RESPONSES OF GAS EXCHANGE AND THE ANTIOXIDANT SYSTEM OF SOYBEAN CULTIVARS TO OZONE AND/OR SULFUR DIOXIDE

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(ABSTRACT)

Soybean cultivars (*Glycine max* (L.) Merr.), "Dare", "Williams" and "Essex", with differential sensitivity to ozone (O_3) based on visible injury were exposed once to 0.20 $\mu l\ l^{-1}$ O_3 and/or 0.70 $\mu l\ l^{-1}$ sulfur dioxide (SO_2) for 4 hr. The cv Dare was considered sensitive, cv Williams intermediate and cv Essex tolerant to O_3. Cultivars exposed to filtered air served as controls. Gas exchange measurements were conducted and antioxidant metabolites (reduced and oxidized glutathione: GSH, GSSG; reduced and oxidized ascorbate: AA, dHAA) concentrations and enzymes (glutathione reductase: GRase; ascorbate peroxidase: APase; superoxide dismutase: SOD) activities were analyzed.

Gas exchange rates of all cultivars were significantly inhibited by pollutants exposure. The cv Essex maintained significantly higher net photosynthesis (Pn) at the end of O_3 exposure, during the SO_2 exposure and in the first 2 hr of O_3/SO_2 fumigation. During O_3/SO_2 exposure, the estimated pollutant fluxes were 50% and integrated doses were 25-30% of O_3 or SO_2 when fumigated singly. During O_3 fumigation, cv Dare exhibited a higher O_3 flux and integrated O_3 dose. However,

cv Essex showed a higher O_3/SO_2 peak flux in O_3/SO_2 fumigation. The cv Williams showed the lowest pollutant flux in all treatments.

Ozone fumigation imposed a substantial, but statistically insignificant, effect on some antioxidant components. Relative to the controls, O_3 exposure resulted in increases of GRase and SOD activity in cv Dare. In cv Williams, decreases of GSSG and SOD total activity and increase of dHAA were observed. Decreases of AA and SOD activity and increases of GSSG, dHAA and APase activity in cv Essex were found. The SO_2 exposure resulted in increases of glutathione, particularly GSSG, in all cultivars. The cv Dare responded with increases in AA, APase specific activity and SOD activity. In cv Williams, AA and SOD specific activity increased and APase activity decreased. Decline of SOD activity in cv Essex was found after SO_2 exposure. The O_3/SO_2 fumigation resulted in increases of glutathione, particularly GSSG, and GRase activity, in all cultivars. Declines of dHAA and SOD activity in cv Williams were found. The cv Essex responded with a decline of AA and increases of dHAA and specific activity of APase and SOD.

Ozone and SO_2, singly or in combination, inhibited gas exchange rates in all cultivars, however, cv Essex was the least affected. Stomatal conductance was inhibited greater by O_3 than by SO_2 fumigation. Conversely, Pn was suppressed more by SO_2 than by O_3. The O_3/SO_2 fumigation, however, suppressed Pn and Cs substantially and to a greater extent than individual pollutants. Under the pollutant dose and fumigation profile used in these studies, no consistent responses of different antioxidant components to O_3 and/or SO_2 correlated with differential sensitivity of these soybean cultivars as determined from foliar symptomology.

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I. Introduction

Air pollution resulting from nature and human activities, imposes a considerable threat to many organisms on earth. Plants, suffering from the assault of gaseous pollutants, show a series of malfunctions with different severities in physiological and/or biochemical processes leading to losses in harvest or growth. The most prevalent pollutants in the atmosphere are ozone and sulfur dioxide. These two toxic gases may exert a selective pressure on plants which must acclimate or adapt in order to survive. Plant breeding of pollutant-tolerant cultivars can certainly hasten this process.

Numerous cultivars have been developed in a single plant species to increase productivity and disease resistance under different environmental conditions, it is worthwhile to investigate factors that contribute to an observed differential sensitivity to pollutants in these cultivars. Incorporation of such knowledge into genetic engineering programs may assist in developing cultivars with high resistance to pollutants. Such studies will provide a better understanding of pollutant interactions with basic plant processes and the responses of specific defense mechanisms in plants to minimize the toxic effects of pollutants.

Both O_3 and SO_2 enter the plant leaf only through open stomata. These gases, singly or in combination, have been found primarily to negatively affect photosynthesis and stomatal conductance in many plant species (Atkinson and Winner, 1989; Beckerson and Hofstra, 1979a, b; Chevone and Yang, 1985; Hill and Littlefield, 1969; Kropff, 1987; Muller *et al.*, 1979; Reich *et al.*, 1985; Saxe and Murali, 1989; Sij and Swanson, 1974). Although the specific mechanism(s) of inhibition is (are) not known, it is important to characterize gas exchange rates in

plants subjected to pollutant stress to assess the role of these processes in species or cultivar tolerance.

Generation of superoxide radical ($O_2^{\cdot -}$) and hydroxy radical ($^{\cdot}OH$) occurs after O_3 enters the plant (Grimes *et al.*, 1983). Formation of sulfite (SO_3^{2-}), bisulfite (HSO_3^-) and sulfate (SO_4^{2-}) ions occur after SO_2 absorption through stomata (Silvius *et al.*, 1975). The sulfite ion may be primarily responsible for SO_2 toxicity, although Tanaka and Sugahara (1980) have suggested that SO_2 toxicity is partly due to the $O_2^{\cdot -}$. The dismutation of $O_2^{\cdot -}$ to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) is one mechanism for plant to reduce the damages induced by $O_2^{\cdot -}$. However, H_2O_2 itself is toxic and can inhibit many enzyme activities, such as the Calvin-cycle sulfhydry (SH) enzymes. A proposed system for the scavenging of H_2O_2 by Foyer and Halliwell (1976): photosystem I → ferredoxin → NADP → glutathione → ascorbate system received much attentions (Fig. 1).

Ozone and SO_2, singly or in combination, have been found to affect (or not affect) photosynthesis and stomatal conductance in many plant species. Changes in the components of H_2O_2-scavenging system under O_3 and SO_2 stress, singly or in combination, were also reported. This study was designed to characterize the responses of gas exchange and the antioxidant system of soybean cultivars to O_3 and SO_2, singly or in combination.

II. Objectives

The general objectives of these studies were to characterize the responses of gas exchange and the antioxidant system of three soybean cultivars with differential foliar sensitivity to O_3. Specific objectives were to:

1. Investigate the gas exchange characteristics of three soybean cultivars under O_3, SO_2 or O_3/SO_2 stress.

2. Investigate and compare responses of the antioxidant metabolites of three soybean cultivars under O_3, SO_2 or O_3/SO_2 stress.

3. Investigate and compare responses of the antioxidant enzymes of three soybean cultivars under O_3, SO_2 or O_3/SO_2 stress.

4. Determine if any antioxidant metabolites and/or enzymes are responsible for the differential sensitivity of these three soybean cultivars under O_3, SO_2 or O_3/SO_2 stress.

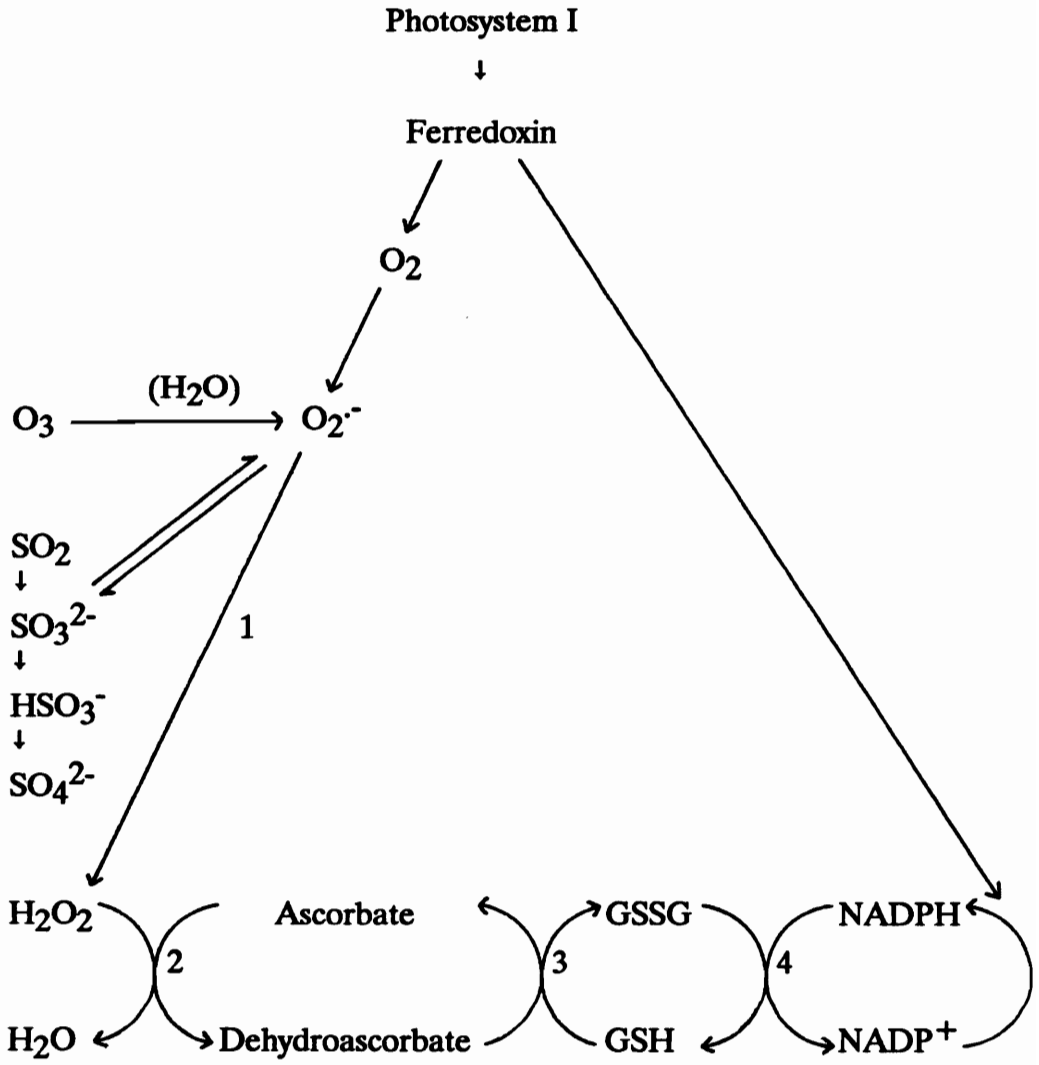

Fig. 1. H_2O_2-scavenging system in plant chloroplasts. 1 = Superoxide dismutase (SOD). 2 = Ascorbate peroxidase (APase). 3 = Dehydroascorbate reductase. 4 = Glutathione reductase (GRase). GSH = reduced glutathione. GSSG = oxidized glutathione.

