

Nutrient absorption by pea plants during dinitrogen fixation. 2. Effects of ambient acidity and temperature

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Summary

Nutrient uptake and biological dinitrogen fixation were studied, using effectively nodulated pea plants. These plants were grown hydroponically for six weeks at different acidities of the nutrient solutions, i.e. pH 4.0, 5.5 and 7.0. The pH of the root medium was kept constant continuously by automatic titration and the temperature was maintained at 13 °C throughout. Although the nitrogen content of the plants, grown at pH 4.0 and 7.0, was lower in comparison with that of plants of the pH 5.5 treatment, no nitrogen deficiency symptoms were observed. In each of the treatments about the same number of nodules was formed, but they were smallest in the pH 5.5 treatment. Nevertheless, the total amount of dinitrogen fixed per plant at pH 5.5 was larger than that at higher or lower acidity. This was due to a higher nitrogenase activity per unit of nodule weight, which could be ascertained by an *in vivo* acetylene reduction test.

In all cases more nutritive cations than anions were absorbed, resulting in net proton extrusion by the roots. Both cation and anion uptake and hence the extent of proton release were affected substantially by ambient acidity. Plants grown at pH 7.0 generated 2.3 times as much acidity than plants grown at pH 4.0.

The symbiotic dinitrogen fixing process was repressed by raising the temperature to 25 °C. This resulted in a cessation of dry matter production and proton release, and accelerated the onset of maturation.

It was concluded that positive effects of proton release by dinitrogen-fixing legumes on mobilization and absorption of soil phosphates are restricted to those cases in which plants are grown on non-acidic soils with low pH-buffering and low phosphate-fixing capacities. In this respect, selection for genotypes with an intensive soil exploring root system and a substantial alkaline nutrient uptake may offer some perspectives.

Introduction

As was described in a previous publication on this subject (van Beusichem, 1981) pea plants take up more nutritive cations than anions during utilization of symbiotically fixed dinitrogen. This so-called alkaline nutrient uptake pattern has an acidifying influence on the ambient root medium. Rhizosphere acidity generation through ammonium nutrition can improve the solubility and hence the efficiency of phosphates in soils (Blair et al., 1971; Riley & Barber, 1971; Smiley, 1974; Soon & Miller, 1977).

It would be of agronomic significance in many countries having the disposal of rock phosphate resources, when legumes could acquire nitrogen through an effective symbiotic machinery while, additionally, the efficiency of alkaline rock phosphate fertilizers could be improved as a result of rhizosphere acidity generation.

For the proton extrusion to play an important role as a phosphate mobilizing mechanism in soils, three conditions must be satisfied:

- that leguminous plants are provided with an intensive soil exploring root system, so that contact possibilities between roots and immobile phosphates are favourable;
- that soils on which the plants are grown have very low pH-buffering and phosphate-fixing capacities;
- that cation absorption is much in excess of anion absorption and that the plants are fast starters and growers.

Big differences exist between legume species, both in rooting characteristics and nutrient uptake pattern. In agricultural practice it is often possible to favour root growth by proper soil management, either when a bad-rooting crop is grown or when soil physical conditions prevent optimal root development. It is evident, however, that improving the physical environment will not significantly influence the extent of rhizosphere acidity generation by the roots. From experiments done by Aguilar S. & van Diest (1981) the conclusion can be drawn that application of small quantities of available phosphorus and combined nitrogen results in a better use of rock phosphate by leguminous plants, probably through a stimulation of initial root growth and nodule development. It is not unlikely that utilization of nitrogen stored in the seeds has the same effect. As the intensity of the proton extrusion pump operation is the result of the difference in absorption of nutritive cations and anions, it is to be expected that proton extrusion can be stimulated through enhancement of nutrient absorption or through shifts in nutrient uptake pattern. Plant growth and thus nutrient uptake can be stimulated by raising the temperature, provided that illumination is not limiting. Nutrient uptake patterns of plants can shift in dependence of the pH of the root medium. It is widely known that absorption of nutritive cations is favoured as the pH of the rhizosphere is higher and that anion absorption is favoured at higher ambient acidities. This implies that a higher proton extrusion by the roots is to be expected as soil pH is higher. Because in tropical and subtropical regions acidic ultisols and oxisols are predominant, the question is relevant, whether un-

