Methods for Assessing the Effects of Mixtures of Chemicals
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Biochemical Mechanisms of Combined Action of Atmospheric Pollutants upon Plants

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ABSTRACT

Atmospheric pollution as a consequence of industrial and economic activity by society is the principal cause of damage to plants. The major forms SO_2, NO_x, and O_3 may act in combination in a variety of ways: additive, synergistic, or antagonistic. An understanding of the molecular mechanisms of such interactions is only now emerging as the individual effects of such pollutants upon vegetation are still being investigated. The major synergistic interaction appears to take place between SO_2 and NO_x, causing considerable additional loss of potential growth of plants. There appear to be two main explanations of these reductions.

Normal pathways of nitrogen metabolism within plants are utilized as a mechanism to mitigate the consequences of NO_x pollution. Nitrite reductase is normally induced to combat excessive quantities of nitrite anions in the chloroplast. Normally SO_2 fumigation alone has little individual effect upon this enzyme but the extra presence of NO_x has the ability to prevent the necessary detoxifying induction. The products of SO_2 and NO_x in solution also appear to be capable of generating much larger quantities of free radical agents than normally occur within active photosynthetic membranes. This has been shown by the specific changes in light-induced quenching of 9-aminoacridine (9-AA) fluorescence. This technique and other such probes (nuclear magnetic resonance (NMR), electron spin resonance (ESR), etc.) may be of value in the future by providing a means of assessing the consequences of the combined effects of atmospheric pollution and ultimately in devising suitable toxicity tests.

1 INTRODUCTION

The principal atmospheric world-wide pollutants are SO_2, NO_x, and O_3. The first two are often emitted from the same source and therefore frequently occur together. The third is formed as a consequence of a combination of oxides of nitrogen, atmospheric hydrocarbons and high light intensities and may appear in association with the others or some distance away. All are phytotoxic to varying

extents, especially in combination with each other. A number of reviews have appeared recently on their combined damaging effects (e.g. Mansfield and Freer-Smith, 1981), but the summarized interactions gathered by Ormrod (1982) shown in Table 1 demonstrate that the nature of the interaction may be additive, synergistic (i.e. more than additive), antagonistic, or a mixture of the three depending on which parameter and pollutant combination is followed. In the case of $SO_2 + NO_2$, synergism is normally detected. The consequences of this are better illustrated in terms of potential growth losses shown by a series of long-term mixed-pollutant studies of grasses (Table 2).

This paper attempts to analyse some of the underlying biochemical 'lesions' which may account for these growth reductions and the extreme phytotoxicity shown by mixtures of atmospheric pollutants. Unfortunately there have been no biochemical studies of O_3 in combination with either SO_2 and/or NO_x, and individual photosynthetic studies using O_3 are few. Furthermore the observed effects of O_3 on plants are more unspecific by comparison with studies on either SO_2 or NO_x and experiments with O_3 are technically very difficult to undertake. The best review of the effects of O_3 on plant tissue at the moment is that of Mudd (1982). Consequently the only combination that is considered in detail here is that of $SO_2 + NO_2$ although the addition of O_3 can only aggravate some of the disturbances because O_3 and $SO_2 + NO_2$ are believed to elicit similar responses at the molecular level.

2 EFFECT ON PHOTOSYNTHESIS WITHIN CHLOROPLASTS

2.1 Overall Events

Photosynthesis within chloroplasts is the initial and major process to be affected by the products of SO_2 in solution (Hällgren, 1978; Ziegler, 1975). High values of pH around 8–9 tend to give sulphite ions as the major product of SO_2 in the aqueous stromal phase of chloroplasts and consequently *in vitro* studies of SO_2 at likely sites of action are carried out using sulphite. Similarly, the products of NO_x in solution are nitrate and nitrite although nitric oxide only shows limited solubility.

Figure 1 outlines the important features of chloroplast metabolism that may be susceptible to damage by SO_2, NO_x, or O_3. Initially, light induces a flow of electrons by virtue of the photosystems which in turn leads to the generation of a proton gradient across the thylakoid membrane. The alkalinization brought about in plastid stroma normally enhances the activity of enzymes within the Calvin cycle located there. Any detrimental acidification due to the products of SO_2 and NO_x pollution may consequently have an inhibitory effect upon processes such as CO_2 fixation. Proton gradients across thylakoid membranes are harnessed by chloroplast coupling factors ($CF_0 + CF_1$) to form ATP (Mitchell, 1966). The products of atmospheric pollution may also interfere with one or more

Table 1 Reported interactions upon various plant functions by atmospheric pollutant mixtures[a]

Mixture	Interaction	Characteristic affected
$SO_2 + O_3$	Additive	Photosynthesis, growth (especially leaves)
	Synergistic	Leaf appearance, growth (especially roots)
	Antagonistic	Growth, fungal infection, leaf appearance
	Mixed	Nematode infection
$SO_2 + NO_2$	Additive	Photosynthesis, growth
	Synergistic	Photosynthesis, growth (especially pollen tubes), enzymic activity
$NO_2 + O_3$	Synergistic	Pollen tube growth
$SO_2 + NO_2 + O_3$	Synergistic	Photosynthesis

[a] Taken from Ormrod (1982).