CHAPTER 1. LITERATURE REVIEW

I. Gas Exchange

1. Introduction

Plants absorb the energy in sunlight and convert it into chemical energy. During this process, named photosynthesis, carbohydrates are synthesized from carbon dioxide (CO_2) and water (H_2O), as carbon and hydrogen donors, respectively, through a series of enzyme-catalyzed reactions in chloroplasts and oxygen (O_2) is produced. The process of respiration, which breaks down organic molecules such as carbohydrates and fats into CO_2 with the release of free energy, is the opposite of photosynthesis. In plants, photorespiration can occur only in the light, which takes place in the chloroplasts, peroxisomes and mitochondria, and is different from normal respiration in that free energy is used but not released.

Gas exchange in plants represents a balance between the uptake of CO_2 during photosynthesis and the release of H_2O from the leaf surface. Passage of gases, with low cuticular permeability, into or out of plants is mostly governed by the stomatal conductance. During light period, CO_2 uptake rate is much greater than CO_2 production since plant growth requires biomass production from photosynthesis directly or indirectly. Thus, the CO_2 gas exchange rate is expressed as a net result between photosynthesis and respiration, both light and dark. However, when plants are subjected to stresses, such as temperature, drought, nutrition or pollution (gases, heavy metals, salt), gas exchange rates (net photosynthesis and transpiration) will be altered. The effects of gaseous pollutants,

O_3 and SO_2, singly or in combination, on gas exchange of plants will be discussed next.

2. Ozone

The ozone layer in the stratosphere serves to protect the earth from ultraviolet light damage, but O_3 at ground level is responsible for the major air pollution injury to plants. It is formed through a series of reactions involving nitrogen dioxide, atmospheric oxygen and hydrocarbons produced from the combustion of fossil fuels in transportation and electrical energy generation.

Background levels, as well as higher concentrations, of O_3 have been reported to reduce crop growth and yield (Heck *et al.*, 1984), although Heagle *et al.* (1974) showed that soybean can sustain some ozone injury without loss of yield. Net photosynthesis and stomatal conductance were two of the most sensitive physiological processes to O_3 (Taylor *et al.*, 1988). Injury may not necessarily occur on the leaves when crop photosynthesis is reduced by O_3 (Hill and Littlefield, 1969).

Oat, barley, wheat, tobacco, corn, sugar beet, Swiss chard, pinto bean, lima bean, bush bean, cauliflower, potato and tomato exposed to 0.4~0.9 $\mu l\ l^{-1}$ O_3 typically exhibited photosynthesis reduced to 40 to 70% of the control within 30 to 90 min (Hill and Littlefield, 1969). After termination of O_3, photosynthesis normally began to increase within 30 min. Within 3 or 4 hr, recovery was usually only partial and by the next morning was complete or nearly complete. The authors certainly used much higher O_3 concentrations for plant fumigation than those found during peak O_3 concentration in the ambient air. Exposure of Norway spruce (*Picea abies*) to 0.064, 0.166, 0.336, 0.452, or 0.693 $\mu l\ l^{-1}$ O_3 showed that the

2 highest O_3 concentrations immediately caused photosynthesis and transpiration reduction while at 0.166 and 0.336 $\mu l\ l^{-1}$ O_3, photosynthesis and transpiration declined 1.5 hr and 2.5 hr, respectively, after O_3 exposure was imposed (Saxe and Murali, 1989). Recovery of photosynthesis and transpiration was very slow and even lower than the average response during exposure. Other reports also showed a considerable decline of photosynthesis (22 to 60% reduction) in various plants by high O_3 concentrations for a short period of time (2 to 4 hr) (see Darrall, 1989).

Low concentrations of O_3 were applied to soybean (Reich and Amundson, 1985; Reich *et al.*, 1986b), hybrid poplar (Reich, 1983; Reich and Amundson, 1985), sugar maple and northern red oak (Reich and Amundson, 1985; Reich *et al.*, 1986a) and wheat (Lehnherr *et al.*, 1988) for a long period of time (5 to 62 days). The O_3 concentrations, ranging from 0.01 to 0.14 $\mu l\ l^{-1}$, were representative of those found in mildly or moderately polluted ambient air of the United States. Reduction of net photosynthesis (Pn) increased as O_3 exposure concentration increased, and a linear relationship between Pn and O_3 concentration was found (Reich *et al.*, 1986b). Ormrod *et al.* (1982) observed that net photosynthesis was depressed significantly by O_3 above a threshold of 0.05 $\mu l\ l^{-1}$ in *Vicia faba* cv Dylan (field bean); and the depression of photosynthesis increased as O_3 concentration increased, reaching a maximum depression of 15-20% at 0.3 $\mu l\ l^{-1}$ O_3. Similarly, spring barley (*Hordeum vulgare* cv Klaxon) exposed to 0.05, 0.10 or 0.15 $\mu l\ l^{-1}$ O_3 for 7 hr per day for 12 days exhibited a decreased carbon exchange rate with increasing concentration of O_3 and fell to 60% of the control at 0.15 $\mu l\ l^{-1}$ O_3 (Rowland-Bamford *et al.*, 1989). On the other hand, exposure to low concentrations of O_3 had no immediate, direct effects on Pn, whereas chronic exposure to similar levels did reduce CO_2 exchange in hybrid poplar leaves,

although the decreased Pn was partially due to accelerated aging in leaves exposed to O_3 (Reich, 1983). Some reports demonstrated no effects on photosynthesis of soybean or *Pinus* sp. by long-term O_3 fumigation (0.05 to 0.15 $\mu l\ l^{-1}$ for 35 to 86 days; cited in Darrall, 1989).

Roper and Williams (1989) found that grapevine leaves fumigated with 0.2 $\mu l\ l^{-1}$ O_3 for 5 hr showed no significant change in net CO_2 assimilation rate and a significant reduction in stomatal conductance. Accordingly, internal CO_2 concentration also declined significantly. The authors suggested a greater stomatal limitation of photosynthesis by the pollutant. However, under higher O_3 exposure (0.6 $\mu l\ l^{-1}$) the authors suggested a greater mesophyll limitation to photosynthesis. Lehnherr *et al.* (1988) exposed wheat to 0.07 or 0.10 $\mu l\ l^{-1}$ O_3 and concluded that the decrease in stomatal conductance with increasing O_3 concentration did not account for the decline in Pn since stomatal conductance was less affected by O_3 than Pn. They suggested that Pn in O_3 treated leaves was limited by ribulose bisphosphate carboxylation.

Stomatal conductance was reduced by O_3 in most studies with some exceptions (Evans and Ting, 1974; Freer-Smith and Dobson, 1989; Olszyk and Tibbitts, 1981) showing an increase response. Hill and Littlefield (1969) showed that stomatal width and transpiration were rapidly reduced by O_3 treatment in many plants. Beckerson and Hofstra (1979a, 1979b) also demonstrated that O_3 decreased stomatal conductance in white bean, radish, cucumber and soybean. Exposure of field-grown cotton to non-charcoal filtered (NF) or double ambient O_3 resulted in 15.5% and 30% lower maximum conductance, respectively, than those in charcoal-filtered chambers (Temple, 1986). Stomatal conductance of soybean cv Hodgson was 20-30% lower when exposed to 0.13 $\mu l\ l^{-1}$ O_3 compared

to 0.01 $\mu l\ l^{-1}$ O_3 (Reich *et al.*, 1985). Species with higher stomatal conductances, which led to a higher potential for pollutant uptake, exhibited greater negative responses to similar O_3 treatment (Reich and Amundson, 1985). However, differential sensitivity to O_3 cannot always be attributed to stomatal responses, but possibly to photosynthetic capability (Coyne and Bingham, 1982; Elkiey *et al.*, 1979).

An increase of stomatal conductance to O_3 exposure was found in Norway and Sitka spruce (Freer-Smith and Dobson, 1989), Scots pine (Skärby *et al.*, 1987), pea (Olszyk and Tibbitts, 1981) and in bean (Evans and Ting, 1974). There was no good explanation for the increased stomatal responses to O_3, however, environmental conditions and plant physiological status have great influences on stomatal movement.

