Effects of Atmospheric NO₂ on Azolla–Anabaena Symbiosis

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Cultures of the water fern *Azolla pinnata* R. Br. exposed for 1 week to atmospheric NO₂ (50, 100 or 200 nl l⁻¹) induced additional levels of nitrate reductase (NAR) protein and nitrite reductase (NiR) activity. At low concentrations of NO₂ (50 nl l⁻¹), nitrate derived from NO₂ provides an alternative N source for *Azolla* but does not affect rates of acetylene reduction. However, the symbiotic relationship between *Azolla* and its endosymbiont, *Anabaena azollae*, is only affected adversely by high concentrations (100 and 200 nl l⁻¹) of atmospheric NO₂. The resultant decrease in rate of growth, nitrogen fixation, heterocyst formation, and overall nitrogen cycling are probably due to the additional accumulation of N products derived from higher levels of atmospheric NO₂. Parallel increases in levels of polyamines suggest that *Azolla* partially alleviates these harmful effects by incorporating some of the extra NO₂-induced N into polyamines.

**Key words:** *Azolla–Anabaena* symbiosis, nitrogen dioxide pollution, nitrogen metabolism, polyamines.

INTRODUCTION

Nitrogen oxides (NO and NO₂) are major atmospheric pollutants emitted in combustion gases and amounts of both are projected to increase globally despite various national control measures; roughly doubling over present emissions by the year 2025 (MacKenzie and El-Ashry, 1988). Ambient concentrations of NO₂ in air over rural areas of Europe are normally below 50 nl l⁻¹ but well above global background levels of 20 nl l⁻¹ (Robinson and Robbins, 1972). NO₂ reacts with unburnt hydrocarbons in bright sunlight to form phytotoxic O₃, but it is itself also phytotoxic to plants although less so than SO₂, O₃, or NO (Wellburn, 1988, 1990). Visible plant injury does not normally occur at low concentrations of NO₂ but poorer growth and productivity are frequently observed (EPA, 1991).

The ability to reduce and incorporate nitrate into organic compounds is almost universal among higher plants (Runge, 1983). Both reduced and oxidized forms of N are taken up by plant roots, but dissolution of atmospheric NO₂ in the apoplast of mesophyll cells also produces nitrate (Zeevaart, 1976) and this may be an additional source of plant N. Indeed, assimilation of ¹⁵NO₃⁻ into organic N has been demonstrated in both beans (Rogers, Campbell and Volk, 1979) and spinach (Yoneyama and Saskawa, 1979). Furthermore, levels of NaR (EC 1.6.6.2) activity are enhanced after exposure of several plant species to atmospheric NO₂ (Zeevaart, 1974; Winesle et al., 1987).

*Azolla pinnata* R. Br. is a small aquatic fern containing a symbiotic N₂-fixing cyanobacterium, *Anabaena azollae* (sp.) which provides the association with its total N requirement (Peters, 1977) and this allows *Azolla* to grow in relatively low N environments. It has also proved to be an interesting lower plant system for study of the effects of atmospheric pollutants like SO₂ because large amounts of uniform study material are available (Hur and Wellburn, 1993). There are as yet no reports of the effects of atmospheric NO₂ on the symbiosis between *Azolla* and *Anabaena*, but the process of N₂-fixation is already known to be more sensitive to atmospheric SO₂ than, for example, photosynthesis (Hällgen and Huss, 1975; Richardson and Nieboer, 1983). Unfortunately, previous studies of the effects of NO₂ on N₂ fixation conflict with each other. Atmospheric NO₂ is claimed to inhibit nitrogenase activity in *Phaseolus* nodules (Srivastava and Ormrod, 1986) but to increase specific root nodule activity in soya (Gupta and Narayanan, 1992).

