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Contamination of Ammonium-Based Nutrient Solutions by Nitrifying Organisms and the Conversion of Ammonium to Nitrate

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Conversion of ammonium to nitrate and contamination by nitrifying organisms are often assumed not to be significant in ammonium-based nutrient solutions. To assess this assumption, maize (*Zea mays*) and pea (*Pisum sativum*) were grown under greenhouse conditions in aeroponic, hydroponic, and sand-culture systems containing 2 mM ammonium chloride as the sole nitrogen source and evaluated for the activity of contaminating nitrifying organisms. In all three culture systems, root colonization by nitrifying organisms was detected within 5 d, and nitrate was detected in the nutrient solution within 10 d after seedling transfer. In sand culture, solution nitrate concentration reached 0.35 mM by the end of the 17-d experiment. Consistent with the microbial ammonium oxidation sequence, nitrite was detected earlier than nitrate and remained at lower levels throughout the experiment. Nitrate was found in significant quantities in root and shoot tissues from seedlings grown in ammonium-based nutrient solutions in all of the solution culture systems. Maize seedlings grown in an ammonium-based hydroponic system contained nitrate concentrations at 40% of that found in plants grown in nitrate-based solution. Determination of nitrate (or nitrite) levels in the nutrient solution was the weakest indicator of the activity of nitrifying organisms. A bioassay for the presence of nitrifying organisms in combination with tissue analysis for nitrate was a better indicator of microbial conversion of ammonium to nitrate in nutrient solution culture. The results have implications for the use of ammonium-based nutrient solutions to obtain plants suitable for research on induction of nitrate uptake and reduction or for research using solution culture to compare ammonium versus nitrate fertilization.

Nitrogen uptake and assimilation has been an area of intense research for decades. Questions related to the apparent efficiency of ammonium versus nitrate fertilization have been studied, as have the biochemistry and physiology of nitrogen uptake and assimilation (Errebhi and Wilcox, 1990; Smith et al., 1990; Alexander et al., 1991; Macduff and Jackson, 1991). Many researchers have chosen to use solution culture techniques to compare ammonium- and nitrate-based nutrient solutions, assuming that the confounding effects of nitrogen oxidation and reduction reactions carried out by soil-borne microorganisms are eliminated or negligible in solution culture (Heberer and Below, 1989; Alexander et al., 1991). The potential for the contribution of microorganisms to the observed experimental results in assimilation of nutrient ions (e.g. phosphate) in nonsterile roots grown hydroponically has been considered (Barber et al., 1976). Little has been reported, however, regarding contamination by nitrifying organisms of solutions used to study nitrogen assimilation by plants. In part, this is because of problems with detection of nitrifying bacteria (Soriano and Walker, 1968) and a lack of a convenient radioactive tracer for nitrogen. Bloom and colleagues (Smart and Bloom, 1988; Bloom, 1989; Bloom and Sukrapanna, 1990) have considered the possibility of nitrification occurring in ammonium-based nutrient solutions, but found no evidence of activity of nitrifying organisms at lower ammonium levels (50–600 μM) than typically employed in nutrient solutions (1–2 mM). Smart and Bloom (1988) removed the plants from the nutrient solution after the experiment, and the solution was monitored for several months for the appearance of nitrate. Under these circumstances, there was no change in nitrate concentration. However, as noted by these authors, if the site of colonization was not the bulk solution, but rather root surfaces, the nitrifying organisms would have been removed with the roots and nitrification activity would go undetected.

Two genera of bacteria, *Nitrosomonas* and *Nitrobacter*, are primarily responsible for oxidation of ammonium in soils (Haynes, 1986). However, several other organisms, both fungal and bacterial, are noted for their capacity to oxidize ammonium. *Nitrosomonas* catalyzes the oxidation of ammonium to nitrite. *Nitrobacter* converts nitrite to nitrate; however, this reaction can occur spontaneously in some soils (Bartlett, 1981). Nitrifying bacteria are broadly distributed in nature and have even been found in dust particles (Brock, 1966). However, they are very difficult to culture under laboratory conditions (Soriano and Walker, 1968). Because both genera are obligate aerobes, it has been generally assumed that they would not proliferate in an aquatic environment such as hydroponic culture.