3. Sulfur Dioxide

Coal-burning electric power plants contribute most of the sulfur dioxide emission into the ambient air while home heating and volcanic eruption contribute a small portion annually. However, a recent (1991) volcanic eruption (Mt. Pinatubo) in the Philippines was estimated to emit 15-20 million tons of SO_2 over a two-week period compared to 15 million tons annually from electrical utilities in the United States. Although effective control of SO_2 emission may be achieved in some countries, many older plants or newer factories in developing countries are without the technology (Treshow and Anderson, 1989). In the United States, the Shenandoah National Park has the worst air pollution of any park in the country. Most of it is blown by winds that carry acids produced by factories and utility plants

in the Midwest (New York Times, cited in Star Tribune, May 12, 1991). The Clean Air Act will require the 111 largest sulfur-emitting electric utility plants, most of which are in the Midwest, in 22 states to meet stricter standards for sulfur emission. By the year 2000 emission of SO_2 will be cut by 12 million to 14 million tons annually. Half of the total SO_2 reductions would occur by Jan. 1, 1995 (New York Times, cited in Star Tribune, Oct. 22, 1990).

Photosynthesis was inhibited by SO_2 fumigation in many plant species in most studies and it may be reduced without accompanying visible damage of the leaf tissue (Muller *et al.*, 1979; Price and Long, 1989). Field-grown soybean, cv. Wells, exposed to 0.79, 0.30 or 0.12 $\mu l\ l^{-1}$ SO_2 for 24 fumigations ranging from 3 hr to 6 hr 40 min within 48 days exhibited photosynthesis reductions to 37-63%, 63-83% and 90-137%, respectively, of control plants (Muller *et al.*, 1979). A delayed photosynthetic response to SO_2 was observed in plants exposed to 0.79 $\mu l\ l^{-1}$ SO_2 and was more pronounced at 0.30 $\mu l\ l^{-1}$ SO_2. Another soybean cultivar, Wayne, exposed to 0.18 to 1.1 $\mu l\ l^{-1}$ SO_2 had a decreased photosynthetic rate with increasing SO_2 concentration and the reduction in photosynthesis was highly correlated with the total amount of SO_2 absorbed (Carlson, 1983). Takemoto and Noble (1982) found that Pn of soybean strain T219 decreased by as much as 45% over a 2 hr period in the presence of 0.50 or 0.75 $\mu l\ l^{-1}$ SO_2, but was not significantly different between those exposed to 0.25 $\mu l\ l^{-1}$ SO_2 and of control plants.

Inhibition of photosynthesis in *Vicia faba* by 0.3 $\mu l\ l^{-1}$ SO_2 occurred within the first 20 min and a stable photosynthetic rate was obtained within 2 hr (Kropff, 1987). Similar results have been found by Black and Unsworth (1979), Darrall (1986), Sij and Swanson (1974) and Sisson *et al.* (1981). As a result, a dose-

response relationship is not valid for the short-term (from 2 to 8 hr) exposure to SO_2 (Darrall, 1989) although an almost linear decline of photosynthesis in *Diplacus aurantiacus* and *Heteromeles arbutifolia* exposed to various SO_2 concentrations for 8 hr was reported (Winner and Mooney, 1980a).

Enhancement of photosynthesis by sub-inhibitory SO_2 concentrations has been reported (Muller *et al.*, 1979; Winner and Mooney, 1980b). Photosynthesis of soybean cv. Wells was 18% and 37% higher in the treatment plants exposed to 0.079 $\mu l\ l^{-1}$ SO_2 than in the control plants, respectively, at the beginning of and after 4 hr fumigation (Muller *et al.*, 1979). Increase of photosynthesis in *Atriplex* species exposed to 0.5 $\mu l\ l^{-1}$ SO_2 was temporary and decline of photosynthesis occurred eventually during fumigation (Winner and Mooney, 1980b).

Changes in stomatal conductance in response to SO_2 were variable depending on species, SO_2 concentrations, exposure regime and environmental conditions. Opening (Black and Black, 1979a, b; Black and Unsworth, 1980; Majernik and Mansfield, 1970; Mansfield and Majernik, 1970; Unsworth *et al.*, 1972) or closure (Atkinson and Winner, 1989; Kondo and Sugahara, 1978; Sij and Swanson, 1974; Winner and Mooney, 1980a) of stomatal conductance induced by SO_2 have been reported. Most of these studies were conducted with either *Vicia* or *Phaseolus* plants. There are only two studies of stomatal response of soybean plants to SO_2. Field-grown soybean, cv. Wells, fumigated with 0.79, 0.30 or 0.12 $\mu l\ l^{-1}$ SO_2 showed a decreased stomatal conductance (10-16%) in all treatments (Muller *et al.*, 1979). Stomatal conductance to CO_2 of soybean, cv. Wayne, declined with increasing SO_2 at 300, 450 and 600 ppm CO_2, with larger initial conductance and larger reductions occurring at lower CO_2 concentrations (Carlson, 1983). Stomatal conductance at 1200 ppm CO_2 for non-fumigated plants

was less than 25% of that observed at 300 ppm and did not significantly decrease with increasing SO_2 concentrations.

4. Ozone and Sulfur Dioxide

In the atmosphere, gaseous pollutants coexist in various ratios depending on environmental conditions and industrial and human activities. As a result, it is difficult to identify the main component causing major damages to plants unless the pollutant concentrations and environmental conditions are properly monitored. Fortunately, symptoms induced by each gaseous pollutant are distinctive. Ozone and SO_2 are, at present, the two most pervasive air pollutants (Gardner and Ormrod, 1976). Studies involving a single pollutant, although seeming to be unrealistic, have an importance to assess and understand the effects and damaging mechanisms that pollutants imposed on plants. Thus, a better understanding of how plants respond to combined air pollutants can be achieved by subjecting plants to combined pollutant stresses.

There are not many studies of the combined effects of O_3 and SO_2 on plants compared to either pollutant alone. Most studies have focused on leaf damages induced by O_3 and SO_2 (Applegate and Durrant, 1969; Gardner and Ormrod, 1976; Heagle and Johnston, 1979; Hofstra and Ormrod, 1977; Jacobson and Colavito, 1976; Macdowall and Cole, 1971; Menser and Heggestad, 1966; Olszyk and Tingey, 1985; Tingey and Reinert, 1975). Ozone and SO_2 treatment reduced photosynthetic rate in hybrid poplar (Jensen and Noble, 1984) and field bean (*Vicia faba* L. cv. Dylan) (Ormrod *et al.*, 1982). In soybean cultivar Essex, Chevone and Yang (1985) observed a 67% decrease in carbon dioxide exchange rate under 0.2 and 0.7 $\mu l\ l^{-1}$ O_3 and SO_2, respectively, for 2 hr.

Leaf diffusive resistance of yellow poplar exposed to 0.15 and 0.25 $\mu l\ l^{-1}$ O_3 and SO_2, respectively, for 5 hr/day for 5 days at 80% R.H. displayed a greater variation with a larger range of increases and decreases than those exposed to either O_3 or SO_2 alone (Jensen and Roberts, 1986). The response of stomata of petunia (*Petunia hybrida* Vilm.) to 0.4 $\mu l\ l^{-1}$ O_3 and 0.8 $\mu l\ l^{-1}$ SO_2 at 50% R.H. exhibited a rapid increase in leaf diffusion resistance (Elkiey and Ormrod, 1979).

Increased stomatal resistance of *Phaseolus vulgaris* L. cv. Sanilac, cucumber (*Cucumis sativus* L. cv. National Pickling), radish (*Raphanus sativus* L. cv. Champion) and soybean (Glycine max (L.) Merr. cv. Harosoy 63) exposed to the gas mixture of 0.15 $\mu l\ l^{-1}$ O_3 and SO_2 was found (Beckerson and Hofstra, 1979a, b). The combination of 0.13 $\mu l\ l^{-1}$ O_3 and 1.23 $\mu l\ l^{-1}$ SO_2 induced only stomatal closure in garden pea (*Pisum sativum* L. cv. Alsweet) (Olszyk and Tibbitts, 1981).

Olszyk and Tingey (1986) found that stomatal conductance of garden pea (*Pisum sativum* L. cv. Alsweet) was reduced with exposure to 0.11 and 0.12 $\mu l\ l^{-1}$ O_3 and SO_2, respectively. They indicated that stomatal conductance was related to the integrated dose of pollutants; however, stomatal responses with combinations of O_3 and SO_2 were not dependent solely on the integrated dose of pollutants, but a metabolic synergistic effect appears to exist. Simultaneous application of 0.2 and 0.7 $\mu l\ l^{-1}$ O_3 and SO_2, respectively, to soybean cultivar Essex for 2 hr resulted in an 11% decrease in stomatal resistance (Chevone and Yang, 1985).

Generally, plant photosynthesis is inhibited by O_3 or SO_2 at various concentrations for short-term or long-term exposures. Some exceptions have been found where air pollutants show no effect or enhance plant photosynthesis. Most studies of the response of stomatal conductance to O_3 or SO_2 exposure have

demonstrated an inhibitory effect by the air pollutants. Exceptions of a stimulative effect on stomatal conductance have also been reported.

II. Metabolites

1. Introduction

In the oxygen-containing atmosphere, production of oxidants is unavoidable. As industrial technology advanced, air pollution problems also increased. Some gaseous pollutants will generate or induce production of oxidants under aqueous conditions. Plants and animals have equipped themselves with the oxidant-ridding mechanisms. Antioxidant metabolites, glutathione and ascorbic acid, are abundant in the chloroplasts and other cell compartments to function as oxidant scavengers (Foyer and Halliwell, 1976). Both are present mostly in their reduced states. The oxidized forms of glutathione and ascorbic acid, GSSG and dehydroascorbate, respectively, would be rapidly reduced to maintain a high ratio of reduced/oxidized by an efficient reducing mechanism which links these metabolites together as a cycle.

