NON-IONIC NITROGEN NUTRITION OF PLANTS

Nutrient uptake and assimilation and proton extrusion during utilization of urea or symbiotically fixed nitrogen

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Proefschrift
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Stellingen

I

De controverse in de literatuur met betrekking tot de geldigheid van het Dijkshoorn - Ben Zioni recirculatiemodel in planten zal voortduren totdat gedetailleerde gegevens beschikbaar komen over de ionensamenstelling van en ionenflux door xyleem en floeem van diverse plantesoorten waarvan bovendien zowel het opnamepatroon van kationen en anionen als de verdeling van de nitraatreductase-aktiviteit over de diverse organen ondubbelzinnig vaststaat.

Ben Zioni, A., Y. Vaadia, and S.H. Lips. Physiol. Plant. 24, 288-290 (1971).

Findenegg, G.R., M.L. van Beusichem, and W.G. Keltjens. Abstr. 4th Congr. Eur. Soc. Plant Physiol. (Strasbourg), 463-464 (1984).

II

Definitieve uitspraken over de landbouwkundige betekenis van het alkalische ionenopname-patroon van stikstofbindende vlinderbloemigen voor de benutting van alkalische ruwfosfaten kunnen slechts worden gedaan op grond van gegevens omtrent de ruimtelijke variabiliteit van de bodem-pH gedurende de groei van deze planten onder (proef)veldomstandigheden.

Aguilar S., A. and A. van Diest. Plant and Soil 61, 27-42 (1981).

Bekele, T., B.J. Cino, P.A.I. Ehlert, A.A. van der Maas, and A. van Diest. Plant and Soil 75, 361-378 (1983).

III

De gebruikelijke bepaling van de acetyleenreductiesnelheid door geïsoleerde wortelknolletjes of genoduleerde wortelstelsels, berekend uit een veronderstelde lineariteit tussen twee meetmomenten tijdens een 60-90 minuten durende toets, is in vele gevallen een onjuiste maat voor de aktuele aktiviteit van het enzym nitrogenase.

Minchin, F.R., J.F. Witty, J.E. Sheehy, and M. Müller. J. exp. Bot. 34, 641-649 (1983).

IV

Uit de waarneming dat in xyleemsap van *Ricinus communis* L. nitraat het merendeel van de electronegatieve lading vertegenwoordigt en dat bovendien het nitraatgehalte in de bladeren veel lager is dan dat in de wortels, mag nog niet de conclusie getrokken worden dat in deze plant de nitraatreductie hoofdzakelijk plaatsvindt in de bovengrondse delen.

Kirkby, E.A. and M.J. Armstrong. Plant Physiol. 65, 286-290 (1980).
Keltjens, W.G. Proc. 9th Int. Plant Nutr. Colloq. (Coventry) 1, 283-287 (1982).

V

Het is, in afwachting van gedetailleerde onderzoekingen daaromtrent, verstandig om als hulpmiddel bij de bestudering van het transport van opgeloste stoffen door het xyleem de 'pressure bomb' te beschouwen als 'toy' en niet als 'tool'.

Simpson, R.J., H. Lambers, and M.J. Dalling. Physiol. Plant. 56, 11-17 (1982).

VI

Mannitol is geen universeel toepasbaar extern osmoticum ter bestudering van de regulatie van volume en turgor van wortelcellen.

Cram, W.J. Physiol. Plant. 61, 396-404 (1984).

VII

Bij het bepalen van het effekt van een van de economisch optimale kunstmeststikstofgift afwijkende gift dient zowel de afwijking in gebruikte stikstofhoeveelheid als in verkregen opbrengst, beide uitgedrukt in bijvoorbeeld guldens per hectare, in rekening te worden gebracht.

Neeteson, J.J. In: The assessment of nitrogen fertilizer requirement (Proc. 2nd meeting). Institute for Soil Fertility, Haren, Groningen (1984).

VIII

De verklaring voor de waarneming van Ikeda en Osawa, dat slechts die plantesoorten die bij éénzijdige ammoniumvoeding ernstige groeireduktie vertonen in staat zijn selectief nitraat uit een ammoniumnitraat-oplossing op te nemen, moet gezocht worden in het nagenoeg afwezig zijn van zowel nitraat- als ammonium-assimilerende enzymen in de wortels van deze planten.

Ikeda, H. and T. Osawa, J. Japan. Soc. hort. Sci. 50, 225-230 (1981).

IX

De nitraatreductase-aktiviteit in de wortel wordt waarschijnlijk niet geïnduceerd door het substraat maar gestimuleerd door de produkten.

Mengel, K., P. Robin, and L. Salsac. Plant Physiol. 71, 618-622 (1983).

X

Het aan de man brengen van de eerste kunstmeststoffen vertoont treffende overeenkomsten met het kolengraafprojekt van RSV.

James Musprat & Co, Liverpool. An address to the agriculturists of Great Britain, explaining the principles and uses of artificial manures (1845).

XI

Het is zowel uit muziekpedagogisch als uit didactisch oogpunt sterk aan te bevelen in concertstukken die worden gecomponeerd of gearrangeerd ter uitvoering door amateur-orkesten, de dynamische tekens slechts op te nemen in de partitie.

XII

De tekst van het Koninklijk Besluit no. 31 d.d. 19 december 1978 suggereert dat vanaf 1 februari 1979 de leer der voeding van herbivoren en vegetariërs mede tot het studiegebied van de algemene bodemkunde wordt gerekend.

Koninklijk Besluit no. 31 d.d. 19 december 1978.
Van Dale, Groot Woordenboek der Nederlandse Taal, tiende druk, deel II, pag. 1874 (1976).

XIII

De mogelijkheden van vele 'blieb-blieb' apparaten worden schromelijk overschat door deze aan te duiden met de functie van diegenen die dergelijke apparatuur bedienen; tekstverwerkers zijn mensen!

XIV

Met de aankondiging "Het laatste rondje" in sommige café's omstreeks het sluitingsuur, wordt kennelijk iets anders bedoeld dan gezegd.

Stellingen behorend bij het proefschrift:
Non-ionic nitrogen nutrition of plants.
M.L. van Beusichem, 10 oktober 1984.

Aan de nagedachtenis
van mijn vader

Woord vooraf

Bij het afronden van deze studie maak ik gaarne van de gelegenheid gebruik, allen, die op enigerlei wijze bijgedragen hebben tot de totstandkoming van het proefschrift, te bedanken.

Allereerst zijn dit mijn ouders die mijn studie, eerst aan de Hogere Landbouwschool te Dordrecht en vervolgens aan de Landbouwhogeschool, mogelijk hebben gemaakt. Het doet mij dan ook erg veel verdriet dat mijn vader de officiële afronding van deze studie niet meer heeft mogen meemaken.

Mijn promotor, prof. Findenegg, ben ik veel dank verschuldigd voor de waardevolle discussies en adviezen en het zeer kritisch redigeren van de diverse manuscripten. Beste Günter, het is verheugend vast te stellen dat onze samenwerkings-curve na een 'lag phase' reeds lang in de exponentiële fase verkeert. Het is mijn oprechte wens dat dit nog lang zo blijft.

Willem Keltjens, onze, overigens onbetaalde, neventaken lieten niet toe dat we kamergenoten bleven. Ik denk met veel genoegen aan de leerzame periode-in-de-kelder terug.

Jaap Nelemans, jouw enthousiasme, toewijding en nauwkeurigheid hebben gezorgd voor een voortdurende stroom van juiste en reproduceerbare resultaten. Door jouw hulp bij de vele en uiteenlopende experimenten en analyses heb je een wezenlijke bijdrage geleverd aan de totstandkoming van een groot deel van dit proefschrift, waarvoor mijn hartelijke dank.

Alle vakgroepmedewerkers, maar in het bijzonder Karel van Gaalen, Piet Jansen, Kees Kok, Bart Matser, Wim Menkveld, Inge Stakman, Ina Steenstra, Karin Sijlmans en Bert Wittich wil ik graag bedanken voor de zeer veelzijdige hulp.

Tenslotte wil ik mijn oprechte dank betuigen aan al diegenen die, zowel in de werk- als in de privé-sfeer, steeds weer het geduld blijken te kunnen opbrengen om langdurig in gezelschap te verkeren vân en/of samen te werken mêt iemand, wiens persoonlijkheidsstructuur daartoe lang niet altijd uitnodigt.

Contents

Abstract

This thesis encompasses six papers, dealing with mainly ionic balance aspects of non-ionic nitrogen nutrition of plants. In most cases urea nutrition or symbiotic N_2-fixation were compared with NH_4^+- or NO_3^--supply with respect to nutrient uptake and assimilation.

From ionic balance and proton release data it was established that maize and sugar-beet plants are able to absorb urea as an undestructed molecule. Results of xylem sap analyses learned that urea, like NH_4^+, is almost quantitatively metabolized in the roots.

Complete ionic uptake balances, including direct measurements of respective H^+- and OH^-/HCO_3^--release from the roots of N_2-fixing and NO_3^--supplied pea plants are presented. Excess nutrient cation over anion uptake and hence H^+-release by N_2-fixing plants increased at higher pH of the nutrient solution. When such plants were grown in soil, cation uptake also exceeded anion uptake, but root growth was severely reduced at low soil pH. This effect could be eliminated completely by liming. Root growth was not inhibited when NO_3^- was the form of N-nutrition.

In soils, mineralized N may confuse the comparison between NO_3^--nutrition and N_2-fixation. It is suggested that the relative contribution of N_2-fixation to the total N-accumulation in plants reflects the point of time at which (^{15}N-)NO_3^- in the soil was depleted and the N_2-fixing process started.

Different ionic uptake patterns of plants in relation to the form of nitrogen nutrition necessarily invoke essential differences in both inorganic and organic chemical composition of the xylem sap of these plants. Complete xylary ionic balances and data about partitioning of the nitrogenous compounds in xylem saps allowed the conclusion that N_2-fixing pea plants belong to the group of amide-transporting legumes and that in NO_3^--supplied pea plants no phloem transport of cation-organate is necessary for the regulation of intracellular pH and electroneutrality.

1 Introduction

1.1. General

All organisms have to absorb chemical compounds from the environment which are essential for growth and metabolism. The supply and absorption of these compounds may be defined as nutrition of the organism. From a nutritional point of view, higher plants behave distinguished from man, animals and heterotrophic microorganisms. Being carbon- and nitrogen-autotrophs, higher plants require exclusively inorganic nutrients for the synthesis of cellular components or as an energy source. The mechanisms and reactions involved in the conversion of nutrients to cellular material and to compounds used for energetic purposes may be defined as plant biochemistry or plant metabolism. Nutrition and metabolism are very closely interrelated processes.

This interrelationship becomes even more clear when nutrition and metabolism of carbon compounds and other nutrients are considered in terms of regulation of intracellular electroneutrality and intracellular pH. Most nutrients are supplied and taken up by plants in either cationic or anionic form. Terrestrial vascular plants generally take up unequal amounts of nutrient cations and anions when expressed in terms of charge equivalents (de Wit et al., 1963). However, during ion uptake, electroneutrality is maintained in the root tissue as well as in the ambient medium by respective organic anion synthesis or breakdown in the root, and extrusion of H^+- or OH^--ions from the root (Houba et al., 1971; Breteler, 1973a). From this simplified conception the conclusion can already be drawn that uptake of nutrient cations and anions is directly related to organic anion metabolism in order to maintain intracellular electroneutrality and to keep the tissue pH between narrow limits. Also H^+- or OH^- ions must be extruded into the rooting medium in order to account for the excess cationic or anionic nutrient uptake and hence for the maintenance of external electro-

neutrality. This implies that, during nutrient uptake, plants may exibit an external acidification or alkalinization. It will thus be clear that in studies concerning the equilibrium between nutrients taken up by plants, four groups of compounds contribute to this dynamic equilibrium or, in other words, play a dominant role in the complete ionic balance of a plant. These groups are: (a) inorganic cations; (b) inorganic anions; (c) organic anions; (d) H^+- and OH^--ions.

In the following sections of this Chapter the most important aspects concerning the ionic balance of plants will be reviewed in more detail. In section 1.2 attention is paid to the ways of classification of plant nutrients and how these nutrients can be classified according to their physiological functions and their contribution to the ionic balance. Section 1.3 encompasses the main processes affecting the organic anion content of a plant and which are thus directly related to organic anion metabolism. Because the form of nitrogen nutrition decides to a great extent the nutrient uptake pattern and hence the internal and external ionic balance of plants, a special section (1.4) is devoted to this subject. The net extrusion or uptake of H^+-ions by the root tissue represents only a small fraction of all trans-membrane proton fluxes in plant cells. Nevertheless, in section 1.5 an attempt is made to emphasize the mechanistic nature of the electrogenic proton pump in plant cells, because this process may be considered as a primary step in inorganic plant nutrition and hence in the regulation of pH and electroneutrality. In the last section of this Chapter (1.6) an overall picture is presented on the localization of the intracellular pH regulation in relation to the form of nitrogen nutrition and the sites of nitrogen assimilation within the plant.

1.2. Classification of plant nutrients

Plant nutrients can be classified according to their: (a) essentiality for normal metabolism and reproduction; (b) content in plant material; (c) biochemical behaviour and physiological functions in the plant.

ad a. Arnon and Stout (1939) proposed three criteria which must be

met in order to consider an element on essential plant nutrient. These are: (a) the element is directly involved in plant metabolism, *e.g.* as a constituent of an essential metabolite or required for the action of an enzyme system; (b) a deficiency of the element makes it impossible for the plant to complete its life cycle; (c) this deficiency is visually specific for the element in question. Based on these criteria, the following chemical elements are considered to be essential for higher plants: C, H, O, N, P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, and Cl. For some elements, such as Na, Si, and Co there is no general agreement with respect to their essentiality for all plants. It is not unlikely that, as a result of further progress in the grade of purification of chemicals and in the refinement of analytical techniques, other elements may be shown sooner or later to be essential for higher plants. This implies that elements which may be added to the abovementioned group will belong to the subgroup of nutrients which are needed in plants in relatively small amounts.

ad b. Based on their content in plant material and the amount needed for normal metabolism and reproduction, nutrients may be divided into macronutrients and micronutrients. The following elements are widely known as macronutrients: C, H, O, N, P, S, K, Ca, and Mg, while Fe, Mn, Cu, Zn, Mo, B, and Cl are considered to be micronutrients. The division of plant nutrients into macro- and micronutrients based on the abovementioned criteria, may be arbitrary because in a sense these criteria are contradictory: in many cases the content of micronutrients, such as Cl, Fe, and Mn is far in excess of their physiological requirements and can even reach phytotoxic concentrations in the leaf tissue (Eaton, 1966; Walter et al., 1974; Tanaka and Yoshida, 1970; Tanaka et al., 1973; Bussler, 1958; Morgan et al., 1966; Ohki, 1977). Plants can exhibit toxicity symptoms caused by essential elements but also by non-essential elements such as Al and F (Foy et al., 1978; Brewer, 1966). It will be clear that the nutrient content of plants or plant organs is not always a reflection of the quantity of that particular nutrient needed to sustain physiological and biochemical processes. This phenomenon may be confusing in studies concerning ionic relations in plants, particularly when the content of the element in question

is in the order of one or more of the macronutrients. Omission of the micronutrients in ionic balance research is therefore only allowed when these nutrients are present in negligible amounts on an equivalent basis and, consequently, minimally contribute to the ionic balance of a plant or plant organ.

ad c. A classification of plant nutrients according to their physiological functions and biochemical behaviour (Table 1) provides information about which elements may not be neglected in multi-element research, in particular concerning the ionic balance of plants.

Nutrient Element	Uptake	Biochemical Functions
1st group C, H, O, N, S	In the form of CO_2, HCO_3^-, H_2O, O_2, NO_3^-, NH_4^+, N_2, SO_4^{2-}, SO_2. The ions from the soil solution, the gases from the atmosphere.	Major constituent of organic material. Essential elements of atomic groups which are involved in enzymic processes. Assimilation by oxidation-reduction reactions.
2nd group P, B, Si	In the form of phosphates, boric acid or borate, silicate from the soil solution.	Esterification with native alcohol groups in plants. The phosphate esters are involved in energy transfer reactions.
3rd group K, Na, Mg, Ca, Mn, Cl	In the form of ions from the soil solution.	Non-specific functions establishing osmotic potentials. More specific reactions in which the ion brings about optimum conformation of an enzyme protein (enzyme activation). Bridging of the reaction partners. Balancing anions. Controlling membrane permeability and electro-potentials.
4th group Fe, Cu, Zn, Mo	In the form of ions or chelates from the soil solution.	Present predominantly in a chelated form incorporated in prosthetic groups. Enable electron transport by valency change.

Table 1. Classification of plant nutrients (Mengel and Kirkby, 1982)

From such a subdivision of the nutrients information can be obtained about the electrical stage in which they are taken up and also whether the assimilation of a nutrient coincides with alterations in its electrical charge and hence causes charge transitions. Both uptake of cationic and anionic nutrients and conversion of nutrients into organic compounds affect the ionic balance of a plant.

1.3. The ionic balance of plants

The main cations present in plant material are K^+, Na^+, Ca^{2+}, and Mg^{2+}. The contribution of free NH_4^+ to the total cation content is mostly very small due to its rapid incorporation in low-molecular neutral organic compounds, even when NH_4^+ is the sole nitrogen source (Martin, 1970; Yoneyama and Kumazawa, 1974; Ivanko and Ingversen, 1971a,b). Organic cations are quantitatively negligible in plant material, perhaps with the exception of some amines and some basic amino acids, and micronutrients are mostly of minor importance with respect to their contribution to the total cation content. In normal cases the total cation content in plant material can thus be expressed as the sum of the charge equivalents of K^+, Na^+, Ca^{2+}, and Mg^{2+}.

The situation with respect to the anionic composition is somewhat more complicated. The main anions taken up by plants are Cl^-, $H_2PO_4^-$, NO_3^-, and SO_4^{2-}. Chloride is the only anion which remains, after its absorption by the plant root, quantitatively in the inorganic electronegative stage (Clarkson and Hanson, 1980). Phosphate is present in the plant tissue in both inorganic and organic form. However, in phosphorylated compounds, such as nucleic acids and adenosine esters, the phosphate groups preserve their electronegative charge. Therefore, the total P content of a plant should be considered to contribute to the ionic balance as monovalent $H_2PO_4^-$ (de Wit et al., 1963). In this respect, $H_2PO_4^-$ behaves completely different from NO_3^- and SO_4^{2-}. Nitrate and sulphate are at least partly reduced in the plant tissue, followed by incorporation in electrically neutral organic compounds. This implies that the electronegative charge originating from NO_3^- and SO_4^{2-} is transferred to other compounds during the reduction process and hence only a frac-

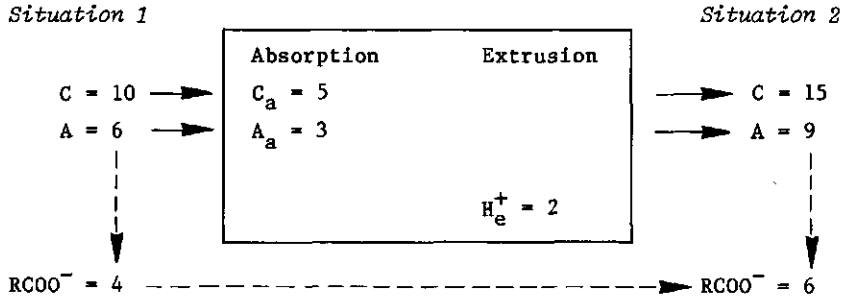
tion of the absorbed NO_3^- and SO_4^{2-} contributes to the inorganic anion content. Because anionic micronutrients (B, Mo) are present in plant material predominantly in undissociated or chelated form and can thus be neglected as ionic constituents, the conclusion is justified that the total inorganic anion content of a plant can be expressed as the sum of the charge equivalents of $H_2PO_4^-$ (total P), Cl^-, NO_3^-, and SO_4^{2-} .

In all cases the total cation content ($C = K^+ + Na^+ + Ca^{2+} + Mg^{2+}$) of a plant exceeds more or less the total inorganic anion content ($A = H_2PO_4^- + Cl^- + NO_3^- + SO_4^{2-}$). The difference between C and A is thought to be related about stoichiometrically to the amount of organic anions (Arnon, 1939; Ulrich, 1941; Pierce and Appleman, 1943; Jacobson and Ordin, 1954; Dijkshoorn, 1962; van Egmond, 1975). In other words, the (C-A) value of a plant is a reflection of the organic anion content (Houba et al., 1971). Because organic phosphates are already included in the calculation of A, the (C-A) value refers more specifically to the dissociated carboxylic acids in the plant. Therefore, the term carboxylates is often preferred to the term organic anions. Nutrient uptake and assimilation processes affect the ionic balance of a plant and thus the carboxylate content. As a result of an excess nutrient cation over anion uptake and of nitrate and sulphate reduction, carboxylates will accumulate in the plant in order to maintain electroneutrality and to buffer the initially formed OH^--ions. On the other hand, an excess nutrient anion over cation uptake and ammonium assimilation is accompanied for the above reasons by a decrease in the size of the carboxylate pool through decarboxylations.

The different processes which influence the carboxylate content of a plant and which can operate simultaneously, are: (a) excess nutrient cation over anion uptake; (b) excess nutrient anion over cation uptake; (c) nitrate reduction; (d) sulphate reduction; (e) ammonium assimilation.

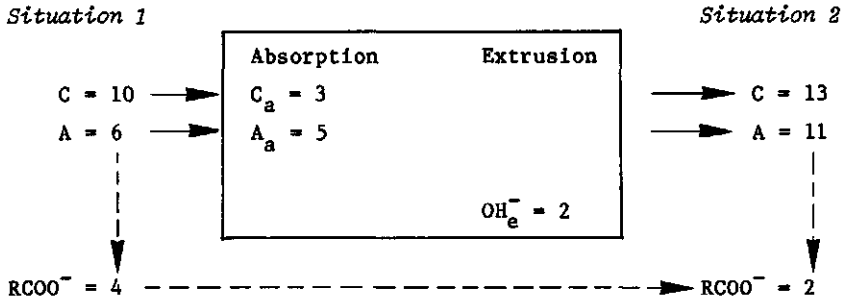
ad a. Differential requirements of plants for nutrients and supply of major nutrients in either cationic or anionic form often result in non-equivalent uptake of cationic and anionic nutrients. In order to maintain electroneutrality in the growth medium, plants absorb or extrude H^+- or OH^--ions in amounts equivalent to the dif-

ference between cation and anion uptake. This aspect of electroneutrality regulation will be treated in detail in section 1.4. The differential uptake of cation and anionic nutrients will oblige the plant to keep processes operative in order to maintain the intracellular electroneutrality as well. This electroneutrality maintenance is achieved by pH-dependent changes in the size of the carboxylate pool through carboxylation and decarboxylation reactions. These reactions are often summarized as the biochemical pH-stat (Davies, 1973a,b). Although the nature of this pH-stat will be treated in detail in section 1.5, it will be clear already that the overall result of an excess nutrient cation over anion uptake can be summarized by: (a) a net extrusion of H^+-ions from the roots into the growth medium and (b) the production of carboxylates, both amounts being equivalent to the difference between cations and anions taken up. Excess nutrient cation over anion uptake is often referred to as an alkaline nutrient uptake pattern of a plant. The next schematic picture demonstrates the interrelations between the processes mentioned above (all units represent arbitrary equivalent units per plant).



ad b. It is obvious that in case a plant absorbs more anionic than cationic nutrients, processes to keep the tissue pH within narrow limits and processes to maintain electroneutrality in the external solution as well as in the plant interior, will proceed in opposite direction as in case of the above example of excess nutrient cation over anion uptake. The next scheme illustrates that an excess nutrient anion over cation uptake, often referred to as an acidic nutrient uptake pattern, is accompanied by: (a) a net extrusion of

OH^--ions from the roots into the growth medium and (b) a decrease in the amount of carboxylates, both equivalent to the difference between anions and cations taken up.



ad c. As was already mentioned before, reduction of NO_3^-, followed by incorporation in electroneutral organic compounds, implies a transfer of electronegative charge from NO_3^- to other constituents. There is a general agreement that in the first instance OH^--ions are formed according to the reaction

$$NO_3^- + 8[H] \longrightarrow (H_3N) + 2H_2O + OH^- \qquad (1)$$

This initial alkaline effect of nitrate reduction is buffered by pH-dependent activation of carboxylases, yielding carboxylates according to the reaction

$$OH^- + RH + CO_2 \longrightarrow RCOO^- + H_2O \qquad (2)$$

The overall effect of nitrate reduction in the plant, as can be summarized by the equations (1) and (2), is the formation of organic compounds which contain the nitrogen in the reduction stage of NH_3, and the production of an equivalent amount of carboxylates.

$$NO_3^- + 8[H] + RH + CO_2 \longrightarrow (H_3N) + RCOO^- + 3H_2O \qquad (3)$$

When nitrate is already reduced in the root, it is possible that a fraction of the initially formed OH^--ions is extruded directly into the growth medium. In such a case these OH^--ions take over the role

in intracellular electroneutrality and pH regulation and is carboxylation of less importance.

ad d. In a chain of reactions analogous to the nitrate reduction process, sulphate reduction also yields carboxylates. This process is summarized by the equation

$$SO_4^{2-} + 8[H] + 2RH + 2CO_2 \longrightarrow (H_2S) + 2RCOO^- + 4H_2O \qquad (4)$$

This means that any (divalent) sulphate ion which is reduced, yields an organic compound which contains sulphur in the reduction stage of H_2S, and an equivalent amount of carboxylates.

ad e. Incorporation of ammonium in electroneutral organic compounds may coincide with transfer of positive charge from ammonium to other constituents. It is thought that in such cases carboxylates serve as proton acceptors so that assimilation of NH_4^+ in the plant tissue is attended with an equivalent decrease in the size of the carboxylate pool, according to the reactions

$$NH_4^+ \longrightarrow (H_3N) + H^+ \qquad (5)$$

$$H^+ + RCOO^- \longrightarrow RH + CO_2 \qquad (6)$$

$$NH_4^+ + RCOO^- \longrightarrow (H_3N) + RH + CO_2 \qquad (7)$$

Since it is widely accepted that ammonium assimilation occurs mainly in roots (Martin, 1970; Ivanko and Ingversen, 1971a,b; Yoneyama and Kumazawa, 1974) it is most likely, however, that a major fraction of the acidity produced during incorporation of NH_4^+ is extruded directly into the growth medium. Acidification of the rooting medium to the extent of 1.1 to 1.25 H^+ extruded per NH_4^+ entering (Becking, 1956; Breteler, 1973a) are consistent with this supposition. This means that decarboxylation is probably of minor importance in overcoming intracellular disturbances of electroneutrality and pH in plants supplied with ammonium as the sole source of nitrogen.