Molina and Rovira (1964) and, more recently, Klemedtsson et al. (1987) have examined the interaction of roots and nitrifying bacteria in soils. Both studies concluded that although interactions were complicated by the effects of other organisms, nitrifying organisms had significance in plant nitrogen availability both in recycling plant exudates and in competing with roots for limited nutrients.

The capacity for the absorption of nitrate by root cells appears to be induced by the exposure of roots to nitrate concentrations as low as 10 μM (MacKown and McClure, 1988). For research on induction of nitrate uptake capacity in roots, plants are commonly grown in ammonium-based

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Abbreviation: DDI, distilled deionized.

nutrient solution under nonsterile conditions, assuming that there is not significant conversion of ammonium to nitrate.

We have grown plants in aeroponic solution culture using ammonium-based nutrient solution to obtain large quantities of roots to be used in studies on nitrate uptake and induction. In connection with this work, we became concerned about the assumption that conversion of ammonium to nitrate was insignificant. Here, we report the results of experiments that indicate that contamination by nitrifying organisms and the conversion of ammonium to nitrate is a significant problem in ammonium-based solutions used for aeroponic, hydroponic, and sand culture.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. hybrid WF9 × MO17) and pea (*Pisum sativum* cv Alaska) seeds were surface sterilized in 1% sodium hypochlorite for 10 min, rinsed in DDI water, and soaked in 5% hydrogen peroxide for 1 min, followed by a second DDI water rinse. Seeds were germinated in glass baking trays (33 × 23 × 5 cm) on blotter paper soaked with 80 mL of 0.1 mM $CaCl_2$. Trays were covered with plastic wrap and incubated in the light at 28°C. Maize seedlings were transferred to solution culture 4 d after germination, and pea seedlings were transferred 7 d after germination. Pea roots were not inoculated with *Rhizobium* bacteria, and no nodulation was detected.

Culture Systems

Aeroponic tanks and the tanks used for hydroponic or sand culture were washed with sodium hypochlorite and rinsed with DDI water. Pots (modified 3-gallon plastic pails) used for sand culture were surface sterilized as above and filled to approximately 2 cm below the overflow pipe with clean, coarse silica sand. Prior to planting, the sand was leached with distilled water 12 times over a 2-d period, and the leachate was discarded. Plants grown aeroponically were suspended over the solution and were continuously misted with nutrient solution, but did not grow into direct contact with the solution. Hydroponically grown maize and pea were transplanted into a rigid plastic grid with roots submerged in a well-aerated nutrient solution. For sand culture, seedlings were transplanted into pots (four pots per solution reservoir). Nutrient solution was pumped into the pots five times per day, and the solution was allowed to drain into the solution reservoir. Nutrient solutions were not changed during the course of the experiment because preliminary experiments indicated that nutrient depletion was minimized by the large solution-to-plant ratio.

To accommodate differences in nutrient solution volumes in the three culture systems, tanks were planted at an approximately equivalent density of four plants to 10 L of solution: 40/100 L for sand culture, 32/80 L for aeroponic culture, and 48/120 L for hydroponic culture. DDI water was added as needed to maintain constant volume.

The basic nutrient solution contained 1 mM $CaCl_2$, 0.25 mM KCl, 0.1 mM $MgSO_4$, 40 μM NaH_2PO_4, 1 μM $MnSO_4$, 3 μM H_3BO_4, 0.1 μM Na_2MoO_4, FeEDTA 20 ppm, 1 μM $ZnCl_2$, and 0.1 μM $CuCl_2$. Nitrogen was provided as 2 mM NH_4Cl (ammonium-based nutrient solution) or 1 mM $Ca(NO_3)_2$ (nitrate-based nutrient solution). Samples were withdrawn throughout the experiment for monitoring of ammonium concentration. Ammonium chloride was added when ammonium concentrations dropped below 1.7 mM. Addition of nitrate was not required, because nitrate concentrations never fell below 1.5 mM. The pH of the nutrient solution was maintained at 6.0 by the addition of 1 M KOH or 1 M HCl as required. Each solution culture system and nitrogen source was replicated three times for both maize and pea. One tank in each system containing ammonium-based nutrient solution was maintained without plants to serve as a control.

Analysis and Assays

Solution and tissue samples for nitrate analysis and root bioassay were taken on the same day at 2- to 4-d intervals. All nitrate and nitrite analyses were performed using a Technicon model II Continuous Flow Analyzer. pH was determined with a Corning model 12 pH meter. Ammonium concentration was measured using a Wescan model 360 Ammonium Analyzer.