The experiments described here describe the effects of ambient and above ambient levels of atmospheric NO₂ on the symbiotic relationship between *Azolla* and *Anabaena* in terms of nitrogen metabolism in order to elucidate those mechanisms involved in NO₂ toxicity as it relates to N₂-fixation and, if possible, resolve conflicting reports already in the literature.

MATERIALS AND METHODS

*Plant material and fumigation conditions*

Culture and growth conditions for *Azolla* have been described earlier (Hur and Wellburn, 1993). Briefly, *Azolla pinnata* free of contaminating epiphytic microorganisms was grown at 23 °C (day) or 18 °C (night) in 250 ml flasks containing 100 ml of Hoagland's solution (minus N plus double phosphate amounts) and added micronutrients in an exposure system previously described by Hur and Wellburn (1993). Filters of either activated charcoal or Purafect™ or...
"dummy" glass beads of similar porosity were fitted to exposure flasks so that five clean-air control (filtered) and five exposure (fumigated) treatments could stand side-by-side at the same temperature and in the same light flux (230 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), 16 h light, 8 h dark cycle) in the exposure system. Concentrations of NO\(_2\) in the whole chamber were controlled by the use of a fine needle valve on the gas supply and monitored by an NO\(_2\) Analyser (Monitor Labs., USA, Model 8840). Rafts of Azolla were exposed to three different concentrations of NO\(_2\) (50, 100 and 200 nl l\(^{-1}\)) for 1 week each.

**Enzyme and other assays**

*In vitro* levels of NADH–NaR and NiR activities were assayed by the method of Wray and Fido (1990) after grinding of Azolla fronds (0·5 g frozen weight) in 5 ml of Tris-HCl buffer (pH 7·5) containing 50 mm Tris, 10 mm EDTA, 10 mm 2-mercaptoethanol, and 10% (v/v) glycerol. Homogenized material was then ultrasonicated at 18 mm s\(^{-1}\) for 10 s in ice and centrifuged at 20000 g for 15 min at 4 °C. Levels of glutamine synthetase (GS) activity were assayed in the supernatant by the method of Lea et al. (1990) and NADH-dependent glutamate dehydrogenase (GDH) by the modified method of Singh and Srivastava (1983).

Levels of NaR proteins were determined semi-quantitatively using ELISA upon extracts prepared by grinding of Azolla fronds (0·2 g f. wt) in 1·2 ml of extraction buffer (50 mm Tris-HCl; 0·2 m sucrose; 10 mm NaCl; 0·1% NaN\(_3\); 1 mm PMSF; 14 mm 2-mercaptoethanol, pH 8·0). The modified ELISA method of Whitford, Fido and Nottton (1987) was used but the order of the assay was changed with the monoclonal antibody being placed on the plate first.

Determination of mean relative growth rate, heterocyst frequency, N\(_2\)-fixation by ethylene reduction, and protein content were carried out according to the methods already described by Hur and Wellburn (1993).

**Polyamine determination**

Azolla samples (100 mg frozen weight) were homogenized in 2 ml ice cold solution of 5% perchloric acid (PCA) containing 1·5 mm hexamethidine (internal standard, 50 μl in 10 ml 5% PCA). Homogenized material was sonicated as above, placed in ice for 1 h, and the supernatant after centrifugation at 10000 g for 10 min was danylized using a modified version of Coghill and Walters (1990). In this, the supernatant (0·2 ml) was added to a saturated Na\(_2\)CO\(_3\) to bring the pH to 10. Samples were then mixed with 0·4 ml dansyl chloride (7·5 mg ml\(^{-1}\) acetone) and incubated in the dark at 60 °C for 1 h. Excess dansyl chloride was converted to dansylproline by a 30 min incubation with 0·1 ml proline (100 mg ml\(^{-1}\) distilled water) after which the danylized polyamines were extracted in 0·5 ml heptane. Separation of polyamines was carried out by HPLC (Kratos dual T-414 pump system) gradient elution using 30 sec of 60% solvent A (100% methanol) and 40% solvent B (ultrapure-water), rising to 95% A over the next 9·5 min, followed by 97·5% of solvent A maintained for 1·5 min before recycling. The flow rate in the 230 mm ODS2 column (Anachem, 5 μm, 0·5 mm i.d.) remained at 3 ml min\(^{-1}\) throughout. Fluorescence of the eluted danylized polyamines was measured by a fluorescence spectrophotometer (Kratos FS 950) with excitation at 338 nm and emission at 455 nm.