der these conditions the rate of proton release by the roots of dinitrogen-fixing legumes is sufficient to have a positive influence on solubility and thus absorption of soil phosphates by these plants. In literature no quantitative data have been reported on the effect of ambient acidity on the intensity of the proton pump operation by leguminous plants. This paper deals with the effects of ambient acidity and temperature on dry matter production, absorption of nutrients, acidity generation, and dinitrogen fixation by hydroponically grown nodulated pea plants.

Materials and methods

Plant cultivation and chemical plant analysis

All materials and methods, including seedling culture, plant growth, and chemical plant analyses, were completely comparable to those described previously for experiments with dinitrogen-fixing plants (van Beusichem, 1981). The experimental conditions were also the same, except for the environmental factors which were object of this investigation. In some experiments plants were transferred to the phytotron and placed in nutrient solutions at different acidities, i.e. pH 4.0, 5.5 or 7.0. In other cases, experiments were carried out simultaneously in phytotrons at 13 °C and 25 °C, using nutrient solutions which were kept constant at pH 5.5. Plants were grown under constant climatic conditions for a period of 42 days. After 21 days, half the number of plants was harvested.

Acetylene reduction

For the acetylene reduction tests, whole plants were transferred into 1-litre Erlenmeyer flasks. Acetylene purification and incubation of the plants were carried out according to Akkermans (1971). The ethylene production was measured with a Becker 417 gaschromatograph, equipped with a flame ionization detector and a stainless steel column filled with Porapak R, at 80 °C. The ethylene content was calibrated with a standard gas mixture consisting of 100 µl/litre C₂H₄ in nitrogen gas. The heights of the peaks were related to the concentrations of C₂H₂ and C₂H₄ (Hardy et al., 1968).

Results

Production of dry matter

As is shown in Fig. 1 there was no significant difference between dry matter yields of 21-day old plants grown at 13 °C or 25 °C. After the third week the shoot production of plants grown at 25 °C fell in comparison with the 13 °C treatment and root growth stopped completely. Plants grown at 25 °C flowered as soon as the end of the third week.

The influence of the acidity of the root medium on dry matter production of shoots and roots of dinitrogen-fixing plants is given in Fig. 2. Dry matter production during the first three weeks was hardly affected by the pH of the nutrient solution, which ranged from 4.0 to 7.0. After 42 days, however, shoot yield

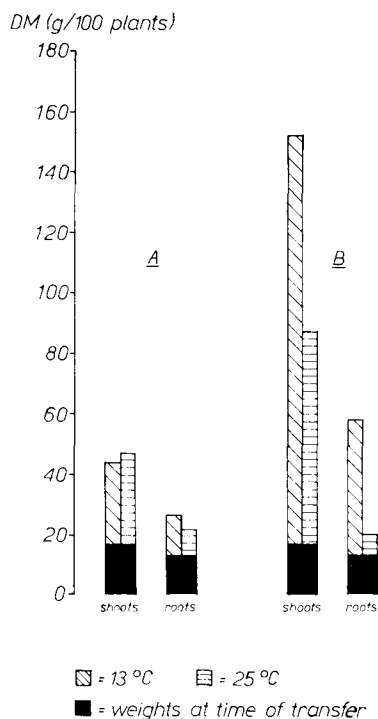


Fig. 1. Dry matter yields of pea plants, 21 (A) and 42 (B) days after transfer to a phytotron at 13 °C or 25 °C. Plants were grown at pH 5.5.