Samples of nutrient solutions were analyzed directly. Nitrate was extracted from tissues by the procedure of Cataldo et al. (1974). Tissue samples were divided into roots and shoots, discarding any residual seed. Tissue was dried to a constant weight in an 80°C oven and ground to pass a 40-mesh screen in a Pichford model 3800 "ball mill." Twenty milligrams of ground tissue were weighed into a 25-mL flask and 10 mL of DDI water were added. Stoppered flasks were incubated in a 45°C water bath for 4 h. Extractant and water blanks were gravity-filtered through Whatman No. 1 and analyzed with the Continuous Flow Analyzer. In preliminary experiments, both nitrate and nitrite were measured. Because no nitrite was detected in tissue samples, only tissue nitrate concentration was determined for the experiments reported here.

The bioassay for nitrifying bacteria on root surfaces used the modified Soriano-Walker media (Soriano and Walker, 1968) specific for nitrifying bacteria. The media consisted of 11 g of Hepes buffer, 1 g of $(NH_4)_2SO_4$, 40 mg of $MgSO_4$, 40 mg of $CaCl_2$, 200 mg of KH_2PO_4, and 0.5 mg of iron citrate per liter. pH was adjusted to 7 prior to dispensing 25-mL aliquots into 125-mL DeLong flasks. Prepared flasks were autoclaved prior to inoculation.

For removal of bacteria from root surfaces, 2 g fresh weight of unwashed, blotted roots were placed in a wide-mouth plastic bottle. Fifty milliliters of sterile DDI water and approximately 20 g of autoclaved 5-mm glass beads were added. The capped bottle was vigorously shaken by hand for 2 min. Five milliliters of the solution was used to inoculate the Soriano-Walker media. A control flask was inoculated with 5 mL of sterile water. Inoculated flasks were placed on a rotating shaker set at 200 rpm and incubated at 28°C. Daily, a 4-mL sample from each flask was gravity-filtered through Whatman No. 42 and analyzed for the production of nitrate and nitrite using the Continuous Flow Analyzer.

The experiment was repeated four times over the course of 6 months. Data shown (± SE) and described are for the

last two experiments, one using maize and one using pea. All time references indicate days from transplant of seedlings into the solution culture systems.

RESULTS

Plant Growth

Observations were made on the growth and morphology of the maize and pea seedlings throughout the experiment. For both species in sand culture, the roots were short and thick, as compared with hydroponically grown roots, which were very long, thin, and unbranched. Roots from aeroponically grown seedlings were intermediate in length and thickness, but exhibited more extensive branching than roots from seedlings grown in the other culture systems. Root weights were recorded at all sampling times throughout the experiments, which lasted for 17 d after transplant to the solution culture systems. For example, for 14-d-old maize seedlings, the fresh weight of the root system was about equal for seedlings grown in sand (1.80 g fresh weight/plant) and aeroponic (1.50 g fresh weight/plant) culture. In contrast, hydroponically grown maize seedlings had less than half of the root mass (0.63 g fresh weight/plant) observed for seedlings grown in sand or aeroponic culture. The type of culture system did not have a significant effect on root mass of the pea seedlings, which averaged 0.9 g fresh weight per plant after 14 d of growth.

Nitrate in Nutrient Solutions

Monitoring the nutrient solution for the presence of contaminating levels of nitrate is a method commonly used to determine if ammonium-grown plants are nitrate free (Bloom, 1989; Peuke and Tischner, 1991). The three solution culture systems used in these studies gave different results with respect to accumulation of nitrite and nitrate in the nutrient solution during growth of maize seedlings (Fig. 1). For maize seedlings grown in sand culture, the nitrate concentration in the ammonium-based nutrient solution exceeded 20 μM within 10 d and reached approximately 0.35 mM by the end of the 17-d experiment. As expected for microbial ammonium oxidation (Haynes, 1986), nitrite was detected at earlier times than nitrate and remained at lower levels throughout the course of the experiment (Fig. 1, inset). In contrast with the situation for sand culture, nitrite and nitrate were barely detectable at the end of the experiment in hydroponic culture (Fig. 1). Solution concentrations of nitrite and nitrate in the aeroponic culture system were intermediate to those found in sand and hydroponic culture (Fig. 1). Accumulation of nitrate in the nutrient solution was also readily detected in controls without plants, particularly in sand culture (data not shown). It appears that contaminating nitrifying organisms can be present on the surface of solid substrates in addition to root surfaces.

