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Effects of Atmospheric O_3 on *Azolla–Anabaena* Symbiosis

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Cultures of water fern *Azolla pinnata* R. Br. exposed for 1 week to either 30, 50 or 80 nl l^{-1} O_3 showed significant reductions in rates of growth and N_2 fixation, and had fewer heterocysts. Although the levels of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) activity were decreased by low concentrations of O_3 exposures (30 or 50 nl l^{-1}), significant increases in levels of the same enzymes were caused by higher concentrations of O_3 (80 nl l^{-1}). Increased levels of total protein, polyamines (putrescine and spermidine), and the xanthophyll-cycle precursor of abscisic acid (ABA), violaxanthin, were also found with higher levels of O_3 (80 nl l^{-1}). Levels of ABA itself were significantly increased by low level O_3 fumigation (30 nl l^{-1}) but significantly decreased by exposure to 80 nl l^{-1} O_3. This may indicate that higher levels of atmospheric O_3 inhibit the final stages of ABA biosynthesis from violaxanthin.

Key words: Abscisic acid, nitrogen assimilation, nitrogen fixation, ozone pollution, polyamines, violaxanthin.

INTRODUCTION

Ozone (O_3) is a major component of photochemical air pollution which causes foliar injury and permanent damage to many plant species including several economically-important crops (Heagle, 1989). Atmospheric O_3 may either increase or decrease stomatal conductance depending upon species, cultivar and exposure conditions (Darrall, 1989) and reduced stomatal conductance has been suggested to be responsible for decreases of photosynthesis by limiting available CO_2 (Saxe and Murali, 1989). However, few studies have been carried out to investigate atmospheric O_3 effects on levels of abscisic acid (ABA) which is principally involved in the regulation of stomatal movements in response to water stress.

Change in plasma membrane permeability is an early event which leads to atmospheric O_3 injury (Heath, 1980). This is mediated by oxidative free radicals which are capable of attacking both lipids and thiol groups of membrane protein although some of them are scavenged by a variety of antioxidative systems beforehand (Heath, 1988). Levels of polyamines are known to increase during exposure to O_3 (Rowland-Bamford *et al.*, 1989) and may also act as free radical scavengers (Smith, 1985). This is supported by the fact that feeding polyamines to plants reduces visible injury caused by atmospheric O_3 (Bors *et al.*, 1989).

Effects of O_3 on nitrogen metabolism are still poorly understood. Changes in nitrogen metabolism as a consequence of accelerated senescence induced by O_3 have been detected (Grandjean and Futhrer, 1989; Adams, Edwards and Taylor, 1990). Furthermore, effects of atmospheric O_3 on nitrogen metabolism may have important consequences for other physiological processes (Manderscheid, Jäger and Kress, 1992).

Azolla is a genus of floating aquatic fern which normally contains a N_2-fixing symbiotic cyanobacterium, *Anabaena* (or *Nostoc*) *azollae* (sp.). This provides the association with its total N requirement (Peters, 1977) and allows *Azolla* to grow in relatively low N environments. There is a close relationship between photosynthesis and N_2 fixation in the *Azolla–Anabaena* symbiosis, photosynthesis in *Azolla* being the source of ATP and reductant for the nitrogenase in *Anabaena*. *Azolla* has also proved to be a useful lower plant system for study of the effects of different atmospheric pollutants. In the case of SO_2, the symbiosis between *Azolla* and *Anabaena* is rapidly disrupted because N_2 fixation is highly sensitive to very low levels of atmospheric SO_2 (Hur and Wellburn, 1993). However, the symbiosis can tolerate quite large additional inputs of N from atmospheric NO_2 before changes (e.g. increased polyamine synthesis) are detected (Hur and Wellburn, 1994). In the experiments with atmospheric O_3 described here, similarities in response to those of atmospheric SO_2, rather than those of NO_2, were detected in different areas of N metabolism but significant changes in ABA levels were also detected which may have wider environmental implications.

