| Available P (ppm) | <5.0[1] |
| pH ($H_2O$) | 4.03 |
| pH (KCl) | 3.65 |
| Cations (meq $100^{-1}$ 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 (ppm) | <5.0[1] |

[1]Adapted from Sudjadi (1984).

sterile soybean seeds were rinsed with sterile water at least three times. They were then germinated on water-agar 0.7-1% for 24-48 hours. Seedlings were transplanted into the rhizotrons filled with 70 g acid soils from Sitiung and incubated for 24 hours. The plants were harvested at 5, 10, and 20 days after treatments (DAT). Upper part of the plant (shoots) was cut with a scissor and oven dried at 70 °C over night. The roots were excised entirely from the soil and nodules were observed and counted. Mass of the dried shoots and the obtained nodules were weighed.

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 Research and Development of Isotopes and Radiation Technology, National Nuclear Energy Agency, Indonesia) were used. In experiments I and II, only one seedling was used per rhizotron, but in experiments III, IV, and V two seedling were used. *B. 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 (1 day after transplanting).

Randomised complete design (RCD) was performed to all experiments. The obtained data were calculated and analysed using Duncan's multiple test (MSTAT-C 1988).

***Experiment I.*** Five levels of $CaCO_3$, equivalent to 0, 0.8, 1.6, 3.3, and 6.7 t $ha^{-1}$ were applied as liming (assuming 1 ha equiva-lent to 2 million kg soil). The visible part of the roots was inspected at 5 DAT. Number of nodules was counted at harvest (20 DAT).

***Experiment II***. In this experiment, $CaCO_3$ 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 roots 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 nodules, six treatments consisting of $H_2O$ (control), and solutions of $CaCO_3$, $K_2HPO_4$ (equivalent to 0.0125 g TSP), TSP, $CaCO_3$+$K_2HPO_4$ and $CaCO_3$+ TSP were carried out. Number and weight of nodules were determined at harvest (20 DAT).

***Experiment IV.*** This experiment comprised three treatments, i.e. $H_2O$ (control) and solutions of $CaCO_3$ and $CaCO_3$+TSP. The plants were harvested at 5, 10, and 20 DAT. At 5 and 10 DAT, the entire roots were excised, number and length of first, second, and third order roots were measured and counted. To determine nodule primordia, the roots harvested at 5 DAT were fixed with glycerol (15 minutes) and cleared by immersing the roots in sodium hypochlorite 6% active chlorine solution for 15 minutes. After clearing, the fixed roots were stained with 0.01% methylene blue (Johnson *et al*. 1996). Primordia of roots and nodules inside root tissue (Fig. 2 at 125X magnifi-cation) were observed under a microscope and counted from the entire root system at 60X magnification. 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 remove the effect of Ca, $K_2CO_3$ (equivalent to 0.075 g $CaCO_3$ per seedling) was used instead of $CaCO_3$ to neutralise acidity. Four treatments, i.e. $H_2O$, $CaCO_3$, $K_2CO_3$ and $K_2CO_3$+TSP were applied. Number and length of first, second, and third order roots were counted and measured from entire roots harvested at 10 DAT. Then, these roots were fixed and coloured in a similar way with that mentioned in experiment IV. Primordia of roots and nodules (Fig. 2 at 125X magnification) were observed and counted from all over the roots under the microscope (magnification 60X ). Number and weight of nodules were determined at 20 DAT.

## RESULTS

### Roots

Calcium, phosphate, and near neutral soil pH were found important to root growth. Applying $CaCO_3$ increased root length and nodule number. The effects

**Fig. 2.** Root (left) and nodule (right) primordia of soybean excised from the plant in a rhizotron experiments and examined under a microscope with magnification of 60X.

were clear and statistically significant at $\alpha = 0.05$ (Table 2). 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 3). Root length 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 shoots increased at 6.7 t $ha^{-1}$ liming. The effect of lime-pellet on root growth was not clear. No effect was obtained on length of main roots by applying $CaCO_3$ to the root tip of the seedling. Although the length of the main roots was highest at the equivalent to 25 kg $CaCO_3$ $ha^{-1}$ applied, the differences with the other treatments were not significant. The effect of lime-pellet on lateral roots was the best at 10 kg $CaCO_3$ $ha^{-1}$ .