The pattern and extent of accumulation of nitrate in the ammonium-based nutrient solutions observed for maize were also observed for pea seedlings grown over a similar period of time (Fig. 2). For both the sand and aeroponic culture system, the nitrate concentration in the nutrient solution rapidly exceeded the 10 μM value, which has been shown to

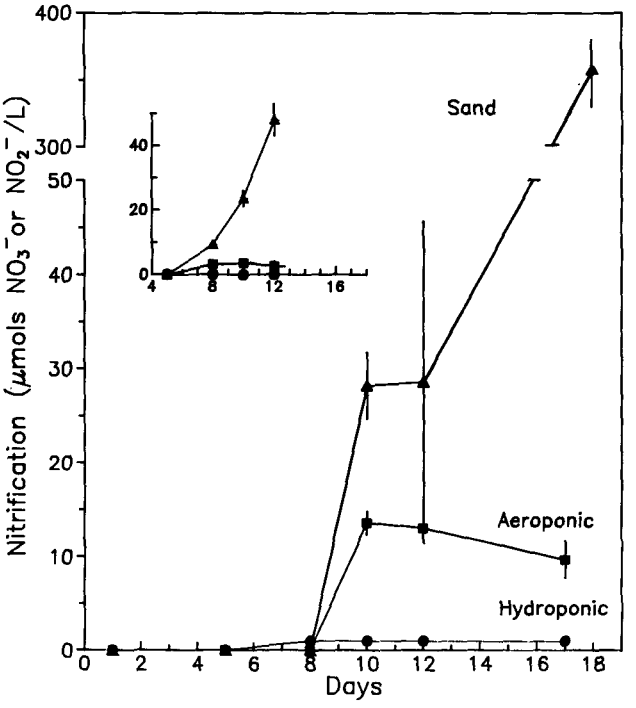

Figure 1. Appearance of nitrate (nitrification) in the ammonium-based nutrient solution as a function of time of growth of maize seedlings in the sand, aeroponic, and hydroponic culture systems. The symbols representing the nitrate concentration in the three culture systems are the same for nitrite concentrations shown in the inset.

be sufficient to induce accelerated rates of nitrate transport in maize roots (MacKown and McClure, 1988).

Nitrate in Plants

Because plants are not known to oxidize ammonium to nitrate, the accumulation of nitrate in tissues reflects the presence of nitrate in the external solution. Nitrate absorbed into roots may be reduced and incorporated into amino acids in epidermal and cortical cells; it may be stored in vacuoles as nitrate; or it may be transported in the xylem to the shoot, where the same options exist, including remobilization to the root (Andrews, 1986; Gojon et al., 1986). The percentage of absorbed nitrate reduced in the root versus the shoot varies with the species and may also be linked to external nitrate concentration as well as the energy status of the plant (Andrews, 1986). Hence, the concentration of nitrate in plant tissue is related to the presence of nitrate in the external solution, but because of variation in the amount of reduction, nitrate in tissue is only a qualitative index of the external nitrate concentration.

Nitrate was found in maize root (Fig. 3) and shoot (Fig. 4, data not shown for all culture systems) tissues from seedlings grown in ammonium-based nutrient solutions in all of the solution culture systems. The hydroponic system, which showed the lowest concentrations of nitrate in the nutrient solution (Fig. 1), resulted in maize roots with the highest nitrate concentrations. Sand culture, while exhibiting the