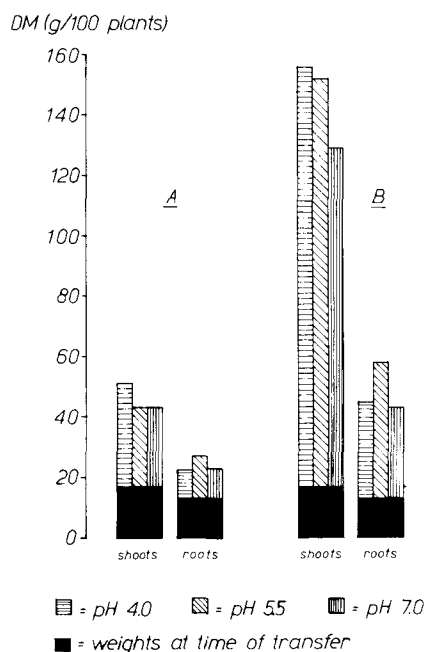


Fig. 2. Dry matter yields of dinitrogen fixing pea plants, 21 (A) and 42 (B) days after transfer to a nutrient solution at pH 4.0, 5.5 or pH 7.0. Plants were grown at 13°C.

at pH 7.0 was significantly lower as compared with that of the pH 4.0 and pH 5.5 treatment. Root growth appeared to be best in the pH 5.5 treatment. No differences in root morphology and shoot colour could be observed between the different treatments. During the whole experimental period plants remained in their vegetative stage.

Absorption of nutrients

It is known from short-term experiments with single-salt solutions that cation absorption is favoured as the pH of the root medium is higher and that uptake of nutritive anions is stimulated at higher ambient acidities. In these experiments with dinitrogen fixing pea plants, this phenomenon was more or less confirmed as can be concluded from the Figs. 3 and 4. During the first three weeks absorption of cationic nutrients was stimulated more at higher pH values (Fig. 3A) than anion absorption was depressed, while sulphate uptake was pH-independent (Fig. 3B). The relatively small amounts of sodium in the plants originated from the sodium hydroxyde which was added in order to keep the pH at the adjusted value. As reflected in the shaded area in Fig. 3C, shifts in uptake of nutritive ions resulted in an increased difference between total cation and anion absorption as the pH of the nutrient solution was higher. The ion uptake picture after a 42 days absorption period is somewhat more complicated (Fig. 4). Plants grown at pH 7.0 had absorbed less potassium than those grown at pH 5.5 (Fig.