MATERIALS AND METHODS

Plant material, growth conditions, and exposure system

Growth conditions for *Azolla pinnata* R. Br. and the pollutant exposure system have been described earlier (Hur and Wellburn, 1993). Dark green and healthy-looking fronds were transferred to fresh culture media 1 week before exposure to one of three relatively low mean concentrations of O_3 (30, 50 or 80 nl l^{-1}) – the highest level being within the range of atmospheric O_3 concentrations experienced in a

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TABLE 1. *Effects of atmospheric O_3 on growth and nitrogen assimilation of* Azolla

Parameter	30 nl l^{-1} O_3		50 nl l^{-1} O_3		80 nl l^{-1} O_3	
	treated	% of control	treated	% of control	treated	% of control
Mean relative growth rate (d^{-1})	0·081* ±0·006	75·0	0·087** ±0·004	76·3	0·059*** ±0·010	51·8
N_2 fixation rate (C_2H_2 reduction) (mmol g^{-1} f.wt h^{-1})	1·45* ±0·08	86·3	1·30*** ±0·05	76·3	1·42** ±0·07	82·6
Heterocyst frequency (%)	20·0*** ±0·24	92·6	20·2** ±0·20	96·2	20·0*** ±0·24	93·0
Glutamine synthetase (GS) activity†	51·6 ±6·39	92·1	57·9 ±5·69	101	83·4* ±7·78	148
Glutamate dehydrogenase (GDH) activity‡	31·1 ±4·38	114	29·9 ±1·21	115	33·4** ±1·11	123
Protein content (mg g^{-1} f.wt)	4·19 ±0·17	95·2	4·16 ±0·19	97·0	5·53*** ±0·21	128
Putrescine level (nmol g^{-1} f.wt)	387·5 ±29·7	110	411·6 ±61·0	115	546·3* ±72·2	147
Spermidine (nmol g^{-1} f.wt)	355·3 ±34·4	112	331·2 ±24·5	98·8	562·9** ±48·3	163

The data are the means of n replicates ± standard errors of the mean, where $n = 5$ (growth rates), 20 (acetylene reductions), 15 (enzyme assays, protein contents and polyamine determinations) and 150 (heterocyst frequencies). The asterisks indicate significant differences in P values between control and fumigation of * < 0·05, ** < 0·01 and *** < 0·001.

† nmoles γ-glutamylhydroxamate formed mg^{-1} protein min^{-1}; ‡ nmoles NADH oxidized mg^{-1} protein min^{-1}.

typical UK summer (UK PORG, 1987). O_3 was generated from pure O_2, using a silent electric discharge apparatus, diluted with activated charcoal- and Purafil®-filtered air, and added to the fumigation chamber between 1000 and 1800 h GMT each day over the week to mimic a natural episode of atmospheric O_3 outdoors. Flow rates of O_3 into the chamber were controlled by fine needle valves and concentrations of O_3 were monitored continuously using an O_3 analyzer (Monitor Labs., USA, Model 8810).

Three different concentrations of O_3 (30, 50 and 80 nl l^{-1}) were used in the chamber at different times and five replicate flasks of each treatment (clean-air controls *vs.* those fumigated) were placed alongside each other in the same light fluence and temperature, etc. Whole *Azolla* fronds in individual flasks were taken as the experimental unit and the Student's *t*-test was used to compare fumigated samples and clean-air controls.

Enzyme assays and other parameters

Measurement of mean relative growth rates, heterocyst frequencies, C_2H_2 reduction rates (N_2 fixation rates), total soluble protein contents, pigment determinations, and levels of GS and GDH activities were carried out according to Hur and Wellburn (1993) and levels of polyamines as described by Hur and Wellburn (1994).

Abscisic acid (ABA) levels

Azolla fronds, freeze-dried for 3 d, were ground to a fine powder in liquid N_2, and shaken overnight at 4 °C with ultra-pure water [extraction ratio 40:1, water (ml): plant dry weight (mg)]. Supernatant fractions, after centrifugation at 4000 *g* for 15 min, were then radioimmunoassayed for ABA as described by Quarrie *et al.* (1988).

RESULTS

Effects of O_3 on the growth of Azolla

Exposure to O_3 significantly reduced the growth rate of *Azolla* at all three concentrations (Table 1). Growth rates of *Azolla* exposed to 30 or 50 nl l^{-1} O_3 were reduced by 25% by comparison to controls but *Azolla* exposed to 80 nl l^{-1} O_3 showed almost 50% less growth.

Effects of O_3 on nitrogen assimilation by the Azolla–Anabaena *system*

N_2 fixation rates in *Anabaena* were decreased by a similar extent (about 20%) at all three concentrations of O_3 (Table 1). Heterocyst frequencies of the *Anabaena* were also significantly decreased to a similar extent (4–7%) by all three O_3 exposures.