**Statistical analysis**

There were three concentrations of NO\(_2\) (50, 100 and 200 nl l\(^{-1}\), each repeated three times), two treatments (control and fumigation) for each concentration, and five replicate flasks per treatment. Whole Azolla fronds in individual flasks were taken as the experimental unit and the data at each concentration were evaluated using ANOVA (Fisher and Yates, 1963).

**RESULTS**

**Effects of NO\(_2\) on the growth of Azolla**

Azolla fronds exposed to low levels of NO\(_2\) (50 nl l\(^{-1}\)) showed a slight increase in growth rate compared to clean-air controls (Table 1) but exposure to higher concentrations significantly reduced growth of Azolla by 7% at 100 nl l\(^{-1}\) and 23% at 200 nl l\(^{-1}\) NO\(_2\).

**Effects of NO\(_2\) on N\(_2\)-fixation rate (C\(_3\)H\(_4\) reduction rate) and numbers of heterocysts in symbiotic Anabaena**

Low levels of NO\(_2\) (50 nl l\(^{-1}\)) had no significant effect on rates of N\(_2\) fixation (Table 1) but Azolla exposed to either 100 and 200 nl l\(^{-1}\) NO\(_2\) showed significant reductions in rates of ethylene reduction (15·1 and 37·9%, respectively, compared to controls).

Exposure to 50 nl l\(^{-1}\) NO\(_2\) slightly increased heterocyst frequency (Table 1) but higher concentrations of NO\(_2\) significantly inhibited heterocyst development. The observed frequency of heterocysts in non-polluted *Anabaena* was in the range of 22·2–23·6%, a value which is similar to that previously reported by Peters (1975).

**Effects of NO\(_2\) on nitrogen assimilation**

Exposure to NO\(_2\) (50 and 100 nl l\(^{-1}\)) significantly increased the levels of NiR activity but not those of the later N-assimilation enzymes (Table 2). Although the levels of NaR activity could not be detected in Azolla by the usual colorimetric methods for *in vitro* NaR assay, ELISA assays (Table 3) clearly demonstrated that Azolla contains considerable levels of NaR proteins and that atmospheric NO\(_2\) significantly increased amounts of these NaR proteins.

Increases in levels of NiR activity were more pronounced at 50 nl l\(^{-1}\) NO\(_2\) (50% increase) than at 100 nl l\(^{-1}\) (14% increase) but changes in ammonium contents were not significant (Table 2). Higher concentrations of NO\(_2\) significantly reduced protein content (14.3% at 100 nl l\(^{-1}\) and 31.7% at 200 nl l\(^{-1}\); Table 1) which indicate that the overall N metabolism of Azolla is adversely affected by NO\(_2\).
Table 1. Effects of atmospheric $\text{NO}_2$ on mean relative growth rates, $\text{C}_4\text{H}_2$ reduction rates (in order to compare $\text{N}_2$ fixation rates), heterocyst frequencies, and total soluble protein contents of Azolla and Anabaena

