ABSTRACT

Since N assimilation is one of the most costly functions of a plant, its efflux before assimilation results in a serious energy cost and loss in efficiency which could decrease yields. Efficient crop production is critical to CELSS. Our objective is to determine the extent of efflux of the N species NO$_3^-$, NH$_4^+$, NO$_2^-$, and urea after uptake, and possible means of regulation. We found that NO$_3^-$ efflux became serious as its substrate level increased. Efflux/Influx (E/I) of NO$_3^-$ was greater in darkness (35%) than in light (14%) and the ratio greatly increased with increased substrate NO$_3^-$ (up to 45% at 10 mM). It seems advantageous to use the lowest possible nutrient concentration of NO$_3^-$. The feasibility of using ClO$_3^-$ as a trapping agent (competitive inhibitor of NO$_3^-$ uptake) for effluxed NO$_3^-$ was assessed and its toxicity determined.

INTRODUCTION

Crop production during extended space flights requires the development of procedures leading to the optimum use of the available energy. Optimizing the utilization of N by crop plants represents an area where significant progress can be made in the CELSS program.

It is well recognized that ion uptake requires ATP and electron flow. The cost of NO$_3^-$ assimilation is particularly high not only due to the energy requirement of uptake but also because of the need for 10 electrons to reduce it to the level of glutamate. In addition are the costs of maintaining pH balance, since each reduction of NO$_3^-$ to the level of NH$_4^+$ produces an OH$^-$. Thus it is not surprising that estimates of respiratory costs of ion absorption range up to 50% of total root respiration and 20% of total plant respiration (1).

Recent reports in the literature indicate that NO$_3^-$ efflux can begin very rapidly after its influx (2,3,4,5,6). In addition, prolonged leakage of NO$_3^-$ occurs from root storage pools dependent upon the N-status of the roots.
Lee and Clarkson (6) estimated that efflux, at external concentrations above 1 mM, could account for up to 40% of the influxed NO$_3^-$.

Thus NO$_3^-$ efflux could represent a significant additional cost to an already energy costly assimilatory pathway. Little is known concerning the regulation of NO$_3^-$ efflux. Recent work of Briskin (12) showed evidence that efflux of NO$_3^-$ utilizes ATP, which could greatly add to the energy cost of NO$_3^-$ assimilation.

Measurement of NO$_3^-$ efflux has been hampered by the lack of easy, rapid, and less costly analytical techniques for detecting the very low concentrations involved in a process with a half-life (t$_{1/2}$) of minutes. The use of $^{15}$N as a tracer is laborious and has problems of sensitivity. $^{13}$N has only a 10 min half-life, must be used at the site of generation (cyclotron), and is very costly to use. We developed an HPLC method which has the sensitivity, ease and low cost required. In addition, we evaluated ClO$_3^-$ as a trapping agent for NO$_3^-$.

This report presents results estimating NO$_3^-$ influx, efflux, and net uptake across several mechanisms of NO$_3^-$ uptake.

MATERIALS AND METHODS

Plant growth. Wheat seedlings were grown hydroponically in a 1/4 strength Hoagland's solution for 8 days in an environmentally controlled growth chamber at 400 µE/m$^2$sec, at 18°C, and 80% relative humidity (13,14). On the 7th day they were transferred into 1/4 strength Hoagland's (loading solution) containing NO$_3^-$ at the concentration to be used in the efflux study (specified in each experiment).

Measurement of NO$_3^-$ efflux. After removal from the loading solution, 10 seedlings were placed in 300 ml of the efflux solution for various periods of time (specified below) containing 0.06 mM Pi at pH 5.8, 0.2 mM CaSO$_4$, and with or without ClO$_3^-$ at the specified concentrations. The seedlings were rinsed
for 2 sec in 300 ml of efflux solution, then placed in 60 ml of efflux solutions for the following times: 10 sec, 30 sec, 1, 2, 5, 10, 15, and 20 min and the amount of NO$_3^-$ released at each time from the roots was determined.

**Measurement of NO$_3^-$ and ClO$_3^-$.** These compounds were measured by HPLC as described previously for NO$_3^-$ (13,14). ClO$_3^-$ was also measured at 210 nm with a UV monitor.

**Uptake rates of NO$_3^-$ and ClO$_3^-$.** Uptake rates were determined as previously described by determining rates of depletion of NO$_3^-$ from substrate solutions, then fitting the rate curves to best fit curves by polynomial analysis using a computer (13).