As will be shown in detail in section 1.4, nitrate-supplied plants often exhibit an acidic nutrient uptake pattern at the expense of carboxylates, while at the same time nitrate (and sulphate) reduction will give raise to the carboxylate content of a plant. The alkaline nutrient uptake pattern of ammonium-supplied plants and the subsequent ammonium assimilation also contribute in opposite direction to the final carboxylate content. During growth, carboxylations and decarboxylations are thus largely mutually neutralized. This implies that additional information must be available about the nutritional history of a plant before the ionic balance or carboxylate content can be used as a 'finger-print' of a plant or plant species.

1.4. The role of nitrogen in the ionic uptake balance

The uptake pattern of nutrient cations and anions depends on the plant species and on several external factors. One of the most important factors influencing the nutrient uptake pattern of a plant is the form of nitrogen nutrition. Because the absorption of nitrogenous ions mostly exceeds that of other ions by far, ammonium nutrition results in an alkaline nutrient uptake pattern (excess uptake of nutrient cations over anions) and thus in extrusion of H^+-ions from the roots into the bathing medium. This phenomenon has been observed with many plant species, provided that they were able to grow with ammonium as the sole source of nitrogen (Breteler, 1973a; Chouteau, 1963; Clark, 1936; Kirkby and Hughes, 1970; Kirkby and Mengel, 1967; Pitman, 1970; Weissman, 1972). When plants are supplied with nitrate as the only nitrogen source, generally an acidic nutrient uptake pattern (excess uptake of nutrient anions over cations) is displayed, associated with alkalinization of the root environment through the extrusion of OH^--ions. Nitrate-fed plants show a very wide range with respect to the extent of alkalinity extrusion by the roots. As will be shown in section 1.6, this range coincides with the relative importance of the various plant parts in nitrate reduction. *Gramineae* generally take up a large excess of nutrient anions over cations, associated with a high rate of OH^--extrusion. This has been shown for *Zea* (van

Beusichem and van Loon, 1978; Keltjens, 1982), *Lolium* (Dijkshoorn, 1962, 1971), *Hordeum* and *Sorghum* (Watanabe et al., 1971). Other plant species produce only small amounts of alkalinity as can be concluded from data obtained by Vickery et al. (1940) with *Nicotinia*, Kirkby and Mengel (1967) with *Lycopersicon*, and Breimer (1982) with *Spinacia*. *Atriplex* (Osmond, 1967) and *Beta* (Houba et al., 1971; Breteler, 1973a; van Egmond, 1975) are well-known examples of plant species, which show an almost neutral nutrient uptake pattern, while some members of the genera *Gossypium* (Bornkamm, 1969; Watanabe et al., 1971) and *Fagopyrum* (de Wit et al., 1963; Pierre et al., 1970; Bekele et al., 1983) have been recognized as species which exhibit even an alkaline nutrient uptake pattern when exposed to media in which nitrate is the only nitrogen source.

When it is difficult or even impossible to translate pH changes in the rooting medium into H^+- or OH^--fluxes, *e.g.* during experiments in pH-buffering soil cultures, the nutrient uptake pattern of such plants can be ascertained afterwards by means of chemical plant analysis, provided that the whole plant (including the roots) is taken into account and that either nitrate or ammonium was the sole source of nitrogen throughout the experiment. When an absolute figure with respect to acidification or alkalinization is required, the plant material should be analyzed for K, Na, Ca, and Mg to calculate the total cation uptake, and for P, S, and Cl to calculate the total anion uptake. The total N amount in the plant should be added to the cations when ammonium was the only nitrogen source, or to the anions when the plant was solely dependent on nitrate as the form of nitrogen nutrition. The difference between uptake of nutrient cations and anions, on a plant basis, provides an accurate estimate of the amount of H^+- or OH^--ions released from the roots during growth. This is summarized in the following equations, in which the subscript 'a' stands for absorption of the ionic nutrients, expressed in equivalents on a plant basis.

Ammonium nutrition

$$H^+\text{-extrusion} = (N + K + Na + Ca + Mg)_a - (P + Cl + S)_a \qquad (8)$$

Nitrate nutrition

$$OH^-\text{-extrusion} = (N + P + Cl + S)_a - (K + Na + Ca + Mg)_a \qquad (9)$$

Since the recognition of the interactions and functional equilibria between nutrients taken up by plants on one hand and the quantities of the same nutrients and their equilibrium in the rooting medium (soil, nutrient solution) on the other (Bear, 1950), many investigations have been carried out to study the effects of ammonium or nitrate nutrition on the uptake of different cations and anions, the ionic balance, and organic acid and carbohydrate metabolism of different plant species, grown hydroponically (Dijkshoorn, 1958; Coïc et al., 1962; Chouteau, 1963; Dijkshoorn et al., 1968; Kirkby, 1968, 1969, 1981; Kirkby & Hughes, 1970; Breteler, 1973a,b; Haynes and Goh, 1978). In addition to ammonium and nitrate, urea is a very important fertilizer in agricultural practice. From a plant nutritional viewpoint it is interesting to know how and to which extent the nutrient uptake pattern of a plant is affected when the nitrogen is applied in molecular form, as compared with NH_4^+ or NO_3^-. However, the number of publications concerning comparative studies, including urea, is very limited (Wallace and Ashcroft, 1956; Kirkby and Mengel, 1967, 1970; Israel and Jackson, 1982). The lack of unequivocal evidence about the effects of urea nutrition on the ionic balance of plants is probably due to experimental difficulties connected with urea hydrolysis. Enzymatic breakdown of this compound to ammonium carbonate (Court et al., 1964) is known to be followed by a preferential uptake of ammonium by plant roots (Ostromeçka, 1961; van Beuischem and van Loon, 1978). This phenomenon confuses the effect of urea absorption and metabolization on ionic plant characteristics. From ionic balance studies, including treatments in which plants were deprived from nitrogen for some time (Houba et al., 1971) it is known that then such plants take up more nutrient cations than anions, resulting in acidification of the nutrient solution. It is to be expected that, when urea decomposition can be avoided completely and hence does not play any role in the ionic relations within the plant, on urea nutrition the following equation will be most likely applicable.

Urea nutrition

$$H^+\text{-extrusion} = (K + Na + Ca + Mg)_a - (P + Cl + S)_a \qquad (10)$$

When urea hydrolysis cannot be prevented, the ultimate effect of urea nutrition on (soil) acidity will be the result of the pH-raising effect of urea hydrolysis on one hand, and the pH-lowering effect due to the acidic nutrient uptake pattern of plants which take up nitrogen as (a mixture of urea and) ammonium. It is obvious that the situation will become even more complicated when part of the ammonium is nitrified. Generally it is not easy to distinguish between the effect of the plant and the effect of the fertilizer on soil acidity (Pierre et al., 1970; Banwart and Pierre, 1975). In Chapter 2 results of experiments on the influence of urea nutrition on the ionic uptake balance are presented and discussed in detail.

Surprisingly, almost no detailed information is available about nutrient uptake patterns of plants which are able to utilize atmospheric nitrogen through the dinitrogen-fixing process. The quantity of nitrogen reduced biologically on a world scale amounts to about 17.2×10^7 tonnes per annum (Chatt, 1976), which is about four times the amount fixed by chemical industry. From an agricultural viewpoint the symbiotic *Rhizobium* bacteria-legume association is of particular significance. Some authors have supposed that an alkaline nutrient uptake pattern occurs in soils (Nyatsanga and Pierre, 1973; Andrew and Johnson, 1976; Israel and Jackson, 1978) but this was based on incomplete plant analysis data. Nevertheless, it is to be expected that plants which are committed to dinitrogen fixation as the sole source of nitrogen nutrition will show a nutrient uptake pattern, obeying an equation analogous to that for urea nutrition.

Dinitrogen fixation

$$H^+\text{-extrusion} = (K + Na + Ca + Mg)_a - (P + Cl + S)_a \qquad (11)$$

Rhizosphere acidity generation by dinitrogen-fixing legumes may be of agronomic significance. Results obtained by van Diest's group (Aguilar S., 1981; Aguilar S. and van Diest, 1981; Bekele et al., 1983) indicate that symbiotic dinitrogen fixation can initiate a

chain of reactions within the plant, leading to acidification of the growth medium in the vicinity of the roots. As a result of this acidification, alkaline rock phosphates when added as a fertilizer might be partially solubilized and hence mobilized. This implies that alkaline rock phosphates are probably more useful phosphate sources for dinitrogen-fixing legumes than for plant species which normally exhibit an acidic nutrient uptake pattern. However, leguminous plant species may vary in their extent of acidity generation (Andrew and Johnson, 1976). Moreover, the ion uptake rate and hence the acidity production is highly dependent on environmental conditions. In Chapter 3 a detailed picture is presented on the nutrient uptake pattern of hydroponically grown dinitrogen-fixing pea plants, as compared with nitrate nutrition. Subsequently, results about the effects of temperature and acidity of the rooting medium on the nutrient uptake pattern and the extent of acidity generation by the roots of dinitrogen-fixing pea plants are presented and discussed in Chapter 4. Both Chapters deal with results, obtained in experiments where the plants were grown at strictly controlled climatic conditions. In order to test whether the effects of ambient acidity on growth and dinitrogen fixation of pea plants, observed in water culture experiments, were convertible to soil conditions, a comprehensive pot experiment was carried out in which pea plants were grown in a sandy soil at different acidities. The results of this experiment are presented in Chapter 5. One of the differences between nutrient solution and soil cultures is that in the latter case the presence of at least small quantities of ammonium and/or nitrate cannot always be prevented. It is widely recognized that combined nitrogen suppresses the symbiotic dinitrogen-fixing process. So far, there is no general agreement about the exact way in which combined nitrogen suppresses both the formation of new nodules and the activity of existing nodules. Limitation of photosynthate supply of the nodules (Houwaard, 1980; Raggio et al., 1965) and changes in the level of the auxin indole-acetic acid (IAA) (Munns, 1968; Tanner and Anderson, 1964; Valera and Alexander, 1965) are thought to be important factors associated with this suppression. Because in our pot experiments all conditions are usually most favourable for mineralization of organic soil nitrogen, it

seemed worth while to get an idea about the effect of nitrate on dinitrogen fixation. For that reason, an additional pot experiment was conducted (Chapter 6) in which labelled ^{15}N-nitrate was used. This tracer technique allows to separate the contribution of dinitrogen fixation and nitrate nutrition at increasing levels of nitrate present in the soil solution.

1.5. Proton extrusion as a primary step in inorganic plant nutrition

The net extrusion or uptake of H^+-ions by the root tissue represents only a small fraction of all trans-membrane proton fluxes in plant cells. Nevertheless, in this section a compilation from the electrophysiological literature is presented, because some knowledge about the mechanistic nature of the electrogenic proton pump in plant cells may provide an insight into the ionic relations in plants. There is a currently held view in the literature that the primary step in the chain of uptake, transport, and metabolization of inorganic ions in the plant is an electrogenic H^+-extrusion process at the plasmalemma of epidermal and cortical cells (Dejaegere and Neirinckx, 1978; Dejaegere et al., 1980; Raven and Smith, 1974, 1976a; 1977; 1980; Smith & Raven, 1974, 1976, 1979).

Although measured cytoplasmic pH values should be interpreted with caution because of a number of experimental difficulties, it seems reasonable to consider a cytoplasmic pH in cortical cells of about 7.0 (Smith and Raven, 1979). Measured values of electrical potential differences across the plasmalemma and of cytoplasmic pH indicate that the proton efflux must be active according to the Nernst equation when the external pH is below 9.0-9.5. This ascertainment, however, is a simplification of the situation because application of Nernst's law is restricted to passive flux equilibrium conditions. On the other hand, it is impossible to measure steady state H^+-fluxes, necessary to establish whether these fluxes obey the Ussing-Teorell criterion or not, because no suitable tracer for H^+ is available. Moreover, it is obvious that it is impossible to remove all protons from the side of the membrane from which their active transport is expected. This removal procedure has been

proved to be a good and simple method to obtain information about the direction of the electrogenic transport of other ions.

For the above reasons, the evidence for an energy-requiring active H^+-efflux pump is mostly indirect and is based on manipulations which change the degree of polarization of membranes and/or on effects of phytotoxins and respiratory inhibitors on membrane potentials and H^+-extrusion. Anderson et al. (1977) did not observe a significant depolarization of the membrane potential of carrot root parenchyma cells after substitution of SO_4^{2-} for Cl^- in the external complete nutrient solution. Apparently the potential does not result from active anion influx. From his data, Pitman (1970) concluded that the release of H^+-ions from low-salt barley roots during salt accumulation must be an active process. This conclusion was based on reasonable estimations and calculations. Depolarization phenomena observed after decreasing the pH of the external solution (Anderson et al., 1977) support the suggestion that an electrogenic proton extrusion pump is operative at the plasmalemma, causing membrane hyperpolarization. There are, however, indications for the existence of two (or more) independent electrogenic mechanisms, operating in plant cells, which seem to act in opposite senses, *e.g.* an additional obligate Cl^-/H^+-symport or Cl^-/OH^--antiport mechanism in which the proton or hydroxyl flux exceeds that of chloride (Anderson et al., 1977; Cram, 1975). Pitman (1970) reported inhibition of both salt uptake and proton release by barley roots after treatment with cyanide meta-chlorophenylhydrazone (CCCP), oligomycin, and arsenite. Similar effects were observed after treatment with 2,4-dinitrophenol (DNP) (Dejaegere and Neirinckx, 1978). After addition of cyanide, carbon monoxyde, or CCCP, a rapid membrane dpolarization in carrot root tissue was detected by Anderson et al., 1977. Based on comparisons between membrane depolarization rates, decay constants of cellular ATP, and rates of pyridine nucleotide reduction, and by taking into account the special architecture of carrot root parenchyma cells, Anderson et al. (1977) could make acceptable that the H^+-extrusion pump is ATP-powered. Slayman et al. (1973) came to the same conclusion after comparing membrane depolarization rates in hyphae of *Neurospora crassa*. Probably, a reversible H^+-ATPase is operative at the plas-

malemma. In response to the resulting electrical potential gradient, cation influx can take place. The approach of considering metabolically dependent H^+-extrusion as the primary step in ion uptake is in agreement with the results of numerous investigations explaining passive or active ion transport in terms of fluxes down or against the electrochemical potential gradient (Etherton, 1963; Higinbotham et al., 1967; Pierce and Higinbotham, 1970; Stepherd and Bowling, 1973).

As a direct result of H^+-extrusion process, OH^--ions are generated in the cytoplasm. In the uptake process, these hydroxyl ions can be exchanged for other ions from the outer solution. Some authors suggest that anion absorption depends on cation absorption because the H^+-extrusion process primarily determines the amount of negative charge generated in the cytoplasm (Dejaegere and Neirinckx, 1978; Dejaegere et al., 1980). As mentioned before, in addition to OH^-/anion-antiport also H^+/anion-symport can occur. These two processes cannot be distinguished experimentally since both result in a hydroxyl ion gradient to drive anion uptake. The buffering capacity of intracellular buffers like bicarbonate, phosphate, hystidine, cysteine, and cystine is by far insufficient to compensate for the changes of internal pH as a result of the differential uptake of nutrient cations and anions (Smith and Raven, 1979). To keep the pH of the cytoplasm within narrow limits, a complicated mechanism is operative in the tissue, as proposed by Davies (1973a,b) in the biochemical pH-stat (Fig. 1). Net hydroxyl generation, associated with an excess nutrient cation over anion absorption, results in an initial increase of cytoplasmic pH. A pH increase in the range of pH 6.0 to 8.0 activates the enzyme phosphoenolpyruvate (PEP) carboxylase and, thus, the production of oxalo-acetic acid (OAA). This compound is converted to dissociated malic acid. When as a result of this reaction, or in response to an excess nutrient anion over cation absorption, the intracellular pH falls to a value of about 6.0, malate is decarboxylated through the

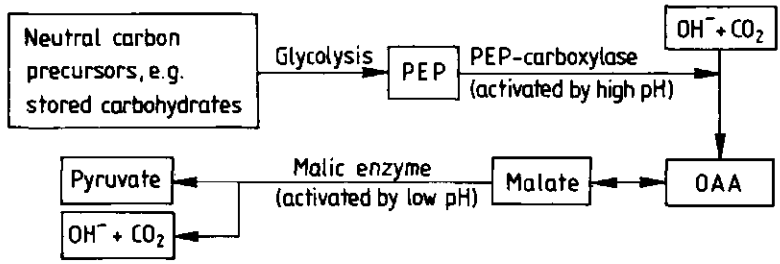


Fig. 1. The biochemical pH-stat (Davies, 1973a,b).

activation of malic enzyme. This process results in the formation of pyruvate, CO_2, and OH^--ions. The action of this biochemical pH-stat or, in other words, the opposite pH responses of enzymes (Hill and Brown, 1978) results in a cytoplasmic pH regulation at about neutral (Bonugli and Davies, 1977).

1.6. Long-distance solute transport in relation to intracellular pH regulation

In the previous sections of this Chapter attention has been paid to the uptake and assimilation processes which determine the ultimate carboxylate content in plants (section 1.3) and the effects of differential uptake of nutrient cations and anions on H^+- or OH^--extrusion from the roots into the growth medium (section 1.4). Uptake and assimilation processes have been mainly considered in relation to internal (section 1.3) or external (section 1.4) electroneutrality regulation, although it is obvious that regulation of external electroneutrality through release of H^+- or OH^--ions by the roots coincides with acidification or alkalinization of the root environment. Regulation of external electroneutrality and external pH are thus indissoluble processes. As was pointed out in the last part of section 1.5, regulation of electroneutrality and pH on an intracellular level are also inseparable processes: within the plant the biochemical pH-stat regulates the pH through decar-

boxylation or carboxylation reactions in order to neutralize initially formed H^+- or OH^--ions. The question arises in which plant parts the biochemical pH-stat is operative or, in other words, in which plant parts processes occur which yield H^+- or OH^--ions as primary reaction products. In an excellent review paper, Raven and Smith (1976b) worked out all possible ways of intracellular pH regulation in dependence of the source of nitrogen nutrition (alkaline *versus* acidic nutrient uptake pattern) and of the site of nitrogen assimilation (in the root, the shoot, or distributed over the whole plant).

Ammonium and urea nutrition, dinitrogen fixation

From the evidence available (section 1.3), Raven and Smith (1976b) concluded that most of the ammonium applied is assimilated in the root tissue. This implies that a relatively simple biophysical H^+-efflux mechanism in the root cortical cells should be sufficient for both internal and external electroneutrality and pH regulation (Fig. 2). When acidic amino acids and their amides are the main nitrogenous compounds to be delivered to the shoot, conversion of these compounds into cell material may involve the production of OH^--ions, which can be readily neutralized by the operation of the

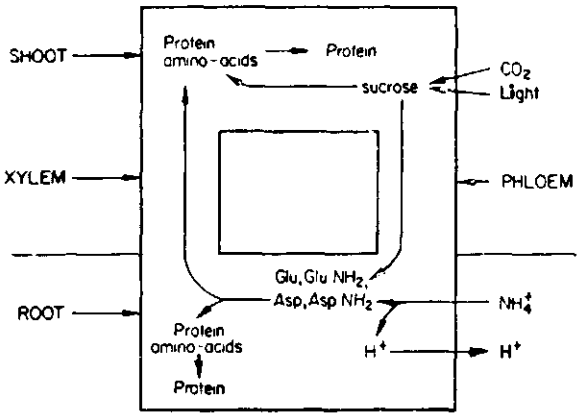


Fig. 2. pH regulation during ammonium assimilation in roots (Raven and Smith, 1976b)

biochemical pH-stat. On the contrary, the presence of relatively more basic amino acids and even some free NH_4^+ in the xylem sap would lead to an excess H^+-production in the shoot during the synthesis of cell material from these solutes. A hypothetical scheme, which allows the neutralization of this excess H^+ in the shoot is shown in Fig. 3. Here the portion of the pH-stat which generates malic acid, operates in the root, the H^+-ions formed after dissociation of this compound are exchanged largely for NH_4^+-ions from

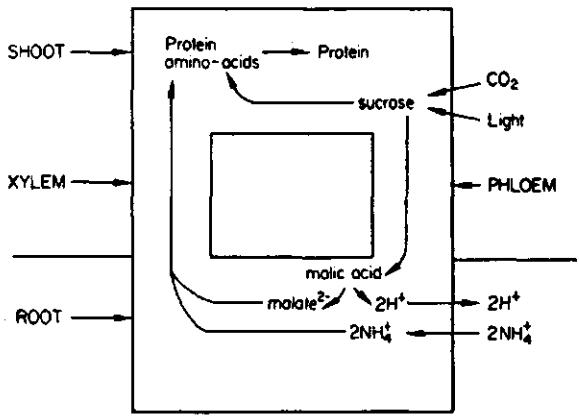


Fig. 3. pH regulation during ammonium assimilation in shoots (Raven and Smith, 1976b)

the outer solution, while the resulting NH_4^+ plus basic amino acids move, together with malate, *via* the xylem to the shoot. In the shoot, malate is converted to pyruvic acid with release of OH^- (Fig. 1) and thus neutralization of excess H^+-ions can occur. The scheme shown in Fig. 3 represents essentially a spatial separation of the two halves of the biochemical pH-stat. Unequivocal evidence in the literature on the xylem sap composition of different plant species, grown on ammonium as the only nitrogen source is so far not adequate to discard the mechanism represented in Fig. 3. However, from the evidence available (section 1.3) it can be concluded that essentially probably all of the H^+ generated during NH_4^+-assimilation is extruded into the growth medium and that most of the ammonium is assimilated in the roots.

It is to be expected that in plants utilizing molecular nitrogen sources, such as urea or atmospheric nitrogen, similar pH-regulating processes will be operative as in ammonium-fed plants since nutrition with one of these molecular nitrogen sources coincide with an alkaline nutrient uptake pattern (section 1.4) and both nitrogen sources are most likely to be assimilated in the root tissue. In order to provide a contribution to a better understanding of pH-regulation in relation to the form of nitrogen nutrition, long-distance transport of nitrogenous solutes in urea- and ammonium-supplied maize plants were investigated. The results are discussed in Chapter 2. Additionally, in a study about the xylary charge distribution and long-distance nitrogen transport in pea plants (Chapter 7), also effectively nodulated and dinitrogen-fixing plants were taken into account.

Nitrate nutrition

It is widely accepted that nitrate assimilation can occur in either the root or the shoot, or in both organs of the plant. The contribution of the roots and the shoots to the total nitrogen assimilation varies extremely with the plant species under consideration (Pate, 1973). Unequivocal evidence for the distribution of the nitrate-reducing capacity over the plant organs is so far very scarcely available in the literature, mainly because of lack of accurate, simple, and confident methods to establish such a distribution. Analyses of bleeding saps for nitrate and total nitrogen provide only a rough indication, and mostly an over-estimate (Breteler and Hänisch ten Cate, 1980; Rufty et al., 1982), of the fraction of nitrate absorbed, which is reduced in the root tissue. Data about the distribution of nitrate reduction over the plant organs, based on *in vivo* or *in vitro* nitrate reductase determinations in different plant species, should also be interpreted with caution since the obtained results depend to a great extent on the age of the different plant parts, thus making extremely high demands upon sampling techniques. Moreover, the nitrate reductase assays provide only a snapshot of the enzyme activity distribution over the plant organs, which may shift depending on plant age. Nevertheless, knowledge about the site of nitrate assimilation within the plant is

essential in order to ascertain the localization of the intracellular pH regulation in the plant.