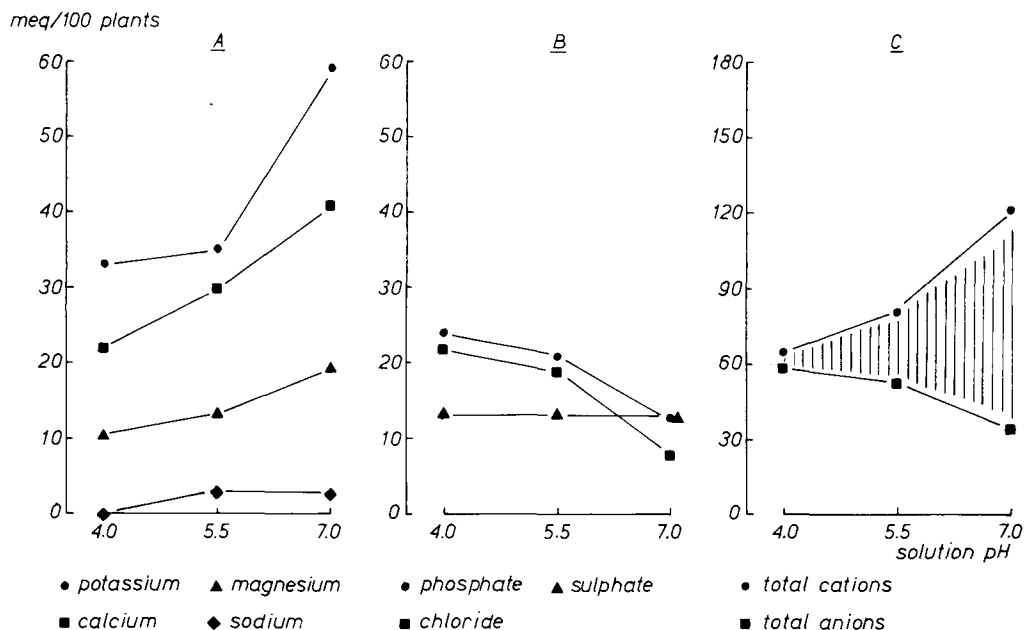


Fig. 3. Cations and anions absorbed by dinitrogen-fixing pea plants grown at pH 4.0, pH 5.5, or pH 7.0, over a three-week period. A: uptake of the different cations; B: uptake of the different anions; C: total cation and anion uptake. Temperature 13°C.

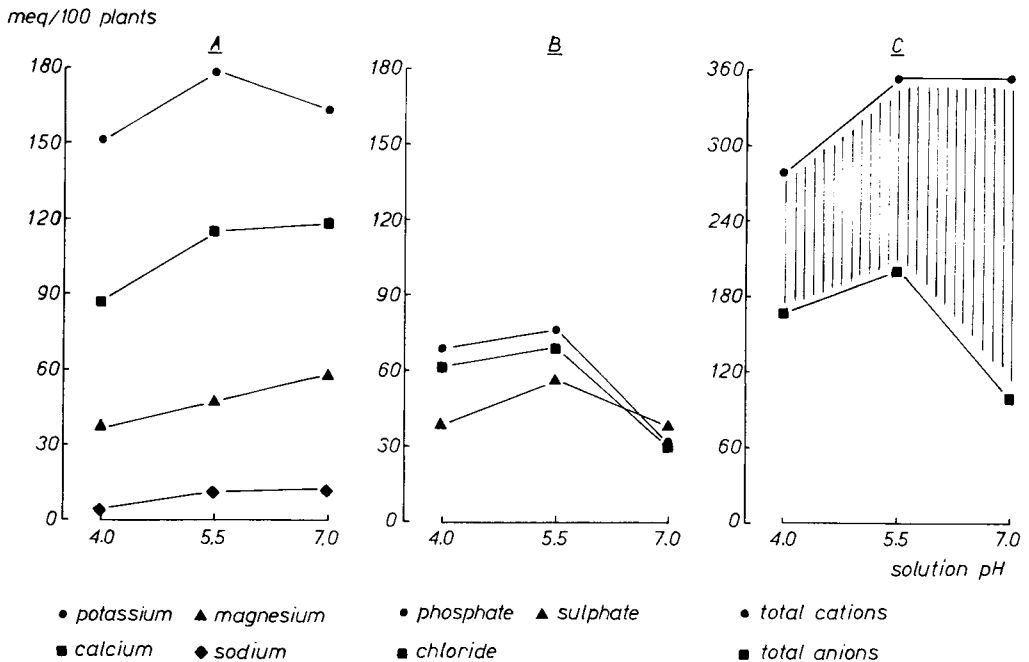


Fig. 4. Cations and anions absorbed by dinitrogen-fixing pea plants grown at pH 4.0, pH 5.5, or pH 7.0, over a six-week period. A: uptake of the different cations; B: uptake of the different anions; C: total cation and anion uptake. Temperature 13°C.

4A) and anion absorption curves show an optimum at pH 5.5 (Fig. 4B). The overall effect of ambient acidity on the extent of alkaline nutrient uptake, however, was comparable with that of younger plants, as can be concluded from Fig. 4C.

Net proton extrusion

In Fig. 5 net proton production by the roots of plants grown at 13 °C and 25 °C are compared. As the proton or hydroxyl/bicarbonate extrusion is a reflection of the dry matter production pattern (van Beusichem, 1981), it is not surprising that the rate of proton release by plants grown at 25 °C decreased after the third week. The curves in Fig. 5 deviate at the time of flowering for the plants grown at 25 °C.