**RESULTS**

**Mechanisms of NO$_3^-$ uptake.** The results in Figures 1 and 2 show several mechanisms for NO$_3^-$ uptake. Uptake as a function of NO$_3^-$ concentration can be determined by either step up or step down, on continuous depletion experiments; Figures 1 and 2 are the results of continuous depletion experiments. In Figure 1, one mechanism is readily seen between about 0.2 and 0.7 mM. This is commonly referred to as Mechanism I in the literature (14). The rates above 0.7 mM are largely undefined but are referred to in the literature as Mechanism II. Another mechanism is indicated in Figure 1 at concentrations below 0.1 mM and it is readily seen when the data are plotted between 0 and 0.1 mM (Fig. 2).

**Comparison of uptake of NO$_3^-$ and ClO$_3^-$.** The comparative uptake of NO$_3^-$ and ClO$_3^-$ is shown in Figures 3 and 4 at initial concentrations of 0.5 and 1 mM. Wheat plants deplete the NO$_3^-$ concentration very efficiently to near zero (Fig. 1), whereas depletion of ClO$_3^-$ is not straightforward. The ability to take up ClO$_3^-$ is continuously lost with time (Fig. 4).

**Toxic effects of ClO$_3^-$ on NO$_3^-$ uptake.** The increasingly toxic effects of ClO$_3^-$ on NO$_3^-$ uptake with time is seen in Table 1.
Comparative effects of pretreatments of ClO$_3^-$ and NO$_3^-$ on their uptakes. The comparative effects of pretreatments of ClO$_3^-$ and NO$_3^-$ on their uptakes are shown in Table 2. Pretreatments varying in time had little effect on subsequent uptake of NO$_3^-$, whereas increasing time of pretreatments greatly decreased ClO$_3^-$ uptake.

ClO$_3^-$ as a competitive inhibitor of NO$_3^-$ uptake. Double reciprocal plots of rates vs concentrations show evidence that ClO$_3^-$ is a competitive inhibitor of NO$_3^-$ uptake (Fig. 5).

NO$_3^-$ efflux. Figure 6 shows a typical example of a determination of NO$_3^-$ efflux. Our results matched quite closely those reported in the literature showing two different early losses of NO$_3^-$, one with a $t_{1/2}$ of less than 10 sec and another with a $t_{1/2}$ of minutes. After one min, the second set of rates approximate an apparent first order reaction. Extrapolation to $t_0$ gives an estimate of the rate of NO$_3^-$ efflux. At a concentration of 1 mM NO$_3^-$, efflux varied consistently between 2.0 and 2.5 $\mu$mol/gxh.

Effect of increasing concentrations of NO$_3^-$ and ClO$_3^-$ on NO$_3^-$ efflux. Efflux in the presence of increasing concentrations of NO$_3^-$ and ClO$_3^-$ is shown in Figure 7. As expected, efflux increased with increasing concentrations of NO$_3^-$ and ClO$_3^-$.

Influx, efflux, and net uptake of NO$_3^-$.

Influx, efflux, and net uptake of NO$_3^-$.

Effect of light and dark on influx, efflux, and net uptake of NO$_3^-$.

NO$_3^-$ efflux was similar in plants in darkness and in light; however, influx and net uptake were much greater in light (Table 4). Thus, in darkness 35% of the influxed NO$_3^-$ was effluxed, while in light the proportion effluxed was reduced to 14%.
DISCUSSION

It is important to continue to develop information concerning the mechanisms of uptake of nutrient ions both to understand the reactions of the plants to changing concentrations and also for planning optimum concentrations of nutrient solutions for maximum efficiency.

Mechanisms of NO$_3^-$ uptake. At least three mechanisms of NO$_3^-$ uptake are present, one between 0 and about 0.05 to 0.08 mM with a $K_m$ of ca 0.012 to 0.018 mM, and another between 0.1 and about 0.7 mM with a $K_m$ of ca 0.025 to 0.04 mM (Figs. 1 and 2). The latter is the typical mechanism reported in the literature (14). At concentrations above 1 mM, the mechanisms are largely undefined and are difficult to determine because efflux becomes such an important component (discussed below).

Comparison of uptake of NO$_3^-$ and ClO$_3^-$. In a determination of NO$_3^-$ efflux, very low concentrations of NO$_3^-$ are present in the external solution. At these low levels of NO$_3^-$ (see Fig. 3), the wheat plant can very efficiently absorb the NO$_3^-$ as it is effluxed into the external solution. Hence, ClO$_3^-$ has been used as a trapping agent for the effluxed NO$_3^-$. Although much work has been reported on the effects of ClO$_3^-$ on NO$_3^-$ uptake, the analytical procedures reported to separate ClO$_3^-$ from NO$_3^-$ in the solutions were in some cases non specific, i.e., ion electrodes (3). Radioactive ClO$_3^-$ was also used (3) which presents problems of low specific activity and sticking to glassware. We developed an HPLC method which effectively separates NO$_3^-$ from ClO$_3^-$ and both can be measured simultaneously. In addition, much of the reported literature did not discriminate between kinetic effects and toxic effects of ClO$_3^-$ on NO$_3^-$ uptake (15).