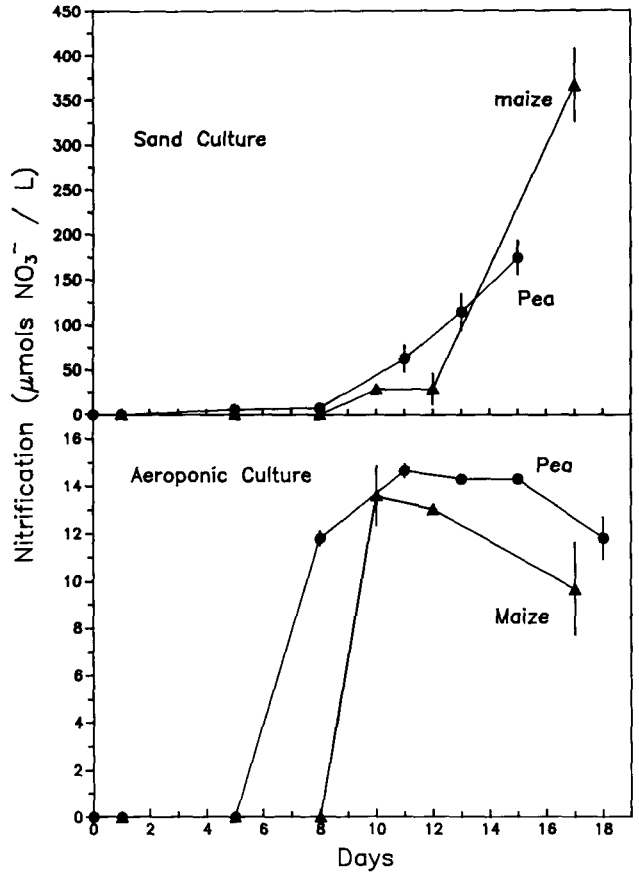

Figure 2. Nitrate accumulation (nitrification) in the ammonium-based nutrient solution as a function of time of growth of maize and pea seedlings in sand and aeroponic culture. Data for maize have been replotted from Figure 1 for comparative purposes.

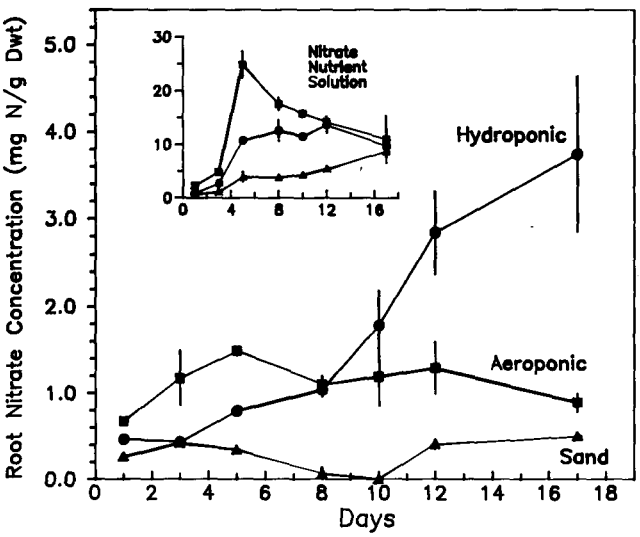

Figure 3. Accumulation of nitrate in maize roots as a function of time of seedling growth in ammonium-based nutrient solution culture. The inset shows the results for maize seedlings grown in the nitrate-based nutrient solution with the symbols corresponding to the same three culture systems as indicated on the main graph.

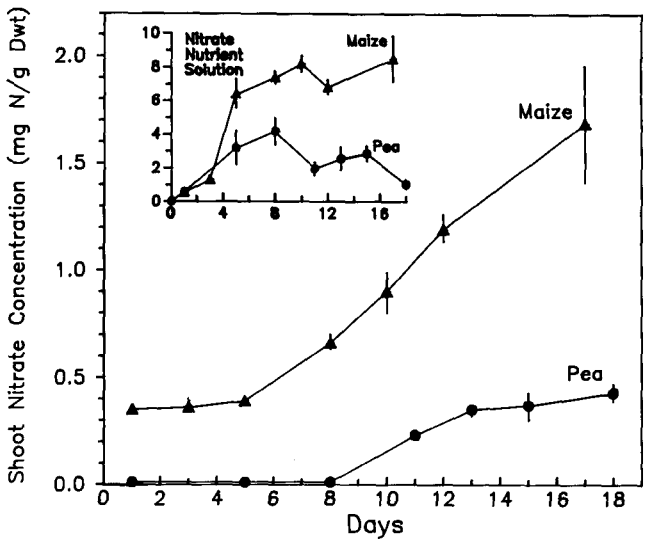

Figure 4. Comparison of the accumulation of nitrate in maize or pea shoots as a function of time of seedling growth in ammonium- or nitrate-based (inset) hydroponic culture.

highest accumulation of nitrate in the nutrient solution, showed the lowest nitrate levels in maize root tissues. The aeroponic system was intermediate to hydroponic and sand culture in nitrate accumulated in maize roots (Fig. 3). Maize plants grown in a nitrate-based nutrient solution also showed the lowest nitrate levels when grown in sand culture (Fig. 3, inset). For maize seedlings, the type of solution culture system clearly influenced the pattern of accumulation and possibly the degree of reduction of nitrate in root tissue. Pea seedlings also exhibited differences in root nitrate concentrations associated with the type of solution culture systems, but they were not as pronounced as those observed in maize (data not shown).