Table 2. Percentage increases or reductions (relative to controls in clean air) in growth of four grasses after being exposed for 140 days in winter to atmospheres containing 68 ppb NO_2, 68 ppb SO_2, or 68 ppb $NO_2 + 68$ ppb SO_2[a]

Plant and parameter	NO_2	SO_2	$NO_2 + SO_2$
Dactylis glomerata L.			
Leaf area	+21	−5	−72
Dry weight of green leaves	−7	−28	−83
Dry weight of roots	−11	−37	−85
Poa pratensis L.			
Leaf area	−17	−28	−84
Dry weight of green leaves	−29	−39	−88
Dry weight of roots	−47	−54	−91
Lolium multiflorum Lam.			
Leaf area	+1	−22	−43
Dry weight of green leaves	−10	−28	−65
Dry weight of roots	+35	+7	−58
Phleum pratense L.			
Leaf area	+30	−11	−82
Dry weight of green leaves	+14	−25	−84
Dry weight of roots	+1	−58	−92

[a] Taken from Ashenden (1979) and Ashenden and Williams (1980).

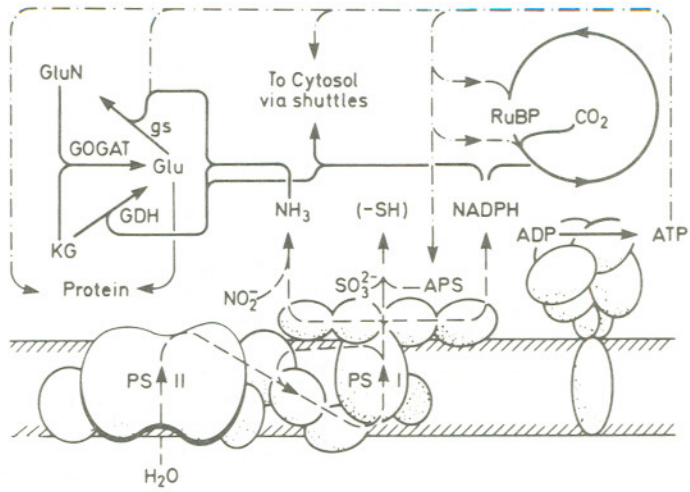

Figure 1 Schematic outline of important photosynthetic events which may be sensitive to the products of SO_2, NO_x or O_3 in solution. The dashed lines signify electron flow from water to various components and the alternate dashed and dotted lines illustrate the various processes which are dependent upon an adequate supply of ATP from the coupling factor particles

of these processes with a consequential reduction of ATP-requiring events. Over the longer term this is likely to be translated into reductions of protein or carbohydrate synthesis and thereby poor growth of plants. The following sections give an outline of those processes known to be affected by pollutants such as SO_2, NO_x, and O_3.

2.2 Ultrastructural Changes

Both SO_2 and NO_x affect chloroplast ultrastructure by causing swelling of the lumen within the thylakoids of chloroplasts (Figure 2). O_3 is less specific and affects the cristae of mitochondria in a similar manner (Lee, 1968). It would appear that these swellings are reversible over the short term if the fumigation is removed, but are permanent if exposure is prolonged. Swelling of this type is thought to be due to ionic disturbances and acidification caused directly by products of the pollutants (Spedding *et al.*, 1980) but it may be symptomatic of changed lipid characteristics which are revealed during the dehydration procedures of sample preparation for electron microscopy.

2.3 Mechanisms of Entry

Active transport of pollutant products such as sulphite and sulphate into the chloroplast takes place by means of the phosphate translocator of plastid

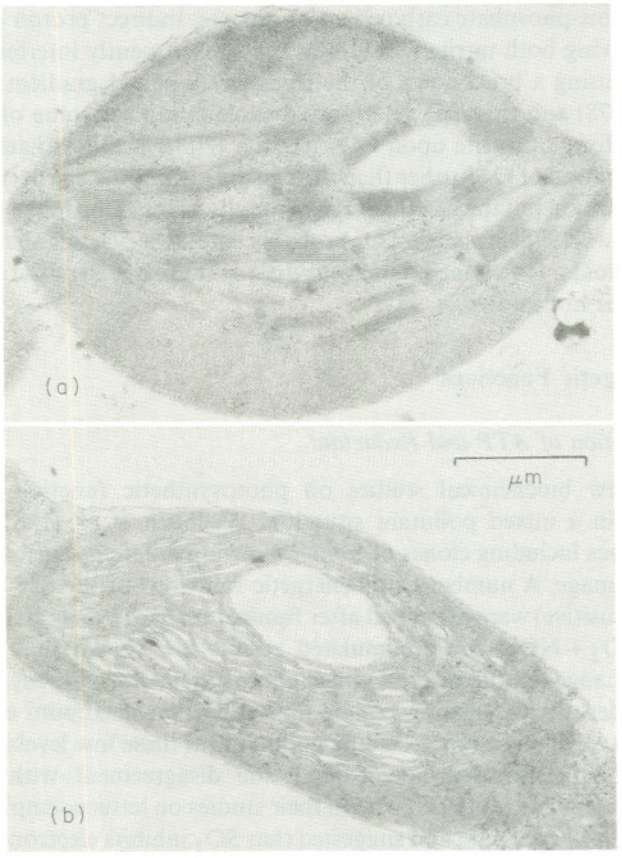

Figure 2 Electron micrographs taken from experiments described by Wellburn *et al.* (1972) showing bean chloroplasts from (a) clean air control tissue as compared with (b) tissue fumigated for 2 hours with 250 ppb SO_2

envelopes (Hampp and Ziegler, 1977; Mourioux and Douce, 1978) and additional orthophosphate appears to enhance the influx of sulphur anions.