Toxic effects of ClO$_3^-$. The results showed that toxicity symptoms, as shown by decreased rates of uptake of NO$_3^-$ and ClO$_3^-$, were apparent after 1 h (Fig. 4, Tables 1 and 2). Toxic effects of ClO$_3^-$ were greater toward its own
uptake than towards NO$_3^-$ uptake. We found that little toxicity occurred during the 20 min period of the efflux experiments.

CI$_3^-\text{O}$ as a competitive inhibitor of NO$_3^-$ uptake. The results verified that CI$_3^-\text{O}$ was a competitive inhibitor of NO$_3^-$ uptake as earlier reported (3) (Fig. 5). Apparently the NO$_3^-$ transporter discriminated effectively between NO$_3^-$ and CI$_3^-\text{O}$ since proportionately much larger concentrations of CI$_3^-\text{O}$ were required to inhibit NO$_3^-$ uptake. In summary, CI$_3^-\text{O}$ could be used as a trapping agent for effluxed NO$_3^-$ for short time experiments.

NO$_3^-$ efflux. The method for determining efflux was based on some excellent work done by Lee and Clarkson (6) and Shone and Flood (16) who used cereal roots to determine the kinetic parameters required to measure efflux of NO$_3^-$. They found that the combined $t_{1/2}$ of release of NO$_3^-$ in the surface film attached to the roots from the external solution and from the root free space was ca 7 sec. In addition, their results showed that the $t_{1/2}$ for cytoplasmic release of NO$_3^-$ was ca 4 min. A semilog plot of efflux rate vs time resulted in a linear regression line after 1 min, since at that time the first two kinetic parameters had passed through ca 9 half-lives. In our results, the relative contributions of these parameters would be very small in relation to the amount of NO$_3^-$ efflux. The rates of efflux we measured are similar to those reported for cereals by workers using $^{13}$N-NO$_3^-$ at NO$_3^-$ concentrations of 1.5 to 5 mM (3,4,6).

**Effect of increasing concentrations NO$_3^-$ and CI$_3^-\text{O}$ on NO$_3^-$ efflux.** As expected, efflux increased with increasing concentrations of NO$_3^-$ (Fig. 7). In the region of Mechanism II of uptake (0.2 mM), efflux was a significant deterrent to N use efficiency. Here 15% of the NO$_3^-$ influxed was effluxed. Net NO$_3^-$ uptake remained quite constant as external NO$_3^-$ increased beyond 0.2 mM. This occurred because, although influx increased, efflux correspondingly increased. The ratio of efflux/influx, expressed as a percentage, increased up to 45% at 10 mM NO$_3^-$. These results help explain results from Raper's
laboratory which showed that net uptake changed little as \( \text{NO}_3^- \) concentrations increased to high levels (11). These workers also presented evidence for significant levels of efflux at increasing concentrations of \( \text{NO}_3^- \).

**Effect of light and dark on influx, efflux, and net uptake of \( \text{NO}_3^- \).**

Since efflux is a function of the concentration of \( \text{NO}_3^- \) in the cytoplasm, light and dark periods had a profound effect on the ratio of efflux/influx (Table 4). Although efflux was the same in light or dark, influx was greatly increased in light along with the rate of \( \text{NO}_3^- \) reduction (17,18). Therefore, the relative percentage of \( \text{NO}_3^- \) effluxed is much less in light.

**Effect of increasing concentrations \( \text{ClO}_3^- \) on \( \text{NO}_3^- \) efflux.** Higher rates of \( \text{NO}_3^- \) efflux were detected as \( \text{ClO}_3^- \) increased in the efflux solution (Fig. 7). This could be because of \( \text{ClO}_3^- \) serving as a trapping agent or because of an unknown effect. The increase in efflux rate with 5 and 10 mM \( \text{ClO}_3^- \) was sufficiently large to be somewhat uncertain. We are currently evaluating this result.

**Effect of \( \text{NH}_4^+ \) on \( \text{NO}_3^- \) efflux.** The literature is confusing on this issue with results of little if any effect of \( \text{NH}_4^+ \) on \( \text{NO}_3^- \) efflux (5,6), and of large effect (4). We have not yet examined the interactions of the different N species on \( \text{NO}_3^- \) efflux.