Nitrate reduction in the roots involves both the biochemical pH-stat and OH^--ions released from the roots into the growth medium. A portion of the excess OH^--ions generated in the roots is thought to be neutralized by the biochemical pH-stat while the rest is extruded in order to neutralize electrically the excess nutrient anion over cation uptake. Slight net OH^-- or H^+-production in the shoot, associated with respective conversions of acid amino acids and their amides or an excess of basic amino acids can be neutralized by the biochemical pH-stat, similar with that discussed with respect to ammonium assimilation.

The picture of pH regulation is more complicated in plant species where the shoots represent the major site of the nitrate reduction. It is obvious that in such plants initial OH^--generation and OH^--extrusion are spatially separated processes. As was already mentioned in section 1.4, nitrate-fed plants show a very wide range with respect to the extent of alkalinity extrusion by the roots. In quantitative terms, it appears that OH^--extrusion from the roots is of less importance when nitrate assimilation occurs predominantly in the shoots (Smiley, 1974). This suggestion has been confirmed by measurements of the ratio of carboxylates to organic nitrogen in the plant, and the ratio of OH^--extrusion to nitrate assimilated (Breteler, 1973a; Coïc, 1971; Houba et al., 1971; Israel and Jackson, 1982; Keltjens, 1982; Kirkby, 1969; Kirkby et al., 1981). Storage of all the organic acid anions, formed in the biochemical pH-stat, in the shoot cell vacuoles might lead to osmotic problems (Cram, 1976). Some plant species are able to eliminate excess osmotic solutes in the shoot tissue by means of precipitation in an osmotically inactive form. Another way is the translocation of carboxylates in the phloem. Following the work of Dijkshoorn (1958), Ben Zioni et al. (1971) have proposed a mechanism by which nitrate reduction in the shoot may control nitrate uptake by the roots. This process, which incorporates recirculation of K^+ within the plant, may be described as follows (Fig. 4): K^+ and NO_3^-, the ions taken up in largest amounts by the root, are translocated in the xylem to the shoot, where for every NO_3^--ion reduced an equivalent

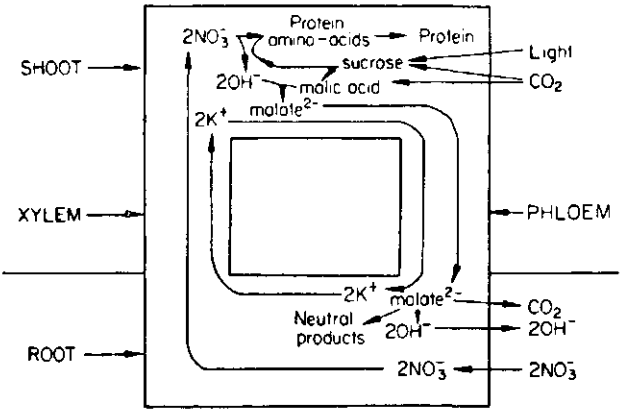


Fig. 4. pH regulation during nitrate assimilation in shoots (Raven and Smith, 1976b)

of malate is formed. Some of the K^+ originally accompanying this NO_3^- is then transferred together with malate *via* the phloem to the root system. Here the malate is decarboxylated, and the OH^- produced is stoichiometrically exchanged for further uptake of NO_3^-. The K^+ remaining in the root, together with this NO_3^-, is transported upwards and the cycle is repeated. This mechanism is currently popular and has acquired a position in many textbooks on plant nutrition. For the Ben Zioni-Dijkshoorn model to play a significant role as a nitrate uptake control mechanism two conditions must be satisfied: (a) that nitrate reduction occurs primarily in the upper plant parts; (b) that a considerable portion of the anion charge arising from the assimilation of nitrate (and sulphate) is directed toward OH^--efflux, *i.e.* that nutrient anion uptake is much in excess of nutrient cation uptake. From their data, Kirkby and Armstrong (1980) concluded that in *Ricinus communis* both these criteria are met. However, a recent detailed investigation of the ionic uptake balance in this plant species, together with a thorough examination of the ionic constituents in the xylem and phloem fluid, proved that the above conclusion was somewhat premature and that in *Ricinus communis* the recirculation scheme (Fig. 4) is only of minor importance (van Beusichem et al., 1985). It appears that mostly sufficient organic nitrogen can be transported up the xylem

to account for the deficiency of organic acid anions relative to organic nitrogen in the shoot without the need to invoke the Ben Zioni-Dijkshoorn scheme. This is consistent with results obtained by Keltjens (1982), which clearly indicate that in a diversity of plant species nitrate reduction in the root is sufficient to account for the OH^--extrusion, without the necessity for OH^--generation from carboxylates, originating from the phloem. Results of split-root experiments with maize in which ^{86}Rb was used as a physiological substitute for ^{42}K (Keltjens, 1981) and of experiments with sorghum, in which K-contents of shoots were considered in relation to the dry tissue-pH (Findenegg et al., 1982) confirmed the absence of the necessity of this xylem-phloem cation recirculation scheme. In Chapter 7 the necessity for the operation of the recirculation scheme is tested for nitrate-supplied pea plants. Final judgement on the applicability of the Ben Zioni-Dijkshoorn model must still await further experimentation to clear up the precise location of nitrate (and sulphate) reduction in plants with varying carboxylate to organic anion ratios in their shoots. Moreover, the recirculation of organic nitrogen within the plant has to be investigated in detail because it confuses xylary partitioning of nitrogenous compounds as a measure for the fraction of absorbed nitrate that is reduced in the root. Recent reports (Simpson et al., 1982; Lambers et al., 1982) indicate that in wheat organic nitrogen recirculation might be substantial.

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2 Urea nutrition of young maize and sugar-beet plants with emphasis on ionic balance and vascular transport of nitrogenous compounds

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Urea nutrition of young maize and sugar-beet plants with emphasis on ionic balance and vascular transport of nitrogenous compounds

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Summary

In a water culture experiment ammonium and urea as nitrogen sources for maize and sugar-beet plants were compared. The form of nitrogen nutrition did not significantly affect the production of dry matter, but both plant species absorbed considerably more nitrogen when they were supplied with ammonium.

In all cases experimental data of cumulative net proton extrusion by the roots showed a close agreement with calculated values for excess absorption of supplied nutritive cations, thus providing evidence for the ability of maize and sugar-beet plants to absorb urea as an undestructed molecule, at a rate sufficient for growth.

The xylem exudates of both ammonium- and urea-supplied maize plants were found to be almost free from these nitrogen sources, allowing the conclusion that urea and ammonium are almost quantitatively metabolized in the roots.

Differences in the fractionation of organic nitrogen compounds in the xylem exudates of ammonium- and urea-supplied maize plants allowed the assumption that urea is assimilated via a metabolic pathway other than enzymatic breakdown followed by incorporation of ammonium.

Introduction

The form of nitrogen nutrition has an important impact on the nutrient-element balance in plants. Many investigations have been carried out to study the effects

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of nitrate or ammonium nutrition on the uptake of different cations and anions, the ionic balance, organic acid and carbohydrate metabolism of different hydroponically grown plant species (Breteler, 1973; Chouteau, 1963; Coïc et al., 1962; Clark, 1936; Dijkshoorn et al., 1968; Houba et al., 1971; Kirkby, 1968, 1969; Kirkby & Hughes, 1970). The number of publications concerning comparative studies of nitrogen sources, including urea, is very limited (van Beusichem & van Loon, 1978; DeKock, 1970; Kirkby & Mengel, 1967, 1970; Wallace & Ashcroft, 1956). One of the experimental problems encountered in urea nutrition research is the chemical oxidation or enzymatic breakdown of this compound to ammonium carbonate or ammonia (Court et al., 1964; Gausman & Batteese, 1966), followed by a preferential absorption of ammonium by the plant roots (van Beusichem & van Loon, 1978; Ostromeçka, 1961). When urea is compared with other nitrogen sources with respect to its effect on the nutrient-element balance in plants, decomposition of this compound must be avoided or at least be quantified.

From studies in soils it is known that urease inhibitors cannot prevent ureolysis completely (Kiss et al., 1975). Also in nutrient solutions urea hydrolysis can occur (Müller, 1961) and this confuses the effect of urea absorption and metabolization on plant characteristics. Negative checks on the ammonium content in nutrient solutions supplied with urea are not sufficient to conclude that all nitrogen was taken up as urea (Kirkby & Mengel, 1967), because of the preferential uptake of ammonium from urea-ammonium mixtures which results in a very low, if detectable, ammonium concentration in the nutrient solution. The flushing technique, described by Wallace & Ashcroft (1956), is not a guarantee for urea uptake either. The slight increase of the pH of the urea-containing nutrient solutions, observed by these authors, was probably a reflection of the urea hydrolysis process.

In short-term experiments urea uptake characteristics can be investigated with ^{14}C-urea (Mitsui & Kurihara, 1962) or twofold ^{15}N-labelled urea (Hentschel, 1976). Determination of both urea and radio activity present in the xylem exudate of urea-supplied bean plants allowed the conclusion that urea was taken up as an undestructed molecule and was not metabolized in the roots (Hentschel, 1976). Similar conclusions were drawn by Mitsui & Kurihara (1962) after comparison of uptake of ^{14}C of urea and ammonium carbonate and its incorporation in ethanol soluble constituents of wheat roots and rice plants.

The only way to avoid urea decomposition in long-term water culture experiments seems to be the application of an intensive renewing scheme. Van Beusichem & van Loon (1978) described an experiment in which ammonium, nitrate, and urea nutrition of 26-day old maize plants were compared under greenhouse conditions, without renewing of the nutrient solutions. From data about the dry matter production, the ionic balance, the nitrogen content and the amounts of acidity or alkalinity which were necessary to keep the pH constant, both in urea containing nutrient solutions with and without plants, they concluded that under such experimental conditions in the urea treatment all nitrogen was taken up as ammonium by these plants. In the experiment described in the present pu-

blication the influence of intensive renewing of a urea- or ammonium-containing nutrient solution on the dry matter production, nitrogen content and ionic balance of young maize and sugar-beet plants, in relation to acidification of the nutrient solution, was studied. Furthermore, the effect of these nitrogen sources on the longitudinal translocation of nitrogenous compounds in maize plants was investigated.

Materials and methods

Plant cultivation

Experiment 1. Seeds of *Beta vulgaris* L. cv. Polyrave were germinated in quartz sand moistened with demineralized water. After three weeks the seedlings were transferred to Mitscherlich pots (inner diameter 20 cm, height 22 cm) all containing 7 litres of a well-aerated 0.5 mmol/l calcium sulphate solution. The pots were covered with perforated lids in which the seedlings were held in place by means of foam plastic (4 seedlings/pot).

Seeds of *Zea mays* L. cv. Prior were germinated in sieves filled with wetted gravel. Each sieve was placed on a Mitscherlich pot containing a well-aerated 0.5 mmol/l calcium sulphate solution.

All pots (16 with sugar-beet and 12 with maize seedlings) were placed in a growth cabinet where the experimental conditions were: temperature 20 °C, photoperiod 14 h day^{-1}, light intensity 40.3 W m^{-2}, and relative air humidity 70-75 %. One week after germination the number of maize seedlings was reduced to 15 per pot. At the same time the calcium sulphate solution in all pots was replaced by a complete nutrient solution which contained either ammonium or urea as the sole source of nitrogen. The acidity of both nutrient solutions was adjusted at pH 5.5. The composition of the nutrient solutions is given in Table 1. In all pots the nutrient solutions were renewed daily. The amounts of acidity produced by the plants were determined daily by back titration of the used solutions to the initial pH value with 0.1000 or 0.2000 mol/l NaOH by means of an automatic titration equipment (Radiometer PHM 64 pH meter/TTT 60 titrator), operating an automatic burette (Radiometer ABU 13). The plants were grown under constant climatic conditions for a period of 21 (maize) or 28 (sugar-beet) days.

Experiment 2. For the exudation experiment seedlings of *Zea mays* L. cv. Prior, which were germinated for two weeks in quartz sand moistened with demineralized water, were transferred to two 65-litre boxes (surface 5000 cm^2, height 13 cm). Each box contained 60 litres of a 0.5-strength Hoagland solution (Table 1) which was circulated and consequently aerated by an electric pump with a capacity of about 15 litres per minute. Each box contained 12 selected maize plants. The boxes were placed in a growth chamber maintained at 22 °C. The light intensity during the 14-h photoperiod was 37.5 W m^{-2} while the relative air humidity varied between 70 and 75 %. The pH of the nutrient solutions was

Table 1. Chemical composition (meq l^{-1}) of the nutrient solutions used in the experiments.

	Urea[1]	K	Na	Ca	Mg	NH_4	H_2PO_4	Cl	NO_3	SO_4
Experiment 1										
Ammonium solution	–	1.0	0.5	0.5	0.5	2.0	1.0	0.5	–	3.0
Urea solution	2.0	1.0	0.5	0.5	0.5	–	1.0	0.5	–	1.0
Experiment 2										
0.5 Hoagland soln	–	3.0	–	5.0	2.0	–	0.5	–	7.5	2.0
Zero-N solution	–	3.0	–	5.0	2.0	–	0.5	5.0	–	4.5
Ammonium solution	–	3.0	–	5.0	2.0	4.0	0.5	5.0	–	8.5
Urea solution	4.0	3.0	–	5.0	2.0	–	0.5	5.0	–	4.5

Trace elements in all solutions (mg l^{-1}): Fe 4.6; B 0.5; Mn 0.5; Zn 0.05; Cu 0.02; Mo 0.01.
[1] As NH_4 equivalent.

measured daily and kept between 5.5 and 6.0 by adding appropriate amounts of 0.1 mol/l HCl. The nutrient solutions were renewed weekly. After 35 days of growth the solutions were replaced by a 0.5-strength Hoagland solution without nitrogen (Table 1). After a further 5 days of growth on this zero-N medium the boxes were filled with complete nutrient solutions which contained either urea or ammonium as the sole source of nitrogen (Table 1). At the same time all plants were decapitated about 5 cm above the root system. About 10 cm of PVC tubing was attached to the cut stump to allow the exudate to collect. During 32 hours xylem sap was removed at 30-minute intervals, using a syringe, and stored in plastic vials at −20 °C immediately after sampling.

Analytical methods

Experiment 1. At harvest time all plants were separated into shoots and roots prior to chemical analysis. The roots were washed for 1 minute in 0.01 mol/l HCl and then rinsed twice with demineralized water. The weighed fresh plant material was partly dried at 70 °C for a period of 24 hours. Subsequently, the dry weights were determined and the samples were ground for analyses. Subsamples were analysed for total nitrogen, potassium, sodium, calcium, magnesium, phosphate, chloride, nitrate, and sulphate. These analyses were performed as described previously (van Beusichem, 1981). Free ammonium was determined by steam distillation in a 1:1 (v/v) mixture of 0.05 mol/l $Na_2B_4O_7$ and 0.1 mol/l NaOH (pH 11) in a Parnas-Wagner apparatus after extraction of fresh plant material with cold 70 % (v/v) ethanol in a cooled Bühler homogenizer. The distillate was collected in 1 % H_3BO_3 (w/v) followed by automatic titration with 0.0100 mol/l $KH(IO_3)_2$.

All results represent the mean values of three (maize) or four (sugar-beet) replicates.

Experiment 2. Xylem exudates collected from 4 plants over a 8-h period were taken together and treated as one sample. At the end of the experiment (32 h af-

ter decapitation) the exudate samples were weighed and the individual root systems were dried at 70 °C to constant weight.

The xylem exudates were analysed for total nitrogen, glutamine, asparagine, ammonium, urea, pH, and ash alkalinity. For the total nitrogen determination the sap was destructed at 360-380 °C in a 30:1 (v/w) H_2SO_4-salicylic acid mixture and 0.2 g Se-mixture (Merck 8030) after nitration at room temperature for at least 2 h (Eastin, 1978). The amide groups of glutamine were hydrolyzed at 100 °C for 3 h in a phosphate buffer at pH 6.5 (6.800 g KH_2PO_4 + 0,556 g NaOH per litre), while treatment of the exudate with 0.625 mol/l H_2SO_4 at 100 °C for 3 h resulted in hydrolysis of the amide groups of glutamine + asparagine. Ammonium in the exudates was determined as described above. In the hydrolysates and the destruates ammonium was determined by steam distillation in 0.1 mol/l NaOH (glutamine) or 12,5 mol/l NaOH (glutamine + asparagine, and total nitrogen).

Urea in the exudates was determined colorimetrically, using acidified diacetyl monoxime and thiosemicarbazide as reagents and phenylmercuric acetate as a urease inhibitor (Kyllingsbaek, 1975).

The excess cation content in the exudates (ash alkalinity) was determined by treatment of 1 ml of exudate with 5.0 ml 0.100 mol/l NaOH at 550 °C for 3 h. After cooling to room temperature 10.0 ml 0.1000 mol/l HCl was added and the excess acid was titrated at 60-80 °C to pH 5.0 with 0.1000 mol/l NaOH, with methyl red-bromocresol green as an indicator.

All results represent the mean values of three replicates.

Results

Experiment 1

Production of dry matter

In Table 2 dry matter yields of maize and sugar-beet plants, grown with ammonium or urea as the sole source of nitrogen nutrition, are compared. The small (not significant) positive effect of ammonium nutrition on the yield of stems + leaves in combination with a reverse effect on the production of roots resulted in a higher shoot-root ratio for ammonium-supplied plants as compared with

Table 2. Dry matter yields (g/100 plants) of shoots and roots and shoot-root ratios of maize and sugar-beet plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source.

	Maize		Sugar-beet	
	NH_4	urea	NH_4	urea
Shoots	107.34	92.90	15.49	14.97
Roots	38.17	46.80	5.14	5.85
Whole plants	145.51	139.70	20.63	20.82
Shoot:root	2.81	1.99	3.01	2.56

Table 3. Nitrogen content (mmol/kg DM) of shoots, roots and whole maize and sugar-beet plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source. Uptake data are expressed as mmol/100 plants.

	Maize		Sugar-beet	
	NH_4	urea	NH_4	urea
Shoots	3317	2209	3308	2292
Roots	2635	1406	3436	2618
Whole plants	3138	1940	3340	2384
Uptake	457	271	69	50

urea-supplied plants. This phenomenon was most clear for maize plants.

Big differences were observed in morphology of maize roots in dependence of the supplied nitrogen source. Roots of ammonium-supplied plants were stubby and brown-coloured, whilst urea-supplied plants were provided with a well-developed and white-coloured root system with many thin roots.

Nitrogen

In Table 3 the nitrogen contents in ammonium-supplied and urea-supplied maize and sugar-beet plants are given. Shoots and roots of both plant species contained considerably more nitrogen when supplied with ammonium than with urea. The lower nitrogen content in the shoots of urea-supplied plants did not lead to visible symptoms associated with nitrogen deficiency.

Total absorption of nitrogen as urea was much lower than as ammonium, indicating that urea is not so readily taken up as ammonium.

Inorganic chemical composition

Table 4 shows the effects of the nitrogen acquisition on the contents of the main inorganic nutritive elements in shoots and roots of maize and sugar-beet plants.

Maize. Substitution of urea for ammonium sulphate in the nutrient solution resulted in a substantial increase in the total inorganic cation content (ΣC) in the shoots. This was mainly due to a much higher potassium accumulation in urea-supplied plants, although also a positive effect of urea nutrition on the content of the divalent ions calcium and magnesium was observed. In the roots only potassium contributed to a higher total inorganic cation content in the urea treatment. Both shoots and roots of urea-supplied plants contained more of all inorganic anions (ΣA) than ammonium-supplied plants with the exception of sulphate, of which the content in the shoots of ammonium-supplied plants was about two times as high as in the urea treatment.

Sugar-beet. The higher total inorganic cation content in the shoots of urea-supplied plants in comparison with the ammonium treatment was mainly the result of a higher content of potassium, calcium, and magnesium. Surprisingly, ammonium nutrition resulted in a higher sodium accumulation in the shoots. The same picture was observed for the roots, although at a lower level. Phosphate and sulphate contributed to a higher total inorganic anion content in the shoots

Table 4. Chemical composition (meq/kg DM) of shoots and roots of maize and sugar-beet plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source. Σ C, Σ A = total cation and anion content, respectively.

	Maize				Sugar-beet			
	NH_4		urea		NH_4		urea	
	shoots	roots	shoots	roots	shoots	roots	shoots	roots
K	1052	631	1883	972	722	441	1674	685
Na	0	43	2	26	764	180	386	73
NH_4	21	16	0	3	0	0	0	0
Ca	72	34	190	42	208	28	524	46
Mg	134	74	260	71	509	149	709	251
Σ C	1279	798	2335	1114	2203	798	3293	1055
H_2PO_4	448	351	847	468	1080	377	1221	331
Cl	330	140	487	307	298	199	289	153
SO_4	104	81	59	127	45	0	106	0
Σ A	882	572	1393	902	1423	576	1616	484
Σ (C-A)	397	226	942	212	780	222	1677	571

of urea-supplied plants in comparison with the ammonium treatment. In the roots a reverse effect of the nitrogen acquisition on the total inorganic anion content was observed, due to a lower phosphate and chloride accumulation. In sugar-beet roots no sulphate could be detected.

Absorption of nutritive ions

The amounts of the different ionogenic nutrients taken up by maize and sugar-beet plants are presented in Table 5. These values are calculated from the Tables 2 and 4 using total nitrogen data (Table 3) for the calculation of ammonium absorption by the ammonium-supplied plants. Since urea was supplied in molecular form, nitrogen data were not included in the calculation of the ionic uptake balance for urea-supplied plants. Sulphate absorption was calculated as the sum of sulphate and organic sulphur, the latter being estimated as 5.4 % of the organic nitrogen (total N minus NH_4-N) amount (Dijkshoorn & van Wijk, 1967).

In all cases differential uptake of cations and anions resulted in an alkaline nutrient uptake pattern (C_a-A_a), which was more pronounced for ammonium-supplied than for urea-supplied plants. In both plant species ammonium absorption was only partly compensated for by a higher potassium, calcium, and magnesium accumulation in the urea-supplied plants; in ammonium-supplied sugar-beet plants sodium uptake was even higher than in the urea treatment. Substitution of urea for ammonium sulphate in the nutrient solution resulted in a small decrease in sulphate absorption by the maize roots, but a considerable positive effect on the accumulation of phosphate and chloride was observed. The overall effect was that urea-supplied maize plants had absorbed more nutri-

Table 5. Nutrient absorption (meq/100 plants) by maize and sugar-beet plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source. C_a, A_a = total cation and anion absorption, respectively.

	Maize		Sugar-beet	
	NH_4	urea	NH_4	urea
K	137	220	13	29
Na	2	1	13	6
NH_4	457	0	69	0
Ca	9	20	3	8
Mg	17	28	9	12
C_a	622	269	107	55
H_2PO_4	61	101	19	20
Cl	41	59	6	5
SO_4	39	26	5	5
A_a	141	186	30	30
C_a-A_a	481	83	77	25

tive anions (A_a) than ammonium-supplied plants. The uptake of the different anions by sugar-beet plants was not affected by the source of nitrogen nutrition.

Net proton extrusion

Both maize and sugar-beet plants extruded considerably more acidity when grown on an ammonium-containing nutrient solution as compared with urea nutrition (Figs 1 and 2). The differences in dry matter production characteristics between both plant species are clearly reflected in the proton production curves. The calculated values for excess cation absorption until harvest corresponded well with the respective cumulative amounts of base necessary to adjust the pH of the nutrient solutions at the initial value (Table 6).

Experiment 2

Longitudinal transport of water and nitrogenous compounds

In Fig. 3 the cumulative production of bleeding sap by maize plants is given.

Table 6. Calculated and recorded alkaline nutrient uptake (mmol/100 plants) by maize and sugar-beet plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source.

	Maize	Sugar-beet
Ammonium		
C_a-A_a (calculated)	481	77
H^+ efflux (recorded)	496	86
Urea		
C_a-A_a (calculated)	83	25
H^+ efflux (recorded)	69	16

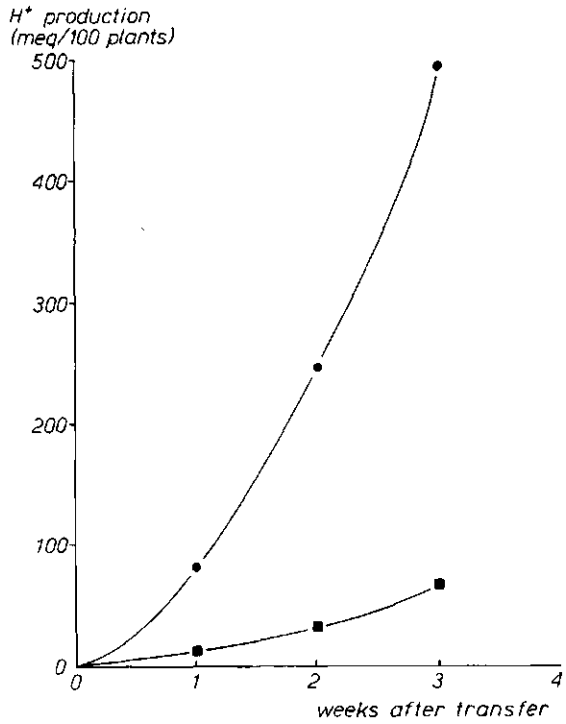

Fig. 1. Cumulative net proton production by maize plants, grown on a nutrient solution with either ammonium (●) or urea (■) as the sole nitrogen source.