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 second order roots was increased significantly by Ca. The increase in number of first and third order roots was not significant. The effect of Ca on root growth was more pronounced than neutralising soil acidity (Table 5).

Apparently, P has only had a positive effect on root growth in the presence of Ca (Table 4). Number of first, second, and third order roots were significantly (at $\alpha$ = 0.05) increased by 1.3, 1.8, and 11 times over the control. A similar response 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. 3).

**Table 2. The effect of liming the soil on root growth and nodulation of soybean growing in acid soil, in rhizotron experiments.**

| Lime ($CaCO_3$) rate (t $ha^{-1}$) | Main root length at 5 DAT (cm) | Number of nodules per plant 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 significantly different according to Duncan's multiple test at $\alpha = 0.05$, with nine replications (MSTAT-C 1988; DAT = days after treatment.

**Table 3. The effect of $CaCO_3$ as a liming agent and equivalent to lime-pellet on root length, number of nodules, and shoot dry weight of soybean in acid soil in rhizotron experiments.**

| $CaCO_3$ rate (kg $ha^{-1}$) | Root length at 5 DAT (cm) | | Number of nodules per plant at 20 DAT | Shoot dry weight at 20 DAT (mg $plant^{-1}$) |
|---|---|---|---|---|
| | Main | Lateral | | |
| Liming ($CaCO_3$ mixed with the soils) | | | | |
| 0 | 1.96c* | 0.111b* | 3.2b* | 73 |
| 800 | 4.80b | 0.169b | 5.0b | 84 |
| 1600 | 6.14b | 0.166b | 4.2b | 77 |
| 3300 | 8.70a | 0.175b | 5.4b | 88 |
| 6700 | 9.12a | 0.299a | 8.2a | 106 |
| Imitation of lime-pellet (applied near root tip) | | | | |
| 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 significantly different according to Duncan's multiple test at $\alpha = 0.05$ and 0.1 respectively, with five replications (MSTAT-C 1988).

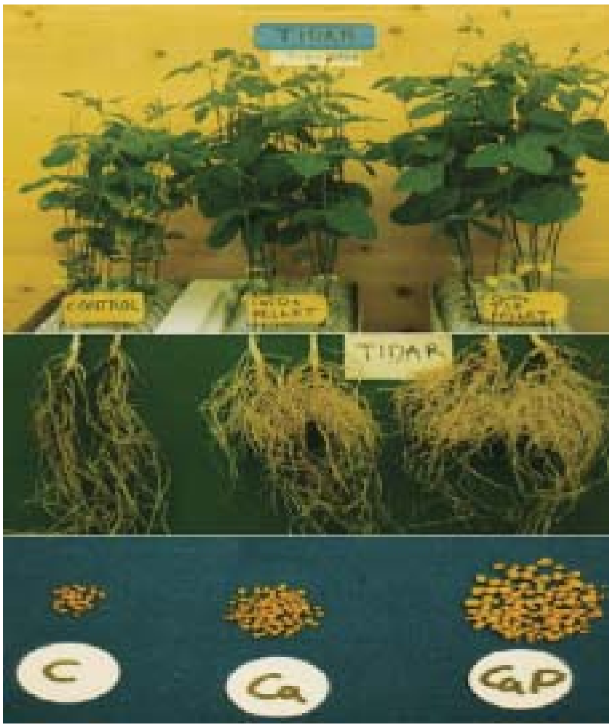


**Fig. 3.** Growth of shoots (top), roots (middle), and nodules (bottom) of soybean in acid soils of Sitiung, West Sumatra, treated with P (TSP) fertiliser and or $CaCO_3$ in a rhizotron experiments.

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

| Treatment | 5 DAT[1] | | | 10 DAT[1] | | | | | | 20 DAT[2] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lenght of first order roots (cm) | Nodule primordia | | First order roots | | Second order roots | | Third order roots | | Number of nodule | Nodule fresh weight (mg) | |
| | | Number | Number per cm of root lenght | Number | Lenght (cm) | Number | Lenght (cm) | Number | Lenght (cm) | | per nodule | per plant |
| $H_2O$ | 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 statistically not significantly different according to Duncan's multiple test at $\alpha$ = 0.1, 0.05, and 0.01 respectively. [1]Number of replication = 3; [2]Number of replication = 4 (MSTAT-C 1988).