**Ramifications.** As the concentration of \( \text{NO}_3^- \) increases in the external solution, efflux becomes an increasingly important energy cost to the plant. Not only is energy needed for \( \text{NO}_3^- \) influx, it now appears that \( \text{NO}_3^- \) efflux may utilize ATP (12). This results in an almost doubling of the cost of absorption at higher concentrations of \( \text{NO}_3^- \). On this basis, it would seem that a nutrient solution for crop growth in CELSS should be optimized at the lowest concentrations possible. In addition, it is increasingly critical to determine the regulation of efflux of N compounds since it is not known how \( \text{NO}_3^- \), \( \text{NH}_4^+ \), \( \text{NO}_2^- \), and urea influence each others efflux. Will total efflux
decrease if the total concentration of N in the nutrient solution is made up of the four species instead of only NO$_3^-$ and NH$_4^+$?
REFERENCES


3. Deane-Drummond CE, ADM Glass 1983a Short-term studies of nitrate uptake into barley plants using ion-specific electrodes and $^{36}$ClO$_3^-$. I. Control of net uptake by NO$_3^-$ efflux. Plant Physiol 73:100-104

4. Deane-Drummond CE, ADM Glass 1983b Short-term studies of nitrate uptake into barley plants using ion-specific electrodes and $^{36}$ClO$_3^-$. Regulation of NO$_3^-$ efflux by NH$_4^+$. Plant Physiol 73:105-110


Table 1. Effect of pretreatment of ClO$_3^-$ on NO$_3^-$ uptake. The initial concentration of substrate NO$_3^-$ was 0.6 mM.

<table>
<thead>
<tr>
<th>Hours Pretreatment</th>
<th>[ClO$_3^-$] 1 mM</th>
<th>NO$_3^-$ Uptake (µmol/gxh) 5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>1</td>
<td>6.2</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>5.3</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>4.9</td>
<td>2.3</td>
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</table>
Table 2. Effect of pretreatment of ClO$_3^-$ or NO$_3^-$ on their uptakes.

<table>
<thead>
<tr>
<th>Hours Pretreatment</th>
<th>NO$_3^-$ Uptake (at 1 mM) (μmol/gxh)</th>
<th>ClO$_3^-$ Uptake (at 1 mM) (μmol/gxh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.3</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>6.8</td>
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Table 3. Efflux, net uptake and influx of NO$_3^-$ See Materials and Methods for procedures.

<table>
<thead>
<tr>
<th>[NO$_3^-$] (mM)</th>
<th>Efflux</th>
<th>Net Uptake</th>
<th>Influx</th>
<th>Efflux Influx</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
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<td>6.5</td>
<td>7.6</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>2.5</td>
<td>8.5</td>
<td>11.0</td>
<td>23</td>
</tr>
<tr>
<td>10.0</td>
<td>7.7</td>
<td>9.5</td>
<td>17.2</td>
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</table>
Table 4. Effect of light and dark treatments (24 h) on NO$_3^-$ efflux, net uptake and influx. See Materials and Methods for procedures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Efflux (μmol/gxh)</th>
<th>Net Uptake</th>
<th>Influx (μmol/gxh)</th>
<th>Efflux %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light*</td>
<td>1.3</td>
<td>7.9</td>
<td>9.2</td>
<td>14</td>
</tr>
<tr>
<td>Dark</td>
<td>1.0</td>
<td>1.8</td>
<td>2.8</td>
<td>35</td>
</tr>
</tbody>
</table>

*plants induced in 0.2 mM NO$_3^-$ for 24 h light or dark.
FIGURE LEGENDS

Fig. 1. NO₃⁻ uptake from 0 to 1000 µm. See Materials and Methods for procedures.

Fig. 2. NO₃⁻ uptake from 0 to 100 µm. See Materials and Methods for procedures.

Fig. 3. Depletion of NO₃⁻ from 0.5 and 1.0 mM substrate solutions. See Materials and Methods for procedures.

Fig. 4. Depletion of ClO₃⁻ from 0.5 and 1.0 mM substrate solutions. See Materials and Methods for procedures.

Fig. 5. ClO₃⁻ as a competitive inhibitor of NO₃⁻ uptake. See Materials and Methods for procedures.

Fig. 6. Semilog plot of NO₃⁻ efflux vs time. See Materials and Methods for procedures.

Fig. 7. Effect of ClO₃⁻ on NO₃⁻ efflux. See Materials and Methods for procedures.