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Effects of Atmospheric NO_2 on *Azolla–Anabaena* Symbiosis

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Cultures of the water fern *Azolla pinnata* R. Br. exposed for 1 week to atmospheric NO_2 (50, 100 or 200 nl l^{-1}) induced additional levels of nitrate reductase (NaR) protein and nitrite reductase (NiR) activity. At low concentrations of NO_2 (50 nl l^{-1}), nitrate derived from NO_2 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 NO_2. The resultant decreases 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 NO_2. Parallel increases in levels of polyamines suggest that *Azolla* partially alleviates these harmful effects by incorporating some of the extra NO_2-induced N into polyamines.

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

INTRODUCTION

Nitrogen oxides (NO and NO_2) 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 NO_2 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). NO_2 reacts with unburnt hydrocarbons in bright sunlight to form phytotoxic O_3 but it itself is also phytotoxic to plants although less so than SO_2, O_3, or NO (Wellburn, 1988, 1990). Visible plant injury does not normally occur at low concentrations of NO_2 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 NO_2 in the apoplast of mesophyll cells also produces nitrate (Zeevaart, 1976) and this may be an additional source of plant N. Indeed, assimilation of $^{15}NO_2$ 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 NO_2 (Zeevaart, 1974; Wingsle *et al.*, 1987).

Azolla pinnata R.Br. is a small aquatic fern containing a symbiotic N_2-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 SO_2 because large amounts of uniform study material are available (Hur and Wellburn, 1993). There are as yet no reports of the effects of atmospheric NO_2 on the symbiosis between *Azolla* and *Anabaena*, but the process of N_2-fixation is already known to be more sensitive to atmospheric SO_2 than, for example, photosynthesis (Hällgen and Huss, 1975; Richardson and Nieboer, 1983). Unfortunately, previous studies of the effects of NO_2 on N_2 fixation conflict with each other. Atmospheric NO_2 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 NO_2 on the symbiotic relationship between *Azolla* and *Anabaena* in terms of nitrogen metabolism in order to elucidate those mechanisms involved in NO_2 toxicity as it relates to N_2-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 microorganism 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 and Purafil™ or

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'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 μmol m^{-2} 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 μm 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 Notton (1987) was used but the order of the assay was changed with the monoclonal antibody being placed on the plate first.

Determinations 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 hexanediamine (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 dansylated using a modified version of Coghlan and Walters (1990). In this, the supernatant (0·2 ml) was added to a saturated Na_2CO_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 dansylated 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 dansylated 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_2H_2 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 NO_2 on mean relative growth rates, C_2H_2 reduction rates (in order to compare N_2 fixation rates), heterocyst frequencies, and total soluble protein contents of* Azolla *and* Anabaena

	NO_2 fumigation (nl l^{-1})					
	50		100		200	
Parameter	Value	%†	Value	%†	Value	%†
Mean relative growth rates (d^{-1})	0·098 ± 0·004	103	0·093* ± 0·004	93	0·088*** ± 0·005	77·2
C_2H_2 reduction rates (mmol g^{-1} f. wt h^{-1})	1·51 ± 0·07	94·4	1·35* ± 0·05	84·9	0·95*** ± 0·06	62·1
Heterocyst frequencies (%)	22·4 ± 0·20	101	22·9* ± 0·20	97	18·8*** ± 0·21	82·8
Protein contents (mg g^{-1} f. wt)	4·47 ± 0·19	103	4·08 ± 0·15	85·7	3·10*** ± 0·71	68·3

† 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

	50 nl l^{-} NO_2		100 nl l^{-1} NO_2	
Activity or Product	Clean-air controls	Fumigated	Clean-air controls	Fumigated
NiR†	1·34 ± 0·07	2·01* ± 0·18	1·39 ± 0·03	1·59* ± 0·06
GS‡	54·4 ± 5·4	59·4 ± 2·5	63·5 ± 2·7	56·0 ± 2·7
GDH§	25·2 ± 0·84	28·1 ± 1·49	21·0 ± 1·09	29·7 ± 3·71
NH_4^+‖	6·34 ± 5·4	5·61 ± 0·38	6·00 ± 0·60	6·24 ± 2·7

† μmoles NO_2^- reduced mg protein^{-1} h^{-1}; ‡ nmoles γ-glutamylhydroxamate formed mg protein^{-1} min^{-1}; § nmoles NADH oxidized mg protein^{-1} min^{-1}; ‖ μmoles g^{-1} f. wt. 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*

	Absorbance at 450 nm	
Titer	Clean-air control	Fumigated
No dilution	1·62 ± 0·06	1·68 ± 0·06
1 : 1 ($\frac{1}{2}$ dilution)	1·11 ± 0·05	1·29* ± 0·02
1 : 3 ($\frac{1}{4}$ dilution)	0·90 ± 0·04	1·18** ± 0·04
1 : 7 ($\frac{1}{8}$ dilution)	0·87 ± 0·05	1·15** ± 0·04

Serial dilutions of the crude extracts (0·2 g 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 ** < 0·01.