Fumigations with O_3 at 30 or 50 nl l^{-1} had no apparent effect on levels of glutamine synthetase (GS) activity but they rose significantly with 80 nl l^{-1} O_3 (48% greater than controls, Table 1). Responses of glutamate dehydrogenase (GDH) activity were similar and again significantly higher at 80 nl l^{-1} O_3 (23%). Significant increases in total soluble protein also occurred at 80 nl l^{-1} (27%, Table 1).

Effects of O_3 on levels of polyamines in Azolla

Cellular levels of ammonium and spermine in *Azolla* exposed to O_3 were unaffected (data not shown) but levels

TABLE 2. *Effects of atmospheric O_3 on abscisic acid (ABA), violaxanthin and antheraxanthin contents of* Azolla

Parameter	30 nl l^{-1} O_3		50 nl l^{-1} O_3		80 nl l^{-1} O_3	
	treated	% of control	treated	% of control	treated	% of control
Abscisic acid (ABA) (μg g^{-1} f.wt)	2·13*** ±0·02	116	1·73 ±0·05	95·2	1·60* ±0·08	84·7
Violaxanthin (μg g^{-1} f.wt)	8·87 ±0·28	107	9·64* ±0·41	117	10·1* ±0·42	121
Antheraxanthin (μg g^{-1} f.wt)	15·9* ±0·54	120	15·8*** ±0·52	126	14·0 ±0·42	95·2
Violaxanthin/Antheraxanthin ratios	0·56 ±0·02	88·9	0·61 ±0·02	96·8	0·72*** ±0·02	126

The data are the means of 15 replicates ± standard errors of the mean and asterisks indicate significant differences in *P* values between control and fumigation of * < 0·05 and *** < 0·001.

of putrescine and spermidine were significantly higher after fumigation with 80 nl l^{-1} O_3 (47 and 63%, respectively, Table 1).

Effects of O_3 on pigment levels and their ratios in Azolla

There were no significant changes in levels of chlorophyll *a* and *b* or most carotenoids to increasing O_3 concentrations (data not shown) but violaxanthin levels were significantly higher at 50 or 80 nl l^{-1} O_3 (Table 2). Antheraxanthin levels were increased significantly at 30 and 50 nl l^{-1} O_3, but were slightly lower than controls at 80 nl l^{-1} O_3. Consequently, higher violaxanthin/antheraxanthin ratios were found in *Azolla* exposed to 80 nl l^{-1} O_3 (Table 2).

Effects of O_3 on the levels of ABA (abscisic acid) in Azolla

Levels of ABA were increased significantly at 30 nl l^{-1} O_3 but significantly lower at 80 nl l^{-1} O_3 (Table 2).

DISCUSSION

The responses of *Azolla* to atmospheric O_3 reported here are similar to those induced by atmospheric SO_2 described earlier (Hur and Wellburn, 1993). Both gases inhibit rates of growth and N_2 fixation, and reduce the frequencies of heterocysts even at levels in the atmosphere normally regarded as below ambient for semi-urban conditions. Such findings rule out the possible use of the *Azolla–Anabaena* system as a specific bioindicator for sulphur deposition in the field although there is no doubt that this system is highly sensitive to certain types of air pollution such as SO_2, O_3, and also mixtures of SO_2 and NO_2 (unpublished data). In a sense, the similarity in response of N_2 fixation, as indicated by C_2H_2 reduction rates and reduced heterocyst frequencies, to atmospheric O_3 and SO_2 (and mixtures of SO_2+NO_2) conforms to the mechanism of inhibition we postulated earlier whereby the barrier to inwards diffusion of O_2 of the heterocyst envelopes is destabilized by air pollutant-induced free radicals (Hur and Wellburn, 1993).

Inhibition of N_2 fixation by atmospheric O_3 (80 nl l^{-1} relative to 'controls' containing 30 nl l^{-1}) has been detected earlier in ladino clover (*Trifolium repens* L. cv. Tillman) using the ^{15}N method (Montes *et al.*, 1983) but linear decreases in N_2-fixation rates with increasing O_3 levels were not found in our study. This may be partly due to structural differences between the *Azolla–Anabaena* system and clover and partly due to the accessibility and subsequent reactions of O_3 with cell components and their fate inside cells. When O_3 enters solution in plant tissues, it rapidly decomposes to oxyradicals and peroxides in the liquid layer surrounding the mesophyll cells which react with the components of cell wall and plasma membranes (Heath, 1988). Consequently, intercellular O_3 levels are close to zero over a wide range of external atmospheric O_3 concentrations (Laisk, Kull and Moldau, 1989). If these oxyradicals and peroxides then penetrate as far as the heterocyst without being scavenged away then the integrity of the heterocyst envelope as a barrier to O_2 will be disrupted and the mechanism of N_2 fixation inside much reduced as a result.