**Table 5. Effect of neutralising soil acidity on number and length of first, second, and third order roots per plant of soybean seedlings at 10 DAT in acid soils in rhizotron experiments.**

| Treatment | Soil pH ($H_2O$) at 5 DAT | First order roots | | | Second order roots | | | Third order roots | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Number | Length (cm) | | Number | Lenght (cm) | | Number | Length (cm) | |
| | | | total | per root | | total | per root | | total | per root |
| 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 statistically not significantly different according to Duncan's multiple test at $\alpha$ = 0.05 with five replications (MSTAT-C 1988); DAT = days after treatment.

## Nodules

The effects of Ca and P on number and weight of nodules were significant (Table 4, 6, 7). Using four soybean varieties, it was found that there were no specific effects of the soybean varieties (Table 6). Compared to the control, number of nodules on cv. Tidar was increased more by $CaCO_3$ (4.4 folds) than by TSP (2.6 folds). The importance of Ca on nodulation is also shown in Table 5. 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 had no effect on number of nodules compared to $CaCO_3$ treatment (Table 6). 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 6). Table 4 also shows that the total weight of nodules increased significantly from 19 mg with $CaCO_3$ alone to 60 mg with $CaCO_3$+TSP. The importance of P on growth and function of nodules was confirmed by the results in Table 7. There was no increase in weight of nodules obtained by applying $K_2CO_3$ alone.

The weight of individual nodules (size of nodule) was also increased by P. Nodules obtained from plants treated with TSP, $CaCO_3$+TSP, and $CaCO_3$+$KH_2PO_4$were heavier than those in the control and $CaCO_3$ treatments (Table 6).

## 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 the effect

**Table 6. Nodulation of soybean cv. Tidar, 214, 23D, and 231A in acid soils of Sitiung, West Sumatra with different treatments in rhizotron experiments.**

| Treatment | Number of nodules per plant | | | | Weight of nodules per plant (mg) | | | |
|---|---|---|---|---|---|---|---|---|
| | Tidar | 214 | 23D | 231A | Tidar | 214 | 23D | 231A |
| Control (acid soil) | 3.5d | 4.3c | 2.3c | 0.0c | 7d (2.0) | 9e (2.0) | 6e (2.6) | 0e (0.0) |
| $CaCO_3$[1] | 15.3b | 21.4a | 18.0a | 17.8a | 29c (1.9) | 50c (2.3) | 59c (3.2) | 50c (2.8) |
| $KH_2PO_4$[2] | 5.4d | 6.6bc | 0.8c | 0.0c | 12d (2.2) | 31d (4.7) | 2e (2.4) | 0e (0.0) |
| TSP[3] | 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) | 82a (4.0) | 95a (5.1) | 76a (4.1) |
| $CaCO_3$+ TSP | 17.6ab | 21.8a | 21.1a | 20.7a | 43b (2.5) | 66b (3.0) | 71b (3.3) | 62b (3.0) |

Values followed by the same letter at the same column are statistically not significantly different according to Duncan's multiple test at $\alpha$ = 0.05, with eight replications (MSTAT-C 1988). Values in bracket are weight of individual nodule.
[1]$CaCO_3$ was applied 0.075 g/seed (50 kg $ha^{-1}$) containing Ca 0.0276 g.
[2]$KH_2PO_4$ was toxic (too acid) for seedlings, some seedlings did not grow.
[3]TSP was applied 0.0125 g/seed (10 kg $ha^{-1}$) containing Ca 0.0022 g.