The difference in cumulative net proton extrusion by dinitrogen-fixing pea plants grown in nutrient solutions of different acidities is presented in Fig. 6. For plants grown at pH 5.5, the alkaline nutrient uptake can be estimated by automatic registration of the amounts of hydroxide necessary to keep the pH constant. These values correspond well with the results of chemical plant analyses (van Beusichem, 1981). At pH 4.0 and 7.0 discrepancies were observed between the amounts of titrated hydroxide and excess cation over anion absorption. In order to obtain well comparable data, all values for the acidity generation in de-

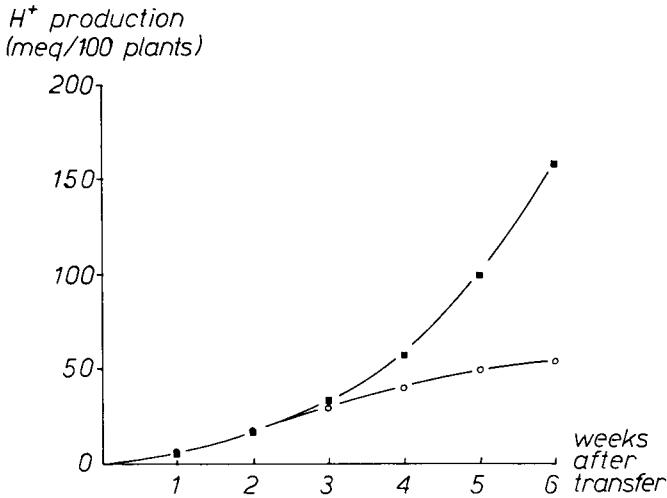


Fig. 5. Cumulative net proton production by dinitrogen-fixing pea plants, grown at 13 °C (■) or at 25 °C (○). Acidity of the nutrient solution pH 5.5.

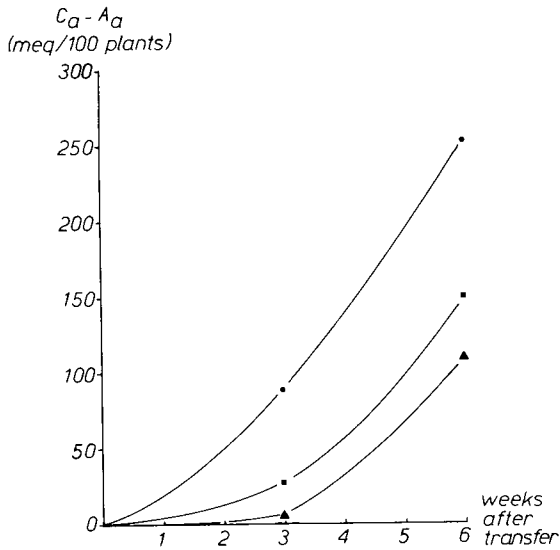


Fig. 6. Cumulative excess cation over anion uptake by dinitrogen-fixing pea plants, grown at pH 4.0 (▲), pH 5.5 (■), or pH 7.0 (●). Temperature 13°C.

pendence of the solution pH are expressed as excess cation over anion absorption (Fig. 6). A negative correlation was found between acidity of the root medium and net proton production by the roots. The cumulative net proton extrusion over the six-week period at pH 7.0 was 2.3 times as high as that at pH 4.0 (253 and 109 meq per 100 plants, respectively).

Nitrogen

Nitrogen contents in both shoots and roots of dinitrogen-fixing plants, grown at different temperatures and acidities, are given in Table 1. For comparison, data for nitrate-supplied plants are added. The nitrogen content in both shoots and roots of plants grown at 13 °C and pH 5.5 did not differ significantly for the nitrate or dinitrogen-fixing treatments. The same was observed for the production of dry matter (van Beusichem, 1981). From these observations the conclusion was drawn that under the described experimental conditions the dinitrogen-fixing process was not rate-limiting for optimal growth. A decrease or increase of ambient acidity as well as an increase in temperature during the experimental period caused a drastic drop in nitrogen content of the plants, both in shoots and roots. The total amount of dinitrogen fixed on a plant basis over a period of 21 or 42 days was lower when the pH was lowered to 4.0 or raised to 7.0 (Table 2). This effect was most pronounced in the pH 7.0 treatment as a result of a lower dry matter production (Fig. 2) and a lower nitrogen content (Table 1) in comparison with the other treatments.