2. Ozone

Glutathione level of four bean (*Phaseolus vulgaris*) cultivars (two sensitive and two insensitive to O_3) exposed to 0.28~0.32 μl l^{-1} O_3 for 8 hr declined substantially in the two sensitive cultivars, whereas in the two insensitive cultivars the decline of glutathione was significant, but small (Guri, 1983). Spinach plants (*Spinacia oleracea*) fumigated with 0.3 μl l^{-1} O_3 for 20 hr had a decreased glutathione level (Tanaka *et al.*, 1985). Exposure of six spinach cultivars to 0.2 μl l^{-1}

O_3 for 27 hr revealed that two cultivars showed a higher tolerance to O_3 with a higher level of glutathione than the other cultivars. At 0.1 $\mu l\ l^{-1}$ O_3 for 7 days, glutathione level of spinach plants was less affected (Tanaka *et al.*, 1985). A rapid loss of glutathione in whole leaves was observed during 0.2 or 0.4 $\mu l\ l^{-1}$ O_3 exposure of *Sedum album* leaves for 2 hr (Castillo and Greppin, 1988). No effect of 0.037 $\mu l\ l^{-1}$ O_3 on glutathione of spruce needles was found (Mehlhorn *et al.*, 1986) but a trend for red spruce needles, exposed to increasing amounts of ambient O_3, to have higher glutathione was reported (Hausladen *et al.*, 1990).

The ascorbate level of four bean cultivars declined significantly after O_3 fumigation and no differences in ascorbate level were found among cultivars (Guri, 1983). When spinach plants were fumigated with O_3, ascorbate level decreased (Tanaka *et al.*, 1985). Spinach cultivars that were O_3-tolerant had a higher level of ascorbate after O_3 exposure. A low concentration of O_3 (0.1 $\mu l\ l^{-1}$) had less effect on the level of ascorbate in spinach (Tanaka *et al.*, 1985). Exposure of *Sedum album* to different O_3 concentrations (0.2, 0.4, and 0.6 $\mu l\ l^{-1}$ for 2 hr) always resulted in an increase in the level of total ascorbate in the extracellular space (Castillo and Greppin, 1988). However, in whole leaves of *Sedum album* plants, the ascorbate level decreased after exposure to O_3. Lee *et al.* (1984) found that leaf ascorbate level increased in the O_3-resistant and declined in the O_3-sensitive cultivars of both soybean and snapbean during O_3 exposure. Hausladen *et al.* (1990) found no effect of O_3 on ascorbate level in red spruce needles.

3. Sulfur Dioxide

Glutathione level in SO_2-damaged spruce needles was always higher than healthy needles except immediately after flushing (abstract from Grill and

Esterbauer, 1973). Several perennial plants from SO_2-polluted areas contained an increased glutathione level (Grill *et al.*, 1979, 1982). Chiment *et al.* (1986) observed an increase in glutathione level of soybean leaves after various concentrations of SO_2 exposures; the maximum glutathione increase occurring at 0.3 $\mu l\ l^{-1}$ SO_2, both higher and lower SO_2 levels producing smaller glutathione increases. Increase of glutathione during SO_2 exposure occurred in an insensitive but not in a sensitive cultivar of *Pisum sativum* (Alscher *et al.*, 1987). Low concentration of SO_2 (0.004~0.012 $\mu l\ l^{-1}$) stimulated glutathione level in 2-year-old needles of spruce (Mehlhorn *et al.*, 1986).

A 35% increase of ascorbic acid in 2-year-old needles of spruce exposed to 0.004~0.012 $\mu l\ l^{-1}$ SO_2 was found (Mehlhorn *et al.*, 1986).

4. Ozone and Sulfur Dioxide

Only one study investigated the effect of O_3 and SO_2 on production of antioxidants in needles of fir (*Abies alba* Mill.) and spruce (*Picea abies* (L.) Karst) (Mehlhorn *et al.*, 1986). The authors found that total glutathione level of both fir and spruce increased significantly in response to 0.037 $\mu l\ l^{-1}$ O_3 and 0.004~0.012 $\mu l\ l^{-1}$ SO_2 compared to those treated with filtered air. Ascorbate level in 2-year-old spruce needles increased significantly after O_3 and SO_2 treatment. Apparently low concentrations of air pollutants induce an increase in antioxidant production.

III. Enzymes

1. Introduction

As antioxidant metabolites scavenge the oxidants, enzymes play an important part in the system. Ascorbate peroxidase and glutathione reductase, found in the chloroplast and other cell compartments, are involved in the scavenging cycle. Superoxide dismutase is present in the chloroplast, mitochondria and cytoplasm to dismutate the superoxide radical ($O_2^{\cdot -}$) to form hydrogen peroxide (H_2O_2) which is then reduced to H_2O by ascorbate and ascorbate peroxidase. Reduction of oxidized glutathione (GSSG) with NADPH and glutathione reductase produces glutathione which in turn reduces oxidized ascorbate (dehydroascorbate).

2. Ozone

Ozone did not affect significantly the activity of glutathione reductase (GRase) in four bean cultivars (Guri, 1983) and in *Sedum album* (Castillo and Greppin, 1988); however, a low O_3 concentration (0.07 μl l^{-1}) induced an increase in GRase activity in spinach leaves (Tanaka *et al.*, 1988). The GRase activity was significantly higher in the two O_3-insensitive bean cultivars before and after O_3 exposure than in the two O_3-sensitive cultivars (Guri, 1983). Exposure of spinach to O_3 resulted in an increase of GRase activity in the first 4 hr and a final decline at the end of 8 hr. No difference in GRase activity among spinach cultivars with differential O_3 sensitivity after O_3 fumigation was found (Tanaka *et al.*, 1985).

When spinach plants were fumigated with 0.5 μl l^{-1} O_3, ascorbate peroxidase (APase) was inactivated; however, increase of APase activity was found

in spinach plants when exposed to 0.1 μl l^{-1} O_3 (Tanaka *et al.*, 1985). Increase of APase activity was also found in *Sedum album* after 0.4 μl l^{-1} O_3 exposure for 2 hr (Castillo and Greppin, 1986).

Activity of superoxide dismutase (SOD) in maize was unaffected 24 hr after O_3 fumigation (Matters and Scandalios, 1987). However, decline of SOD activity in spinach plants exposed to 0.5 μl l^{-1} O_3 was found (Tanaka *et al.*, 1985). Red spruce needles exposed to increasing amount of ambient O_3 displayed a decline in SOD activity (Hausladen *et al.*, 1990).

3. Sulfur Dioxide

Fumigation of spinach leaves with SO_2 resulted in inactivation of GRase and APase activities and no effect on SOD (Tanaka *et al.*, 1982). On the other hand, Shimazaki *et al.* (1980) observed a 60% reduction of SOD activity in spinach leaves after SO_2 exposure.

4. Ozone and Sulfur Dioxide

No studies on the effect of O_3 and SO_2 on antioxidant enzymes is available in the literature. Effects of air pollutants on antioxidant enzyme activity of plant species and/or cultivars are varied depending on the pollutant concentrations and the sensitivity of the plants. Low concentrations of air pollutants could induce, while high concentrations could inactivate, some antioxidant enzyme activities. Other factors which can affect plant responses to air pollutants are the environmental conditions during exposure and the fumigation regime.

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CHAPTER 2. RESPONSES OF GAS EXCHANGE RATES AND THE ANTIOXIDANT SYSTEM OF SOYBEAN (*Glycine max* (L.) Merr.) CULTIVARS TO OZONE

Introduction

Through photochemical reactions, ozone (O_3) is generated in the surface troposphere. The photolytic cleavage of nitrogen dioxide (NO_2), emitted principally in the U.S. from the transportation industry, by UV light results in nitric oxide (NO) and atomic oxygen (O) formation. The reaction of molecular oxygen (O_2) with O forms O_3; and the reverse reaction of O_3 with NO produces O_2 and NO_2, which completes the reaction cycle. However, hydrocarbons, also emitted primarily from the transportation industry, may compete with O_3 for NO. This reaction generates peroxyacyl nitrate (PAN), an eye irritating and phytotoxic pollutant, but more importantly, leads to decreased NO concentrations and prevents the reverse reaction. Under such conditions, tropospheric O_3 concentrations may increase from background levels of 0.02 to 0.04 $\mu l\ l^{-1}$ to episodic levels in excess of 0.15 $\mu l\ l^{-1}$.

Annual crop yield reduction in the United States caused by O_3 alone has been estimated to be about 15 % which amounts to one to two billion dollars in loss (Heck *et al.*, 1983). Recently, forest decline in Europe and the United States have become a major concern and are believed to be caused by O_3 and other

factors such as acidic precipitation, drought, soil nutrients and plant pathogens (Chevone and Linzon, 1988; Freedman, 1989; Treshow and Anderson, 1989).