**Table 7. Effect of neutralising soil acidity by $CaCO_3$ or $K_2CO_3$ on formation of nodule primordia and on nodule per plant of soybean.**

| Treatment | Soil pH ($H_2O$) at 5 DAT | Nodule primordia at 10 DAT | | Nodule at 20 DAT | | |
|---|---|---|---|---|---|---|
| | | Number | Number per cm of first order root lenght | Number | Fresh weight (mg) per nodule | Fresh weight (mg) per plant |
| Control | 4.9 | 16b[1] | 0.14 | 3.0d[2] | 1.3 | 4d |
| $CaCO_3$ | 7.3 | 34ab | 0.25 | 8.0b | 1.6 | 13c |
| $K_2CO_3$ | 6.9 | 31ab | 0.25 | 3.0d | 1.7 | 5d |
| $K_2CO_3$+TSP | 5.8 | 48a | 0.33 | 5.0c | 3.6 | 18b |

Values followed by the same letter at the same column are statistically not significantly different according to Duncan's multiple test at $\alpha$ = 0.05; [1]Number of replication = 5; [2]Number of replication = 6 (MSTAT-C 1988); DAT = days after treatment.

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*. 1981a).

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 near 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 7). However, a more significant increase was found when $CaCO_3$ was added as well. The treatment of $CaCO_3$ + TSP increased the number of nodule primordia by a factor of 3.7 (Table 4) and $K_2CO_3$ + TSP by a factor of 3.0 (Table 7) 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 the root tissue. Brockwell *et al*. (1985) suggested that optimum nodulation and N fixation are functions of early colonisation of the plant rhizospheres by *B. 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).

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. 3 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 soil.

Besides increasing number of nodule primordia, P also had a great influence on growth and function of nodules as shown 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 6). 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.

## CONCLUSION

The number of nodule primordia of soybean was increased significantly by the addition of P. Quantification of nodule and root primordia inside the roots at an early stage (5 days after inoculation), using a microscope with 60-time magnification, gave more direct data compared to observation of the emerging nodules. Moreover, by using the rhizotron system, the intact roots can be easily released from the soil matrix, and hence nodule primodia can be quantified from whole root systems.

Phosphate plays an important role either on the initiation of nodule formation or on the development and function of the nodules of soybean grown in the acid soils. In addition, in these soils, Ca and P are the most limiting nutrients for BNF of soybean.

## REFERENCES

Alva, A.K., D.G. Edwards, C.J. Asher, and S. Suthipradit. 1987. Effects of acid soil infertility factors on growth and nodulation of soybean. Agron. J. 79: 302-306.

Beck, D.P. and D.N. Munns. 1984. Phosphate nutrition of *Rhizobium* sp. Appl. Environ. Microbiol. 47: 278-282.

Bhuvaneswari, T.V., B.G. Turgeon, and W.D. Bauer. 1980. Early events in the infection of soybean (*Glycine max* L. Merr) by *Rhizobium japonicum*. I. Localization of infectible root cells. Plant Physiol. 66: 1027-1031.

Blamey, F.P.C., D.G. Edwards, and C.J. Asher. 1983. Effect of aluminium, OH:Al and P:Al molar ratios, and ionic strength on soybean root elongation in solution culture. Soil Sci. 136: 197-207.

Brady D.J., C.H. Hecht-Buchholz, C.J. Asher, and D.G. Edwards. 1990. Effect of low activities of aluminium on soybean (*Glycine max*). I. Early growth and nodulation. p. 329-344. *In* M.L. van Beusichem (Ed.). Plant Nutrition-Physiology and Applications. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Brockwell, J., R.R. Gault, D.L. Chase, G.L. Turner, and F.J. Bergersen. 1985. Establishment and expression of soybean symbiosis in a soil previously free of *Rhizobium japonicum*. Aust. J. Agric. Res. 36: 397-409.

Cassman, K.G., A.S. Whitney, and K.R. Stockinger. 1980. Root growth and dry matter distribution of soybean as affected by phosphorus stress, nodulation and nitrogen source. Crop Sci. 20: 239-244.

Cassman, K.G., A.S. Whitney, and R.L. Fox. 1981a. Phosphorus requirements of soybean and cowpea as affected by mode of N nutrition. Agron. J. 73: 17-22.

Cassman, K.G., D.N. Munns, and D.P. Beck. 1981b. Growth of *Rhizobium* strains at low concentration of phosphate. Soil Sci. Soc. Am. J. 45: 520-523.

Cassman, K.G., P.W. Singleton, and B.A. Linquist. 1993. Input/output analysis of the cumulative soybean response to phosphorus on an Ultisol. Field Crops Res. 34: 23-36.

DeMooy, C.J. and J. Pesek. 1966. Nodulation responses of soybeans to added phosphorus, potassium and calcium salts. Agron. J. 58: 275-280.