Over the 17-d growth period in the ammonium-based nutrient solution, the nitrate concentration in roots (Fig. 3) and shoots (Fig. 4) steadily increased to values that reached 40 and 20% (respectively) of those for the same tissues of maize plants grown with the nitrate-based nutrient solution. This striking accumulation of nitrate in tissues occurred despite the fact that the concentration of nitrate in the nutrient solution remained at low levels, particularly compared with those observed for sand and aeroponic culture (Fig. 1). Pea seedlings also accumulated nitrate to significant levels when grown in ammonium-based nutrient solutions, and the time course of nitrate accumulation was similar to that observed for maize (Fig. 4). In hydroponic culture using an ammonium-based solution, the presence of low concentrations of nitrate in the ammonium-based nutrient solution was not indicative of the nitrate status of the plant.

Bioassay for Nitrifying Organisms

A bioassay was used to detect the presence of nitrifying organisms on roots of seedlings grown in the ammonium-based nutrient solution (Table I). Root samples were agitated in sterile water to remove microorganisms. A sample of the

Table I. *Bioassay for the presence of nitrifying organisms on the roots of maize or pea seedlings growing in ammonium-based nutrient solution culture*

Samples of the population of microorganisms removed from roots were incubated in the Soriano-Walker selection medium for 12 d. An increase in the initial nitrate and nitrite concentration in the incubation flask of between 20 and 50% was scored with a +. An increase of greater than 50% was scored with a ++.

Culture System	Organism	Days after Planting						
		0	1	3	5	8	10	12
Aeroponic	Maize	−	−	+	+	++	+	++
	Pea	−	−	++	+	+	+	++
Hydroponic	Maize	−	−	−	+	+	+	+
	Pea	−	−	−	−	+	+	+
Sand culture	Maize	−	+	++	+	++	++	++
	Pea	−	−	++	++	+	++	++

population of microorganisms removed from the roots was cultured in a medium that is selective for nitrifying organisms. The presence of nitrifying organisms was assayed by detecting the appearance of nitrite and nitrate over time in the culture medium. Nitrifying organisms were detected on roots of plants grown in the ammonium-based nutrient solution for the aeroponic and sand culture systems within 3 d of growth of maize or pea seedlings (Table I). For the hydroponic culture system, nitrifying organisms were not detected until 5 to 8 d after the beginning of the experiment, and the amounts detected remained at lower values throughout the experiment than those observed for the other nutrient culture systems. Low but detectable levels of nitrifying organisms were associated with roots of maize and pea seedlings grown in the nitrate-based nutrient solution (data not shown).

DISCUSSION

Detection of nitrate in the ammonium-based nutrient solution and in plant tissues in conjunction with a bioassay for the presence of nitrifying organisms revealed that plants grown in commonly used solution culture systems were contaminated by, and sustained growing populations of, nitrifying organisms. The solution culture system exhibited differences in timing and apparent degree of microbial colonization of the plant species studied (Table I). The results showed that nitrate can accumulate in plants grown under greenhouse conditions in ammonium-based nutrient solutions and the concentration of nitrate in root and shoot tissues can readily exceed that required for the induction of accelerated nitrate transport. Hence, plants grown in this way may not be useful in research on the induction of nitrate transport or for comparative research using solution culture to investigate the efficiency of ammonium versus nitrate fertilization.