Plastid envelopes are also permeable to nitrite, ammonia, and unionized nitrous acid but not to ammonium ions (Heber and Purczeld, 1978). As ammonia may move across the envelope freely in either direction, the ionization relationship with ammonium, in the stroma for example, will be disturbed as the protons are left behind causing an acidification of the space vacated. However, when both an anion and its neutral protonation product (e.g. NO_2^- and HNO_2) can permeate a membrane barrier, shuttle transfer of protons will abolish the pH gradient across the membranes. In the light, alkalinization of the stroma normally activates

ribulose-1,5-bis-phosphate carboxylase/oxygenase. Indirect proton uptake via a shuttle involving both nitrite and HNO_2 may consequently interfere with such events by causing a breakdown of the trans-envelope pH gradient (Heber and Purczeld, 1978) and this may be a partial explanation for some of the known inhibitory effects of nitrite upon CO_2 fixation (Hiller and Bassham, 1965).

The penetration of O_3 further than the plasma membrane has been the subject of some discussion but the fact that O_3 can rapidly affect chlorophyll fluorescence (Schreiber *et al.*, 1978) means that O_3 probably enters the plastids and gains access to the thylakoids. How much damage it does as it crosses the plastid envelopes and stroma is not known.

2.4 Bioenergetic Functions

2.4.1 Formation of ATP and Reductant

Relatively few biochemical studies on photosynthetic functions have been undertaken in a mixed pollutant situation. Wellburn *et al.* (1981) fumigated various grasses including clones of *Lolium* showing different sensitivities to SO_2 pollution damage. A number of bioenergetic functions of the S23 cultivar (the most SO_2-sensitive) were examined after fumigation for 11 days at 250 ppb SO_2, NO_2, or $SO_2 + NO_2$; the accumulated results are shown in Figure 3. No significant changes of the rates of either photosystem I or photosystem II were detected under any fumigation regime indicating that maximum electron flow capabilities appear to be unaffected by pollutants at these low levels. Differences in pollutant concentration may explain the disagreement with reports by Shimazaki and Sugahara (1979) who in their studies on lettuces employed SO_2 at levels between 1 and 2 ppm and suggested that SO_2 inhibits electron transfer at a site close to the reaction centre of photosystem II.

Examination of the rates of ascorbate/diaminodurene-dependent ATP formation under similar conditions (Figure 4) is more revealing. Significant reductions of cyclic photophosphorylation below those in unpolluted *Lolium* (S23 clone) were given by $SO_2 + NO_2$ treatments, while NO_2-polluted air alone gave enhanced rates of ATP formation. Similar results were given in the longer term (68 ppb for 20 weeks) for different SO_2-sensitive clones of *Lolium* and also other grasses (Figure 4). This indicates an individual sensitivity to SO_2 in all clones and consequently any resistance characteristics are not to be found in the area of ATP formation. A possible explanation of this behaviour has been given by Cerović *et al.* (1983) who have definitively identified competitive inhibition between sulphite and orthophosphate at the site of ATP formation on the coupling factor particles.

Estimations of the reduced ATP levels and the energy charge ratios (Figure 3) are also in accordance with the observed reduction in the rates of cyclic photophosphorylation due to SO_2 and NO_2, but significantly higher total ATP

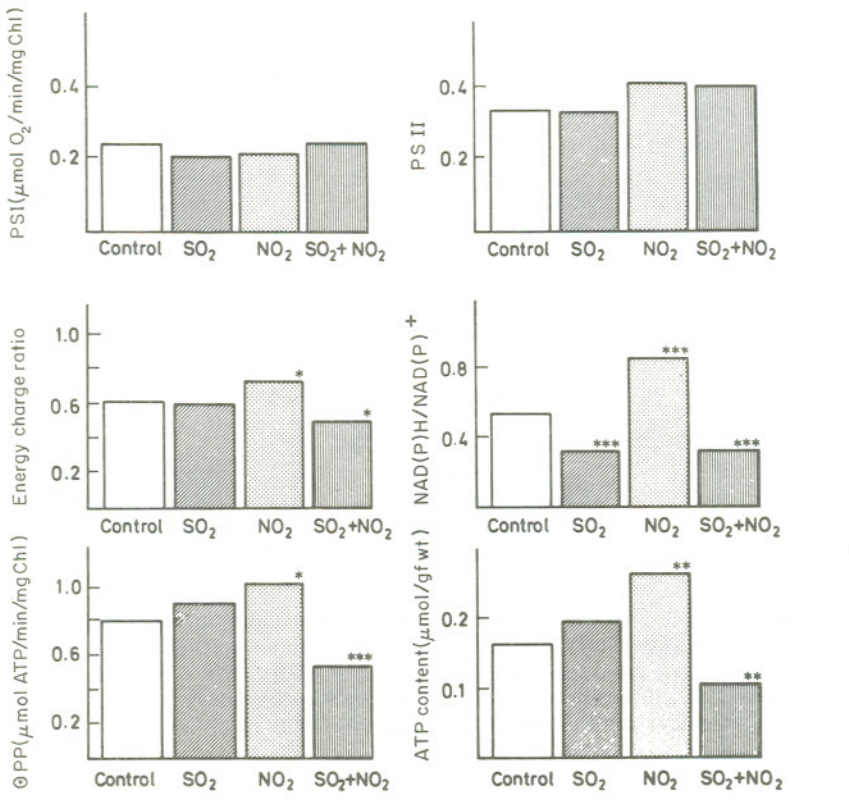

Figure 3 Effect upon various cellular parameters of treating *Lolium perenne* L. (S23 clone) with clean air or with air containing SO_2 and/or NO_2 (250 ppb) for 11 days (***$P < 0.01$, **$P < 0.05$, *$P < 0.10$)

amounts and energy charge ratios for NO_2 fumigation alone were also noted.