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NO$_2$ Fumigation (nl l$^{-1}$)</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Mean relative growth rates (d$^{-1}$)</td>
<td>0.098 ± 0.004 103</td>
<td>0.093 ± 0.004 93</td>
<td>0.088*** ± 0.005 772</td>
<td></td>
</tr>
<tr>
<td>$\text{C}_4\text{H}_2$ reduction rates (mmol g$^{-1}$ f.w. h$^{-1}$)</td>
<td>1.51 ± 0.07 944</td>
<td>1.35 ± 0.05 849</td>
<td>0.95*** ± 0.06 62.1</td>
<td></td>
</tr>
<tr>
<td>Heterocyst frequencies (%)</td>
<td>22.4 ± 0.20 101</td>
<td>22.9 ± 0.20 97</td>
<td>18.8*** ± 0.21 82.8</td>
<td></td>
</tr>
<tr>
<td>Protein contents (mg g$^{-1}$ f.w.)</td>
<td>4.47 ± 0.19 103</td>
<td>4.08 ± 0.15 857</td>
<td>3.10*** ± 0.71 68.3</td>
<td></td>
</tr>
</tbody>
</table>

† Relative to controls. The data are the means of $n$ replicates ± standard errors of the mean, where $n$ = 5 (growth rate), 20 (acetylene reduction), 150 (heterocyst frequency) and 15 (protein content). The asterisks indicate significant differences in $P$ values between control and fumigation of * < 0.05 and *** < 0.001.

Table 2. Effects of NO$_2$ on levels of enzyme activity associated with nitrogen assimilation and NH$_4^+$ content during symbiosis between Azolla and Anabaena

<table>
<thead>
<tr>
<th>Activity or Product</th>
<th>50 nl l$^{-1}$ NO$_2$</th>
<th>100 nl l$^{-1}$ NO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean-air controls</td>
<td>Fumigated</td>
</tr>
<tr>
<td>NiR†</td>
<td>1.34 ± 0.07</td>
<td>2.01* ± 0.18</td>
</tr>
<tr>
<td>GS†</td>
<td>54.4 ± 5.4</td>
<td>59.4 ± 2.5</td>
</tr>
<tr>
<td>GDH§</td>
<td>25.2 ± 0.84</td>
<td>28.1 ± 1.49</td>
</tr>
<tr>
<td>NH$_4^+$¶</td>
<td>6.34 ± 5.4</td>
<td>5.61 ± 0.38</td>
</tr>
</tbody>
</table>

† µmoles NO$_2$ reduced mg protein$^{-1}$ h$^{-1}$; † µmoles γ-glutamylhydroxamate formed mg protein$^{-1}$ min$^{-1}$; § µmoles NADH oxidized mg protein$^{-1}$ f.w. The data are the means of five replicates, ± the standard errors of the means, and the asterisks indicate significant difference in $P$ values between filtered and polluted air where $P < 0.05$.

Table 3. Effects of NO$_2$ (100 nl l$^{-1}$ for 1 week) on the formation of nitrate reductase protein in Azolla as measured by ELISA assays using pea NaR antibodies

<table>
<thead>
<tr>
<th>Titer</th>
<th>Absorbance at 450 nm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Clean-air control</td>
<td>Fumigated</td>
</tr>
<tr>
<td>No dilution</td>
<td>1.62 ± 0.06</td>
<td>1.88 ± 0.06</td>
</tr>
<tr>
<td>1:1 (1 dilution)</td>
<td>1.11 ± 0.05</td>
<td>1.29* ± 0.02</td>
</tr>
<tr>
<td>1:3 (3 dilution)</td>
<td>0.90 ± 0.04</td>
<td>1.12** ± 0.04</td>
</tr>
<tr>
<td>1:7 (7 dilution)</td>
<td>0.87 ± 0.20</td>
<td>1.15** ± 0.04</td>
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</table>

Serial dilutions of the crude extracts (0.2g frozen weight in 1.2 ml extraction buffer) were carried out to find an optimum concentration of NaR protein in extracts from Azolla. Data are the means of five replicates ± standard errors of the mean. Asterisks indicate significant difference in $P$ value between filtered and polluted air where * < 0.05 and ** < 0.001.