Ozone has been found to decrease (or not affect) photosynthesis (Ps) and stomatal conductance (Cs) of many plants in either greenhouse, laboratory, open-top chamber or field studies (Gillespie and Winner, 1989; Hill and Littlefield, 1969; Reich and Lassoie, 1984; Reich and Amundson, 1985; Reich *et al.*, 1986; Temple, 1986; Yang *et al.*, 1983). Some exceptions have been reported where O_3 treated plants had a higher Ps or Cs than the controls (Evans and Ting, 1974; Freer-Smith and Dobson, 1989). As demonstrated in many reports, O_3 uptake is principally controlled by the stomata (Rich *et al.*, 1970; Tibbitts and Kobriger, 1983), and the magnitude of influx is proportional to the O_3 concentration (Taylor *et al.*, 1982). Ozone also may affect stomatal aperture (Adedipe *et al.*, 1973; Hill and Littlefield, 1969) which in turn will reduce its entry into the leaf interior. After O_3 enters the plant, it can decompose in the cell walls or plasmalemma generating radical products, such as superoxide radical ($O_2^{\cdot -}$) and hydroxy radical ($^{\cdot}OH$) (Grimes *et al.*, 1983). The free radicals may be most damaging to the tissue or O_3 itself may cause damage. Laisk *et al.* (1989) observed that there was zero O_3 concentration in the leaf intercellular air space because no O_3 could be monitored on the upper sunflower leaf surface when gas uptake was through the lower leaf surface. Laisk's report was the first study designed to measure O_3 in the intercellular spaces. Most earlier reports only assumed that the intercellular O_3 concentration was zero when calculating the O_3 flux (Taylor *et al.*, 1982). It is noteworthy that an intercellular O_3 concentration of zero was measured for only one minute during the application of

the pollutant. It is not known if the intercellular concentration would increase during longer fumigation periods.

The visible injury caused by O_3 occurs only on the upper leaf surface unless the concentration is very high. The high surface/volume ratio in the palisade layer of mesophyll cells may, in part, be responsible for the site of injury (Heath, 1975; Thomson *et al.*, 1966). The degree of injury severity is dependent on plant cultivars, species, developmental stage and environmental conditions which influence leaf physiology and can affect the O_3 action against plant tissues. Apparently, the differential sensitivity to pollutants that exists among plants may occur for genetic, physiological and/or biochemical reasons (Butler and Tibbitts, 1979a, b; Butler *et al.*, 1979; Dean, 1972; Engle and Gabelman, 1966; Thorne and Hanson, 1976).

There is no definitive evidence of the primary site of attack by O_3 on plant cells. However, phospholipids (Mackay *et al.*, 1987) and proteins in the plasmalemma (Perchorowicz and Ting, 1974) appear susceptible, since O_3 tends to cause lipid peroxidation and oxidation of the surface proteins on the membrane. Also, O_3 can pass the plasmalemma and attack subcellular organelles, such as chloroplasts and mitochondria, as evidenced by shrinkage of chloroplasts (Thomson *et al.*, 1966) and swelling of mitochondria (Swanson *et al.*, 1973). As a result, metabolic pathways in these organelles may be affected. All these changes caused by O_3 do not necessarily produce visible injury, but certainly can affect plant growth and productivity.

The possible production of $O_2^{\cdot-}$ or $^{\cdot}OH$ (Grimes *et al.*, 1983) during O_3 decomposition imposes a great threat to the plant tissue. However, all aerobic life

on earth has defense mechanisms against O_2 toxicity and the formation of $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2) and $^{\cdot}OH$. Superoxide dismutase (SOD), catalase and peroxidase scavenge the $O_2^{\cdot -}$ and H_2O_2 (Harper and Harvey, 1978; Kelly and Latzko, 1979; McCord and Fridovich, 1969), and prevent the formation of $^{\cdot}OH$. Superoxide dismutase exists in organelles like chloroplasts and mitochondria (Asada *et al.*, 1973; Baum and Scandalios, 1979; Baum and Scandalios, 1981; Jackson *et al.*, 1978) and is found also in cytoplasm (Baum and Scandalios, 1979; Foster and Edwards, 1980) and peroxisomes (Sandalio and Del Río, 1988); catalase is present in peroxisomes (Halliwell, 1974) and peroxidase, especially ascorbate peroxidase (APase), in chloroplasts and the apoplastic space (Castillo and Greppin, 1986; 1988; Nakano and Asada, 1981).

Because of compartmentalization, reactions of these enzymes with O_3 products might not occur in an ordered series. Either the enzymes may be transported out of their compartment to scavenge the toxic O_3 products or the toxic oxidants may diffuse into the enzyme location to be detoxified. If O_3 decomposes in the cell wall or plasmalemma area (Castillo and Greppin, 1988; Laisk *et al.*, 1989) and generates free radicals which can be removed by ascorbate (AA) or possibly SOD which diffuses from cytosol, then further damage caused by $O_2^{\cdot -}$ is eliminated. The toxicity of H_2O_2 is minimized by APase which catalyzes the reaction of AA and H_2O_2 to form H_2O and dehydroascorbate (dHAA). Ascorbate is an abundant antioxidant compound in plant tissue (Halliwell, 1982) that is located in the same organelle or cytoplasmic spaces as APase (Castillo and Greppin, 1986, 1988; Nakano and Asada, 1981). Hydrogen peroxide may also be detoxified by catalase in the peroxisome if H_2O_2 is transported into it. The oxidation ability of H_2O_2 tends to target many

compounds inside the cell, especially the free sulfhydryl compound such as thiol enzymes of the Calvin cycle (Kaiser, 1976; Kaiser, 1979; Charles and Halliwell, 1980).

Ascorbate may be regenerated from dHAA by dehydroascorbate reductase with glutathione (GSH) serving as the electron donor (Foyer and Halliwell, 1976). In the process, GSH forms oxidized glutathione (GSSG) which is then reduced by NADPH and glutathione reductase (GRase) (Foyer and Halliwell, 1976). This coupled ascorbate-glutathione cycle is proposed as a primary scavenging system in plant tissue for oxidants. When SOD activity and AA levels were increased by O_3 exposure, they were found to be higher in young leaves than in newly matured leaves, and higher in O_3-resistant compared to susceptible genotypes (Lee and Bennett, 1982; Lee *et al.*, 1984; Tanaka *et al.*, 1985). However, contradictory results in terms of O_3 stress stimulating SOD activity also have been reported (Chanway and Runeckles, 1984a, b; Matters and Scandalios, 1987).

At present, the mechanism of ozone toxicity to plants is not definitively known, especially those events which occur during the initial stages of pollutant exposure. In addition, the basis for differential ozone sensitivity among genotypes within a plant species is inadequately understood. The objectives of this research were to investigate various physiological and biochemical responses of soybean plants to short-term O_3 exposures. Soybean (*Glycine max* (L.) Merr.) cultivars: Dare, Williams and Essex were selected for study because of their different sensitivity to O_3. Based on foliar symptomology, Dare is considered sensitive (Howell *et al.*, 1979; Heagle, 1979) Williams is considered moderately sensitive

and Essex is considered moderately tolerant (Heagle and Letchworth, 1982). To understand possible mechanisms involved in the differential sensitivity to O_3 among soybean cultivars, gas exchange rates, enzyme activities of SOD, APase and GRase and antioxidant metabolite levels of ascorbate and glutathione were measured when exposed to O_3 stress.

Materials and Methods

Plant Material

A potting mixture of Weblite: vermiculite: Altavista soil (sandy loam): sand (5:5:1:1 V:V:V:V:) was used for growing plants. Soybean seed (*Glycine Max* (L.) Merr.), cultivars: Dare, Williams and Essex, was sown in 15 cm-diameter plastic pots containing the growing mixture and 8 gm Osmocote (14:14:14 N:P:K, a controlled release fertilizer, Sierra Chemical Company, Milpitas, CA) per pot. All seedlings were kept in a greenhouse supplied with charcoal filtered air (< 0.025 $\mu l\ l^{-1}$ O_3) under a 16 hour photoperiod with supplemental lighting provided by high pressure sodium lamps (1000W Ceramalux, Philips Lighting Corp., Bloomfield, NJ) to extend the normal day length. Environmental conditions in the greenhouse were 55% ± 10% relative humidity, 28 ± 5 °C temperature and 900 ±100 $\mu E\ m^{-2}\ s^{-1}$ photosynthetic active radiation (PAR) supplemental lighting. Soybean seedlings were thinned to 2 uniformly developed plants per pot at the fully-opened primary leaf stage. All plants were well-watered throughout their growing period. Three-week-old plants with a new, fully expanded, second trifoliate leaf were used for all experiments.

Pollutant Application

Prior to O_3 exposure, plants were acclimated overnight in the Continuously Stirred Tank Reactors (CSTRs) (Heck, Philbeck and Dunning, 1978) beginning late in the afternoon (1700) under dark conditions with the impellers and exhaust system operating. Timer-controlled metalarc lamps (1000W, Sylvania, GTE Products Corp., Manchester, NH) which provided 550 ±

100 μE m^{-2} s^{-1} PAR at plant canopy height, were activated in the morning (0500), 5.5-6 hr before starting a fumigation (1030-1100). The CSTRs were equipped with environmental controls to maintain the temperature and relative humidity at 26 ± 3 °C and 65% ± 10%, respectively.

Ozone was generated from pure O_2 (Industrial Gas & Supply Company, Radford, VA) by UV light using a Welsbach ozonator (Model T-408, Welsbach Ozone System Corp., Philadelphia, PA). Ozone concentration within a CSTR was controlled by a mass flow controller (Sierra Instruments, Inc., Carmel Valley, CA) and monitored with a UV photometric O_3 analyzer (Model 49, Thermo Electron Instruments, Hopkinton, MA) which was calibrated every 2 weeks with a photocal 3000 ozone calibrator (Columbia Scientific Industries, Austin, TX). A data acquisition system (Keithley Data Acquisition & Control, Cleveland, OH) linked to an IBM computer through a software program (written by Farhoood Moselehi, Virginia Tech, VA) provided a time-shared monitoring between control and fumigated chambers every 3 minutes. Ozone concentration profiles were plotted on a color graphics CRT display in real time and the information was stored on computer diskette.

For the purpose of uniformity, each pot was selected to have two seedlings of similar developmental stage and leaf size. Although the CSTR chambers were equipped with an environmental control system, the external environmental conditions still imposed some effects on the chambers. Preliminary studies suggested that more uniform results were obtained from plants which were acclimated to the CSTR chambers for 16 hr compared to those that were not acclimated.