Most of the other significant disturbances in N assimilation caused by atmospheric O_3 probably reflect reduced N_2 fixation. For example, increases in levels of GS activity might have been predicted but additional formation of polyamines and extra GDH activity, both of which are characteristic of oxidative stress, are likely to exacerbate the problem by draining away available N despite the fact that extra protein is formed. Robinson *et al.* (1991) have suggested that GDH is involved in the catabolism of glutamine, having a regulatory function between nitrogen and carbon metabolism. This enzyme is involved in NADH formation and in the supply of carbon skeletons to the tricarboxylic acid cycle under conditions when carbon is limited. Further evidence of this hypothesis is supported by the observation that O_3-induced increases of GDH are due to increased oxidative deamination rates of glutamate. (Manderscheid *et al.*, 1992).

Polyamines are known to play an important role in maintaining membrane permeability and rigidity (De Agazio, Gardinia and Grego, 1987) and respond to O_3-induced Ca^{2+}/K^+ imbalances or pH changes in the cell wall by stabilizing membranes which re-establishes ionic balances across the plasmalemma (Smith, 1985; Heath, 1988).

Consequently, increased levels of polyamines in *Azolla* may indicate a metabolic response which minimizes damage at the initial site of injury.

Stomatal responses to atmospheric O_3 are complex and may operate at several different physiological levels (Winner, 1989). Most experimental studies of crop plants have found that stomata close and transpiration rates decline in response to O_3 exposure (Olszyk and Tingey, 1986; Reich and Lasoie, 1984) but there are also reports of increased stomatal conductance in response to O_3 exposure (Olszyk and Tibbits, 1981). ABA is now recognized to be a major regulator of stomatal aperture and the significant changes in levels of ABA may well be a response to atmospheric O_3. It has been known for some time, for example, that applications of exogenous ABA to primary bean leaves close stomata and reduce the phytotoxicity of O_3 (Fletcher, Adepipe and Ormrod, 1972). Consequently, increased levels of endogenous ABA at low levels of O_3 may also protect *Azolla* by restricting access of the pollutant. However, when levels of ABA are lower in *Azolla* at high levels of O_3, this protection may have been lost. Moreover, reductions in ABA levels due to O_3 (80 nl l^{-1}) are not confined to *Azolla*. Significant depressions in ABA levels in both well-watered tomato and cherry leaves have also been detected recently in the presence of similar levels of O_3 (A. Sier and W. J. Davies, pers. comm.).

Low ratios of violaxanthin/antheraxanthin at 30 and 50 nl l^{-1} O_3 also imply that the scavenging (i.e. by the xanthophyll cycle) of free radicals induced by air pollutants is effective. However, the situation is reversed at high O_3 levels (80 nl l^{-1}) when violaxanthin/antheraxanthin ratios are significantly increased. Little is yet known about the effects of O_3 on ABA biosynthesis, especially in relation to violaxanthin. Our observation of reduced levels of ABA may be related to the increases in violaxanthin and violaxanthin/antheraxanthin ratios in *Azolla* at high levels of O_3. Recently, Parry and Horgan (1991) have suggested that 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin are potential precursors of ABA and both are derived from all-*trans*-violaxanthin. Consequently, if ABA levels fall in *Azolla* due to high levels of O_3, this may be because the synthesis of 9′-*cis*-neoxanthin from all-*trans*-violaxanthin is inhibited by higher levels of atmospheric O_3.

Further studies are needed to investigate O_3-induced stomatal changes in relation to bulk ABA biosynthesis. The possibility that ABA acts as a metabolic messenger which triggers physiological responses (e.g. changes in levels of free Ca^{2+}) towards atmospheric O_3 is worth investigating. ABA levels in plants rise in dramatic fashion in certain cellular compartments in response to water stress and minor changes in water potential may blot out the detection of specific ABA responses to air pollutants. This may well have been the problem when Kobriger, Tibbits and Brenner (1984) failed to relate stomatal apertures to ABA levels when peas were fumigated with O_3 and SO_2. By using *Azolla*, which floats on water, this possibility is excluded.

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