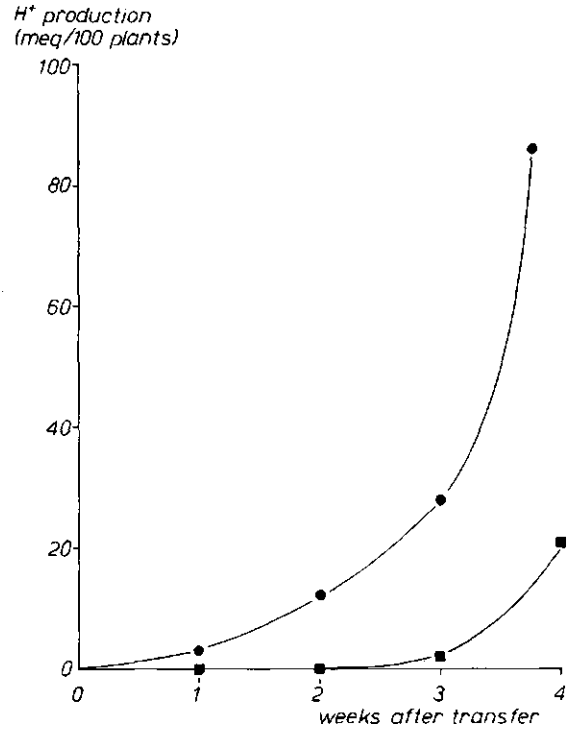

Fig. 2. Cumulative net proton production by sugar-beet plants, grown on a nutrient solution with either ammonium (●) or urea (■) as the sole nitrogen source.

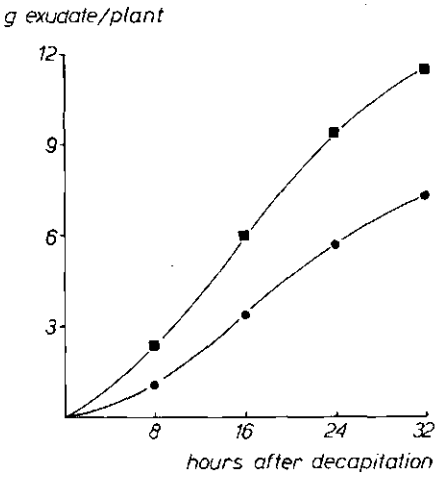

Fig. 3. Cumulative exudation by maize plants, grown on a nutrient solution with either ammonium (●) or urea (■) as the sole nitrogen source.

The exudate production curves of both ammonium- and urea-supplied plants show a lag phase during the first hours and are almost linear during the period between 8 and 24 hours after decapitation. After 24 hours the exudation rate decreased slightly. The same picture was observed for the translocation rates of the different nitrogenous compounds (Fig. 4). In the linear part urea-supplied plants exuded 1.5 times as much as ammonium-supplied plants (3.5 versus 2.3 g per plant per 8 h). As can be concluded from Table 7 longitudinal transport of total nitrogen through urea-supplied plants during the 'steady state' period was two times as high as through ammonium-supplied plants. The results show

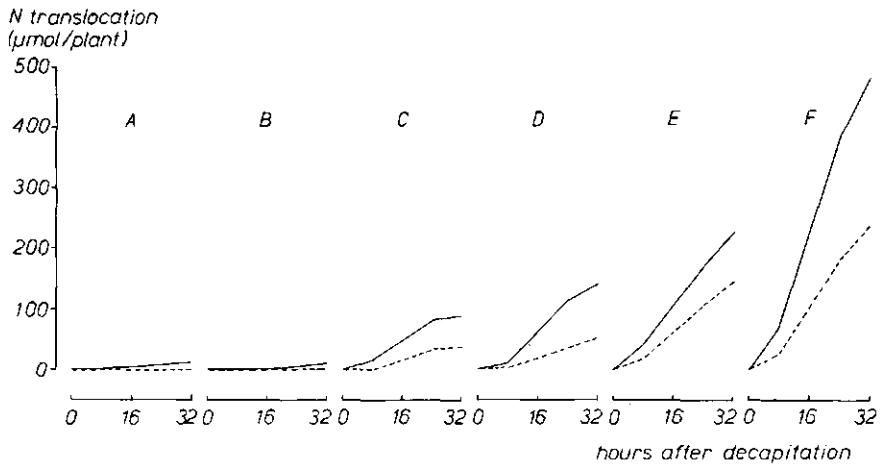

Fig. 4. Time course of longitudinal transport of nitrogenous compounds through maize plants, grown on a nutrient solution with either ammonium (---) or urea (——) as the only nitrogen source. A: urea-N; B: NH_4-N; C: glutamine-N; D: asparagine-N; E: rest-N; F: total N.

Table 7. Longitudinal transport of nitrogenous compounds through maize plants, grown on a nutrient solution with either ammonium or urea as the sole nitrogen source. Values represent the period of stationary water and solute transport (8-24 h after decapitation).

	Ammonium		Urea	
	μmol/plant	%	μmol/plant	%
Urea-N	0	0	7	2
Ammonium	2	1	3	1
Glutamine-N	35	22	67	21
Asparagine-N	35	22	104	33
Rest-N	87	55	133	43
Total-N	159	100	314	100

clearly that in the roots both ammonium- and urea-nitrogen were almost quantitatively metabolized and recovered as amides and other nitrogenous compounds, which contributed for 97-99 % of the longitudinal transport of nitrogen. Values for rest-N in Table 7 probably represent negatively charged amino acids, since in all cases these values corresponded stoichiometrically with ash alkalinity data.

Big differences in age and nutritional status of the maize plants used in the ionic balance experiment (1) and in the exudation experiment (2) do not allow comparisons of nitrogen contents in the plant shoots (Table 3) with rates of translocation of nitrogenous compounds through the xylem (Table 7).

Discussion

Experiment 1

Dry matter production

The results presented show clearly that under the described experimental conditions total dry matter production of maize and sugar-beet plants was not significantly affected by the form of nitrogen nutrition (Table 2). It should be pointed out, however, that ammonium is not the most beneficial nitrogen source for sugar-beet (Ulrich & Mostafa, 1980). This may explain the relatively slow start and low yields of these plants.

The big visual differences in maize-root morphology between the treatments were reflected in the dry weights of the roots, although not as drastically as reported for rough lemon and bush bean (Wallace & Ashcroft, 1956), tomato (Kirkby & Mengel, 1967), and white goosefoot (Kirkby, 1967). This confirms earlier findings that maize is a good grower when supplied with ammonium as the sole source of nitrogen (van Beusichem & van Loon, 1978). Results of recent work by Ikeda & Osawa (1981) show that only plants which do not show growth inhibition when supplied with ammonium are able to take up this compound preferentially from ammonium-nitrate mixtures and thereby causing a substantial acidification of the ambient medium. Tolerancy to ammonium ions seems

thus to be based on the potential of the plant root to sustain an intensive proton extrusion pump operation.

Ionic balance

Both maize and sugar-beet plants showed an alkaline nutrient uptake pattern when supplied with either ammonium or urea as the sole source of nitrogen (Figs 1 and 2). Moreover, calculated values for excess cation uptake (Table 5) corresponded well with the respective amounts of net proton production by the roots (Table 6). Since partial hydrolysis of urea and subsequent uptake of ammonium would have yielded values for recorded proton production exceeding those for calcultated excess cation uptake, the conclusion is justified that under the described experimental conditions no urea decomposition had occurred. These results provide thus evidence for the ability of maize and sugar-beet plants to absorb urea as an undestructed molecule, at a rate sufficient for growth.

Ammonium-supplied plants had absorbed considerably more nitrogen than urea-supplied plants (Table 3). This implies that when both nitrogen sources are absorbed by a common mechanism, as supposed by Hentschel (1976), the affinity of the uptake system is different for ammonium ions and urea molecules. From the results of the present ionic balance experiment evidence for the existance of a common uptake system for ammonium and urea cannot be obtained.

Experiment 2

Xylem transport of nitrogenous compounds

Examination of xylem exudates of many ammonium-supplied plant species has learned that in the root tissue the absorbed ammonium ions are readily incorporated in organic compounds, probably via the GS/GOGAT (glutamine synthetase/glutamine α-oxoglutarate amino transferase) pathway (Lea & Miflin, 1974; Miflin & Lea, 1976).

$$\text{glutamate} + NH_3 + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i$$

$$\text{glutamine} + \alpha\text{-oxoglutarate} + NAD(P)H + H^+ \xrightarrow{GOGAT} 2\,\text{glutamate} + NAD(P)^+$$

$$NH_3 + \alpha\text{-oxoglutarate} + ATP + NAD(P)H + H^+ \longrightarrow \text{glutamate} + ADP + P_i + NAD(P)^+$$

The very low ammonium concentration in the xylem sap of ammonium-supplied plants (Fig. 4, Table 7) is a clear reflection of this phenomenon.

Furthermore, the results presented in Fig. 4 and Table 7 provide evidence for an almost complete metabolization of urea in the roots. At least two pathways for assimilation of urea can occur, including hydrolytic decomposition catalysed by urease followed by incorporation of ammonium, and direct incorporation of urea via the reversal of the ornithine cycle.

arginine + fumarate	→ argininosuccinate
argininosuccinate + AMP + $2P_i$	→ aspartate + citrulline + ATP
citrulline + P_i	→ ornithine + carbamoylphosphate
ornithine + urea	→ arginine + H_2O
urea + fumarate + AMP + $3P_i$	→ aspartate + carbamoylphosphate + ATP + H_2O

When urea is assimilated via enzymatic breakdown, fractionation of nitrogenous compounds in the xylem exudates of urea- and ammonium-supplied plants should be similar, since ammonium is one of the products of urease activity. However, the contribution of amides and rest-N (amino acids) to the total transport of nitrogen through the xylem differed for both treatments (Table 7). This allows the assumption that in the root tissue urea is assimilated via the reversal of the ornithine cycle or another mechanism including the conversion of ornithine into arginine, rather than via urea hydrolysis followed by ammonium incorporation via the GS/GOGAT mechanism.

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3 Nutrient absorption by pea plants during dinitrogen fixation. 1. Comparison with nitrate nutrition

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Nutrient absorption by pea plants during dinitrogen fixation. 1. Comparison with nitrate nutrition

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Summary

In experiments with pea plants, grown hydroponically for six weeks with nitrate as the only nitrogen source, an acidic nutrient uptake pattern was observed. When effectively nodulated plants were fixing dinitrogen, however, cation absorption exceeded anion absorption resulting in an alkaline ion uptake pattern.

Maintaining the ambient temperature at 13 °C allowed the comparison of the effects of nitrogen acquisition on nutrient absorption and proton or hydroxyl/bicarbonate excretion by the roots, since plants both similar in dry matter yields and in nitrogen content were obtained.

Both for nitrate-supplied and dinitrogen-fixing plants the amount of excreted alkalinity or acidity, achieved by automatic titration, corresponded well with the respective excess absorption of nutritive anions or cations.

Some physiological and agronomic consequences of the alkaline nutrient uptake pattern and acidity generation by dinitrogen-fixing legumes are discussed.

Introduction

It is widely known that availability of nutrients for plants depends on many soil characteristics. One of the main factors affecting the solubility of nutrients such as phosphorus and micro-nutrients is the acidity of the soil. Deficiency of most nutrients can be induced by a high soil pH. Nutrient availability is generally favoured at a low soil pH, but sometimes solubility is enhanced to such an extent that some elements reach phytotoxic concentrations. Iron deficiency in soya bean crops grown on alkaline soils, generally referred to as lime-induced chlorosis, as well as aluminium toxicity in several crops grown on acidic soils, can be considered as world-wide problems in this respect.

An interesting property of a plant is its ability to change the pH of the rhizosphere during growth. Rhizosphere acidity or alkalinity generation is influenced by the operation of ion uptake processes. Vascular land plants generally take up unequal amounts of nutritive cations and anions when expressed in terms of charge equivalents. It has been found that the amount of excreted H^+ or OH^-/HCO_3^- by the roots is stoichiometrically equal to the respective excess cation or anion absorption (Breteler, 1973a; van Beusichem & van Loon, 1978; van Egmond & Aktas, 1977). Through this mechanism, electroneutrality of both plant and environment is maintained. As a consequence of rhizosphere acidity or alkalinity generation, plants are able to affect solubility of nutrients and hence their avialability and uptake. This phenomenon was clearly demonstrated by van Egmond & Aktas (1977), showing that Fe-efficient plant species and cultivars, grown hydroponically with nitrate as a nitrogen source, respond to iron stress by lowering the pH of the nutrient solution. In soils, this mechanism would enable the plant to mobilize precipitated iron compounds.

Because fertilizers can also change the pH of the soil, it is not easy to distinguish between the effects of the plant and the effect of the fertilizer on soil pH. Therefore, Pierre and co-workers gathered many data on the cation-anion balance of field-grown crops in order to separate the effects of crops and of fertilizers on soil acidity (Banwart & Pierre, 1975; Pierre & Banwart, 1973; Pierre et al., 1970).

In order to predict the extent of the H^+ or OH^-/HCO_3^- excretion process, it is necessary to have an insight in nutrient absorption characteristics of plants and in both external factors and internal processes, affecting the cation-anion uptake balance.

It is to be expected that the nutrient uptake pattern is affected by the ambient acidity. Up till now, this item has received little or no attention in literature. Data on the effects of the root medium pH on the cation-anion uptake balance and related H^+ excretion will be treated in a subsequent publication (van Beusichem, 1982). More information is available about the relations between nitrogen nutrition of plants and effects on the ambient pH. The uptake pattern of cations and anions and the resulting ionic balance in plants is influenced strongly by the nitrogen source, although differences exist between plant species. Because absorption of nitrogenous ions is substantial, ammonium nutrition results in an alkaline nutrient uptake pattern (net H^+ excretion by the roots), regardless of plant species. In contrast, nitrate nutrition generally results in an acidic nutrient uptake pattern (net OH^-/HCO_3^- excretion by the roots), but big differences exist between plant species with respect to the amounts of excreted alkalinity. Gramineae generally take up a large excess anions over cations. Some plant species, especially members of the Compositae, Polygonacea, Solanaceae, and Chenopodiaceae families, show a low acidic or even a neutral ion uptake pat-

tern when exposed to media in which nitrate is the only nitrogen source.

Many investigations have been carried out to study the effects of ammonium or nitrate nutrition on the uptake of different cations and anions, the ionic balance, organic acid and carbohydrate metabolism of different hydroponically grown plant species (Breteler, 1973a, 1973b; Chouteau, 1963; Clark, 1936; Dijkshoorn et al., 1968; Houba et al., 1971; Kirkby, 1968, 1969; Kirkby & Hughes, 1970). The number of publications concerning comparative studies of nitrogen sources, including urea, is very limited (van Beusichem & van Loon, 1978; van Beusichem & Neeteson, 1982; Kirkby & Mengel, 1967; Wallace & Ashcroft, 1956), may be partly because of experimental difficulties connected with hydrolysis of urea. When decomposition of urea in the nutrient solution is avoided, sugar-beet and maize plants show an alkaline nutrient uptake pattern, corresponding with acidification of the root medium (van Beusichem & Neeteson, 1981). Almost no information is available concerning nutrient uptake patterns of dinitrogen fixing plant species. Some authors have supposed that an alkaline uptake pattern occurs in soils, but this is based on incomplete plant analysis data (Andrew & Johnson, 1976; Israel & Jackson, 1978; Nyatsanga & Pierre, 1973). To the best of the author's knowledge, no quantitative data are available on the ionic balance of hydroponically grown dinitrogen-fixing plants. The present study deals with the effects of the nitrogen source (nitrate supply versus dinitrogen fixation) on dry matter production, nitrogen content, and ionic balance of young pea plants in relation to alkalinization or acidification of the nutrient solution.

Materials and methods

Seedling culture

Seeds of *Pisum sativum* L. cv. Rondo were surface-sterilized with 3 % hydrogen peroxide (v/v) for 30 minutes. The desinfected seeds were washed intensively with demineralized water and germinated on wetted filter paper at 20 °C in the dark.

After 10 days, selected seedlings were transferred to Mitscherlich pots (inner diameter 20 cm, height 22 cm) containing 6.5 litres of nutrient solution. The composition of the nutrient solution is given in Table 1 (pre-treatment). The pots were covered with perforated lids in which the seedlings were held in place by means of foam plastic (6 seedlings/pot). The pots were placed in a growth chamber maintained at 22 °C. The light intensity during the 14-h photoperiod was 13.5 J cm^{-2} h^{-1} while the relative air humidity varied between 70 and 75 %. The nutrient solutions were aerated continuously and the pH was adjusted daily at a value between 6.0 and 6.5.

Six days after transfer of the plants to the growth chamber, 10 ml of a dense suspension of *Rhizobium leguminosarum* were added to each of the pots. When

Table 1. Chemical composition (meq l^{-1}) of the nutrient solutions used in the experiments.

	K	Ca	Mg	H_2PO_4	Cl	NO_3	SO_4
Pre-treatment	2.5	2.5	2.5	2.5	2.5	–	2.5
Nitrate solution	2.5	4.0	2.5	2.5	–	4.0	2.5
Zero N solution	2.5	4.0	2.5	2.5	4.0	–	2.5

Trace elements in all solutions (mg l^{-1}): Fe 4.6; B 0.5; Mn 0.5; Zn 0.05; Cu 0.02; Mo 0.01.

in the subsequent experiment nitrate was used as a nitrogen source, the plants were inoculated with the strain P8, resulting in an ineffective symbiosis. The strain PF2 was used when the plants were committed to dinitrogen fixation; this strain accomplished an effective symbiosis.

Root nodules became visible in all pots about 4 days after inoculation. From this time onward the level of the nutrient solution was lowered daily by removing about 750 ml per pot. At the end of the pre-treatment period, 14 days after transfer to the growth chamber, about 10 cm of the nodulated root systems were above the solution level.

Plant growth

After the pre-treatment period, 40 plants were selected for each treatment and transferred to a 72-litre PVC box (surface 3000 cm^2, height 24 cm) containing 30 litres of a nutrient solution. The solution contained either nitrate as a nitrogen source or no combined nitrogen (Table 1). In the latter case the plants were solely dependent on symbiotic dinitrogen fixation as a nitrogen source. The solutions were circulated and consequently aerated by an electric pump with a capacity of about 15 litres per minute. The acidity of each nutrient solution was adjusted to pH 5.50 and kept constant by a pH-meter with pH-stat equipment (Radiometer T.T.T.2), operating an automatic burette (Radiometer A.B.U.12). The burette contained 0.1000 *M* NaOH (dinitrogen fixation) or 0.1000 *M* H_2SO_4 (nitrate nutrition). This technique was used successfully and described in detail by Breteler (1973a). The whole system was set up in a phytotron where the experimental conditions were: temperature 13 °C, photoperiod 16 h day^{-1}, light intensity 14.5 J cm^{-2} h^{-1}, and relative air humidity 70 %.

The plants were grown under constant climatic conditions for a period of 42 days. After 21 days the nutrient solutions were renewed and 20 plants of each treatment were harvested.

Only two treatments could be carried out at the same time. Each treatment combination was repeated two or three times. Repeats of experiments yielded data similar to those presented here.

Plant analysis

The plants were separated into shoots and roots prior to chemical analysis.

When the root system was nodulated the nodules were collected quantitatively. The roots were washed for 1 minute in 0.01 *M* HCl and then rinsed twice with demineralized water. Shoots, roots, and nodules were dried at 70 °C for a period of 24 hours. Subsequently, the dry weights were determined and the samples were ground for analyses.

Subsamples were analyzed for total nitrogen, potassium, sodium, calcium, magnesium, phosphate, chloride, nitrate, and sulphate. Total N, K, Na, Ca, Mg, and H_2PO_4 were determined after wet digestion of the samples in concentrated sulphuric acid and hydrogen peroxide (Lindner & Harley, 1942) in the presence of salicylic acid. In the diluted digests total nitrogen was measured colorimetrically by the indophenol-blue method (Novozamsky et al., 1974). K, Na, and Ca were determined by flame emission spectrometry, and Mg by atomic absorption spectrometry. H_2PO_4 was determined colorimetrically, using ammonium molybdate as a reagent, potassium antimonyl tartrate as a catalyst and ascorbic acid as a reductant. For the determination of Cl, NO_3, and SO_4 other subsamples were extracted with demineralized water (1:50, w/v). In the filtered extracts Cl was determined coulometrically with an Ag anode at constant current, NO_3 potentiometrically with a NO_3 selective electrode, and SO_4 turbidimetrically with $BaCl_2$ and Tween 80. For detailed description of the analyses see van Schouwenburg & Walinga (1979).

Results

Production of dry matter

In Fig. 1 the results are given of dry matter yields of shoots and roots of nitrate-supplied and dinitrogen-fixing pea plants. Both shoot and root production of the two treatments differed by less than 20 %, provided that the temperature in the phytotron was maintained at 13 °C throughout. Plants grown for six weeks produced four (nitrate nutrition) to five (dinitrogen fixation) times as much dry matter as plants grown for a three-week period. This may indicate that over the experimental period plants of both treatments were in their exponential phase of growth.

Inorganic chemical composition

Table 2 shows the results of the chemical plant analyses. The influence of the nitrogen acquisition is clearly reflected in the total inorganic cation content (C) in the shoots which was highest in nitrate-supplied plants at both harvests. In comparison with dinitrogen-fixing plants these plants had accumulated much more potassium. In the three-week-old plants also calcium contributed substantially to the higher cation content in nitrate-supplied plants. The cation content in the roots was about the same for both nitrogen sources. Substitution of chloride for

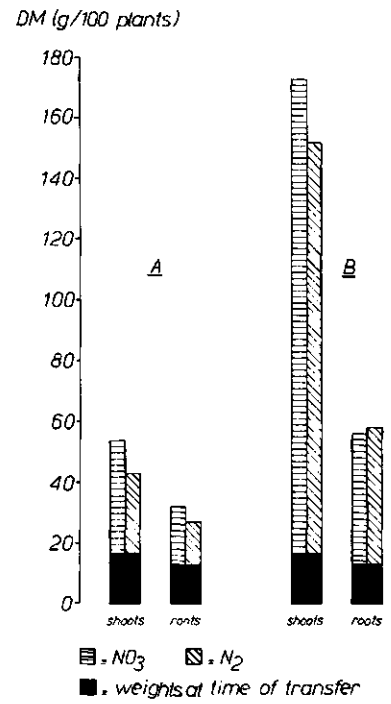

Fig. 1. Dry matter yields of pea plants, 21 (A) and 42 (B) days after transfer to a nutrient solution with (NO_3) or without (N_2) combined nitrogen.

Table 2. Chemical composition (meq/kg DM) of shoots and roots of pea plants after 21 and 42 days growth on a nitrate-containing solution (NO_3) or on a nutrient solution without combined nitrogen (N_2).

	At transfer		21 days after transfer				42 days after transfer			
			NO_3		N_2		NO_3		N_2	
	shoots	roots	shoots	roots	shoots	roots	shoots	roots	shoots	roots
K	1049	1601	1245	1103	1000	1128	1282	905	1033	1016
Na	35	17	28	47	56	71	21	35	56	76
Ca	470	456	1054	488	784	396	766	490	693	431
Mg	385	259	466	298	411	192	374	166	307	172
ΣC	1939	2333	2793	1936	2251	1787	2443	1596	2089	1695
H_2PO_4	283	780	314	728	371	742	270	556	320	731
Cl	257	240	153	64	480	243	101	52	423	231
NO_3	0	0	232	192	0	0	153	145	0	0
SO_4	220	428	124	300	112	257	86	224	108	249
ΣA	760	1448	823	1284	963	1242	610	977	851	1211
$\Sigma (C - A)$	1179	885	1970	652	1288	545	1833	619	1238	484

nitrate in the nutrient solution and nitrate reduction in the roots and shoots of the plants resulted in a somewhat lower total inorganic anion content (A) in nitrate-supplied plants than in dinitrogen-fixing plants. The anionic composition of the roots of dinitrogen-fixing plants did not change with age.