The computer-controlled O_3 dispensing system in the CSTR chambers required approximately 20 min for the concentration to reach the designated value (0.20 $\mu l\ l^{-1}$) at which point, the fumigation time was initialized as zero and measurements were taken at intervals of 30 min. Ozone concentration was 0.20 ± 0.02 $\mu l\ l^{-1}$ for fumigated and 0.015 ± 0.005 $\mu l\ l^{-1}$ for control chambers. The total O_3 fumigation time was 4 hr. Following termination of O_3 addition to the CSTRs, after which O_3 concentration decreased to < 0.025 $\mu l\ l^{-1}$ within 3 min, plants were allowed to recover in charcoal filtered air for 2 hr inside the CSTRs. Control plants were exposed to charcoal filtered air for all 6 hr.

Gas Exchange Measurements

A Li-Cor 6000 Portable Photosynthesis System (Li-Cor, Inc., Lincoln, NE) was used for gas exchange measurements. This system was preprogrammed for various input parameters, such as leaf area, boundary layer conductance (BLC), pressure and chamber volume, and for output parameters, such as PAR, temperature, relative humidity, ambient CO_2 concentration, net photosynthesis, stomatal conductance, intercellular CO_2 concentration and transpiration. A 1 L cuvette with a fixed area insert was used with each CSTR chamber (control and fumigated chambers) and the environmental conditions within the cuvette during measurement were: 26 ± 2 °C, 65% ± 5% RH, 550 ± 100 $\mu E\ m^{-2}\ s^{-1}$ PAR, 1.68-2.23 cm s^{-1} BLC (depending on the fixed area insert used) and 350 ± 20 ppm CO_2 concentration. The temperature difference between the air and leaf was about 1.5 °C. Specially designed CSTR chamber doors fitted with two 82 cm long neoprene coated gloves (The Germfree Laboratories, Inc., Miami FL) were used

to provide an undisturbed environment inside the CSTRs when taking measurements.

Before starting a fumigation, gas exchange measurements were taken using the center leaflet of the 2nd trifoliate leaf. Measurements were conducted throughout the fumigation period (4 hr) and 2 hr after terminating fumigation at intervals of 30 min. Repeated fumigations (at least 5 times) were performed to obtain a sufficient sample size ($n \geq 10$) for statistical analysis using the general linear models procedure for a repeated measures analysis of variance with a nested design (cultivar nested within treatment) (Cole and Grizzle, 1966, cited in SAS/STAT™ user's guide).

Metabolite and Enzyme Analyses

Prior to fumigation, either the right or left leaflet of the 2nd trifoliate was collected from the control and treated plants. Plant samples were also collected at 2 hr and at the end of fumigation (4 hr). All samples were weighed, wrapped in aluminum foil and submerged in liquid N_2. The time from collecting to freezing was less than 20 sec for each leaflet. Whole leaflets from 2 plants were used for enzyme analyses and a 0.50 ± 0.08 g leaflet from a single plant was used for metabolite analyses. All samples were stored in liquid N_2 or at -70 °C prior to extraction. Sufficient sample size ($n \bullet 4$) was used for statistical analysis using the general linear models procedure of least-square means.

All chemicals used for metabolite and enzyme analyses were obtained from Sigma Chemical Co., St. Louis, MO unless otherwise specified. For metabolite analyses, plant samples were crushed in a mortar containing liquid

nitrogen and quickly poured into 15-ml centrifuge tubes (Corex, Corning) submerged in an ice bath. Three ml of ice cold 6% metaphosphoric acid (Aldrich Chemical Company, Inc., Milwaukee, WI) with 1 μM EDTA was added to each tube. A Polytron (PT 10-30, Kinematica, Kriens, Luzern) was used to initially grind and mix the plant tissue with the extracting solution at a low speed (15 sec) and then to homogenize the tissues at a full speed (60 sec). After homogenation, the samples were centrifuged at 31,000 x g for 15 min (Model J-21B, Beckman Instruments, Inc., Palo Alto, CA). The supernatants were filtered through 0.45 μm millipore filters and stored in 3.5 ml cryo-store vials (Perfector Scientific, Inc., Atascadero, CA) either in an ice bath for immediate analyses or in a freezer (-10 °C) for later analyses.

For enzyme analyses, polyvinylpolypyrrolidone (PVPP) was added, in a ratio of 1:2 (W/W) of the plant sample, into 15-cm centrifuge tubes. Plant samples were crushed in liquid N_2 and poured into the prechilled tubes. A cold solution containing 6.8 mM D-isoascorbic acid and 2 mM polyvinylpyrrolidone (PVP-10) in 0.1 M Tris-Cl buffer, pH 6.9, was added to the tube in a ratio of 3 ml:1 g plant leaf weight. The samples were ground using a Polytron at a low speed (30 sec) to mix the solution , tissues and PVPP and at a high speed (60 sec) to homogenize the mixture. During homogenation, the tubes were immersed in an ice bath. Samples then were centrifuged at 31,000 x g for 20 min using a Beckman centrifuge. Supernatants were carefully pipetted into 3.5 ml vials in an ice bath to keep sample temperature below 10 °C. Sephadex G-25 columns (PD-10, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) were washed with 0.1 M phosphate buffer, pH 8.0, equilibrated with 0.1 M potassium phosphate buffer with 0.2 mM EDTA, pH 7.8, and centrifuged in 30-ml centrifuge tubes (Corex,

Corning) using a Sorval Rotor centrifuge at 3,000 rpm for 5 min. Plant tissue extracts were added to the column 1 ml at a time and desalted at 3,000 rpm for 5 min. The desalting process was used to minimize possible interference from small molecular weight compounds, such as phenolics, during SOD analysis. The desalted extracts were stored in 1.5 ml vials either in an ice bath for immediate analysis or in a freezer for later analysis.

Ascorbate Analysis

Ascorbate was analyzed by the methods of Watada (1982) and Lee *et al.* (1984). A high-performance liquid chromatography (HPLC) (Model 740 Spectra-Physics, Santa Clare, CA) system with a Model 770 spectrophotometric detector set at 245 nm, a Spectra-Physics System I Computing Integrator and a Linear chart recorder (Model 385, Linear Instrument Corp., Irvine, CA) were used for the separation, detection, integration and calculation of AA concentration. A 250 x 4.6 mm Whatman PartiSphere C_{18} reversed phase analytical column of 5 μm particle size (Whatman Inc., Clifton, NJ), a 250 x 4.6 mm Whatman Solvecon precolumn and a 70 x 2.1 mm Whatman anion exchange guard column were used in the HPLC system for ascorbate separation. The mobile phase was 2% ammonium phosphate ($NH_4H_2PO_4$) adjusted to pH 2.8 with concentrated phosphoric acid (H_3PO_4) filtered and prepared at least one day before usage. The flow rate of the eluting solvent was 1 ml min^{-1} and the column pressure was 1800 $\pm$ 10 psi for each single analysis. The filtered supernatants of plant extract were pipetted in a volume of 1 ml into 1.5 ml cryo-store vials containing 5 mg dithiothreitol (DTT) (final concentration was 30 mM), mixed well and incubated at room temperature for 24 hr under dark conditions. The DTT is a reductant

which will reduce dHAA in the plant extract to AA (Okamura, 1980) and the total ascorbate concentration can be obtained. Twenty μl of plant extract with or without DTT or a standard ascorbate solution (4.54 mM ascorbate and 15 mM DTT in 6% metaphosphoric acid and 1 μM EDTA, diluted in a factor of 2 to obtain a series of 4.54, 2.27, 1.14, 0.57 and 0.28 mM solutions) was injected into the HPLC system and the total and reduced ascorbate concentrations of plant extract were interpolated from the least squares fit for standard curve of peak areas. The dHAA concentrations were calculated from the subtraction of the reduced ascorbate from the total ascorbate concentrations.

Glutathione Analysis

Glutathione was analyzed by the methods of Brehe and Burch (1976) and Griffith (1980). The following reagents were prepared before analysis. Reagent 1 consisted of 110 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 15 mM EDTA, 0.04% BSA (bovine serum albumin) and 0.3 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] in double distilled H_2O (ddH_2O), pH ~6.9, and was stored in a dark bottle at 4 °C. Reagent 2 consisted of 1 mM EDTA, 0.02% BSA and 50 mM imidazole (prepared from 1 M imidazole in ddH_2O as stock solution with 1 N HCl to adjust pH to 7.2) in ddH_2O, pH ~7.12, and was stored at 4 °C. Immediately prior to analysis, a 5% $Na_2HPO_4 \cdot 7H_2O$ buffer solution (adjusted with phosphoric acid to pH 7.5), a 9 mM NADPH solution in ddH_2O (cooled on an ice bath), a 5 mM GSSG solution in 0.01 N HCl were prepared. Commercial yeast glutathione reductase was added to each ml of reagent 2 to provide an activity of 1.5 units/mol.