General levels of reductant which are available are indicated by the assays of the NAD(P)H/NAD(P)$^+$ ratios (Figure 3). Both SO_2 and $SO_2 + NO_2$ treated tissues had lower levels of reduced cofactors than unpolluted tissues, while those treated with NO_2 appeared to have an excess of available reductant even in the presence of enhanced nitrite-reducing capability.

Taken together, these indications of changes in the capacity for ATP and general levels of reductant show that, although levels of NO_2 alone may be beneficial, in the case of $SO_2 + NO_2$ pollution exactly the reverse holds. These plants appear to be more incapacitated bioenergetically than those which have experienced only SO_2 fumigation.

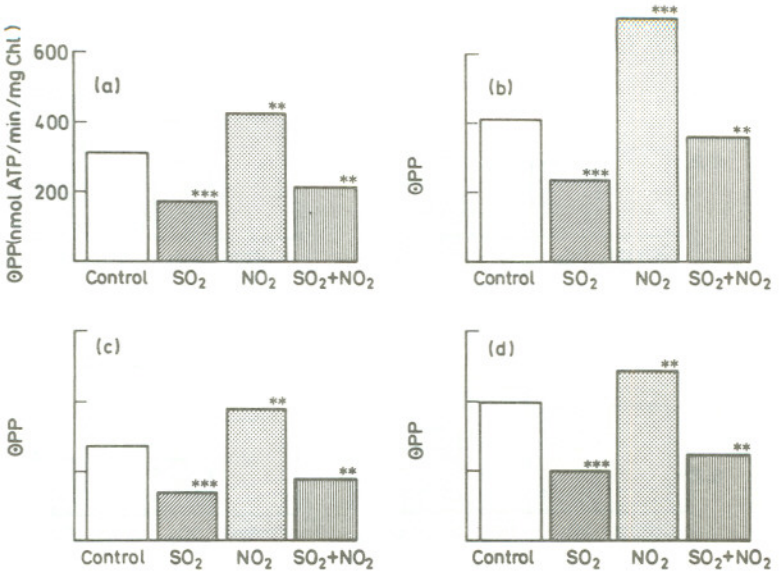

Figure 4 Rates of ascorbate/diaminodurene-dependent photophosphorylation (OPP) in extracts from grass clones exposed to clean air or to air containing SO_2 and/or NO_2 (68 ppb) over a period of 20 weeks (SD $\pm$ 10 %, $^{**}P < 0.05$, $^{***}P < 0.01$). (a) SO_2-sensitive *Lolium* S23; (b) SO_2-sensitive *Phleum pratense*; (c) SO_2-tolerant *Lolium* S23 'Bell resistant'; (d) SO_2-tolerant *Lolium* 'Helmshore'. From Wellburn *et al.* (1981).

2.4.2 Generation of ΔpH

In a chemiosmotic explanation of ATP formation, electron flow is responsible for the creation of a proton gradient which, in turn, is harnessed by the coupling factor complex ($CF_1 + CF_0$) to form ATP (Mitchell, 1966). At the same time electrons reduce nitrite or NAD^+ to form either ammonia or NADPH. These are then consumed by the glutamine synthetase/glutamate synthase (GOGAT) and Calvin cycles, respectively (Figure 1).

Various methods for the determination of the ΔpH generated across photosynthetic membranes in the light and the dark exist but one of the most convenient, based upon the uptake of fluorescent amines, was suggested by Schuldiner *et al.* (1972). This uptake is dependent upon the dissociation constant of the amine and the number of ionizable amines in the probe. 9-AA was found to have highly suitable properties for these measurements and has recently been widely adopted. The quenching of 9-AA fluorescence by illuminated thylakoid suspensions is partly due to the redistribution of the probe in response to the proton gradient and also to increased binding of the probe to thylakoid membranes (Haraux and Kouchkovsky, 1979). Corrections can be made for

binding of the probe by measuring light-induced quenching as a function of chlorophyll concentration which allows meaningful assessments of ΔpH to be made (Slovacek and Hind, 1981). Leaving that possibility aside, the technique has the additional advantage that for comparative purposes the relative percentage change on light-induced quenching quickly provides an indication of differential change across thylakoid membranes due to different treatments with respect to protons.

Using a fluorescence apparatus very similar to that described by Mills *et al.* (1978) with identical filters and light intensities, we have followed the procedure and calculations of Slovacek and Hind (1981) to employ the technique of 9-AA light-induced fluorescence quenching to follow the effects of O_3, sulphite, sulphate, nitrite, and nitrate singly and in combination upon thylakoid preparation from oats (Robinson and Wellburn, 1983).