Effects of NO$_2$ on polyamines of Azolla

Putrescine was significantly increased in Azolla exposed to 100 nl l$^{-1}$ NO$_2$ (Table 4) whereas spermine content was significantly reduced at 50 nl l$^{-1}$ NO$_2$. No significant changes in levels of spermidine or homospermidine (a polyamine found in Rhizobium bacteria and N$_2$-fixing cyanobacteria) could be detected.

Discussion

Most atmospheric NO$_2$ enters plants through stomatal apertures and enters the apoplast of mesophyll cells (Mansfield and Freer-Smith, 1981). Reaction of NO$_2$ with aqueous phases then forms mainly nitric acid which ionizes to nitrate and protons over the normal pH range of the apoplast (pH 5.5–7 according to Hartung, Radin and Hendrix, 1988). All studies of the subsequent cycling, partitioning and removal of NO$_2$-derived N products have indicated that the deposited N from atmospheric NO$_2$ is metabolized by the normal N assimilation pathway involving the enzymatic reduction of nitrate to nitrite to NH$_4^+$ before incorporation into amino acids using the GS/GOGAT pathway (Wellburn, 1990).

NaR protein is substrate-induced (Beevers and Hageman, 1969) and levels of activity are regulated by fresh enzyme synthesis and breakdown rather than by activation and inactivation of the original protein (Remmler and Campbell, 1986). Zeevaart (1974) first demonstrated induction of NaR
activity by NO₃ in peas grown only on an ammonium-based medium (like Azolla) but, apart from the studies of Srivastava and Ormrod (1986) and Gupta and Narayanan (1992) on legumes, there is no other information on the effects of NO₃ on N₂ fixation.

No signs of NaR activity in Azolla symbiosis could be detected by the in vitro colorimetric NaR assay of Wray and Fido (1990), which performs normally for cereals, legumes, lettuce, and Lemna. This suggests that either Azolla maintains very low levels of NaR or it exists naturally as an inactive form, or, more likely, some unidentified inhibitor interferes with the assay. Nevertheless, our ELISA studies clearly show that Azolla does contain detectable levels of NaR protein which are significantly increased by NO₃ exposure.

Fumigation with NO₃ also enhances levels of activity of NiR in Azolla. This may be a consequence of nitrite accumulation resulting from the increased levels of NaR protein in polluted Azolla similar to that in Pisum sativum (Gupta and Beevers, 1983). Wodziński, Labeda and Alexander (1977) have already demonstrated that cyanobacteria are very sensitive to nitrite which is formed by mixtures of NO₃ and NO. However, negative feedback of NH₄⁺ on both transcriptional control of NaR synthesis and overall N assimilation has been reported in bryophytes (Woodin and Lee, 1987; Padidam, Vankateswarlu and Johri, 1991). Therefore, in the long term, accumulation of NH₃ and NH₄⁺ derived from NO₃ probably exceeds the capacity of bryophytes to remove these products.

In the Azolla–Anabaena system, assimilation of NH₃ by the endosymbiotic is regulated by the host and N₂ fixed by Anabaena is transferred to Azolla where it is subsequently metabolized via the GS/GOGAT pathway (Braun–Howland and Nierzwicki-Becker, 1990). Therefore, it is vital that any negative feedback effects of extra NO₃-derived NH₃ and NH₄⁺ on N assimilation are minimized if the symbiosis is to remain effective and for N₂ fixation to continue to operate. This is supported by our results concerning NH₃ assimilation, protein content, and N₂ fixation. Levels of ammonium, produced as consequence of NO₃-induced nitrate assimilation, are reduced at low concentrations of atmospheric NO₃ (50 nl⁻¹) and only accumulate at relatively high concentrations of NO₃ (100 and 200 nl⁻¹), partially because of reduced rates of NH₄⁺ assimilation through the GS/GOGAT cycle, and this eventually causes significant decreases in total protein content. These effects of higher levels of atmospheric NO₃ are also linked to the pronounced inhibition of N₂ fixation rates. The importance of NH₄⁺ and glutamine levels in regulating N₂ fixation is already known (Haselkorn, 1986). Consequently, excess NH₄⁺ arising from the higher levels of atmospheric NO₃ in Azolla probably acts as a signal to Anabaena to cut back N₂ fixation.