The difference between total cation and total anion content (C-A) is thought to be related about stoichiometrically to the amount of carboxylates (Houba et al., 1971). At both harvests the (C-A) value in the shoots of nitrate-supplied plants was 1.5 times as high as that in the shoots of dinitrogen-fixing plants (Table 2). The same picture was observed for the roots, although less pronounced. In alle cases much more carboxylates accumulated in the shoots than in the roots.

Nutrient absorption

The amounts of the different nutrients taken up during periods of 21 and 42 days are given in Table 3. These values are calculated from Table 2 and Fig. 1, taking into account the amounts absorbed during the pre-treatment period and using total nitrogen data (Table 5) for the calculation of nitrate absorption. Sulphate absorption was calculated as the sum of sulphate and organic sulphur, the latter being estimated as 5.4 % of the organic nitrogen amount (Dijkshoorn & van Wijk, 1967).

Differential uptake of cations and anions resulted in an alkaline nutrient uptake pattern (C_a-A_a) by dinitrogen-fixing plants and an acidic uptake pattern (A_a-C_a) by nitrate-supplied plants (Table 3). Nitrate absorption was partly re-

Table 3. Nutrient absorption (meq/100 plants) during 21 and 42 days growth on a nitrate-containing solution (NO_3) or on a nutrient solution without combined nitrogen (N_2). C_a, A_a = total cation and anion absorption, respectively. Figures in parenthesis represent total amounts of cations accumulated in the shoots.

	21 days after transfer		42 days after transfer	
	NO_3	N_2	NO_3	N_2
K	63	35	232	178
Na	1	3	4	12
Ca	58	30	145	116
Mg	24	13	63	47
C_a	146(118)	81(65)	444(390)	353(286)
H_2PO_4	24	21	62	76
Cl	1	19	11	70
NO_3	249	0	736	0
SO_4	19	13	55	56
A_a	293	53	864	202
$C_a - A_a$		28		151
$A_a - C_a$	147		420	

placed in dinitrogen-fixing plants by chloride uptake. However, in spite of the fact that chloride was completely substituted for nitrate in the nutrient solution of dinitrogen-fixing plants, chloride uptake of these plants could account only for about 8 % of the total nitrate absorption of nitrate-supplied plants. This indicates that chloride and nitrate are taken up by different mechanisms or, in case one mechanism is operative, that the affinity of the uptake system for both ions is different. Phosphate and sulphate absorption was not much affected by the form of nitrogen nutrition. Another way for compensating the amount of negative charge of nitrate is the suppression of cation uptake by dinitrogen-fixing plants. At both harvests nitrate-supplied plants had absorbed more of all cations (C_a) than dinitrogen-fixing plants. Between 80 and 88 % of the absorbed cations had been transported to the upper plant parts. Since nitrate absorption was only compensated for about 13 % by a lower potassium, calcium, and magnesium accumulation in the dinitrogen-fixing plants, this mechanism does not seem to play a dominant role. This implies that a mechanism through which pea plants, irrespective of the nitrogen source, aim at a constant internal pH only by way of changes in the absorption of non-nitrogenous ions, is not likely. The origin of these non-specific shifts in ion uptake is still unknown.

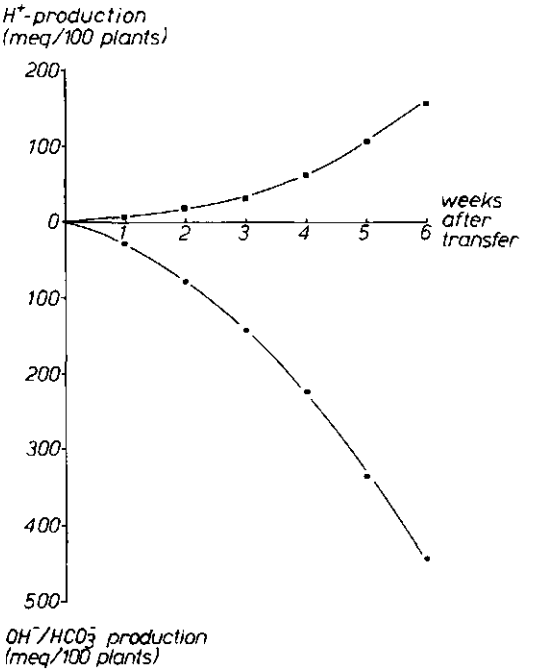

Fig. 2. Cumulative net hydroxyl/bicarbonate and proton production by pea plants grown on a nitrate-containing solution (●) or on a nutrient solution without combined nitrogen (■), respectively.

Net proton or hydroxyl/bicarbonate extrusion

Pea plants grown on a nitrate-containing nutrient solution extruded a considerable amount of alkalinity as a result of excess anion over cation absorption (Fig. 2). In contrast, when plants were fixing dinitrogen, the nutrient uptake pattern was shifted, resulting in net proton excretion by the roots.

The curves presented in Fig. 2 are redrawn form the recorder sheets of the pH-stat equipment. At both harvests the amounts of acid or base necessary to keep the pH of the solutions at 5.50 corresponded well with the respective calculated values for excess anion or cation absorption (Table 4; Breteler, 1973a).

Nitrogen

In Table 5 the nitrogen contents in both shoots and roots of nitrate-supplied and dinitrogen-fixing pea plants are compared. The nitrogen content in both organs did not differ significantly for both treatments.

Because of a somewhat higher dry matter production in the nitrate treatment (Fig. 1), the total amount of nitrate taken up by the plants over the 42 days was about 13 % higher than the total amount of dinitrogen fixed during that period (Table 6).

Table 4. Calculated and recorded acidic and alkaline nutrient uptake (meq/100 plants) by pea plants after 21 and 42 days growth on a nitrate-containing solution (NO_3) or on a nutrient solution without combined nitrogen (N_2).

	Days after transfer	
	21	42
NO_3		
A_a-C_a (calculated)	147	420
OH^- efflux (recorded)	141	444
N_2		
C_a-A_a (calculated)	28	151
H^+ efflux (recorded)	33	158

Table 5. Nitrogen content (mmol/kg DM) of shoots and roots of pea plants after 21 and 42 days growth on a nitrate-containing solution (NO_3) or on a nutrient solution without combined nitrogen (N_2). Initial nitrogen content: 1848 (shoots) and 1867 (roots) mmol/kg DM.

	21 days after transfer		42 days after transfer	
	shoots	roots	shoots	roots
NO_3	3927	2907	3668	3824
N_2	4051	3065	3593	2838

Table 6. Nitrate absorption (NO_3) and dinitrogen fixation (N_2) (mmol N/100 plants) by pea plants during 21 and 42 days.

	Days after transfer	
	21	42
NO_3	249	736
N_2	198	653

Discussion

To obtain comparable results with respect to the influence of the nitrogen source on net proton or hydroxyl/bicarbonate extrusion by the roots it is desirable that the environmental conditions are chosen in such a way that both dry matter production and relative mass increment rate are in the same order of magnitude.

Maintaining the ambient temperature at 13 °C throughout, in combination with the prevailing lighting conditions (16 h day^{-1}; 14.5 J $cm^{-2}h^{-1}$), yielded morphologically comparable plants in both treatments. Over the experimental period no colour differences were observed, indicating that the dinitrogen-fixing plants did not suffer from possible limitations in nitrogen supply. Since dry matter production of nitrate-supplied plants was less than 20 % higher than that of dinitrogen-fixing plants the conclusion is justified that for comparative purposes temperature and illumination were 'in balance'. This conclusion is supported by data obtained from experiments at higher temperature (van Beusichem, 1982).

As is shown in Fig. 3, nutrient absorption characteristics, expressed as excess cation over anion absorption, and dry matter production of the dinitrogen-fixing plants were exponential over the 6-week period. On a relative scale, the dry matter production rate was somewhat lower than the alkaline uptake rate, a commonly observed phenomenon when dry matter production and nutrient absorption are compared. The same picture was observed in the nitrate treatment.

The nitrogen source had no significant influence on the total nitrogen content in the plants (Table 5). This implies that under the earlier described experimental conditions the dinitrogen-fixing process was not rate-limiting for optimal growth. It should be pointed out, however, that nutrient absorption and dinitrogen fixation were studied on plants which were already provided with an effectively nodulated root system. Differences between the treatments, associated with nodule initiation and development, were thus eliminated.

Nutrient absorption data and results from the pH-stat titrations clearly indicate that dinitrogen-fixing plants showed an alkaline nutrient uptake pattern (Fig. 2, Table 3). It is interesting to speculate on some physiological as well as agronomic aspects connected with this phenomenon.

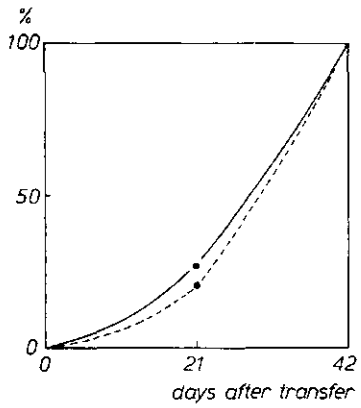

Fig. 3. Relative dry matter production (●) and net proton production (■) by dinitrogen-fixing pea plants.

In most cases, including this experiment, nitrate nutrition leads to an acidic nutrient uptake pattern by the roots, i.e. an excess anion over cation absorption. In apparent contrast, some authors reported that in nitrate-supplied plants inorganic cations in the xylem sap were almost completely balanced by inorganic anions. This phenomenon was found in a diversity of plant species, such as maize (Dijkshoorn, 1971), tomato (Wallace et al., 1971), dwarf bean (Breteler & Hänisch ten Cate, 1978), and castor oil plants (Kirkby & Armstrong, 1980). Equivalent xylem transport of inorganic cations and anions only occurs under conditions of adequate nitrate supply, so that nitrate deposition in the xylem is substantial. This implies that nitrate reductase activity must at least partly be located in the upper plant parts. Possible charge imbalances can be eliminated by a xylem-phloem recirculation of cations as proposed in the Dijkshoorn-Ben Zioni model (Dijkshoorn, 1958; Dijkshoorn et al., 1968; Ben Zioni et al., 1971; Kirkby, 1974). In our preliminary experiments, it was found that nitrate-supplied pea plants contained equal amounts of inorganic cations and inorganic anions in the bleeding sap (van Beusichem, to be published). This implies that the abovementioned conditions are satisfied, although it is known that *Pisum* reduces substantial amounts of nitrate in the roots (Pate, 1973), indicating that the extent of cation recirculation in the pea plant is probably low.

To maintain electrically neutral longitudinal ion transport, in dinitrogen-fixing plants quite other processes must be operative than in nitrate-supplied plants. The excess of cationic over anionic nutrient uptake by dinitrogen-fixing plants (Table 3) and the small differences in total cation contents in the roots between both treatments (Table 2) suggest the necessity of organic anion synthesis in the roots and deposition of these compounds in the xylem. This could have an impact on the xylem loading rate of cations as a result of complexing ca-

pabilities of carboxylates and amino acids and their restricted radial movement in the root. The smaller cation accumulation in the upper parts of dinitrogen-fixing plants in comparison with nitrate-supplied plants (Table 3) is possibly a reflection of these processes. Careful examination of the organic chemical composition of bleeding saps of dinitrogen-fixing plants is necessary to get an insight into the dynamics of these uptake and transport phenomena.

Besides these physiological aspects, nutrient uptake patterns and acidity generation by dinitrogen-fixing legumes may have some agronomic significance. As a result of the proton extrusion pump operation, acidification of the rhizosphere can occur under field conditions. Soil rhizosphere acidity is influenced by operation of uptake processes. Variation in acidity of the rhizosphere through ammonium or nitrate nutrition has been shown to influence the solubility of soil phosphorus and thereby causing substantial modification in the amounts of phosphorus absorbed (Blair et al., 1971; Riley & Barber, 1971; Smiley, 1974; Soon & Miller, 1977).

The question arises which consequences rhizosphere acidity generation by dinitrogen-fixing legumes would have for the improvement of the efficiency of naturally occurring or added (rock) phosphates. An important difference between ammonium nutrition and dinitrogen fixation is the amount of acidity excreted by the roots per equivalent of nitrogen absorbed. For ammonium nutrition this value appears to vary between 1.10 and 1.25 for different plant species, such as sugar-beet (Breteler, 1973a), maize (van Beusichem & van Loon, 1978), and tomato (Kirkby & Mengel, 1967). In this experiment the amount of acidity excreted per unit nitrogen fixed was only 0.2 on an equivalence basis. When adequate nitrogen supply is sustained in leguminous plants, great deviations from this value are not likely to occur, so that no drastic effect of dinitrogen-fixing plants on acidification of the soil rhizosphere is to be expected.

In most of the tropical legumes, rhizosphere acidity generation appears to be lower than in temperate species (Andrew & Johnson, 1976). Moreover, in tropical and subtropical regions acidic ultisols and oxisols are predominant. An important aspect in this connection is the finding that the extent of acidity generation is highly pH-dependent (van Beusichem, 1982).

From these considerations the conclusion seems to be justified that it is necessary not to be too optimistic about the agronomic significance of the alkaline nutrient uptake pattern of legumes with respect to phosphate utilization. Probably positive effects are restricted to those cases in which legumes, provided with an intensive soil-exploring root system, are grown on non-acidic soils with very low pH-buffering and phospate-fixing capacities (Aguilar S. & van Diest, 1981). Data reported by Fried (1953), who found that the abilities to take up phosphorus, supplied as rock phosphate, were favoured by leguminous plants in comparison

with gramineous plants, should be considered as the result of a combination of the above-mentioned prerequisites.

Acknowledgements

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

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

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Key-words: acidification, alkaline uptake pattern, nitrogenase activity, nutrient absorption, *Pisum sativum,* proton extrusion, rhizosphere pH, symbiotic N fixation, temperature effect, dinitrogen fixation, pea.

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 poton 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 C_2H_4 in nitrogen gas. The heights of the peaks were related to the concentrations of C_2H_2 and C_2H_4 (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

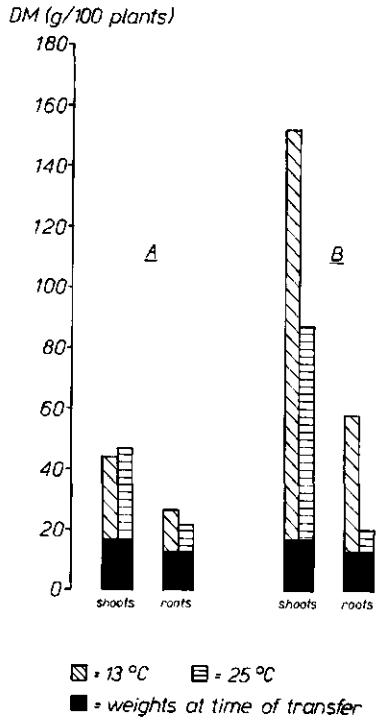

Fig. 1. Dry matter yields of pea plants, 21 (A) and 42 (B) days after transfer to a phytotron at 13 °C or 25 °C. Plants were grown at pH 5.5.

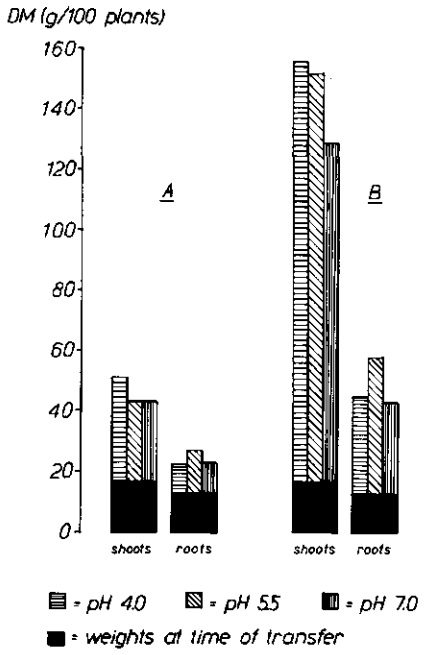

Fig. 2. Dry matter yields of dinitrogen fixing pea plants, 21 (A) and 42 (B) days after transfer to a nutrient solution at pH 4.0, 5.5 or pH 7.0. Plants were grown at 13°C.

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.

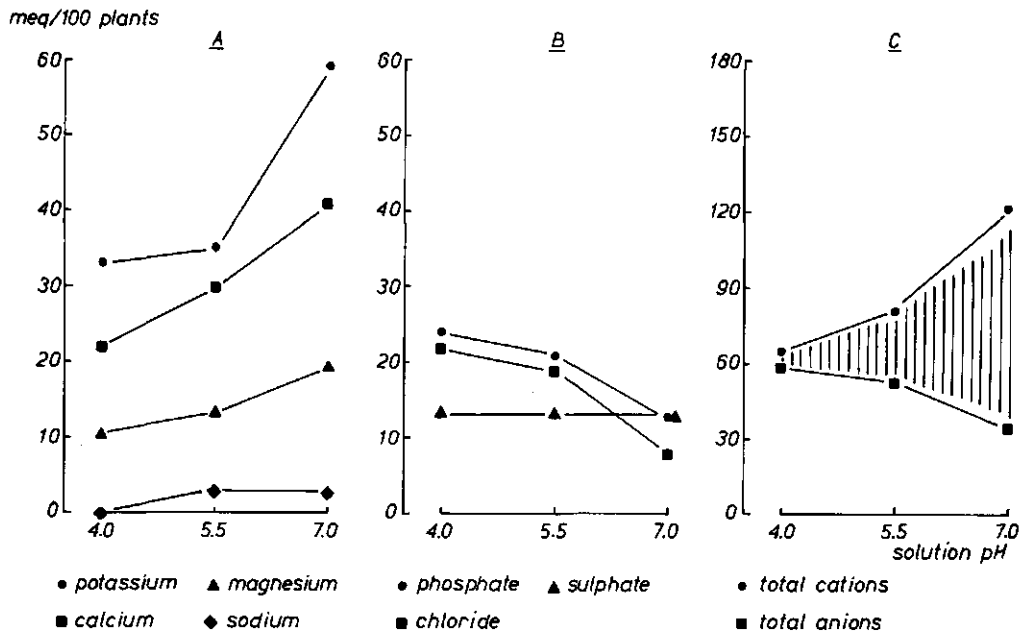

Fig. 3. Cations and anions absorbed by dinitrogen-fixing pea plants grown at pH 4.0, pH 5.5, or pH 7.0, over a three-week period. A: uptake of the different cations; B: uptake of the different anions; C: total cation and anion uptake. Temperature 13°C.

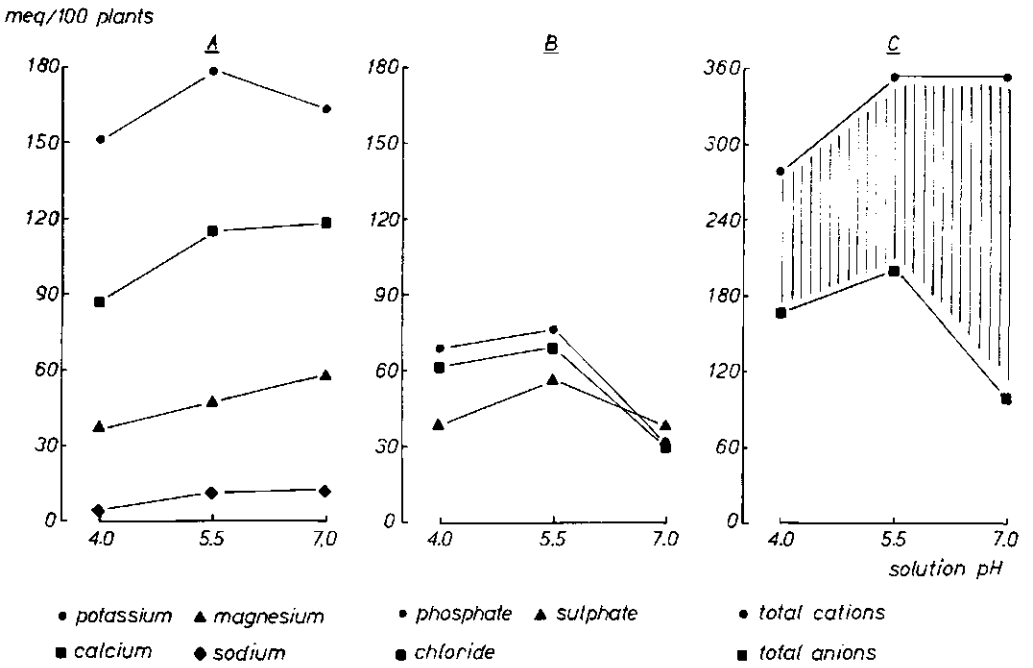

Fig. 4. Cations and anions absorbed by dinitrogen-fixing pea plants grown at pH 4.0, pH 5.5, or pH 7.0, over a six-week period. A: uptake of the different cations; B: uptake of the different anions; C: total cation and anion uptake. Temperature 13°C.

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 nutreint 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 hydroxyde 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 hydroxyde and excess cation over anion absorption. In order to obtain well comparable data, all values for the acidity generation in de-

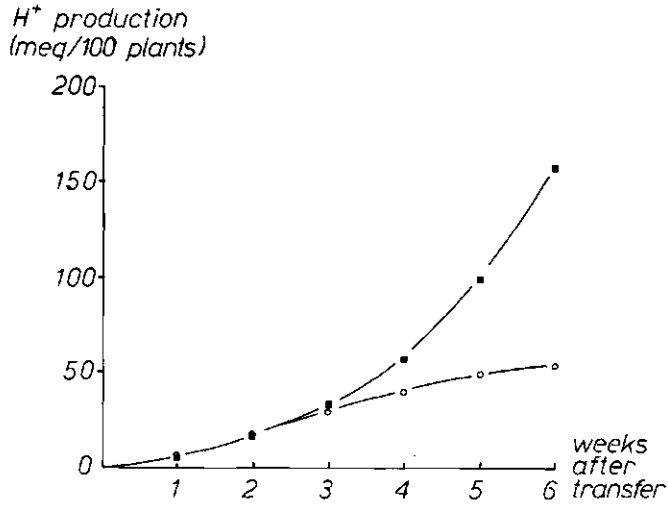

Fig. 5. Cumulative net proton production by dinitrogen-fixing pea plants, grown at 13 °C (■) or at 25 °C (o). Acidity of the nutrient solution pH 5.5.

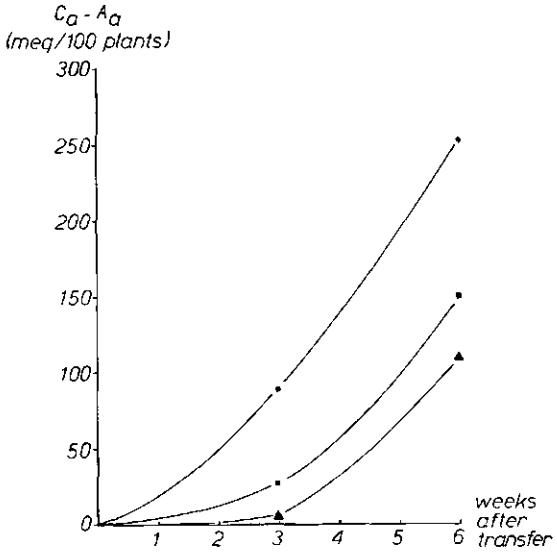

Fig. 6. Cumulative excess cation over anion uptake by dinitrogen-fixing pea plants, grown at pH 4.0 (▲), pH 5.5 (■), or pH 7.0 (●). Temperature 13°C.

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 centent (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-

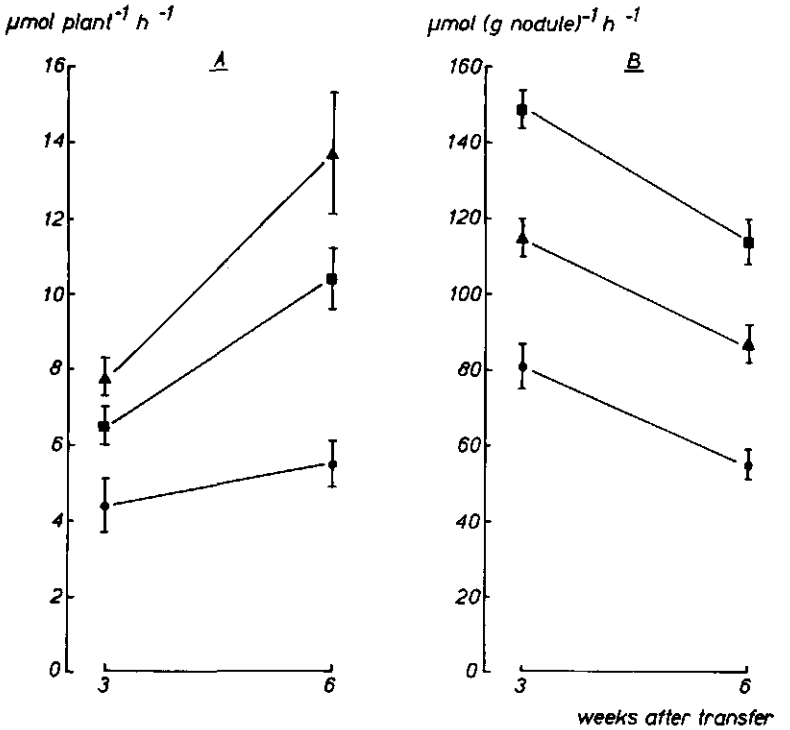

Fig. 7. Acetylene reduction by pea plants grown for three weeks or six weeks at pH 4.0 (▲), pH 5.5 (■), or pH 7.0 (●). A: values expressed on plant basis; B: values expressed on nodule weight basis. Vertical bars represent S.D.s. (n = 4).