Figure 5 illustrates examples of the traces from untreated, sulphite, nitrite or sulphite plus nitrite treated preparations. Typically the level of 9-AA fluorescence of 1.5-ml samples of thylakoid preparations (15 μg of chlorophyll) in Tris–Tricine buffer (pH 8.1) in the dark is considerably above instrumental zero (left-hand side of the traces) and taken to be 100 % relative fluorescence. Upon illumination with red light (> 630 nm) the 9-AA fluorescence of the samples declines in proportion to the formation of pH across the thylakoids. Upon reverting to the dark the signal gradually increases as the proton gradient decays. Repeated signals with

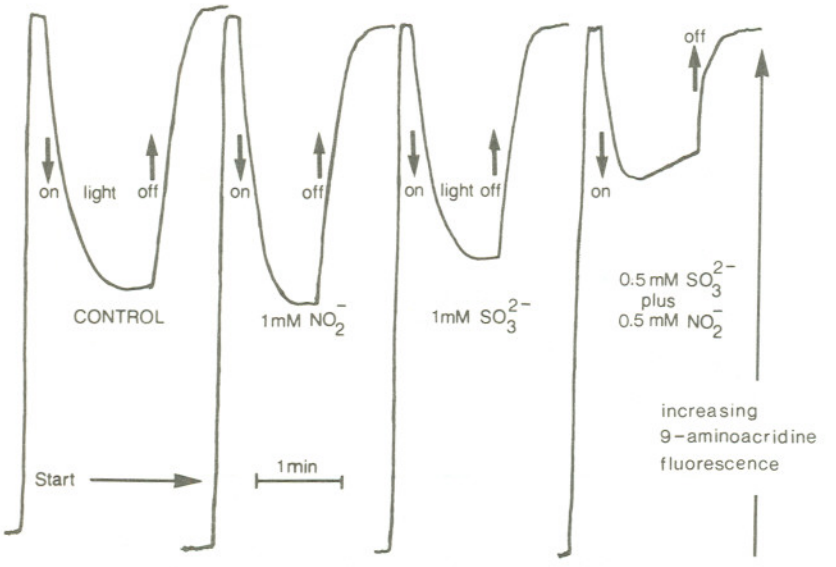

Figure 5 Recording of 9-aminoacridine fluorescence from red light illuminated thylakoid preparations given various anion treatments. From Robinson and Wellburn (1983).

little or no change in properties could be obtained from the same sample following fresh illumination cycles.

Figure 6 shows the means and ranges of maximum quenching of samples treated with different concentrations of sulphate, sulphite, nitrate, and nitrite relative to untreated control quenching of 9-AA fluorescence. With single treatments of any of these ions no significant decline in light-induced 9-AA fluorescence quenching occurred at concentrations of 1 mmol/litre or less. However, in some instances, especially with 0.1–0.5 mmol/litre sulphite, a noticeable increase in quench was observed. This phenomenon is not understood but is thought to be caused by changes in the binding properties between the membranes and 9-AA due to the sulphite and, to a lesser extent, sulphate or nitrite. It is difficult to ascribe any relationship between this response and enhanced effects upon growth sometimes reported for fumigations of whole plants at very low levels of SO_2.

Above 1 mmol/litre, nitrite appeared to be the most (and sulphate the least)

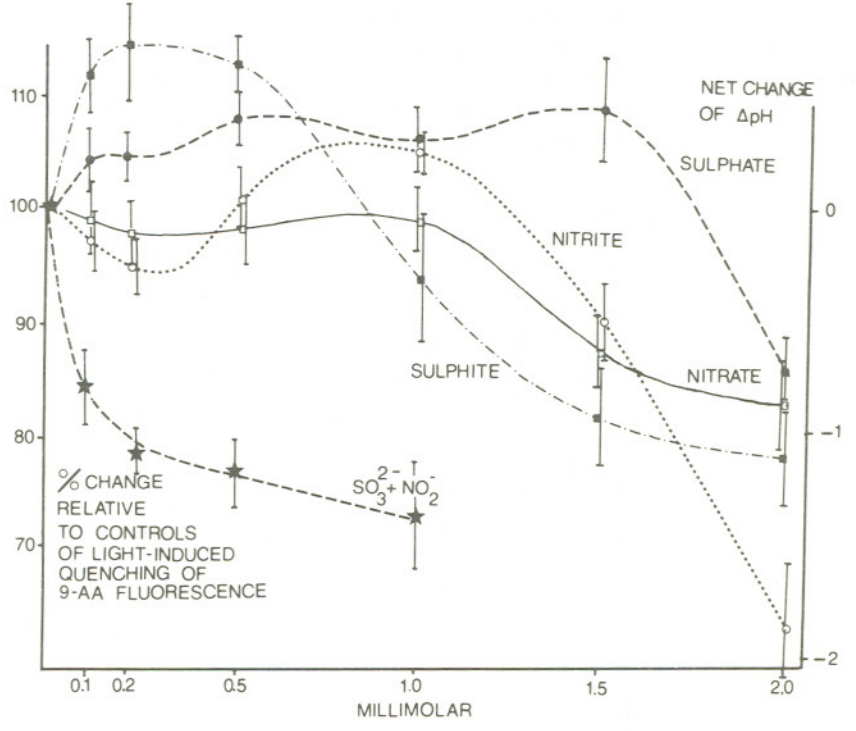

Figure 6 Relative reductions in 9-AA light-induced fluorescence quenching in response to sulphate, sulphite, nitrate, and nitrite and mixtures of sulphite and nitrite by comparison with untreated controls. Approximate changes in ΔpH are shown on the right. From Robinson and Wellburn (1983).

effective ion in promoting a decline in light-induced 9-AA fluorescence quenching by the thylakoid samples. However the most interesting observations were obtained with equal mixtures of sulphite and nitrite. Even at lower concentrations of both compounds (0.1–0.5 mmol/litre) highly significant ($P < 0.001$) differences in the relative decline of quenching from controls and also from individual additions of sulphite or nitrite were obtained. Analysis of variance showed clear 'more than additive' interactions ($P < 0.01$) over the range 0.1–1 mmol/litre if compared with separate additions of 0.1–1 mmol/litre sulphite or nitrite. However, in an analysis of this form, the sum concentration of the mixture is double that of the individual treatments. It may be more appropriate to test 0.1 mmol/litre sulphite + 0.1 mmol/litre nitrite against 0.2 mmol/litre sulphite alone or 0.2 mmol/litre nitrite alone. When such analyses were undertaken for the range of 0.1–0.5 mmol/litre of mixtures, highly significant ($P < 0.01$) interactions were again detected but not at the 1 mmol/litre level. An example of a trace obtained with a mixture of 0.5 mmol/litre sulphite + 0.5 mmol/litre nitrite is included in Figure 5.