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_3 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

TABLE 4. *Effects of fumigation with NO_2 on polyamine content in* Azolla

Polyamines (nmoles g^{-1} f. wt)	50 nl l^{-1} NO_2		100 nl l^{-1} NO_2	
	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	118·4 ± 28·5	43·7* ± 9·4	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	255·3 ± 24·2	271·0 ± 33·0	229·0 ± 10·9	269·8 ± 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.

activity by NO_2 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 NO_2 on N_2 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 NO_2 exposure.

Fumigation with NO_2 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). Wodzinski, Labeda and Alexander (1977) have already demonstrated that cyanobacteria are very sensitive to nitrite which is formed by mixtures of NO_2 and NO. However, negative feedback of NH_4^+ 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 NH_3 and NH_4^+ derived from NO_2 probably exceeds the capacity of bryophytes to remove these products.

In the *Azolla–Anabaena* system, assimilation of NH_3 by the endosymbiont is regulated by the host and N_2 fixed by *Anabaena* is transferred to *Azolla* where it is subsequently metabolized via the GS/GOGAT pathway (Braun-Howland and Nierzwicki-Bauer, 1990). Therefore, it is vital that any negative feedback effects of extra NO_2-derived NH_3 and NH_4^+ on N assimilation are minimized if the symbiosis is to remain effective and for N_2 fixation to continue to operate. This is supported by our results concerning NH_3 assimilation, protein content, and N_2 fixation. Levels of ammonium, produced as consequence of NO_2-induced nitrate assimilation, are reduced at low concentrations of atmospheric NO_2 (50 nl l^{-1}) and only accumulate at relatively high concentrations of NO_2 (100 and 200 nl l^{-1}), partially because of reduced rates of NH_4^+ assimilation through the GS/GOGAT cycle, and this eventually causes significant decreases in total protein content. These effects of higher levels of atmospheric NO_2 are also linked to the pronounced inhibition of N_2 fixation rates. The importance of NH_4^+ and glutamine levels in regulating N_2 fixation is already known (Haselkorn, 1986). Consequently, excess NH_4^+ arising from the higher levels of atmospheric NO_2 in *Azolla* probably acts as a signal to *Anabaena* to cut back N_2 fixation.

The NO_2-induced inhibition of N_2 fixation in *Anabaena* differs markedly from that caused by atmospheric SO_2. Very low levels of atmospheric SO_2 (< 25 nl l^{-1}) lead to free radical mediated destruction of the barrier to O_2 diffusion afforded by the heterocyst envelope which rapidly shuts down nitrogenase activity and nitrogen assimilation (Hur and Wellburn, 1993). On the other hand, it is quite evident that atmospheric NO_2 provides an additional source of N which eventually closes down N_2 fixation only if the NO_2 levels are high enough (i.e. ⩾ 100 nl l^{-1}). This, in part, may explain the reported decreases (Srivastava and Ormrod, (1986) and increases (Gupta and Narayanan, 1992) of N_2 fixation in root nodules of legumes when exposed to atmospheric NO_2. 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 N_2 fixation in soya (Streeter, 1982) and symbiotic lichens (Hällbom and Bergman, 1983). Our results do suggest that low levels of NO_2 (⩽ 50 nl l^{-1}) may benefit *Azolla* while N_2 fixation still operates in *Anabaena* at these ambient levels of NO_2 (unlike SO_2) so that the extra NO_2-derived N may permit extra growth. Indeed, this ability of *Azolla* to use an alternative source of N derived from low levels of atmospheric NO_2 may be a significant general response for many species which fix N_2; 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 NO_2 (Wellburn, 1990). Although polyamine levels change in response to many environmental stresses including atmospheric O_3 and SO_2 (Smith, 1985; Rowland-Bamford *et al.*, 1989; Langebartels *et al.*, 1991), there are no reports of NO_2-induced changes in polyamine levels in lower (or higher) plants. Our results clearly show that NO_2 (100 nl l^{-1}) increases levels of putrescine, a precursor for spermine and spermidine, which is already known to be affected by both N source and content (Preibe, Klein and Jäger, 1978; Smith, 1985).

Consequently, this study has several implications. Firstly, extra NO_2-derived N does not normally increase NH_4^+ levels which would otherwise close down N_2 fixation and *Azolla* may even limit NH_4^+ levels so that the symbiosis continues to operate at ambient levels of atmospheric NO_2. Secondly, any N arising from NO_2 at such levels is additional to that fixed and may also be used for growth and, finally, additional polyamine formation only appears when N throughput is exceeded at high levels of atmospheric NO_2.

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