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 μmol plant^{-1} h^{-1}), but at the end of the experiment the activity was only 0.2 μmol plant^{-1} 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 rasing 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 maintainance 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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5 Nitrogen accumulation in nodulated and non-nodulated pea plants, grown in a sandy soil at different acidities

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NITROGEN ACCUMULATION IN NODULATED AND NON-NODULATED PEA PLANTS, GROWN IN A SANDY SOIL AT DIFFERENT ACIDITIES

Key words: acid soil, cation-anion uptake pattern, nitrate supply, nodulation, pea, pH-shift, Pisum sativum L., root growth, symbiotic N-fixation.

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ABSTRACT

The growth of nitrate-supplied and dinitrogen-fixing pea plants was studied in a pot experiment with a sandy soil in a pH-H_2O range from 3.4 to 5.6. Optimum growth in both treatments occurred at pH 5.0. At low pH, N_2-plants yielded significantly less than NO_3-plants. Planting of nodulated seedlings did not enhance yield in comparison with sowing in inoculated soil, indicating that nodulation was not the most sensitive process in restricting yield. Comparison of the nitrogen contents of shoots of planted and sown N_2-plants allowed the suggestion that the synthesis of nitrogenous compounds was also not limiting yield. At low pH, root growth was severely reduced in dinitrogen-fixing plants in comparison with nitrate-supplied plants. This difference could be explained by the influence of the form of nitrogen nutrition on the cation-anion uptake pattern of the plant and the resulting pH-shift in the rhizosphere. It is to be expected

that in an acid soil under field conditions the indirect effect of nitrate on root growth and nodulation via increase of the pH is more extensive than its direct negative effect on nodulation.

INTRODUCTION

The pH-values of many soils are below those most favourable for dinitrogen fixation. The sensitivity of the legume/Rhizobium symbiotic system to low pH depends on plant species[1,13] and on Rhizobium strain[6]. Growth at low pH has found to be mainly depressed by unsuccessful nodulation and consequent nitrogen deficiency[9,13,14].

The amount and the viability of the Rhizobium present in the soil decreases with falling pH[6,19]. Moreover, the effectivity of the Rhizobium present in acid soil is detrimentally affected by high concentrations of heavy metals such as Mn and Al[7]. Application of selected effective Rhizobium strains to acid soil can enhance dry matter production of legumes[6,19].

Nodulation is very sensitive to low pH especially the second and third day after inoculation[9,14]. Early stages of the nodulation process, corresponding with root hair curling and formation and extension of the infection thread, are most sensitive to acidity. Once formation of the nodules in the cortex has started, growth is not longer inhibited at low pH[9,14,16].

The negative effect of low pH on nodulation can be counteracted to some extent by increasing the calcium concentration[11,16]. This is explained by competition of calcium and hydrogen ions at the infection sites.

At low pH, growth of a legume is substantially increased by nitrogen fertilization[1,13]. This has been explained by assuming that growth of the legume is less sensitive to low pH than are the nodulation and growth of Rhizobium[13].

In the present work these effects of low pH are considered

in an experiment using nodulated seedlings. In the pot experiment carried out the pH of the soil ranged from pH-H_2O 3.4 to 5.6. At each pH the effects of a sowing in inoculted soil, b planting nodulated seedlings, and c nitrate supply on yield and nitrogen contents in pea plants were compared.

MATERIALS AND METHODS

Soil was taken from the A-horizon (5-20 cm) of an acid sandy soil. Results of soil analysis were: pH-KCl 3.4, pH-H_2O 4.1, organic matter 6.8%, organic carbon 4.2%, total nitrogen 0.1%, ammoniumlactate extractable phosphate 0, K-HCl 3.3 mg K/100 g, and Mg-NaCl 1.3 mg Mg/100 g. Based on a titration curve five different pH-values, i.e. pH-H_2O 3.4, 4.0, 4.6, 5.0 and 5.6 were created by adding amounts of $Ca(OH)_2$. To avoid interaction between effects of pH and calcium concentration on nodulation, differences between the amounts of calcium added as $Ca(OH)_2$ were eliminated by compensating amounts of calcium as $CaSO_4$. After increasing the moisture content to 50% of the maximum water-holding capacity the soils were incubated in plastic bags in the dark at 25 °C for six weeks.

After the incubation period the following amounts of nutrients were added per pot (based on 6 kg of dry soil): 0.44 g P, 1.66 g Mg, and 84 mg Fe (as KH_2PO_4, KCl, $MgSO_4$ and $FeCl_3$ solutions) and 15 ml of Hoagland's solution of trace elements. To 1/3 of the pots 250 mg N per 6 kg was added as a $NaNO_3$-solution (code NO_3), while 2/3 of the pots received an equivalent amount of NaCl (code N_2). Mitscherlich pots were filled with an amount of soil, based on 6 kg dry weight, and a surface layer of 500 g quartz sand.

Seeds of Pisum sativum L. c.v. "Rondo" were surface-sterilized with 3% hydrogen peroxide (v/v) for 20 minutes. Desinfected seeds were rinsed in demineralized water and germinated on wetted filter paper at 20 °C in the dark. Seedlings were sprayed with a

dense suspension of Rhizobium leguminosarum, strain PF2, after 7 and 10 days. In each pot, containing soil supplied with $NaNO_3$, and in half of the number of pots containing soil supplied with NaCl, 10 11-day-old seedlings were planted (code p) and 50 ml of a sterilized suspension of the ineffective Rhizobium strain P8 was added. In the other pots, containing soil supplied with NaCl, 10 sterilized seeds were sown (code s) and 50 ml of a suspension of the effective Rhizobium strain PF2 was added (Table 1).

TABLE 1

Scheme of the three different treatments within each of the five pH-levels.

treatment code	nitrogen supply ($NaNO_3$/NaCl)	plant material	inoculum at filling of pots
pNO_3	$NaNO_3$	infected seedlings	sterilized P8
pN_2	NaCl	infected seedlings	sterilized P8
sN_2	NaCl	seeds	PF2

The moisture content was increased to 70% of the maximum water-holding capacity and re-established by weighing once and later twice a day. Each treatment consisted of three replicates. Pots were placed on tables in a climate-controlled glasshouse, where the respective relative air humidity and temperature were 65% and 13 °C. To avoid effects of place in the glasshouse, a rotation scheme was developed and placement of pots changed once and later twice a day. Two weeks after planting or sowing the number of plants was reduced to 5 per pot. Six weeks after planting and another 5 times (every two weeks) 250 mg N/pot as $NaNO_3$ was applied to the pNO_3-pots.

A harvest was taken 18 weeks after planting or sowing. Shoots were separated into seeds and straw and after drying at 70 °C for at least 24 hours, dry weight was determined. The ground material was digested with a H_2SO_4/salicylic-acid mixture[21]. Nitrogen was determined by the indophenol-blue method in the diluted digests[17]. From each pot a soil sample was taken to measure pH-H_2O in a 1:2.5 (w/v) soil-water mixture. The quantity of roots was estimated by separating roots from soil by extensive washing and subsequent drying at 70 °C. This was done in the described experiment and in a special experiment to study the effect of soil acidity and form of nitrogen nutrition (nitrate supply or dinitrogen fixation) on root growth. In this experiment pea seeds were sown in pots filled with a 6 kg of acid soil (pH 4.0), b 6 kg of limed soil (pH 6.1), c a layer of 3 kg of acid soil covered by 3 kg of limed soil (upper half limed), or d the other way round (lower half limed).

RESULTS

Yield and Nitrogen Content

Both in pNO_3-plants and N_2-plants, dry matter yields increased dramatically as the pH increased from pH-H_2O 3.4 to 5.0. In all cases maximum dry matter yields were attained at pH 5.0 (Table 2). At low pH, especially at pH 4.0, dry matter yields of pNO_3-plants were significantly higher than the N_2-plants. Planting nodulated seedlings rather than sowing did not lead to significant differences in dry matter yield (Table 2).

Straw nitrogen content tended to be higher at low pH than at high pH. Apparently, redistribution of nitrogen was most intensive at high pH. Seed nitrogen content in the N_2-plants was lower than in the NO_3-plants, although the differences were not always significant. Percentage seed of dry matter yield (harvest index) tended to be lower in pNO_3-plants in comparison with N_2-

TABLE 2

Influence of pH and nitrate supply on dry matter yield, percentage roots and seeds, nitrogen content of seed and straw and total nitrogen accumulation in shoots of planted (p) or sown (s) pea plants. For explanation of codes see Table 1. All values are given ± S.D. (n = 3).

treatment code		dry matter yield (g/pot)	percentage roots (%)	percentage seeds (%)	N-content seed (%)	N-content straw (%)	N-accumulation in shoot (mg/pot)
pNO_3	3.4	4 ± 1 a[1]	23.0 ± 3.4 c	8.0 ± 6.0 a	4.50 ± 0.30 b	5.20 ± 0.20 c	160 ± 40 a
pNO_3	4.0	78 ± 4 b	9.9 ± 1.3 b	37.7 ± 3.5 b	3.85 ± 0.15 a	1.14 ± 0.18 a	1610 ± 140 b
pNO_3	4.6	113 ± 4 c	9.4 ± 0.6 b	39.3 ± 1.7 b	3.78 ± 0.15 a	1.41 ± 0.14 b	2510 ± 80 d
pNO_3	5.0	125 ± 7 d	7.7 ± 0.1 a	38.4 ± 1.9 b	3.78 ± 0.07 a	1.38 ± 0.05 b	2660 ± 30 d
pNO_3	5.6	99 ± 12 c	9.2 ± 0.6 b	41.0 ± 1.0 b	3.85 ± 0.17 a	1.10 ± 0.10 a	2100 ± 250 c
sN_2	3.4	*0.4*[2] a	–	*0* a	–	8.04 c	*30* a
sN_2	4.0	*31* ± *5* b	*5.3* ± *0.5* a	36.2 ± 3.3 b	3.63 ± 0.12 b	*1.54* ± *0.12* b	*660* ± *70* b
sN_2	4.6	*108* ± *1* d	*6.7* ± *0.4* b	*41.9* ± *0.9* c	3.68 ± 0.20 b	1.25 ± 0.05 a	2360 ± 110 d
sN_2	5.0	112 d	9.8 c	41.1 c	*3.36* a	*1.11* a	*2160* cd
sN_2	5.6	91 ± 15 c	8.8 ± 0.7 c	40.4 ± 2.7 bc	*3.57* ± *0.09* ab	1.13 ± 0.15 a	1830 ± 290 c
pN_2	3.4	*0.4* a	–	*0* a	–	6.81 c	*30* a
pN_2	4.0	*21* ± *3* b	*5.8* ± *0.8* a	42.9 ± 3.0 b	*3.37* ± *0.05* a	1.29 ± 0.06 b	*430* ± *80* b
pN_2	4.6	*90* ± *8* c	*6.4* ± *0.7* a	42.2 ± 1.5 b	*3.60* ± *0.02* b	1.25 ± 0.05 b	*1930* ± *120* c
pN_2	5.0	119 ± 6 d	7.5 ± 0.6 b	42.1 ± 1.9 b	3.63 ± 0.11 b	1.20 ± 0.12 b	2530 ± 150 d
pN_2	5.6	108 ± 14 cd	8.3 ± 0.4 b	*43.5* ± *1.0* b	3.88 ± 0.13 c	*0.96* ± *0.02* a	2310 ± 210 d

1) different letters behind figures indicate significant difference ($P < 5\%$) between pH-treatments.
2) italic printed figures are significantly different ($P < 5\%$) from the PNO_3-treatment at the same pH.

plants (Table 2). As for the effect of pH on yield and total nitrogen accumulation, the overall picture shows that differences between the N_2-treatments were smaller than between N_2- and NO_3-treatments.

Root Growth

In pNO_3-plants the percentage roots of dry matter yield was not influenced above pH 4.0, but in N_2-plants this percentage decreased as the pH fell from 5.0 to 4.0 (Table 2). When the soil was only partly limed the growth of pea plants was not affected by pH when nitrate was supplied, whereas root growth of N_2-plants appeared to be severely inhibited in soil at pH 4.0. Root weight in limed soil was four times as high as in the acid soil, regardless of whether the limed soil was in the lower, upper or in both parts of the pot (Table 3).

Changes in Soil Acidity

By measuring the pH of the bulk soil it could be shown that dinitrogen-fixing plants decreased the pH of the soil (Fig. 1). The pH-shift appeared to be positively correlated with dry matter yield (Fig. 1). In contrast, nitrate nutrition resulted in an increase of soil pH. In the pNO_3-treatments the pH-lowering effect of additional dinitrogen fixation slightly depressed the increase in pH associated with nitrate nutrition.

In an analogous experiment pea was sown in soil inoculated with an ineffective Rhizobium strain. In this case nitrogen accumulation was restricted to 1750 mg N supplied as nitrate (code sNO_3). The pH-shift per gram dry matter produced (proportional to the slope in Fig. 1) appeared to be largest, when only nitrate-nitrogen could be assimilated.

The titration curve of the soil used in this experiment appeared to be linear between pH 3.5 and 5.5, but the buffering capacity increased at higher pH-values. The small pH-shift at

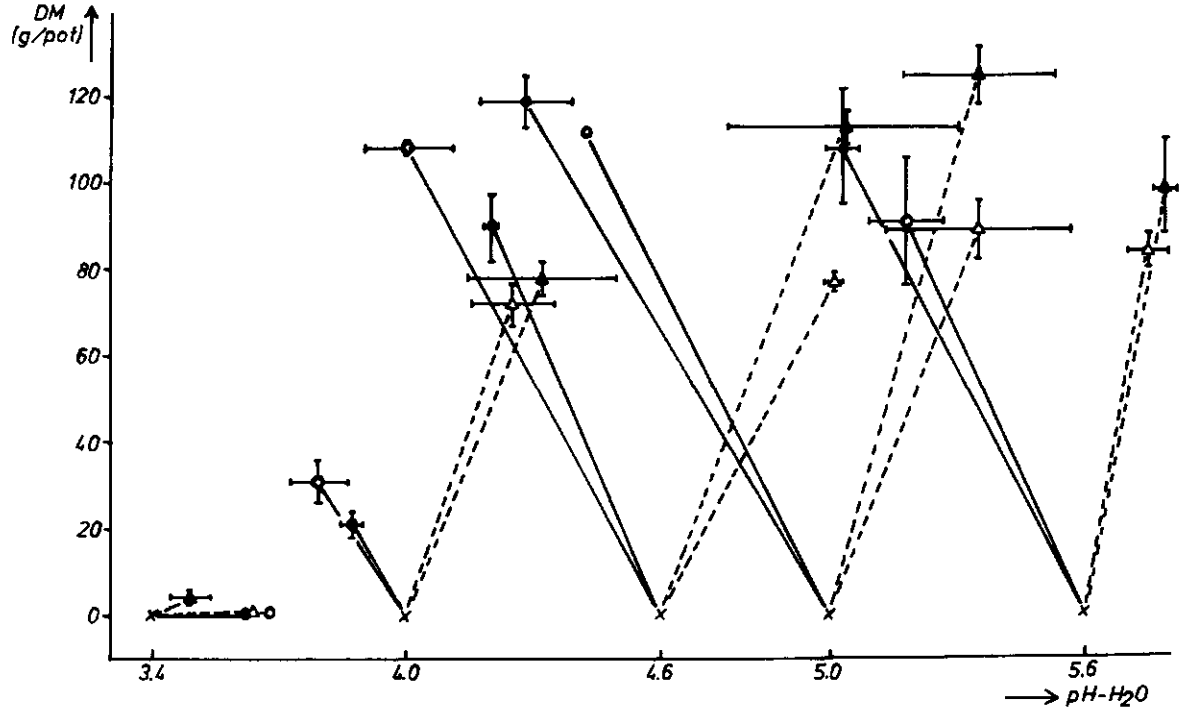

Fig. 1: Influence of pH-H_2O at the onset of the experiment (x) and nitrogen source on the dry matter production of pea (Pisum sativum L.) and on the pH-change during the growing period (horizontal shift). (--- △ = sown, nitrate-supplied; --- ▲ = planted, nitrate-supplied; —— ○ = sown, nitrogen fixation; —— ● = planted, nitrogen fixation; ⊢—⊣ = standard deviation.).

TABLE 3

Influence of liming on the absolute and relative quantity of roots of pea plants in acid (pH 4.0) or limed (pH 6.1) soil. The peas were sown and plants were either supplied with nitrate (sNO_3) or dependent on dinitrogen fixation (sN_2).

part of pot containing limed soil	whole pot (g/pot)	upper half (g/0.5 pot)	lower half (g/0.5 pot)	relative root weights (limid:acid)
sNO_3-plants				
contr. (pH 4)	6.31 ± 0.11			1.3
whole pot	7.96 ± 1.16			
lower half	7.47 ± 1.86	3.20 ± 0.10	4.27 ± 2.24	1.3
upper half	7.47 ± 1.37	3.43 ± 1.20	4.04 ± 1.12	0.9
sN_2-plants				
contr. (pH 4)	1.66 ± 0.41			4.1
whole pot	6.75 ± 0.82			
lower half	3.95 ± 0.64	0.77 ± 0.03	3.18 ± 0.76	4.1
upper half	3.68 ± 1.64	2.97 ± 1.70	0.71 ± 0.33	4.2

pH 5.6 (Fig. 1) is in agreement with these buffering characteristics.

The results indicate a positive correlation between dry matter production and excretion of hydroxyl ions, when nitrogen was taken up as nitrate, and a positive correlation between dry matter production and excretion of protons, when atmospheric nitrogen was fixed.

DISCUSSION

Yield and Nitrogen Content

In this particular experiment maximum yield was obtained at a relatively low pH (pH 5.0). This is in agreement with the

results of a water culture experiment (optimum pH between pH 4.0 and 5.5)[3]. In a field trial highest yields of dinitrogen-fixing pea plants were obtained at pH 6.2[13]. In a natural soil a low pH generally coincides with the presence of only a small amount of effective Rhizobia[19] and a low calcium concentration in the soil solution. Calcium compensates to a certain extent the influence of a low soil pH on nodulation[11,16]. In the present experiment calcium concentration was relatively high and nodulation was facilitated by adding a suspension of effective Rhizobium. This may explain the low pH optimum.

At low pH, prior nodulation resulted in yields of pea plants equal to those of the sN_2-treatments, but yields were significantly lower than those of the pNO_3-treatments (Table 2). The statement that nodulation is more sensitive to pH than is the growth of a legume[9,14] could not be confirmed in this experiment. Moreover, the similar nitrogen contents of the shoots (seeds and straw) of all the plants grown at pH 4.0 or higher (Table 2) indicate that nitrogen availability was not limiting growth under these conditions.

Changes in Soil Acidity

Measuring the pH of the bulk soil it could be shown that nitrate-supplied plants increased, and dinitrogen-fixing plants decreased the pH of the soil (Fig. 1). When nitrogen is taken up as urea[5,10], or when the dinitrogen-fixing process provides nitrogenous compounds[2], generally cation uptake exceeds anion uptake. In contrast, anion uptake generally exceeds cation uptake when the form of nitrogen is nitrate[10]. The charge of excess cation uptake is compensated for by the excretion of hydrogen ions and the charge of excess anion uptake is compensated for by the excretion of hydroxyl or bicarbonate ions[8]. The pH-changes (Fig. 1) can be explained by the influence of the form of nitrogen on the cation-anion uptake pattern.

When only one form of nitrogen is assimilated the resulting

acid or base excretion by the roots appeared positively correlated with the dry matter production[2]. In some high yielding pNO_3-treatments pH-change was smaller than could be expected of nitrate-supplied plants. As already nodulated seedlings were planted and only 17 50 mg N/pot was supplied as nitrate, part of the accumulated nitrogen (Table 2) was most likely delivered by additional dinitrogen fixation. The latter process depressed partly the increase in pH associated with nitrate nutrition. When only a small amount of nitrate is supplied the overall result will be a pH decrease, caused by the alkaline uptake pattern of dinitrogen-fixing plants[18].

In a pot experiment soil is thoroughly rooted and per unit of soil a high dry matter yield is produced. The pH-changes in the bulk soil can thus easily be noticed (Fig. 1)[18]. In the field only small pH-changes will be detectable in the bulk soil, but in the active root zone pH-shifts can still be considerable[20,22].

Root Growth

Root growth in N_2-treatments was inhibited when soil pH decreased from 4.6 to 4.0 during the growing period (Fig. 1). This inhibitory effect was even more drastic when pH of the bulk soil decreased below pH 4.0. This is in agreement with results obtained with pea in water culture in which acidity was maintained constant at pH 4.0. In this case a reduced percentage of roots was found, whereas shoot growth was not influenced[3].

In acid soils both root growth and nodulation will be factors limiting yields of dinitrogen-fixing legumes. In this experiment root growth especially affected yield, but when other conditions than pH are less favourable for nodulation (low calcium concentration, no inoculation) nodulation will be the most important factor causing reduced yields.

Nitrogen Fertilization on Acid Soils

In order to save costs of nitrogen fertilizer and to maximize use of the dinitrogen-fixing process, small applications of nitrogen fertilizer are of interest. In neutral soils it seems advisable to use ammonium rather than nitrate, because nitrate inhibits nodulation even at a low concentration[15]. In acid soils, however, nitrate fertilization appears more favourable because of increase of the rhizosphere-pH, associated with nitrate nutrition. In acid soils the indirect effect of nitrate fertilization on root growth and nodulation via the pH is substantial and is probably of more importance than direct inhibiting effects of nitrate on nodulation.

Nodules will be initiated after depletion of nitrate[4,15]. At this time ion uptake changes from an acidic to an alkaline pattern, resulting in a pH-decrease around the root. Thus nodulation starts when the pH in the rhizosphere has reached a maximum. After a successful (pH-sensitive[14]) infection, further growth of nodules is independent of the pH of the medium. When only a small amount of nitrate is supplied, nodulation will occur early and a decrease in pH of the rhizosphere will be considerable. Probably the inhibitory effect of a low pH on nodule formation can be compensated to some extent by growth of nodules already present. Dinitrogen-fixing legumes lower the pH of the rhizosphere during the course of the growing season. It is probable that the effect of this pH-decrease on root growth and root functioning is underestimated. Further research is needed to distinguish between effects of rhizosphere acidity on yield of legumes via nodulation and via root growth.

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6 Effects of ^{15}N-nitrate fertilization on yield and dinitrogen fixation of vegetative pea plants

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EFFECTS OF ^{15}N-NITRATE FERTILIZATION ON YIELD AND DINITROGEN FIXATION OF VEGETATIVE PEA PLANTS

Key words: heavy nitrogen, N-fertilization, nitrate, nodulation, pea, Pisum sativum L., symbiotic N-fixation.

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ABSTRACT

The dry matter yields and total nitrogen contents in the vegetative shoots of pea plants (Pisum sativum L.), infected with an effective or ineffective strain of Rhizobium leguminosarum, and supplied with increasing amounts of ^{15}N-labelled nitrate fertilizer, were studied in a pot experiment with a sandy soil. In the ineffective strain treatment both dry matter yield and shoot nitrogen concentration increased considerably as the amount of ^{15}N-labelled nitrate fertilizer was increased from 0 to 400 mg N/pot. When plants had been infected with an effective strain the dry matter yield was independent of the nitrate application rate as was the tissue nitrogen concentration which was high in all cases, indicating that nitrogen supply had been sufficient. Only application of 100 mg N/pot in the effective strain treatment resulted in a slightly depressed nitrogen content of the shoot, indicating a suboptimal collaboration of nitrate uptake and assimilation, and dinitrogen fixation. Calculation of the

amount of ^{15}N-labelled combined (NO_3^--)nitrogen which was taken up by the plants, based on the ^{15}N content of the shoot and some simple assumptions, indicated that nodulation was initiated after depletion of nitrate-nitrogen in the soil. The decreasing contributions of dinitrogen fixation to nitrogen accumulation from 65 to 8% as nitrate supply was increased thus reflect the points of time at which nitrogen nutrition of the plant was altered from nitrate uptake to dinitrogen fixation.

INTRODUCTION

The increasing cost of nitrogen fertilizers over the past 15 years and the associated restriction in supply of nitrogen fertilizers to developing countries has drawn attention to the urgent need to focus research on the dinitrogen fixation process in the legume-rhizobium symbiosis. In particular the question has arisen of how different amounts of nitrogen fertilizer affect the nitrogen nutrition and yield of leguminous crops.