At 25 °C the amount of dinitrogen fixed over the whole experimental period appeared to be almost completely the result of nitrogenase activity during the first three weeks (177 and 160 mmol per 100 plants, respectively).

Table 1. Nitrogen content (mmol/kg DM) of shoots and roots of pea plants, after 21 and 42 days growth under different conditions of nitrogen nutrition, pH of the medium, and ambient temperature.

Treatment	21 days after transfer		42 days after transfer	
	shoots	roots	shoots	roots
NO ₃ , 13 °C, pH 5.5	3927	2907	3668	2834
N ₂ , 13 °C, pH 4.0	3399	2690	2485	2465
N ₂ , 13 °C, pH 5.5	4051	3065	3593	2838
N ₂ , 13 °C, pH 7.0	3058	2459	1971	2175
N ₂ , 25 °C, pH 5.5	3380	2841	2238	2051

Table 2. Nitrate absorption or dinitrogen fixation (mmol N per 100 plants) by pea plants after 21 and 42 days growth under different conditions of nitrogen nutrition, pH of the medium, and ambient temperature.

Treatment	21 days	42 days
NO ₃ , 13 °C, pH 5.5	249	736
N ₂ , 13 °C, pH 4.0	176	441
N ₂ , 13 °C, pH 5.5	198	653
N ₂ , 13 °C, pH 7.0	130	290
N ₂ , 25 °C, pH 5.5	160	177

Nitrogenase activity

To get some idea about the dinitrogen-reducing activity of the plants, at both harvests four plants of each pH treatment were used in an acetylene reduction test. The results are summarized in Fig. 7. The dinitrogen-reducing capacity of plants, grown at pH 4.0 and 5.5, increased substantially with age, in contrast to the pH 7.0 treatment (Fig. 7A). At both harvests, plants grown at pH 4.0 showed a higher C_2H_2 -reducing activity than those grown at pH 5.5 or 7.0. In Table 3 the effects of ambient acidity on number and weight of root nodules are compared. The number of nodules per plant was not significantly influenced by the acidity of the nutrient solution. The data clearly indicate that, in all treatments, formation of new root nodules did not take place after the first three weeks. Plants grown at pH 4.0, however, had much more nodule tissue at their disposal than those of the other treatments. Nodule growth was also greatest in the low pH treatment. Synthesis of nodule tissue could not prevent a decrease in C_2H_2 -reducing activity per gram of dry root nodule (Fig. 7B) as the plants were older.

The cessation of the dinitrogen-fixing process at 25 °C, as reflected in the nitrogen data given in Table 2, could be confirmed by estimations of the nitroge-