It is possible to make estimates of the ΔpH values for different light-induced fluorescence determinations for the quenching of 9-AA, but these are subject to corrections to allow for binding errors. On the right-hand side of Figure 6 are shown comparative changes in ΔpH calculated in the manner described by Slovacek and Hind (1981) using their binding corrections and a thylakoid volume of 4.9 μl/mg chlorophyll. Estimates of ΔpH in control preparations under our conditions are in the range 3.5–4.5 pH units but because of the uncertainty over binding corrections and the possibility that these also might change due to the different treatments, the equivalent net changes of ΔpH shown on the right-hand side of Figure 6 should be regarded as only approximate by comparison with the relative decline in 9-AA fluorescence quenching of treatment to control (which are the plotted values).

In Figure 5 the combined treatment of sulphite with nitrite also shows another feature which was also exhibited with studies using O_3. The decay of the 9-AA fluorescence is enhanced in the light, and darkness has the effect of aiding a partial repair so that when the light is initially turned on again most of the quenching recovers but then decays in the light to a greater extent. This is thought to be due to free radical events elicited by the mixture of sulphite and nitrite similar to those shown by O_3 which cause ozonolysis of lipids and oxidation of amino acid residues (Mudd, 1982). This leads to increased leakiness of the thylakoid membrane to protons and thereby reduces the effectiveness of the phosphorylation mechanisms associated with the membrane.

2.5 Stromal Functions

Initial studies using peas and high levels (1 ppm) of SO_2 and NO_2 showed that there was a pronounced synergistic interaction between the pollutants when the

levels of activities of peroxidase, ribulose-1,5-bis-phosphate carboxylase, glutamate dehydrogenase, and various transaminases were determined (Horsman and Wellburn, 1975; Wellburn *et al.*, 1976). However, at much lower concentrations (68 ppb) only changes in glutamate dehydrogenase were found to be affected in a synergistic manner (Wellburn *et al.*, 1981). Another critical enzyme of nitrogen metabolism is nitrite reductase which is localized within the chloroplast stroma and utilizes the electron flow from the photosystems within the thylakoids.

Using plastid preparations from control and polluted grasses, the possibility of changes in the levels of nitrite reductase activity due to SO_2, NO_2, or $SO_2 + NO_2$ in the atmosphere was investigated. The results are shown in Figures 7–9. The time course of responses to different pollutant concentrations using the SO_2-sensitive S23 *Lolium* clone (Figure 7) revealed many interesting features.

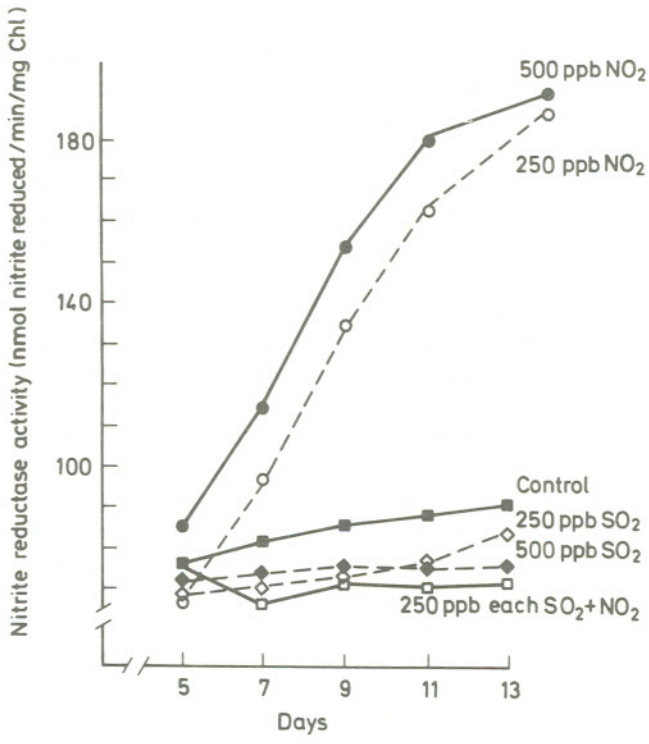

Figure 7 Levels of nitrite reductase activity in extracts from *Lolium perenne* L. (S23 clone) laminae (six weeks old from selfing, before experiment started) which had been exposed to clean air or various combinations of SO_2-polluted and/or NO_2-polluted air for 5–15 days