The identity of the nitrifying microbe(s) was not established, so it is not known with certainty if *Nitrosomonas* and *Nitrobacter* were the organisms involved. We believe that because of the broad distribution noted earlier, these bacterial genera are the most likely candidates for the nitrifying organisms colonizing the roots of seedlings grown in the solution culture systems employed in these studies. Bacterial colonization was evident when roots were examined under the light microscope. Although all of the autotrophic nitrifying bacteria belong to the family Nitrobacteraceae, there are heterotrophic organisms such as fungi in the *Aspergillus* and *Penicillium* genera as well as other bacteria that are capable of oxidizing both ammonium and nitrite in laboratory culture solutions. Whether or not these organisms are of significance in nature is a matter of debate (Haynes, 1986). In the studies presented here, the identity of the organisms is less important than the observation that nitrifying bacteria tend to grow and metabolize best when associated with surfaces such as roots as opposed to floating free in solution (Keen and Prossier, 1987). Keen and Prossier (1987) also noted that colonies of nitrifying bacteria examined by scanning EM were covered by a slime material perhaps serving a protective role in less than optimal environments. Because nitrifying organisms are associated with roots, frequent replacement of the nutrient solution with fresh solution may not significantly reduce the problem of nitrification in ammonium-based solution culture. The initial contamination of the solution culture systems most probably occurred from dust and soil particles common to the greenhouse environment.

Nitrifying bacteria are obligate aerobes and are sensitive to the relatively low oxygen tensions found in nutrient solution culture. However, the use of nitrifying bacteria in submerged fixed-film bioreactors for removal of nitrate in waste water treatment (Al-Haddad et al., 1991) indicates that submerging roots in nutrient solution would not eliminate the activity of these obligate aerobes.

The accumulation of nitrate in the roots and shoots of the maize and pea seedlings grown in the ammonium-based nutrient solution is viewed as clear evidence for the conversion of ammonium to nitrate in the external solution and the absorption of nitrate into the plant. However, nitrate was detected in nitrogen-starved, sterile *Chlorella* culture and in sterile-grown soybean seedlings (Kessler and Oesterheld, 1970; Funkhouser and Garay, 1981). Under stressed conditions, plants may have a mechanism for oxidizing ammonium to nitrate. For the experiments reported here, the plants gave no indication of nitrogen stress, and we assume that the accumulation of nitrate in tissues resulted exclusively from absorption of nitrate from the root apoplast.

We observed that nitrate was accumulated in root tissues before it was detected in the external solution, and the greatest accumulation of nitrate in tissues occurred for the hydroponic culture systems, which exhibited barely detectable solution nitrate levels until the end of the experiment (Figs. 1 and 3). We conclude from these results that at the early stages of colonization of roots by nitrifying bacteria, the nitrate was absorbed from the root apoplast before it accumulated to sufficient levels to alter bulk (80–120 L) solution concentration. As root colonization progressed, the conversion of ammonium to nitrate exceeded absorption by root cells and the concentration in the nutrient solution reached detectable levels. It has been demonstrated that pea roots can deplete nutrient solutions down to 1 to 5 μM nitrate (Oscarson et al., 1989). One micromolar is the approximate detection limit of the Continuous Flow Analyzer as used in this research to measure nitrate.

Differences in the pattern of accumulation of nitrate in

shoots of maize and pea (Fig. 4) are the result of well-established differences in the regulation of nitrate reduction in these species. Temperate legumes such as pea are largely dependent on root assimilation at low nitrate levels (Andrews, 1986). As nitrate levels increase, transport of nitrate to the shoot for assimilation increases. Hence, for pea, we observed a lag in accumulation of nitrate in shoots (Fig. 4). Maize, on the other hand, carries out a larger portion of nitrate assimilation in the shoot, which is independent of external nitrate concentration. A concurrent appearance of nitrate in the roots and shoots would be expected for maize seedlings regardless of the nitrate level in the nutrient solution (Figs. 3 and 4).

Experiments have been conducted to determine if the nitrification inhibitor nitrapyrin (Huber et al., 1977; Feng and Barker, 1989) would prevent the apparent oxidation of ammonium to nitrate in solution culture. Addition of nitrapyrin to the nutrient solution at 9 mg/L did not result in a significant decrease in nitrate accumulation in the hydroponic culture solution or in the tissues of maize seedlings (data not shown). The absence of an effect may be related to technical difficulties in maintaining the solubility of nitrapyrin in solution leading to uncertainties about whether or not the inhibitor is in contact with nitrifying organisms in sufficient concentration to be effective. This research is continuing with all three solution culture systems to which known quantities of *Nitrosomonas* and *Nitrobacter* have been added.

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