For analysis, plant extracts were diluted with buffer at a 1:50 ratio. A standard GSSG solution was diluted to a series of 0, 0.08, 0.16, 0.40, 0.64, and 0.80 nmol with buffer. Each diluted plant extract or the above diluted standard GSSG solutions were added (1 ml) to a microcentrifuge tube containing 40 μl of 2-vinylpyridine, used to react with existing GSH without interferring with the GSSG concentrations in the plant extract, and then were incubated and constantly mixed for 1 hr. The remainder of the diluted samples was used for total glutathione analysis. In a 1 ml polystyrene cuvette (Perfector Scientific, Inc., Atascadero, CA) 400 μl plant extract or diluted standard GSSG solutions, 400 μl reagent 1, 320 μl reagent 2 and 80 μl NADPH solution were added, mixed well and the absorbance change at 412 nm was monitored with a spectrophotometer (Ultrospec II, model 4050, LKB Biochrom Ltd., Cambridge, England) linked to an Apple IIe computer through a reaction rate analysis software program. The reaction rate of the sample was compared to the reaction rate curve of diluted standard GSSG solutions to calculate the total glutathione concentrations of plant extracts. The same procedure was applied to the samples incubated with 2-vinylpyridine to calculate the GSSG concentrations. The reduced glutathione concentrations were calculated from the total GSH - 2 x GSSG concentrations.

Protein Analysis

Protein was analyzed according to the method of Bradford (1976) (also named as Bio-Rad protein assay). A stock dye solution was prepared as follows: 100 mg Coomasie blue G-250, 50 ml 95% ethanol and 100 ml 85% H_3PO_4 were adjusted to 200 ml with ddH_2O. A set of standard bovine serum albumin (BSA) solutions containing 0, 20, 40, 60, 80, or 100 μg protein in 0.1 ml ddH_2O or 50 μl

of plant extracts (prepared for enzyme analyses) with 50 μl ddH_2O were pipetted into test tubes. After diluting the stock dye solution with ddH_2O in 1:4 ratio and filtering through Whatman #1 filter paper, 5 ml was added to the test tubes and mixed thoroughly. Absorbance of standard BSA and sample solutions was measured in 3.5 ml polystyrene cuvettes at 595 nm in a spectrophotometer. Protein concentrations of plant extract were calculated from the coefficients of linear BSA standard curve.

Glutathione Reductase Analysis

Glutathione reductase was analyzed by the method of Schaedle and Bassham (1977). The following reagents were used: a) 0.05 M Tris-Cl buffer, pH 7.5, b) 30 mM $MgCl_2$ in buffer, c) 5 mM GSSG in buffer and d) 1.5 mM NADPH in buffer (freshly made). Into a 1 ml cuvette, 600, 100, and 100 μl of reagents a, b, and c, respectively, were pipetted and then 100 μl of plant extract followed by 100 μl of reagent d were added, mixed rapidly and the absorbance change (decrease) at 340 nm was measured in a spectrophotometer (Ultrospec II). One unit of GRase is the amount of enzyme required to oxidize 1 μmol NADPH per min at 25 °C. The GRase activity on a fresh tissue weight or extraced protein basis was calculated from the NADPH extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Ascorbate Peroxidase Analysis

Ascorbate peroxidase activity was analyzed by the method of Peters *et al.* (1989). The following reagents were used: a) 166 mM Hepes-KOH, pH 7.0, b) 1 mM EDTA, c) 1.5 mM sodium ascorbate, d) 1.0 mM H_2O_2. Into a 1 ml quartz cuvette 600 , 100, and 30 μl of reagents a, b, and c, respectively, were pipetted and

30 μl of plant extract, 140 μl ddH2O and 100 μl of reagent d were added, mixed rapidly and the absorbance change at 265 nm was measured with the spectrophotometer. The enzyme activity on a fresh weight or protein basis was calculated from the ascorbate extinction coefficient of 13.4 mM^{-1} cm^{-1}.

Superoxide Dismutase Analysis

A. Spectrophotometric assay

Superoxide dismutase was analyzed spectrophotometrically after the method of McCord and Fridovich (1969). The following reagents were used: 0.1 M KH_2PO_4 buffer with 0.2 mM EDTA in ddH_2O adjusted with KOH to pH 7.8, 1 mM xanthine in buffer, 0.1 mM cytochrome *c* (titrated with dithionite at 550 nm and corrected for the contribution of oxidized cyt. *c*, personal communication, E. M. Gregory) and xanthine oxidase (diluted to 0.5:1.5 ml buffer). In a 3.5 ml cuvette 1.50 ml buffer, 0.30 ml cytochrome *c*, 0.15 ml xanthine, 1.05 ml ddH_2O, and an amount (10-12 μl) of xanthine oxidase was added repeatedly to give a standard rate change of 0.025 absorbance units min^{-1} at 550 nm. When 0.10 ml plant extract was added, the volume of ddH_2O used was 0.95 ml. For every 6 samples analyzed, the amount of xanthine oxidase added was checked to insure that the standard rate change occurred. The inhibition percentage of SOD in plant extracts as calculated from [(0.025-SOD inhibited rate)/0.025] x 100 was referenced to the standard SOD curve relating percentage inhibition to SOD units. One unit of SOD activity was the amount of enzyme required to inhibit

standard rate of cytochrome *c* reduction by 50%. The amount of plant extract used to obtain an inhibition ranging from 20 to 60% was considered suitable.

B. Slab gel electrophoresis assay

Nondenaturing 7.5% polyacrylamide gels (160 x 160 x 0.75 mm) were used. The separation gel was prepared with 3.75 ml of 30% acrylamide/0.8% N,N'-methylene-bisacrylamide, 3.75 ml of 1.5 M Tris-Cl, pH 8.8 and 7.5 ml ddH_2O, degassed under vacuum for 10-15 min; 0.05 ml of 10% ammonium persulfate (freshly made) and 0.01 ml TEMED was added, mixed gently, and the solution used immediately. The stacking gel was prepared with 0.65 ml of 30% acrylamide/0.8% N,N'-methylene-bisacrylamide, 1.25 ml of Tris-Cl, pH 6.8 and 3.05 ml ddH_2O, degassed; 0.025 ml of 10% ammonium persulfate and 0.005 ml TEMED was added, mixed and used immediately. After loading the separation solutions to the gel electrophoresis casting units, ddH_2O was added to the surface very gently and poured out after the gels had polymerized. Then the stacking gel solution was added onto the top of the separation gels and a Teflon comb was inserted into the solution. After the stacking gels had polymerized, the comb was removed and the wells were rinsed with ddH_2O. Plant extract was mixed with sample buffer containing 1.52 g Tris base, 20 ml glycerol and 1 mg bromphenol blue in 100 ml ddH_2O, pH 6.8 adjusted with 1N HCl in a varied ratio depending on the protein concentration before being loaded to the stacking gels. The total amount of protein of each tissue extract loaded into each well was equal and the final loading volume was adjusted to 100 μl. Standard SOD from bacteria was loaded at 0.5, 1 or 2 μg protein concentration for comparison. The

electrophoresis buffer (15.1 g Tris base, 72.0 g glycine in 1000 ml ddH_2O, diluted 1:5 before use to give pH 8.3) was loaded into the electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) situated on a magnetic stirrer with cold water circulating throughout the system to maintain a constant low temperature (10 °C) and the power supply (American Bionetics, Inc., Hayward, CA) was set at a constant current of 40 mA for 2 gels. The total running time was around 3-4 hr.

Gels were removed from the unit, rinsed with ddH_2O and soaked under dark conditions in SOD staining solution, with or without 1 mM sodium cyanide (NaCN), containing 16 mg nitroblue-tetrazolium, 1 mg riboflavin and 0.2 ml TEMED in 80 ml of 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.8 (SOD buffer) for 60 min (Beauchamp and Fridovich, 1971). Gels were exposed to light after removing the staining solution, washed with SOD buffer, for further clarification if necessary and, after the SOD bands appeared, were transferred to and stored in sealed microseal pouches (Dazey Corp., Industrial Airport, KS) for storage.

Results

Foliar response to O_3

All the treated plants showed typical O_3 injury (stippling) 24 hr after fumigation on the upper surface of older and newly matured leaves. The stippling lesions appeared first along the leaf veins, then gradually spread into interveinal area. The cv Williams appeared to have less stippling on the leaves ($\approx$ 20% of leaf surface) compared to cvs Dare and Essex, which were quite similar in their degree of injury ($\approx$ 25%). Control plants displayed healthy, non-injured leaves.

Gas exchange

The physiological characteristics of the three soybean cultivars at pre-exposure conditions (means of plants in both treated and control chambers) are shown in Table 1. There were no significant differences among net photosynthesis (Pn), stomatal conductance (Cs), internal CO_2 concentration (Ci) or transpiration (Ts); although statistically insignificant, cv Essex had a higher Pn (23.7 vs. $\approx$ 22 mg CO_2 dm^{-2} hr^{-1}) and cv Dare had a higher Cs (1.7 vs. 1.5 cm s^{-1}) than the other cultivars.

In the control plants (Fig. 1B; Table 2), there were small changes of Pn during the 6 hr treatment period with cv Essex showing a slightly higher Pn and a significant decrease of Pn within 4 hr compared to the other cultivars. Net photosynthesis (Pn) was suppressed gradually by O_3 in all three cultivars (Fig. 1A) and attained an equilibrium rate after 3 hr of fumigation. The exception was cv Dare, which showed a continuous decrease in Pn. There was no evidence of recovery of Pn after termination of O_3 application at 4 hr in any cultivars.

Throughout the 6 hr period, cv Essex maintained a slightly higher Pn (Fig. 1A), and exhibited a smaller inhibition at the end of the fumigation, than either cvs Dare or Williams (Table 3). The relative reduction in Pn, compared to control plants, caused by O_3 exposure was 29, 21 and 16%, respectively, in cvs Dare, Williams and Essex.