SO_2 had no direct effect upon the levels of nitrite reductase activity, even at a relatively high concentration (1 ppm), but NO_2 alone induced a significant increase in nitrite reductase activity ($P < 0.01$ after nine days at 250 ppb or after seven days at 500 ppb). This feature was also shown by the SO_2-resistant 'Helmshore' clone after 13 days of fumigation. Most important of all were the effects of $SO_2 + NO_2$. In these instances the presence of SO_2 completely prevented the induction of increased activity by the NO_2. This phenomenon was shown in all of the clones of *Lolium* available (Figure 8a–d) and also in the other grasses even at very low levels of pollutant (Figure 9a–c). After 20 weeks, levels of nitrite reductase activity in plants grown in NO_2-polluted air (68 ppb) were approximately double those in plants grown in clean air. *Lolium perenne* (any clone) or *Dactylis glomerata* samples, after SO_2 fumigation (68 ppb) for the same length of time, showed no significant changes in the levels of nitrite reductase activities, although both *Phleum pratense* and *Poa pratensis* exhibited significant depressions of activity due to the SO_2 treatment. In all grasses the $SO_2 + NO_2$ treatment failed to increase the levels of nitrite reductase in ways similar to those shown by the NO_2 treatment. Indeed, with the exception of the SO_2-resistant S23

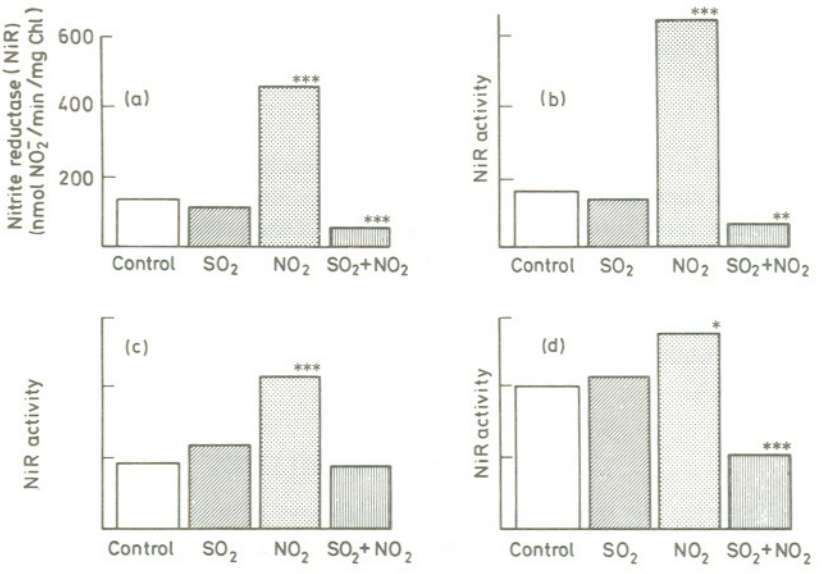

Figure 8 Levels of nitrite reductase activity from *Lolium perenne* L. laminae (6 weeks old after selfing) which had been exposed to clean air or to air containing SO_2 and/or NO_2 (68 ppb) over a period of 20 weeks. (The asterisks indicate the significance of the difference between treatment and control: $*P < 0.1$, $**P < 0.05$; $***P < 0.01$.) (a) *Lolium* S23; (b) *Lolium* S24; (c) *Lolium* S23 'Bell resistant'; (d) *Lolium* 'Helmshore'. NB: (a, b) SO_2-sensitive strains, (c, d) SO_2-tolerant strains

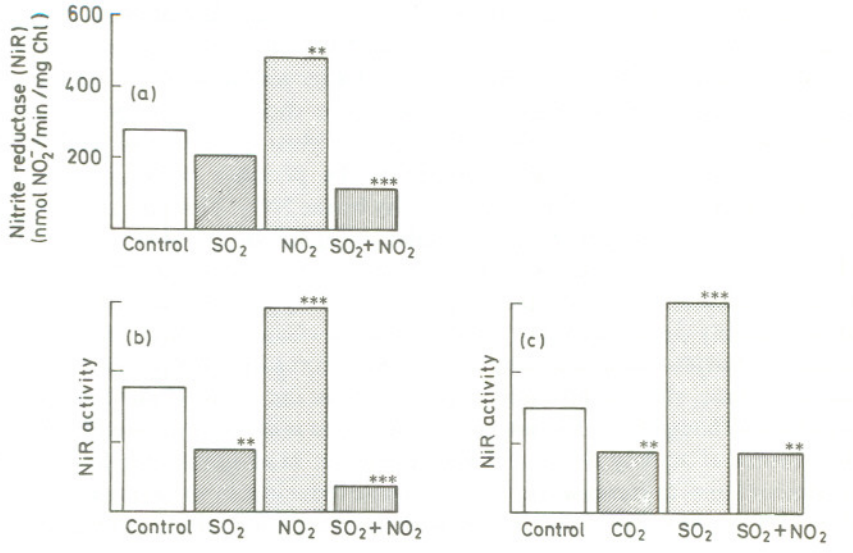

Figure 9 Levels of nitrite reductase activity in different grasses (eight weeks old after germination, before experiment started) exposed to atmospheres containing SO_2 and NO_2 alone (68 ppb) or in combination for 20 weeks (**$P < 0.05$, ***$P < 0.01$). (a) *Dactylis glomerata*, (b) *Phleum pratense*, (c) *Poa pratensis*, all of which are SO_2-sensitive

'Bell resistant' (*Lolium*) clone (Figure 8c), all levels of nitrite reductase were significantly depressed below normal clean air control levels.

The inescapable conclusion to be drawn is that the presence of SO_2 prevents the induction of additional nitrite reductase activity normally associated with NO_2 fumigation. As a consequence the plants, harmed by the SO_2 and unable to properly utilize or detoxify the NO_2, are exposed to damage by both pollutants in one or a number of ways at the same time.