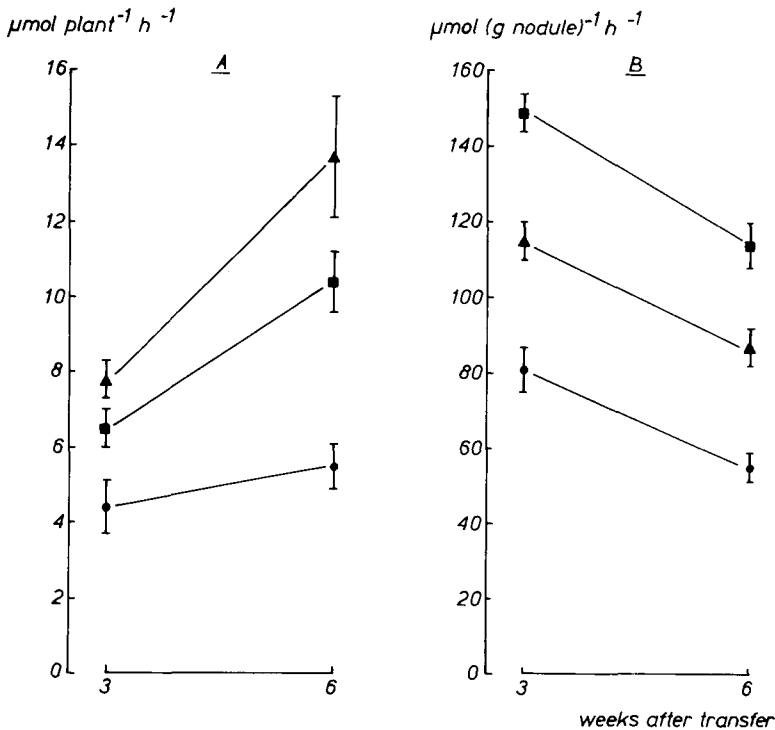


Fig. 7. Acetylene reduction by pea plants grown for three weeks or six weeks at pH 4.0 (▲), pH 5.5 (■), or pH 7.0 (●). A: values expressed on plant basis; B: values expressed on nodule weight basis. Vertical bars represent S.D.s. (n = 4).

Table 3. Number and dry weights of root nodules collected from pea plants after 21 and 42 days growth at pH, 4.0, 5.5 and 7.0.

Treatment	Number per plant		Dry weight (mg/plant)	
	21 days	42 days	21 days	42 days
13 °C, pH 4.0	265 ± 13	288 ± 27	68 ± 8	157 ± 11
13 °C, pH 5.5	248 ± 17	260 ± 31	44 ± 6	74 ± 8
13 °C, pH 7.0	239 ± 21	246 ± 16	54 ± 6	99 ± 6

nase activity; the ethylene production by three-week old plants was still in the same order as that of the other treatments ($4.8 \mu\text{mol plant}^{-1} \text{h}^{-1}$), but at the end of the experiment the activity was only $0.2 \mu\text{mol plant}^{-1} \text{h}^{-1}$.

Discussion

In agricultural practice it is widely known that the biological dinitrogen-fixing process through a symbiosis of *Rhizobium* spp. and leguminous plants is depressed at lower pH values of the soil solution. This problem is discussed thoroughly in literature (Holding & Lowe, 1971; Mulder et al., 1966; Vincent, 1965). Acidic soils are sometimes found to be almost free from *Rhizobium*. When *Rhizobium* species are isolated from acidic or anaerobic soils, they often appear not to be able to achieve effective symbiosis (Holding & Lowe, 1971). It is not yet completely understood whether negative effects of low soil pH values on the symbiotic system are direct effects of a high proton concentration on plant growth, root development and/or symbiotic properties of *Rhizobium*, or are based on enhanced solubility and thus absorption of some heavy metal ions (manganese, aluminium) by plants or micro-organisms. On the other hand, the availability of calcium and molybdenum, both essential elements for symbiosis, can be sub-optimal in acidic soils. It is very difficult, if not impossible, to distinguish experimentally between direct and indirect effects of ambient acidity when soil is used as the growth medium.

Regarding the nodulation process, Mulder and co-workers (Mulder et al., 1966; Lie, 1969), working with hydroponically grown pea plants, found that only the processes taking place during the first two days after inoculation, and hence during the root infection period, were sensitive to acidity. The acid-sensitive period coincided with root nodule initiation, more particularly with root-hair curling (Munns, 1968), and is probably based on pH-dependent activity of pectinase (Munns, 1969). Israel & Jackson (1978) have supposed a competition between protons and calcium at the infection sites as the process responsible for acid-sensitive nodulation. To get around all possible difficulties connected with nodule initiation, all plants used in the experiments described in this paper, were inoculated at pH 6.5. The nutrient solutions were kept at this acidity for eight days. After this 'acid-sensitive' period, plants were transferred to nutrient solutions of different acidities.