The NO₃-induced inhibition of N₂ fixation in Anabaena differs markedly from that caused by atmospheric SO₂. Very low levels of atmospheric SO₂ (< 25 nl⁻¹) lead to free radical mediated destruction of the barrier to O₂ diffusion afforded by the heterocyst envelope which rapidly shuts down nitrogenase activity and nitrogen assimilation (Hurl and Wellburn, 1993). On the other hand, it is quite evident that atmospheric NO₃ provides an additional source of N which eventually closes down N₂ fixation only if the NO₃ levels are high enough (i.e. ≥ 100 nl⁻¹). This, in part, may explain the reported decreases (Srivastava and Ormrod, 1986) and increases (Gupta and Narayanan, 1992) of N₂ fixation in root nodules of legumes when exposed to atmospheric NO₃. However, as the latter authors make no reference to the former contribution, the situation in higher plants remains confused; especially as both nitrate and nitrite are known to interfere with the overall process of N₂ fixation in soy (Streeter, 1982) and symbiotic lichens (Hallbom and Bergman, 1983). Our results do suggest that low levels of NO₃ (50 nl⁻¹) may benefit Azolla while N₂ fixation still operates in Anabaena at these ambient levels of NO₃ (unlike SO₂) so that the extra NO₃-derived N may permit extra growth. Indeed, this ability of Azolla to use an alternative source of N derived from low levels of atmospheric NO₃ may be a significant general response for many species which fix N₂, especially those that occur in ecosystems that are naturally N-limited.

Additional polyamine formation by plants has been suggested as a possible means of absorbing additional N from atmospheric NO₃ (Wellburn, 1990). Although polyamine levels change in response to many environmental stresses including atmospheric O₃ and SO₂ (Smith, 1985; Rowland-Barnford et al., 1989; Langebartels et al., 1991), there are no reports of NO₃-induced changes in polyamine levels in lower (or higher) plants. Our results clearly show that NO₃ (100 nl⁻¹) increases levels of putrescine, a precursor for spermine and spermidine, which is already known to be affected by both N source and content (Prech, Klein and Jäger, 1978; Smith, 1985).

| Polyamines (nmoles g⁻¹ f. wt) | 50 nl⁻¹ NO₃ | | | 100 nl⁻¹ NO₃ | | |
|---|---|---|---|---|---|
| | Clean-air controls | Fumigated | | Clean-air controls | Fumigated | |
| Putrescine | 312.6 ± 20.1 | 320.5 ± 25.9 | | 318.8 ± 22.5 | 404.3 * * ± 17.2 |
| Spermine | 110.4 ± 25.8 | 43.7 * ± 5.4 | | 251.4 ± 48.2 | 197.8 ± 11.6 |
| Spermidine | 342.5 ± 20.1 | 327.4 ± 25.9 | | 390.1 ± 16.7 | 360.2 ± 25.1 |
| Homospermidine | 2553 ± 24.2 | 2710 ± 33.0 | | 2290 ± 10.9 | 2698.8 ± 21.4 |

The data are the means of five replicates ± the standard errors of the means, and asterisks indicate insignificant difference in P values between filtered and polluted air where * < 0.05 and ** < 0.01.
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Consequently, this study has several implications. Firstly, extra NO₂-derived N does not normally increase NH₄⁺ levels which would otherwise close down N₂ fixation and Azolla may even limit NH₄⁺ levels so that the symbiosis continues to operate at ambient levels of atmospheric NO₂. Secondly, any N arising from NO₂ at such levels is additional to that fixed and may also be used for growth and, finally, additional polynamine formation only appears when N throughput is exceeded at high levels of atmospheric NO₂.

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LITERATURE CITED