3 EMERGING POSSIBILITIES

We believe that the more than additive inhibitory effects of low atmospheric concentrations of SO_2 and NO_2 may be explained partly by failure to induce additional nitrite reductase activity and partly by the combined ability of sulphite and nitrite to induce membrane damage. These ions may act by the intermediate formation of free radicals damaging the photosynthetic membrane and preventing sufficient proton gradients, which would normally have allowed extra ATP to be formed. This additional ATP is required to counteract the stromal and cytoplasmic consequences of the two pollutants, deprivation of which results in

significant depressions of growth and reductions of leaf area (e.g. Ashenden, 1979; Bennett *et al.*, 1975) caused especially by the combined action of $SO_2 + NO_2$.

For various reasons mentioned earlier, the biochemical effects of ozone upon photosynthesis have not been studied to the depth of those employing SO_2 and/or NO_x. Nevertheless, the additional presence of ozone can only aggravate the damaging consequences of the combined pollutant action of $SO_2 + NO_2$. A number of techniques are emerging which may allow us to gain a greater insight into these combined mechanisms. Non-destructive methods such as those available for the measurement of leaf chlorophyll fluorescence are capable of providing detailed information on the efficiency of the photosynthetic membranes. Schreiber *et al.* (1978) were able to predict ozone injury 20 hours before visible signs of bean leaf necrosis, using such techniques. The first effect they detected was on the water splitting systems followed by inhibition of electron transport between the photosystems. Clearly, such sensitive techniques could equally well be usefully employed for studies on SO_2, NO_x and their various interactions. The other line to be pursued in the biochemical area is the use of isolated plant cell protoplasts. Precise control of pollutant dosage over the short term in such systems could remove some of the variation experienced at present between different plants and populations, leading to a greater insight into basic disturbances within the cell due to sulphite, nitrite, and ozone.

3.1 Nuclear Magnetic Resonance

Sulphite, nitrate, and nitrite, the major ions produced by SO_2 and NO_x fumigation, are buffered in the plastid by existing stromal proteins and free amino acids such as cysteine, as well as by phosphate-containing compounds. However, some acidification inevitably takes place if these ions rise to higher levels. A reduction in the buffer capacity of plants has been shown in leaves exposed to pollution (Grill *et al.*, 1975; Klein and Jäger, 1976). Furthermore, those lichens which are most sensitive to pollution are generally those with the lowest buffering capacities (Nieboer *et al.*, 1983). A number of indirect methods to determine interplastidic pH values with plants have been employed (e.g. penetration of [^{14}C]methylamine) with reasonable success (Heldt *et al.*, 1973) although the weak acid 5,5-dimethyl-[2-^{14}C]oxazoidine-2,4-dione may be a better alternative as it is not metabolized or adsorbed onto proteins.

For a more rigorous study of pH changes a direct method is required, preferably one that is rapid, non-invasive and non-destructive. Intracellular pH in erythrocytes and yeast (Moon and Richards, 1973; Navon *et al.*, 1979) has been determined using NMR. Using ^{31}P nuclei the technique is showing considerable promise for the determination of cytoplasmic and vacuolar pH levels in plant cells as well (Roberts *et al.*, 1980, 1981). Nieboer *et al.* (1983) point out that, when perfected, similar techniques may also be extremely useful to detect possible reduced stromal pH changes as a consequence of SO_2 and NO_x exposure.

3.2 Electron Spin Resonance

The mechanism of water splitting during photosynthesis involves manganese (Khanna *et al.*, 1981; Radmer and Cheniae, 1977). Manganese has a distinctive ESR spectrum but, unfortunately, bound manganese within chloroplast membranes is ESR silent and is only detected when freed from its functional site. Liberation of manganese is dependent upon the surrounding regions of the thylakoids which allows ESR to be a possible technique for determining the effect of the products of SO_2, NO_x, and O_3 upon photosynthetic membranes and to indicate any damage they may have upon bioenergetic functions. ESR has already been used to detect changes in the membranes of animals by such pollutants (Rowlands *et al.*, 1977).

In a series of preliminary studies ESR has been used as a probe to investigate the possibility of detecting effects due to the products of atmospheric pollution (e.g. sulphite) upon photosynthetic membranes (Wellburn, 1983). Only single ionic species so far have been investigated but studies of the mixed pollutant situation (i.e. sulphite + nitrite) may well be more rewarding as the 9-AA fluorescence quenching study has already indicated.

3.3 Priorities

Very little is known about the crucial points of attack by single pollutants upon plant metabolism and consequently even less is known about combined modes of action. In the past, a number of biochemical studies of fumigated plants have been conducted at unrealistically high levels of fumigation or with over-optimistic levels of likely *in vitro* anions. Clearly there is a need to measure accurately the intraplastidic concentrations of the different active ions such as sulphite or nitrite after the single and mixed fumigations and use these levels in any subsequent *in vitro* investigation.

It is also important with these acidic anions to discriminate between general disturbances of pH and buffering from specific damage, especially that caused by interactions between sulphite and nitrite within photosynthetic membranes. Consequently more basic studies are required upon natural and pollution-generated free radical events and the natural mechanisms by which the chloroplast removes their damaging effects through various antioxidation mechanisms such as superoxide dismutase, glutathione reductase, ascorbate oxidase and α-tocopherol scavenging. Such information and its exploitation may lead to improved selection not just of SO_2-tolerant cultivars of plants but of those with an enhanced ability to cope with mixtures of atmospheric pollutants which are increasingly the rule rather than the exception within the environment.

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