For many reasons it is necessary to be cautious in extrapolating results of water culture experiments to field situations. Nevertheless, in this experiment it has been clearly demonstrated that once the nodulation is successful, no reduction in dry matter production occurs when the plants are grown at pH 4.0 (Fig. 2). Although the nitrogen content of the plants grown at pH 4.0 fell in comparison with plants grown at pH 5.5 (Table 1), this did not lead to shoot colour differences. The nitrogen content in all treatments can be considered as high.

From laboratory studies it is known that nodule initiation rather than dinitrogen fixation is acid-sensitive, but this phenomenon is never exploited for application in acid soils. In the author's department, experiments are currently being carried out to study the effects of planting inoculated seedlings, coating the seeds with calcium carbonate, and the effects of local liming on dry matter production and dinitrogen fixation by pea plants grown on an acid sandy soil (van Beusichem & Langelaan, to be published).

As already discussed previously (van Beusichem, 1981) nutrient uptake patterns and acidity generation by dinitrogen-fixing legumes may have some agronomic importance. Proton extrusion by the roots as a result of excess cation over anion absorption can increase the solubility and thus absorption of soil phosphorus by these plants. An interesting conclusion that can be drawn from this investigation is that the proton extrusion pump of dinitrogen-fixing pea plants operates more intensively as the ambient acidity is lower (Figs. 3C, 4C and 6). Assuming that the nutrient solutions can be considered as ideally behaving non-buffering systems and that uptake patterns of nutritive ions are not affected by variations in the acidity of the root medium, it can be calculated that, if a pH-stat technique had not been used, the pH of the solutions initially adjusted at 4.0, 5.5, and 7.0 would have dropped to 3.6, 2.7, and 2.5 after six weeks as a result of the proton extrusion process. Although many objections can be raised against this excessive simplification, it can be expected that in acidic soils rhizosphere acidity generation is not substantial. The results obtained in this investigation support the previously drawn conclusion (van Beusichem, 1981) that positive effects of the alkaline nutrient uptake pattern of leguminous plants, utilizing symbiotically fixed nitrogen, are restricted to those cases in which legumes are grown on non-acidic soils with low pH-buffering and low phosphate-fixing capacities.

An attempt to stimulate growth and nutrient absorption and thus proton extrusion by raising the temperature from 13 °C to 25 °C failed. After three weeks, the shoot production fell in comparison with the other treatments and root growth stopped completely (Fig. 1). Some *Rhizobium* strains respond to high temperatures by a rapid degeneration of the bacteriod tissue, resulting in shortening of the period of dinitrogen-fixing activity of the root (Pankhurst & Gibson, 1973). From the experiments done by Lie (1974) the supposition seems to be justified that the *Rhizobium* strain PF2 is able to resist long-term exposure to higher temperatures. However, the amount of dinitrogen fixed over the whole experimental period appeared to be almost completely the result of nitrogenase activity during the first three weeks (Table 2). Probably, photosynthetic capacity was

the limiting factor under circumstances of a relative high temperature in combination with a relative low light intensity. This results in competition for photosynthates for growth and maintenance on one hand and for the operation of the symbiotic system on the other hand. In response to the cessation of the dinitrogen-fixing process, the plants redistributed their nitrogen. The observed accelerated transition into maturation can be considered as the overall reaction of the plants on these stress conditions.

In conclusion, it can be said that the extent of acidity generation of dinitrogen-fixing leguminous plants depends strongly on environmental conditions. Probably, the wide adaptation to soil and climate conditions among legume genotypes is partly based on the intensity of the proton extrusion pump operation. Since in most of the tropical legumes rhizosphere acidity generation appears to be lower than in temperate species (Andrew & Johnson, 1976), selection for genotypes with an intensive soil exploring root system and a substantial alkaline nutrient uptake seems to be one of the few measures to improve the phosphate-mobilizing potential of leguminous crops.

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