Seedlings can take up combined nitrogen a few days after germination, but the formation of active root nodules requires several weeks. This is especially the case when the quantity of protein stored in the seed is small. A temporary shortage of nitrogen can thus arise[12]. Indeed, plants solely dependent on dinitrogen fixation as a nitrogen source generally produce lower amounts of dry matter than nitrate-supplied plants, a difference which originates from the first two or three weeks of growth[2,6]. A small application of nitrogen fertilizer might thus be beneficial in overcoming such a temporary shortage of nitrogen.

In water culture experiments Bethlenfalvay et al.[1] demonstrated that a low concentration of ammonium-N (2 mM) can increase the dry matter production of nodulating peas. In both treatments,

with and without ammonium-N, the nodule numbers were similar, but in the ammonium-N treatment both ammonium uptake and dinitrogen fixation contributed to nitrogen nutrition. Only at a high ammonium concentration (> 8 mM) was nodule formation inhibited. In other experiments investigating the effect of nitrate nutrition it has been shown that low nitrate concentrations (2.5-5 mM) inhibit nodulation of field pea severely, while these concentrations are too low to achieve a reasonable yield[9]. Nitrate causes a reduction in the formation of nodules by inhibiting root hair curling and growth of the infection thread[7].

Both application of nitrate and ammonium reduce nitrogenase activity. This has been explained in terms of a shortage of carbohydrates in the nodules, caused by the demand of carbohydrates for assimilation of combined nitrogen[14]. Stimulation of photosyntheses by increasing light intensity[5] or CO_2-supply[11], and addition of sugar to the root medium[16] enhance dinitrogen fixation and compensate for the detrimental effect of combined nitrogen on dinitrogen fixation.

The contribution of the dinitrogen-fixing process to total nitrogen accumulation is dependent on the length of the dinitrogen-fixing period and the nitrogenase activity in the root nodules. Large seeds and high levels of nitrogen fertilization delay the dinitrogen-fixing process, which will only begin when low concentrations of combined nitrogen are present in the root vicinity. Dinitrogen fixation activity is also much dependent on the stop of growth of the plant. After early pod-fill there is a sharp decrease in dinitrogen fixation[4,15]. Inhibition of dinitrogen fixation in the generative stage has been explained by the high demand of fruits for carbohydrates, causing a shortage of carbohydrates in the root nodules.

The aim of the present work was to obtain more information of the relationship between nitrogen fertilization and dinitro-

gen fixation in the vegetative stage of pea plants. A pot experiment has been carried out in which plants infected with an effective strain of Rhizobium leguminosarum and plants solely dependent on nitrate as a nitrogen source have been compared at different levels of nitrate supply. In order to distinguish between dinitrogen fixation and nitrate uptake labelled nitrogen fertilizer has been used.

MATERIALS AND METHODS

Experimental Conditions, Harvest and Analytical Methods

Preparation of soil and seeds, amounts of nutrients added per kg soil and environmental conditions were the same as those used in experiments described elsewhere by Van Beusichem & Langelaan[3]. In the present study 3 kg soil was used (pH-H_2O 6.1), covered with 200 g of quartz sand. Before sowing a dense suspension of an effective (PF2) or ineffective (P8) strain of Rhizobium leguminosarum was added. Levels of nitrogen nutrition were established by applications of 0, 100, 200 or 400 mg N per pot as $Ca(NO_3)_2$, which had a ^{15}N-enrichment of 2,88 atom %. Harvesting was carried out 67 days after sowing; shoots were dried at 70 ^{o}C for at least 24 hours. After determination of the dry weights, total nitrogen contents of the shoots were determined according to Novozamsky et al.[8].

For the ^{15}N-determination the following procedure was used. After destruction with H_2SO_4/salicylic acid of a sample containing 0.5 mmol N, ammonium was distilled into 10 ml 0.1 M HCl. The ammonium was then oxidized to N_2 under vacuum, using NaBrO. Using a sample of the N_2 obtained the ratio $^{29}N_2/^{28}N_2$ was detected by means of a Statron NOI-S emission-spectrograph and the ^{15}N-enrichment of the shoot nitrogen calculated.

Calculation of Contribution of Different Nitrogen Sources

The assumption was made that the 3 seeds per pot, containing a total of 32 mg N, supply 20 mg N to the total shoot nitrogen. The ^{15}N-enrichment of the nitrogen in the shoots, which was not from the seeds (E), could then be calculated as:

$$E = X/(X-20) \cdot N\text{-enrichment of shoot nitrogen},$$

where X was the shoot nitrogen (mg N/pot). In plants infected with the ineffective Rhizobium strain, only combined nitrogen (c) other than the seed nitrogen contributed to nitrogen accumulation. For plants grown with the effective strain, seed nitrogen plus combined nitrogen plus dinitrogen fixation (c+f) represented the nitrogen accumulation. When the soil was inoculated with an ineffective strain, $E=E_c$ where E_c equals the ^{15}N-enrichment of the combined nitrogen of the soil. For a particular level of nitrogen application E_c was assumed to be the same in both treatments. In dinitrogen-fixing plants the fraction of shoot nitrogen not originating from the seeds (X-20), supplied by nitrate nutrition, was calculated as:

$$E_{c+f}/E_c$$

The respective quantitative contribution of nitrate and dinitrogen could be calculated as:

$$(E_{c+f}/E_c)(X-20) \text{ and } (1-E_{c+f}/E_c)(X-20) \text{ mg N/pot}$$

RESULTS AND DISCUSSION

In Table 1 it is clearly demonstrated that infection with the effective strain PF2 significantly affected dry matter yield when no nitrate was added (6.22 versus 4.18 g/pot). Yield was even higher when fertilizer nitrogen was applied, optimum dry

matter yields being achieved at 200 mg N/pot. At this rate of nitrate application inoculation with the effective strain resulted in a significant lower dry matter yield than inoculation with the ineffective strain (Table 1). Using pea plants, comparative effects on yield were found by Bethlenfalvay et al.[1], who varied ammonium concentration in a water culture from 0 to 16 mM; in inoculated plants maximum dry matter yield was attained at a low ammonium concentration (2 mM).

The negative effect of nodulation on yield is often explained by the higher energy requirements of the dinitrogen-fixing system in comparison with nitrate reduction[10]. In dinitrogen-fixing plants extra energy is needed for formation and maintenance of nodules. As at high rates of nitrate application almost all nitrogen in the shoots originated from nitrate (Table 2), significant lower yields of dinitrogen-fixing plants (Table 1) are most likely the result of energy costs, associated with formation and maintenance of inactive nodules.

From the nitrogen content data (Table 1) it can be concluded that nitrogen supply was limiting when no effective symbiosis was ascertained; only at the highest nitrogen level (400 mg N/pot) was the nitrogen content in the shoot high. In plants infected with the ineffective strain nitrogen accumulation in the shoot and nitrate supply were positively correlated. In contrast when soil was inoculated with an effective strain, nitrogen accumulation in the shoots was in the same order in all treatments, except when 100 mg N/pot was added. Very recently, Schilling[13] reported that symbiotic dinitrogen fixation is inhibited more than is nitrate reduction when the supply of carbohydrates is limiting. This is especially the case at the generative stage. In the present experiment dinitrogen fixation together with nitrate reduction appeared to be able to provide sufficient amounts of reduced nitrogenous compounds in the vegetative stage.

Application of 100 mg N as nitrate to the pots which con-

TABLE 1

Effect of amount of nitrogen fertilizer on dry matter yield, nitrogen content and total nitrogen accumulation in the shoots of pea plants, 67 days after sowing. Plants were inoculated with an ineffective or an effective strain of Rhizobium leguminosarum. All values are given ± S.D. (n=3).

NO_3-N added (mg N/pot)	Rhizobium strain	Dry matter yield (g/pot)	Nitrogen content (g/100 g DM)	Nitrogen accumulation (mg N/pot)
0	ineff.	4.18 ± 0.11	1.99 ± 0.21	83 ± 8.7
0	eff.	6.22 ± 0.26	3.81 ± 0.09	237 ± 15.3
100	ineff.	6.95 ± 0.27	1.66 ± 0.07	115 ± 0.1
100	eff.	7.05 ± 0.40	2.67 ± 0.16	188 ± 2.3
200	ineff.	8.60 ± 0.12	2.11 ± 0.12	181 ± 7.8
200	eff.	7.49 ± 0.52	3.14 ± 0.15	235 ± 17.1
400	ineff.	8.07 ± 0.18	3.51 ± 0.09	283 ± 13.4
400	eff.	6.97 ± 0.94	3.39 ± 0.13	235 ± 22.9

tained the effective Rhizobium strain resulted in a significant lower nitrogen content and nitrogen accumulation in comparison with the zero N treatment (Table 1). Such negative effects of a small amount of nitrate have also been found by Oghoghorie & Pate[9], using field pea grown in a low nitrate (2.5-5 mM) nutrient solution. It seems probable that nitrate application delayed nodule formation, resulting in a temporary shortage of nitrogen in the period between depletion of soil nitrate and formation of active nodules. The overall result is that the initial positive effect of nitrate on root and shoot development is altered into a negative effect on total nitrogen accumulation.

When a low ammonium concentration (2 mM) was supplied, maximum nitrogen accumulation was found in pea shoots[1]. Pea appears to form equal amounts of nodules, when ammonium concentration varied from 0 to 8 mM[1]. In soil ammonium is adsorbed to

some extent by organic matter and clay and for this reason it is more gradually depleted than nitrate, the latter being completely in soil solution. The positive efffect of a small dose of ammonium-nitrate on nitrogen accumulation found by Schilling et al.[12] can be explained by assuming that the effect of ammonium-nitrate on nodulation tends to be the result of ammonium-N. Thus only ammonium results in an overall positive effect, because it does not inhibit nodulation.

In all cases the calculated ^{15}N-enrichment of combined inorganic nitrogen in the soil appeared to be lower than the ^{15}N-enrichment of fertilizer nitrogen, which was 2,88 atom % (Table 2). This is partly caused by dilution with an amount of nitrate nitrogen already present in the soil, but also by the activity of micro-organisms, resulting in incorporation of labelled nitrogen and the mineralization of organic matter. At low nitrogen application almost all nitrate present in the soil will be taken up by ineffectively nodulated plants.

When 100 or 200 mg N/pot was supplied, the contribution of nitrate to total nitrogen accumulation in shoots appeared to be the same, regardless of whether the soil had been inoculated with the effective or ineffective strain (Table 2). In these cases obviously all available nitrate was taken up. These results suggest that formation of nodules only started after almost all nitrate was taken up. This is in agreement with results obtained by Munns[7]. Only when 400 mg N/pot was applied did dinitrogen fixation result in a lower total nitrogen accumulation in comparison with plants infected with ineffective strain (Table 1).

In effectively nodulated plants the contribution of symbiotic dinitrogen fixation to the total nitrogen accumulation decreased from 65 to 8% as the amount of fertilizer nitrogen was increased from 0 to 400 mg N/pot (Table 2). These relative contributions seem of minor importance; in the vegetative period both uptake of nitrate and symbiotic dinitrogen fixation

TABLE 2

Effect of amount of ^{15}N-enriched nitrate fertilizer on the ^{15}N-enrichment of the shoot, ^{15}N-enrichment of soil nitrate and on the contribution of nitrate and dinitrogen fixation to the total nitrogen accumulation in shoots of pea plants. All values are given ± S.D. (n=3)

NO_3-N added (mg N/pot	Rhizobium strain	^{15}N-enrichment of shoot (atom %)	^{15}N-enrichment of soil nitrate (atom %)	Contribution soil nitrate (mg N/pot)	Contribution dinitrogen fixation (mg N/pot) (%)
0	ineff.	0	0	63 ± 8.7	
0	eff.	0	0	63	154 ± 15.3 (65 ± 2.3)
100	ineff.	1.08 ± 0.039	1.29 ± 0.073	97 ± 0.8	
100	eff.	0.67 ± 0.070	1.29	98 ± 10.2	70 ± 10.3 (37 ± 5.4)
200	ineff.	1.51 ± 0.034	1.69 ± 0.047	161 ± 7.8	
200	eff.	1.19 ± 0.194	1.69	163 ± 19.2	52 ± 32.7 (21 ± 11.9)
400	ineff.	1.89 ± 0.036	2.04 ± 0.046	263 ± 13.4	
400	eff.	1.69 ± 0.036	2.04	196 ± 23.2	19 ± 0.3 (8 ± 0.9)

seem to be able to provide sufficient nitrogenous compounds. Probably the relative contribution of dinitrogen fixation gives only an indication of the point of time at which nitrate was depleted and the dinitrogen-fixing process started.

A period of shortage of available nitrogen between depletion of nitrate and the presence of active nodules should be avoided. As a low ammonium concentration does not inhibit nodulation[1] it would be advisable to use ammonium fertilizer in combination with a nitrification inhibitor. Application of a small amount of ammonium fertilizer would maximize the use of the symbiotic dinitrogen-fixing machinery, while the same effect on yield and nitrogen accumulation could be achieved by relatively high amounts of nitrate, thus minimizing the contribution of dinitrogen fixation. This point deserves further attention in plant-soil studies.

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7 Xylary charge distribution and nitrogen transport in *Pisum sativum* L. during dinitrogen fixation or nitrate nutrition

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Xylary Charge Distribution and Nitrogen Transport in *Pisum sativum* L. During Dinitrogen Fixation or Nitrate Nutrition

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Summary

Electrical charge distribution and partitioning of nitrogenous compounds in xylem exudates of dinitrogen-fixing and nitrate-supplied pea plants (*Pisum sativum* L.) were compared. Nitrate-supplied plants exuded considerably more xylem sap than dinitrogen-fixing plants.

In the xylem saps of nitrate-supplied plants 91 % of the total cationic charge was balanced by inorganic anions. This small excess inorganic positive charge was compensated by aspartate, citrate, and malate. Total cation concentration in the xylem sap of dinitrogen-fixing plants was twice that of nitrate-supplied plants (70.0 versus 36.6 meq l^{-1}), while an excess cation over inorganic anion concentration of 21.6 meq l^{-1} was found. This charge gap was compensated for 80 % by aspartate and the remaining charge was balanced by citrate, malate, and succinate. In both treatments total cations were balanced within 3 % by inorganic anions plus carboxylates.

Total nitrogen concentration in the xylem saps of dinitrogen-fixing plants was 2.7 times as high as that of nitrate-supplied plants (174 versus 65 mmol l^{-1}). The relative distribution of nitrogen over the various compounds differed for the two treatments. In nitrate-supplied plants 65 % of the nitrogen was translocated in reduced form. Asparagine and glutamine contributed for 45 and 13 %, respectively, to the long distance nitrogen transport. The respective contributions of these amides in the xylem sap of dinitrogen-fixing plants were 70 and 16 %, while 11 % was transported as aspartate. In both treatments very low concentrations of other amino acids were detected and all xylem sap samples were shown to be free from ureides.

Evaluation of data about the distribution of nitrate reductase activity over the plant organs, the fraction of absorbed nitrate reduced in the roots, and the relation between hydroxyl/bicarbonate excretion (= excess anion uptake) and nitrate uptake, allowed the conclusion that in nitrate-supplied pea plants no xylem-phloem cation recirculation is necessary for the regulation of electroneutrality in the root tissue.

Key words: Pisum sativum, amino acids, carboxylates, cation recirculation, inorganic cations and anions, nitrate nutrition, pea, symbiotic nitrogen fixation, xylem transport.

Introduction

Many plant species, including leguminous plants, take up an excess of nutritive anions over cations when abundantly supplied with nitrate as the sole source of nitrogen (Aguilar S. and van Diest, 1981; van Beusichem, 1981; Israel and Jackson, 1982; Riley and Barber, 1969). In apparent contrast, inorganic cations in the xylem sap of nitrate-supplied plants are almost completely balanced by inorganic anions. Xylem

transport of equivalent amounts of inorganic cations and anions only occurs under conditions of adequate nitrate supply, so that nitrate deposition in the xylem is substantial. This phenomenon has been demonstrated for a diversity of plant species, such as maize (Dijkshoorn, 1971), tobacco (Wallace et al., 1971), dwarf bean (Breteler and Hänisch ten Cate, 1978), and castor oil plants (Kirkby and Armstrong, 1980). The apparent discrepancy between excess anion uptake on one side and equivalent upward transport of inorganic positive and negative charge on the other, is thought to be eliminated by transport of cations (mainly potassium) together with carboxylates (mainly malate) from the shoots *via* the phloem to the roots. It has been suggested that in the root tissue these organic anions are decarboxylated to yield bicarbonate which can be exchanged for nitrate. Subsequently, cations originating from the phloem stream can be translocated back to the shoot in association with recently absorbed nitrate (Dijkshoorn, 1958; Dijkshoorn et al., 1968; Ben Zioni et al., 1970, 1971; Kirkby, 1974; Blevins et al., 1978; Kirkby and Armstrong, 1980). The extent of cation recirculation can remain small or be even absent in plants which reduce nitrate predominantly in the roots or when anion uptake is not much in excess of cation uptake (Armstrong and Kirkby, 1979; Israel and Jackson, 1982; Keltjens, 1981, 1982).

To maintain electrically neutral long distance ion transport in dinitrogen-fixing plants, other processes must be operative than in nitrate-supplied plants. An excess cationic over anionic nutrient uptake by dinitrogen-fixing plants (Aguilar S. and van Diest, 1981; van Beusichem, 1981; Israel and Jackson, 1982; Nyatsanga and Pierre, 1973; Raven and Smith, 1976) suggests the necessity of organic anion (carboxylate) synthesis in the roots and deposition of these compounds in the xylem in order to balance the excess inorganic positive charge. Israel and Jackson (1978) suggested that this could have an impact on the xylem loading rate of cations as a result of complexing capabilities of carboxylates and amino acids and their restricted radial movement in the root. Next to their function as a carrier of negative charge, some carboxylates (aspartate, glutamate) can also contribute to the delivery of nitrogen to the shoots.

In the present study both distribution of electrical charge and partitioning of nitrogenous compounds in the xylem sap of dinitrogen-fixing and nitrate-supplied pea plants are compared and evaluated.

Materials and Methods

Plant cultivation

For this experiment, pea plants (*Pisum sativum* L. cv. «Rondo») were grown as described in detail elsewhere (van Beusichem, 1981). When in the subsequent experiment nitrate was used as a nitrogen source, the plants were inoculated with a dense suspension of *Rhizobium leguminosarum*, strain P8, resulting in an ineffective symbiosis. The strain PF2 was used when the plants were committed to dinitrogen fixation; this strain accomplished an effective symbiosis. After the pre-treatment period 2 containers were filled with 30 litres of the nitrate (4.0 mmol l^{-1}) solution and another 2 with 30 litres of the zero-N solution (van Beusichem, 1981). The whole

system was set up in a phytotron where the experimental conditions were: temperature 15 °C, dewing point 14 °C, photoperiod 14 h day^{-1}, light intensity 60 W m^{-2}, constant solution pH 5.50. On each container 40 plants were grown. The solutions were renewed weekly to prevent depletion of any nutrient. After 13 and 26 days of growth, 4 plants of the nitrate treatments were used for an *in vivo* nitrate reductase activity assay. At the onset of the photoperiod of the 32nd day all plants were decapitated about 4 cm above the root system. During 14 h xylem exudate was collected continuously, using Pasteur pipettes, and stored in plastic vials at −20 °C immediately after sampling.

Analytical methods

Nitrate reductase activity in leaves, stems + petioles, and roots was determined by the *in vivo* procedure described by Jaworski (1971). Xylem exudates collected from 20 plants over the first 2 h and those collected over the subsequent two 6-h intervals were treated as 3 separate samples. The saps were analyzed for total nitrogen, pH, inorganic cations (K, Na, Ca, Mg, NH_4), inorganic anions (H_2PO_4, Cl, NO_3, SO_4), amides, amino acids, citrate, malate, succinate, ascorbate, oxalate, formate, and fumarate. For the total nitrogen determination 0.2 ml was digested at 360–380 °C in 1 ml of a 30:1 (v/w) H_2SO_4- salicylic acid mixture and 0.2 g Se-mixture (Merck 3080) after nitration at room temperature for at least 2 h (Eastin, 1978). In the diluted digests nitrogen and phosphorus were determined on a Technicon auto-analyzer. In the diluted saps K, Na, Ca, Mg, Cl, and SO_4 were analyzed as described earlier (van Beusichem, 1981). Nitrate was determined on a Technicon auto-analyzer. Carboxylates were determined by enzymatic procedures, all based on the increase or decrease of the UV-absorption (340 nm) of NAD(P)H as a result of specific enzymatic oxidation or reduction of the substrate. All enzymatic procedures were provided by Boehringer Mannheim GmbH. Amino acids, ureides, and NH_4 were assayed on an amino acid analyzer. Amides were determined as described elsewhere (van Beusichem and Neeteson, 1982). The amount of H_2SO_4 necessary to keep the pH of the nitrate solution at 5.50 was considered to provide an accurate estimate of the excess anion over cation absorption by the nitrate-supplied plants (van Beusichem, 1981). The amount of nitrate taken up during the whole experimental period was calculated from shoot and root tissue analysis for total nitrogen (Eastin, 1978). All results represent mean values of four replicates.

Results

Exudate production and acidity

As was shown previously (van Beusichem, 1981), total dry matter yields did not differ significantly for both treatments, provided that plants were grown at a relatively low temperature. Nevertheless, nitrate-supplied plants exuded considerably more xylem sap than dinitrogen-fixing plants (Table 1). In both treatments the exudation rate was found to be constant during the 1–9 h interval. During this period the

Table 1: Weights (g/100 plants) and pH of xylem exudates collected from pea plants grown for 32 days on a nitrate-containing solution or on a nutrient solution without combined nitrogen.

	nitrate nutrition			dinitrogen fixation		
	hours after decapitation			hours after decapitation		
	0–2	2–8	8–14	0–2	2–8	8–14
Exudate weight	27.04	65.07	20.90	6.08	13.41	8.82
Exudate pH	5.10	4.85	4.86	5.15	5.04	5.25

exudation rate of nitrate-supplied plants was 5 times as high as that of dinitrogen-fixing plants (10.8 versus 2.2 g per 100 plants per h). Over the whole experimental period (14 h) xylem sap production by nitrate-supplied plants was 4 times higher (113.0 versus 28.3 g per 100 plants).

The pH of the xylem exudates decreased from 5.10 and 5.15 to 4.85 and 5.04 in the nitrate-supplied and dinitrogen-fixing treatment, respectively. During the last time interval (8–14 h) sap pH of the dinitrogen-fixing plants rose to 5.25, but in the nitrate treatment the acidity was maintained at pH 4.86 (Table 1).

Xylary ionic balance

In order to make an accurate estimation about the contribution of phosphate and the different organic anions to the ionic balance in the xylem saps, the degree of ionization of these compounds in dependence of the prevailing sap pH was calculated using pK values as tabulated in the Handbook of Chemistry and Physics.

In the period of stationary bleeding (2–8 h) total cation concentration in the xylem sap of dinitrogen-fixing plants was twice as high as compared with the nitrate treatment (70.0 versus 36.6 meq l^{-1}). This was due to a roughly doubling of the concentration of all major contributing cations (Table 2). Substitution of chloride for nitrate in the nutrient solution resulted in an equivalent compensation by means of a higher chloride concentration in the bleeding sap of dinitrogen-fixing plants. But also the

Table 2: Cationic and anionic composition (meq l^{-1}) of xylem exudates collected from pea plants grown for 32 days on a nitrate-containing solution or on a nutrient solution without combined nitrogen.

	nitrate nutrition			dinitrogen fixation		
	hours after decapitation			hours after decapitation		
	0–2	2–8	8–14	0–2	2–8	8–14
Potassium	15.3	18.9	21.8	17.7	34.5	–
Sodium	0.5	0.5	1.3	1.6	1.5	–
Calcium	10.2	12.6	13.4	8.4	22.9	–
Magnesium	3.0	4.6	6.0	5.4	10.9	–
Ammonium	0.0	0.0	0.0	0.2	0.2	0.2
Phosphate	4.2	5.6	8.5	6.8	15.3	18.2
Chloride	1.5	2.4	4.0	11.7	21.2	25.2
Nitrate	18.0	23.2	22.3	0.0	0.0	0.0
Sulphate	2.6	2.8	5.5	4.8	11.9	21.8
Aspartate	0.8	1.1	0.7	6.6	17.3	10.8
Citrate	1.1	0.8	0.6	1.1	2.4	1.6
Malate	0.6	0.2	0.3	1.2	0.5	0.0
Succinate	0.0	0.0	0.0	0.1	0.3	–
Sum cations	29.0	36.6	42.5	33.3	70.0	–
Sum anions	28.8	36.1	41.9	32.3	68.9	–

phosphate and sulphate concentration were drastically enhanced. As an overall result an excess cation over inorganic anion concentration of 21.6 meq l^{-1} in the xylem sap of dinitrogen-fixing plants was found.