Freire, J.R.J. 1976. Inoculation of soybeans. p. 335-380. *In* J.M. Vincent, A.S. Whitney, and J. Bose (Eds.). Exploiting the Legume-*Rhizobium* Symbiosis in Tropical Agriculture. College of Tropical Agriculture Misc. Pub. 145, Department of Agronomy and Soil Sciences, University of Hawaii, Honolulu, USA.

Gates, C.T. 1974. Nodule and plant development in *Stylosanthes humilis* H.B.K.: Symbiotic response to phosphorus and sulphur. Aust. J. Bot. 22: 45-55.

Gates, C.T. and W.J. Muller. 1979. Nodule and plant development in the soybean, *Glycine max* (L.) Merr.: Growth response to nitrogen, phosphorus and sulphur. Aust. J. Bot. 27: 203-215.

Graham, P.H. and J.C. Rosas. 1979. Phosphorus fertilization and symbiotic nitrogen fixation in common bean. Agron. J. 71: 925-926.

Israel, D.W. 1987. Investigation of the role of phosphorus in symbiotic nitrogen fixation. Plant Physiol. 84: 835-840.

Israel, D.W. 1993. Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. Physiol. Plant 88: 294-300.

Johnson, J.F., C.P. Vance, and D.L. Allan. 1996. Phosphorus deficiency in *Lupinus albus*. Plant Physiol. 112: 31-41.

Keyser, H.H. and D.N. Munns. 1979. Tolerance of rhizobia to acidity, aluminium and phosphate. Soil Sci. Soc. Am. J. 43: 519-523.

Leung, K. and P.J. Bottomley. 1987. Influence of phosphate on the growth and nodulation characteristics of *Rhizobium trifolii*. Appl. Environ. Microbiol. 53: 2098-2105.

Lie, T.A. 1969. The effect of low pH on different phases of nodule formation in pea plants. Plant Soil 31: 391-405.

MSTAT-C. 1988. A Software program for the Design, Management, and Analysis of Agronomic Research Experiments. Michigan State University, USA.

Mullen, M.D., D.W. Israel, and A.G. Wollum II. 1988. Effects of *Bradyrhizobium japonicum* and soybean (*Glycine max* (L) Merr.) phosphorus nutrition on nodulation and dinitrogen fixation. Appl. Environ. Microbiol. 54: 2387-2392.

Munns, D.N. 1968. Nodulation of *Medicago sativa* in solution culture. I. Acid-sensitive steps. Plant Soil 28: 129-146.

Munns, D.N., J.S. Hohenberg, T. L. Righetti, and D. T. Lauter. 1981. Soil acidity tolerance of symbiotic and nitrogen fertilized soybeans. Agron. J. 73: 407-410.

Pijnenborg, J.W.M. and T.A. Lie. 1990. Effect of lime-pelleting on the nodulation of lucerne (*Medicago sativa* L.) in an acid soils: A comparative study carried out in the field, in pots and in rhizotrons. Plant Soil 121: 225-234.

Singleton, P.W., H.M. Abdel Magid, and J.W. Tavares. 1985. Effect of phosphorus on the effectiveness of strains of *Rhizobium japonicum*. Soil Sci. Soc. Am. J. 49: 613-616.

Somasegaran, P. and H.J. Hoben. 1995. Handbook for Rhizobia. Methods in legume-*Rhizobium* technology. Springer-Verlag, New York, Inc., USA.

Turgeon, B.G. and W.D. Bauer. 1982. Early events in the infection of soybean *Rhizobium japonicum*. Time course and cytology of the initial process. Can. J. Bot. 60: 152-161

Wade, M.K., D.W. Gill, H. Subagjo, M. Sudjadi, and P. A. Sanchez. 1988. Overcoming soil fertility constraints in a transmigration area of Indonesia. Trop Soil Bulletin Number 88-01. North Carolina State University, Raleigh, NC 2769-7113, USA.

Wan Othman, W.M., T.A. Lie, L. ’t Mannetje, and G.Y. Wassink. 1991. Low level phosphorus supply affecting nodulation, $N_2$ fixation and growth of cowpea (*Vigna unguiculata* L. Walp). Plant Soil 135: 67-74.