In the xylem saps of nitrate-supplied plants 91% of the total cationic charge was balanced by inorganic anions (Table 2). The small excess inorganic positive charge was found to be compensated by aspartate, citrate, and malate. The inorganic charge gap in the xylem sap of dinitrogen-fixing plants was balanced for 80% (17.3 meq l^{-1}) by aspartate and for 15% by citrate, malate, and succinate.

In both treatments total cations in the xylem saps were closely balanced by inorganic anions plus carboxylates (Table 2), indicating that all major contributing compounds had been taken into account.

In all samples, traces (less than 0.05 meq l^{-1}) were found of oxalate, fumarate, formate, and glutamate. All samples proved to be free from ascorbate and ureides (allantoin and/or allantoic acid).

Long distance transport of nitrogenous compounds

During the period of stationary water flux (2–8 h) through nitrate-supplied roots, about 35% of the nitrogen was transported in the nitrate form (Table 3). Asparagine and glutamine were the main organic compounds involved in nitrogen transport, contributing about 45 and 13%, respectively, to the total translocation of nitrogen.

Table 3: Total nitrogen contents and partitioning of nitrogenous compounds in xylem exudates collected from pea plants grown for 32 days on a nitrate-containing solution or on a nutrient solution without combined nitrogen. All data are expressed in mmol N l^{-1}.

	nitrate nutrition			dinitrogen fixation		
	hours after decapitation			hours after decapitation		
	0–2	2–8	8–14	0–2	2–8	8–14
Asparagine	16	30	24	51	126	128
Glutamine	4	9	5	19	28	13
Aspartate	1	1	1	7	19	12
Other amino acids	2	4	6	3	6	8
Nitrate	18	23	22	0	0	0
Sum	41	67	58	80	179	161
Total nitrogen	41	65	59	84	174	161

Total nitrogen concentration as well as its distribution over the different compounds in the xylem sap of dinitrogen-fixing plants differed from those of the nitrate treatment. During stationary bleeding the total nitrogen concentration was 2.7 times as high as that in the sap of nitrate-supplied plants (174 versus 65 mmol l^{-1}). The respective contributions of the amides asparagine and glutamine were 70 and 16%, while 11% was transported as aspartate.

In both treatments only very low concentrations of other amino acids could be

Table 4: Amino acid composition (μmol l^{-1}) of xylem exudates collected during 2–8 h after decapitation of pea plants grown for 32 days on a nitrate-containing solution (NO_3) or on a nutrient solution without combined nitrogen (N_2).

	NO_3	N_2		NO_3	N_2
Threonine	604	473	Isoleucine	213	149
Serine	36	14	Leucine	142	122
Glutamic acid	49	46	Tyrosine	29	27
Glycine	7	9	Phenylalanine	29	24
Alanine	14	33	Ornithine	9	26
Valine	488	381	Lysine	230	205
Cystine	129	575	Histidine	162	299
Methionine	18	10	Arginine	485	583

detected (Table 4), contributing only 6 and 3 % to the translocation of nitrogen in the nitrate and dinitrogen treatment, respectively (Table 3).

In all cases a good agreement was found between the sum of the determined nitrogenous compounds and the results of the total nitrogen determinations (Table 3), indicating that all major contributing compounds had been taken into account.

Nitrate reductase activity

The nitrate reductase activities in the different tissues of nitrate-supplied pea plants are shown in Table 5. The enzyme activity, considered in relation to total fresh weight, provides a rough indication of the importance of the various plant parts in nitrate reduction. At both sampling dates, the roots accounted for about 51 % of the total nitrate reductase activity, while stems + petioles and leaves represented the site of about 31 and 18 %, respectively, of the total nitrate reductase activity.

Table 5: Distribution of nitrate reductase activity over leaves, stems + petioles, and roots of pea plants grown for 13 or 26 days on a nitrate-containing solution.

	13 days after transfer		26 days after transfer	
	nmol NO_2^- $organ^{-1}$ h^{-1}	% distribution	nmol NO_2^- $organ^{-1}$ h^{-1}	% distribution
Leaves	443	17.9	2025	18.5
Stems + petioles	756	31.9	3241	29.7
Roots	1235	50.2	5666	51.8
Whole plant	2434	100.0	10932	100.0

Discussion

Xylary ionic balance

In addition to the large amount of literature which has accumulated on this subject in the last decade (Pate, 1980), this seems to be one of the first reports presenting a very close charge balance in combination with a quantitative identification of all

nitrogenous compounds in the xylem sap of both nitrate-supplied and dinitrogen-fixing leguminous plants (Tables 2 and 3).

Recently, Israel and Jackson (1982) presented charge balances of xylem saps of soybean plants. They found that, irrespective of the form of nitrogen nutrition (dinitrogen fixation, urea or nitrate nutrition), malate balanced about 75 % of the excess inorganic positive charge, while allantoate and aspartate balanced most of the remaining charge. Also in the xylem sap of nitrate-supplied plants an excess inorganic positive charge was observed, although about one-third that of dinitrogen-fixing plants (9.7 versus 3.1 meq l^{-1}). This picture deviates remarkably from the data presented here for pea plants. In the xylem sap of nitrate-supplied plants a close inorganic cation-anion balance was found (Table 2), which is in agreement with results obtained for a diversity of other plant species (Dijkshoorn, 1971; Wallace et al., 1971; Breteler and Hänisch ten Cate, 1978; Kirkby and Armstrong, 1980). The deviation of the present results from those obtained by Israel and Jackson (1982) is probably due to nitrate limitation in their experiments. Their observation that plants supplied with 10 mM nitrate showed an acidification of the nutrient solution supports this assumption. This phenomenon, associated with an excess cation over anion uptake, is most likely to explain by assuming a temporary nitrate depletion. A second interesting difference between the results obtained by Israel and Jackson (1982) and those presented here are the reversal contributions of aspartate and malate as balancing ions in the xylem sap of dinitrogen-fixing soybean and pea plants. Malate balanced 78 % of the excess inorganic positive charge in soybean xylem sap (Israel and Jackson, 1982), while in the xylem sap of pea plants 80 % of the excess inorganic positive charge was balanced by aspartate (Table 2). Aspartate in soybean exudate and malate in pea exudate negligibly contributed as a carrier of electronegative charge. Probably, the absence of a ureide-synthesizing mechanism in the root nodules of pea plants (Tables 3 and 4) implies the necessity of translocating a fraction of the fixed nitrogen in an alternative ionic form, e.g. aspartate instead of allantoate (Reynolds et al., 1982).

Xylem-phloem cation recirculation

Pea plants take up an excess of nutritive anions over cations when abundantly supplied with nitrate as the sole source of nitrogen (van Beusichem, 1981; Fig. 1). In the xylem fluid of these plants inorganic cations and anions are closely balanced (Table 2). This implies that either nitrate reduction in the roots and subsequent nitrate-hydroxyl/bicarbonate exchange, and/or downward transport of cation-carboxylates through the phloem must occur. Fig. 1 shows the linear relationship between nitrate uptake and hydroxyl/bicarbonate excretion by pea roots. These data were taken from several experiments. From the slope constant of the line it is concluded that, supposing that no xylem-phloem cation recirculation takes place, necessarily 53 % of the absorbed nitrate must be reduced in the root tissue for the generation of sufficient

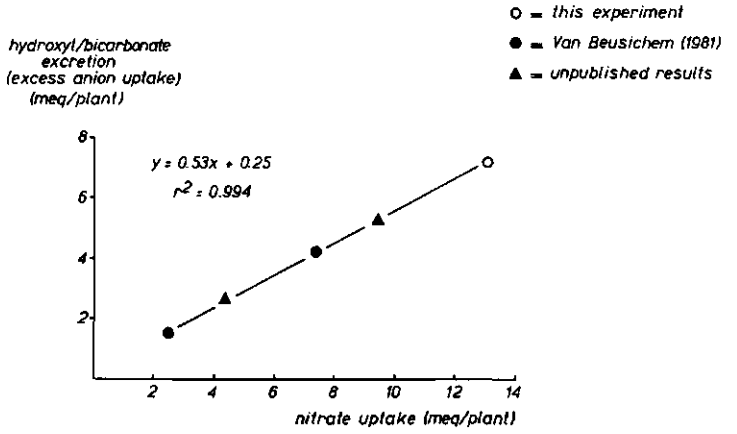

Fig. 1. Relation between hydroxyl/bicarbonate excretion (= excess anion over cation uptake) and nitrate uptake by pea plants supplied with nitrate as the sole source of nitrogen.

hydroxyl/bicarbonate ions in order to sustain the excess anion uptake by nitrate-supplied pea plants. This figure is in very good agreement with the distribution of nitrate reductase activity over the plant organs, showing that 50–52 % of the total enzyme activity was located in the root tissue (Table 5) and not contrasting with data from Table 3 from which can be concluded that even 65 % of the translocated nitrogen was in the reduced form. It has been reported, however, that the relative content of nitrate and reduced nitrogen in the xylem exudate might provide an over-estimate of the percentage reduction of freshly absorbed nitrate in the roots (Breteler and Hänisch ten Cate, 1980; Rufty et al., 1982). Nevertheless, the present observations justify the conclusion that in pea plants, abundantly supplied with nitrate, no xylem-phloem cation recirculation is necessary for the regulation of electroneutrality in the root tissue.

Nitrogen metabolism and translocation of nitrogenous compounds

In both nitrate-supplied and dinitrogen-fixing pea plants asparagine was the main organic nitrogen carrier in the xylem (Table 3). Irrespective of the nitrogen source, about 70 % of the translocated organic nitrogen was in the asparagine form. This may indicate that ammonium assimilation and subsequent nitrogen metabolism is located in the root tissue rather than in the root nodules.

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Samenvatting

Hogere planten hebben voor een goede groei en ontwikkeling, naast water en CO_2, vele voedingsstoffen nodig die door de wortels uit het bodemvocht opgenomen moeten worden om vervolgens naar de bovengrondse delen getransporteerd te kunnen worden. Deze voedingsstoffen komen opgelost in het bodemvocht voor. Wanneer de in de bodem beschikbare hoeveelheid aan één of meer van deze nutriënten niet toereikend is om een goede opbrengst te verkrijgen, kunnen deze bijvoorbeeld als kunstmeststoffen aan de bodem worden toegevoegd. Kunstmeststoffen zijn veelal zouten die opgelost in het bodemvocht gesplitst zijn in positief en negatief geladen ionen. Deze ionen kunnen door de plantewortel worden opgenomen. Zo wordt 'kali' opgenomen als het positief geladen kalium-kation en 'fosfor' in de vorm van een negatief geladen fosfaat-anion. Stikstof is het kwantitatief belangrijkste voedingselement. De voor de plant beschikbare hoeveelheid stikstof in de bodem is doorgaans erg klein, zodat dit element vaak in relatief grote hoeveelheden moet worden toegediend teneinde een behoorlijke opbrengst van een bepaald gewas te kunnen verkrijgen. Stikstof kan door de plant worden opgenomen als het positief geladen ammonium-ion, maar ook als het negatief geladen nitraat-ion. Bovendien kan de stikstof in niet-ionogene, dus moleculaire, vorm aan de plant worden aangeboden. Dit is het geval bij bemesting met ureum. Sommige plantenfamilies zijn in staat om, in nauwe samenwerking (symbiose) met bepaalde micro-organismen, stikstof uit de atmosfeer te binden en te gebruiken voor hun groei en ontwikkeling. Deze biologische stikstofbinding kan dus ook beschouwd worden als een vorm van niet-ionogene stikstofvoeding.

Omdat de behoefte van planten aan de afzonderlijke voedingskationen en -anionen zeer verschillend kan zijn, en omdat bovendien de stikstof in verschillende ladingsvormen kan worden aangeboden en opgenomen, nemen planten doorgaans geen gelijke hoeveelheden voedingskationen en -anionen op. Dit verschil tussen de som van de

opgenomen kationen en die van de anionen wordt door de plant gecompenseerd door het uitscheiden van H^+- of OH^--ionen, waardoor de plant de zuurgraad van de wortelomgeving kan veranderen. Deze pH-veranderingen in het wortelmilieu kunnen van grote praktische betekenis zijn omdat de oplosbaarheid van een aantal voedingselementen sterk afhangt van de pH. Zowel gebreks- als vergiftigingsverschijnselen, waargenomen bij diverse op kunstmatige substraten geteelde groentegewassen, bleken bijvoorbeeld samen te hangen met het onoplosbaar worden of juist zeer aktief worden van bepaalde voedingselementen als gevolg van het niet in de hand (kunnen) houden van de pH van de voedingsoplossing. In de landbouw kan van deze pH-veranderingen gebruik gemaakt worden doordat bijvoorbeeld als gevolg van H^+-uitscheiding door de wortel de van nature in de bodem voorkomende of als kunstmest toegediende moeilijk oplosbare voedingsstoffen beter voor de plant beschikbaar worden.

Deze H^+- of OH^--uitscheiding heeft ook gevolgen voor het milieu binnen de plant. Vele stofwisselingsprocessen zijn zeer gevoelig voor schommelingen in de pH van het celvocht, zodat de plant een mechanisme ter beschikking moet staan om de interne pH binnen nauwe grenzen te kunnen reguleren. Bij de verwerking van opgenomen ionogene stikstof tot aminozuren en eiwitten, wordt de positieve lading van ammonium in eerste instantie overgedragen op H^+- en de negatieve lading van nitraat op OH^--ionen. Het vrij gecompliceerde mechanisme dat binnen de plant pH-veranderingen elimineert, die kunnen ontstaan door uitscheiding van H^+- of OH^--ionen door de wortels en door verwerking van nitraat of ammonium, wordt vaak aangeduid met de 'biochemische pH-stat'. In hoofdstuk 1 worden de achtergronden van het begrip ionenbalans in planten behandeld. Tevens worden de belangrijkste processen die een rol kunnen spelen bij de handhaving van de electroneutraliteit en intracellulaire pH, in afhankelijkheid van het opnamepatroon van voedingsionen en de vorm waarin stikstof wordt opgenomen, aan een nadere beschouwing onderworpen. De hoofdstukken 2 t/m 7 zijn hoofdzakelijk gewijd aan experimenten waarin de niet-ionogene vormen van stikstofvoeding, ureumvoeding en symbiotische stikstofbinding, centraal staan.

In hoofdstuk 2 zijn enkele waterculture-proeven beschreven met betrekking tot de ionenbalans van mais en suikerbiet in afhanke-

lijkheid van de stikstofvormen ureum en ammonium. Door de opname van alle belangrijke voedingskationen en -anionen te bepalen, alsmede door de uitgescheiden hoeveelheid H^+-ionen direkt te registreren met behulp van automatische titratie-apparatuur, kon aangetoond worden dat ureum als zodanig werd opgenomen door de wortels en niet eerst in de voedingsoplossing werd afgebroken (gehydrolyseerd) tot ammoniumcarbonaat. Uit dit onderzoek bleek verder dat zowel mais als suikerbiet beide stikstofvormen goed konden benutten, hetgeen tot uiting kwam in het geringe verschil in drogestof produktie, maar dat vooral mais veel meer stikstof in de vorm van ammonium dan in de vorm van ureum opnam, terwijl ammoniumvoeding tevens een veel grotere H^+-produktie tot gevolg had. Omdat in het bloedingssap van mais zowel ammonium als ureum in verwaarloosbare concentraties voorkwamen, kon geconcludeerd worden dat beide stikstofvormen bijna volledig in de wortel werden omgezet. Verondersteld werd dat de verwerking van ureum in de wortel waarschijnlijk niet via een eenvoudige afbraak tot ammoniumcarbonaat plaatsvindt, gezien de verschillen tussen de ureum- en ammonium-behandeling met betrekking tot de relatieve verdeling van organische stikstofcomponenten in het bloedingssap.

In hoofdstuk 3 worden complete ionenbalansen beschreven van erwteplanten, die in watercultures óf rijkelijk voorzien werden van nitraat, óf voor wat betreft de stikstofvoorziening aangewezen waren op biologische stikstofbinding in symbiose met een bepaalde stam van de bacterie *Rhizobium leguminosarum*. Direkte registratie van de pH-verandering in de voedingsoplossing, onmiddellijk gevolgd door automatische terugtitratie, en berekening van het verschil in opgenomen voedingskationen en -anionen via plantenanalyse kwam in alle gevallen goed overeen. Nitraatvoeding leidde tot een grotere opname van voedingsanionen dan -kationen, gepaard gaand met een OH^--uitscheiding, terwijl stikstofbindende erwteplanten juist meer kationen dan anionen opnamen en dus hun wortelmedium zouden verzuren, althans wanneer de geproduceerde H^+-ionen niet direkt weggetitreerd zouden worden.

De mate van H^+-productie bleek zeer sterk af te hangen van de pH van de voedingsoplossing. Uit proeven, beschreven in hoofdstuk 4, bleek dat de opname van voedingskationen gestimuleerd werd naar-

mate de pH hoger was, terwijl de opname van voedingsanionen juist tegenovergesteld op een verhoogde pH reageerde. De neiging van stikstofbindende erwteplanten om de wortelomgeving te verzuren was dus groter naarmate de pH van de voedingsoplossing hoger was. Deze planten vertoonden overigens geen grote verschillen in drogestof produktie.

In een grond is het praktisch onmogelijk om de pH gedurende de groei constant te houden. Dit geldt zeker voor de pH van de direkte wortelomgeving. Anderzijds bezitten gronden een meer of minder sterk bufferend vermogen, waardoor een zekere mate van H^+- of OH^--uitscheiding door plantewortels niet direkt behoeft te resulteren in een grote verandering van de bodem-pH. De groei, stikstofopname en pH-veranderingen werden nader bestudeerd in een omvangrijke potproef met een humeuze zandgrond, waarbij erwteplanten al of niet nitraatstikstof kregen aangeboden. In het laatste geval werd een aktieve *Rhizobium*-suspensie toegevoegd, zodat deze planten eventueel wel symbiotisch stikstof konden binden. Reeds lang voor de aanvang van de proef was de grond in porties verdeeld die elk op een bepaalde pH waren gebracht. Alle karakteristieken konden dus worden bepaald in afhankelijkheid van een bepaalde evenwichts-pH van de grond bij de aanvang van de proef. De resultaten van deze proef zijn beschreven in hoofdstuk 5. Het bleek dat, onafhankelijk van de stikstofvoeding, de opbrengst een optimum vertoonde bij (uitgangs-) pH 5.0. Vooral bij lage pH-waarden groeiden de stikstofbindende planten slechter dan die welke nitraat aangeboden hadden gekregen; bovendien vond een geringere stikstofaccumulatie plaats in de bovengrondse delen van de stikstofbindende planten. Deze verschijnselen werden waarschijnlijk veroorzaakt door de sterk gereduceerde wortelgroei als gevolg van de lage uitgangs-pH plus de additionele verzuring van de bodem door de stikstofbindende planten. Bekalking van de grond elimineerde de geremde wortelgroei bij stikstofbindende planten volledig. Met nitraat gevoede planten vertoonden in het geheel geen geremde wortelgroei bij een lage uitgangs-pH, waarschijnlijk als gevolg van de waargenomen OH^--uitscheiding door de wortels tijdens de groei. Er werd dan ook geconcludeerd dat de teelt vån en de stikstofbinding dóór leguminosen op zure gronden waarschijnlijk wel perspectieven biedt, mits een be-

scheiden nitraatbemesting wordt toegepast. Het indirecte positieve effect van nitraat op de wortelgroei en de nodulatie via lokale pH-verhoging zal dan het indirecte negatieve effect van nitraat op de infectie van de wortelharen kunnen overschaduwen.

In de grond is altijd wel wat ammonium en/of nitraat aanwezig. Dit is zeker het geval bij een potproef waarin de omstandigheden, ook die voor mineralisatie van organische stof, zo optimaal mogelijk worden gehouden. De opname van ammonium en/of nitraat kan, wanneer men het effect van een uitwendige factor op de stikstofbinding van planten in een grond wil bestuderen, aldus de verkregen resultaten vertroebelen. In hoeverre dit het geval is, werd nagegaan in een proef met de reeds eerder genoemde humeuze zandgrond waaraan toenemende hoeveelheden gelabeld nitraat werden toegediend. Elke pot werd tevens geënt met een suspensie van een effectieve of ineffectieve stam van *Rhizobium leguminosarum* (hoofdstuk 6). Erwteplanten gegroeid op de met de ineffectieve stam behandelde grond vertoonden een vertrouwde responscurve voor wat betreft de drogestof productie, het stikstofgehalte in de spruit en de totale stikstofopname per pot. De drogestof productie van de planten afkomstig van de met de effectieve stam behandelde grond was daarentegen onafhankelijk van de stikstofgift. Mineralisatie van organische stof leverde dus reeds een voldoende hoeveelheid 'starter'-stikstof. De stikstofresponscurve vertoonde echter een deuk bij een matige N-gift, hetgeen verklaard werd door een ongunstig tijdstip waarop de plant moest overschakelen van nitraatopname op stikstofbinding. Gesuggereerd werd dat in het algemeen de relatieve bijdrage van de stikstofbinding aan de totale stikstofaccumulatie, vastgesteld met behulp van toegediend gelabeld nitraat, waarschijnlijk slechts een afspiegeling oplevert van het tijdstip waarop de plant overschakelde van nitraatopname op stikstofbinding. Berekening van het elementrendement zou vervolgens uitsluitsel kunnen geven of dat tijdstip gunstig is geweest.

In hoofdstuk 7 is tenslotte een experiment beschreven waarbij vooral het transport van verbindingen door de houtvaten naar de bovengrondse delen is bestudeerd. Uit het voorgaande is reeds naar voren gekomen dat met nitraat gevoede erwteplanten veel meer voedingsanionen dan -kationen opnemen, resulterend in OH^--uitscheiding

door de wortels. Nauwkeurige analyse van het bloedingssap bracht echter aan het licht dat hierin de concentratie aan kationen niet zoveel verschilde van die van de anorganische anionen. Uit bepalingen van zowel de verdeling van de nitraatreduktase-aktiviteit over wortel en spruit als de fraktie van het opgenomen nitraat dat reeds als organische stikstof in het bloedingssap voorkwam, werd de conclusie getrokken dat de hoeveelheid nitraat die reeds in de wortels werd gereduceerd juist voldoende was om de OH^--ionen te leveren die uitgescheiden werden én om een nagenoeg neutraal transport van kationen en anorganische anionen naar de spruit te bewerkstelligen. Om elektrisch neutraal transport in stikstofbindende planten, die immers méér voedingskationen dan -anionen opnemen, te handhaven, zal in de wortel synthese plaats moeten vinden van organische anionen, die vervolgens in het xyleem uitgescheiden moeten worden. Het bleek dat het negatief geladen aminozuur aspartaat in dit opzicht de belangrijkste functie vervult. Door het transport van aspartaat werd de ladingsbalans in het bloedingssap grotendeels gesloten en tegelijkertijd een deel van de in de wortelknolletjes gebonden stikstof naar de spruit getransporteerd. Het merendeel van de stikstof bleek echter in stikstofbindende erwteplanten als asparagine en glutamine te worden vervoerd. De conclusie was dan ook dat de erwteplant een typische vertegenwoordiger is van de amidetransporterende leguminosen.

Curriculum vitae

De schrijver van dit proefschrift werd op 19 juli 1949 te Rotterdam (Hoek van Holland) geboren. Na de lagere school te Maasdijk en de ULO-B te Naaldwijk te hebben doorlopen, werd in september 1965 een aanvang gemaakt met de landbouwkundige studie. In juni 1968 werd het diploma van de HLS van het KNLC te Dordrecht behaald. In september van datzelfde jaar begon hij de studie aan de Landbouwhogeschool te Wageningen waar in juni 1972 het kandidaatsexamen in de studierichting Bodemkunde en Bemestingsleer cum laude werd afgelegd. De periode 1972-1975 werd besteed aan de doctoraalstudie in dezelfde studierichting, terwijl hij tevens als student-assistent was verbonden aan de vakgroep Bodemkunde en Bemestingsleer.

In september 1975 werd het doctoraalexamen afgelegd met als hoofdvakken de bodemvruchtbaarheid (Prof.dr. A.C. Schuffelen; dr.ir H. Breteler) en de microbiologie (Prof.dr. E.G. Mulder; dr. A.D.L. Akkermans) en als bijvak de organische scheikunde (Prof.dr. H.C. van der Plas; dr. P.J. Lont). Per 1 september 1975 trad hij in dienst van de vakgroep Bodemkunde en Bemestingsleer (thans vakgroep Bodemkunde en Plantevoeding) van de Landbouwhogeschool. Na de instelling, in 1979, van de leerstoel Bodemvruchtbaarheid en Plantevoeding maakt hij als wetenschappelijk medewerker (I) deel uit van de gelijknamige sectie van voornoemde vakgroep met als hoofdtaken het leveren van een bijdrage aan de ontwikkeling en uitvoering van het onderwijs- en onderzoekprogramma van deze sectie. Sinds augustus 1982 is hij tevens studiecoördinator van de studierichting Bodemkunde en Bemestingsleer (N33, oude stijl), c.q. Bodemkunde (L15, nieuwe stijl) en maakt in deze functie deel uit van de Richtings Onderwijs Commissie.