Stomatal conductance of control plants maintained a fairly steady rate which decreased slightly throughout the treatment period (Fig. 2B) and showed a reduction of 15 to 25% in all cultivars (Table 2). In the fumigated plants, cv Dare showed no change in Cs between 0.5 and 1.5 hr of fumigation, whereas cv Essex showed a slight decrease and cv Williams a greater reduction between 1 and 1.5 hr of O_3 application (Fig. 2A). After 1.5 hr, Cs of all cvs was suppressed to a great extent (Fig. 2A) and showed no recovery. The cv Dare had a higher Cs during the exposure period than the other cultivars, which indicated a higher O_3 influx into the leaves. The reduction of Cs by O_3 at the end of fumigation was about 70% for all cultivars (Table 3) and, the relative reduction in Cs, compared to control plants, was 45, 54 and 49%, respectively, in cvs Dare, Williams and Essex.

Control plants mainly retained a constant Ci with a small but significant decline through the end of 6 hr (Fig. 3B; Table 2). Internal carbon dioxide concentration was reduced by O_3 at a slow rate during the fumigation (Fig. 3A). The Ci in cv Dare remained nearly constant between 0.5 and 2 hr of fumigation and decreased after this time. In cv Dare, Ci was higher than that in cvs Williams and Essex, both of which had a similar reduction in Ci during the exposure period (Table 3). No positive trend of recovery of Ci was evident after fumigation ceased

(Fig. 3A). The relative decline in Ci, compared to control plants, caused by O_3 exposure was 5, 16 and 12%, respectively, in cvs Dare, Williams and Essex.

Transpiration of all cultivars was similar and decreased slightly during the 6 hr period in control plants (Fig. 4B; Table 2), while O_3 fumigation resulted in reductions in Ts of approximately 45% in all cultivars (Fig. 4A; Table 3). The relative suppression of Ts, compared to control plants, resulting from O_3 exposure was 36, 36 and 45%, respectively, in cvs Dare, Williams and Essex.

The estimated O_3 flux to the leaf interior, calculated according to Laisk *et al.* (1989) using the diffusivity ratio of 1.68 for H_2O/O_3 (O_3 flux = O_3 concentration x Cs /1.68), in all cultivars is shown in Figure 5A. The cv Dare had a higher O_3 flux during the first 3 hr than either cvs Williams or Essex. The peak of O_3 flux ($\approx$ 75 nmol m^{-2} s^{-1}) was reached 0.5 hr after exposure to 0.2 μl l^{-1} O_3 in all cultivars (Fig. 5A). In cv Dare, O_3 flux was maintained at near peak rates for another hr followed by a steep decline corresponding to decreased stomatal conductance. At 3 hr of fumigation, O_3 flux stabilized at about 47% ($\approx$ 35 nmol m^{-2} s^{-1}) of the maximum. In cvs Williams and Essex, O_3 flux decreased gradually, to about 94% at 0.5 hr after the peak. A steeper decline in O_3 flux occurred between 1.0 and 2.5 hr of fumigation and stabilized at about 42% of maximum uptake rate at 3 hr (Fig. 5A). Integrated O_3 dose (Olszyk and Tingey, 1986) [dose = sum (flux x 1,800 sec hr^{-1}) over 0.5 hr of exposure] during the fumigation period of all cultivars is shown in Figure 5B. The dose increased over time, with cv Dare initially showing a higher dose than the other cultivars at 2 hr of O_3 exposure. At the end of the fumigation, cv Dare had absorbed 12% more O_3 (750 μmol m^{-2}) than cvs Williams and Essex ($\approx$ 650 μmol m^{-2}).

The residual conductance (Cr) to CO_2 was calculated by the method of Gaastra (1959) (cited in Jones, 1985), $1/r_{r1} = A/C_w$ (r_{r1}: residual resistance, A: net assimilation rate and C_w: cell wall concentration of CO_2) where C_w=Ci, on the assumption that the CO_2 concentration is zero at the carboxylation site within the chloroplasts. The Cr exhibited a reduction caused by O_3 exposure in cv Dare throughout the fumigation and recovery period (Fig. 6A). A similar reduction was observed in cv Williams, however, there was a recovery of Cr after 3.5 hr of fumigation. In cv Essex, the Cr was reduced in the first 2 hr of O_3 exposure and maintained at an equilibrium state afterwards. The cv Essex maintained a higher Cr during the O_3 exposure and recovery period than cvs Dare and Williams, indicating a lower resistance to CO_2 uptake in the leaf interior. In control plants, a slight increase in Cr in the first 3 hr was observed in all cultivars followed by a return to pre-exposure levels except cv Dare which showed an increase in Cr at the end of 6 hr (Fig. 6B).

The fractional contributions of stomatal and non-stomatal components to the change in limitation to Pn (σ_s and σ_{ns}, respectively) compared to pre-fumigation, calculated according to Jones (1985) using state function methods, is shown in Figure 7. An increased σ_s was found in all cultivars exposed to O_3 (Fig. 7A) reflecting the decreased stomatal conductance. During the first hr of O_3 fumigation, when Pn and Cs changes were gradual, non-stomatal limitations contributed 65 to 80% of the decrease in Pn (Fig. 7B). However, as the rate of gas exchange inhibition increased between 2 and 3 hr of fumigation, the stomata contributed to a greater extent in limiting Pn, especially in cvs Williams and Essex. By 4 hr of O_3 exposure, non-stomatal factors contributed only 30 to 35% to Pn limitation in cvs Williams and Essex, but still about 56% in cv Dare. The cv

Dare showed the least increase in σ_s and cvs Williams and Essex were similar and approximately 25% higher than cv Dare at the end of O_3 fumigation.

Water use efficiency (WUE) (calculated as Pn/Ts) of O_3-fumigated plants increased slightly for 1.5 hr in cvs Williams and Essex, but declined slightly in cv Dare (Fig. 8A). A dramatic increase in WUE of all cultivars was observed for the rest of the O_3 exposure period except for a substantial decrease at 3.5 hr resulting from an increase in Ts. The cv Dare had the least increase in WUE and cv Essex the greatest. During the recovery period, cvs Williams and Essex showed a relatively constant WUE except for a decrease in cv Williams at 6 hr; and cv Dare showed a variable WUE (Fig. 8A). The WUE of control plants of all cultivars remained relatively constant throughout the 6 hr period except for a slight decrease between 2 and 3.5 hr (Fig. 8B). A lower WUE between 1 and 3.5 hr in cv Williams than in cvs Dare and Essex and a higher WUE in the first 1.5 hr in cv Essex than in cvs Dare and Williams were observed although the differences among cultivars were insignificant.

Metabolites

The antioxidant components of the cultivars at pre-exposure conditions are shown in Table 1. The endogenous levels of total and reduced glutathione (GSH) were significantly higher, 15 and 18%, respectively, in cv Essex compared to cv Dare; cv Williams had intermediate concentrations of these metabolites. The endogenous levels of total and reduced ascorbate (AA) also were significantly higher in cv Essex, 29 to 41% and 16 to 24%, respectively, compared to cvs Dare and Williams. Oxidized glutathione (GSSG) and ascorbate (dHAA) were similar among all cultivars.

There were no significant changes in total, reduced and oxidized glutathione levels in both O_3 and filtered air treatments of all cultivars (Figs. 9, 10; Tables 4, 5). However, GSSG levels were reduced 21% in cv Williams after O_3 exposure (Table 5; Fig. 11A). The ratio of GSH/GSSG remained constant in cvs Dare and Essex and increased in cv Williams after O_3 fumigation (Fig. 12A). In control plants, cv Dare showed a slight increase in the GSH/GSSG ratio at 4 hr, whereas cvs Williams and Essex had increased GSH/GSSG ratios at 2 hr after exposure that returned to near pre-fumigation levels at the end of the treatment period (Fig. 12B). Relative to the control plants (Fig. 13A-C), no changes were found due to O_3 treatment in either total or reduced glutathione; however, GSSG decreased 23% in cv Williams and increased 15% in cv Essex after O_3 exposure.

Total ascorbate levels remained essentially unchanged over time in all control and fumigated plants (Tables 4, 5; Fig. 14). Similar responses were also observed in reduced ascorbate levels (Tables 4, 5; Fig. 15). The dHAA levels declined 26 and 20% (statistically insignificant) in cvs Dare and Williams, respectively, exposed to O_3, but there was no change in dHAA in cv Essex (Table 5; Fig. 16A). However, dHAA increased 40% in cv Dare exposed to filtered air and decreased 39% in cv Williams and 54% in cv Essex (Table 4; Fig. 16B). Although these changes were substantial, they were statistically insignificant because of the considerable variability among plants. As a result of the changes in dHAA, the ratio of AA/dHAA increased slightly in cvs Dare and Williams and decreased in cv Essex after O_3 exposure (Fig. 17A). A dramatic increase of AA/dHAA was found in cv Essex whereas, only a slight decrease and increase of AA/dHAA was observed in cvs Dare and Williams, respectively, exposed to filtered air (Fig. 17B). In O_3-treated plants, the levels of total and reduced

ascorbate, as a percentage of controls, in cvs Dare and Williams remained relatively constant, but decreased in cv Essex (Fig. 18A-B). However, cv Dare exhibited a reduction of dHAA caused by O_3 fumigation relative to the control plants while cv Williams showed a stimulative effect of O_3 on dHAA (Fig. 18C). In cv Essex, because dHAA decreased substantially during the exposure period in control plants but remained nearly constant in the O_3-fumigated plants (Fig. 16), O_3 exposure, relative to the controls, resulted in a 100% greater dHAA concentration (Fig. 18C).

Protein

Total protein concentrations were quite stable and not affected by either treatment in all cultivars (Fig. 19). The cv Williams had the lowest amount of protein ($\approx$ 27 mg g^{-1} fresh wt) while cv Dare had the highest ($\approx$ 32 mg g^{-1} fresh wt).

Enzymes

The endogenous enzyme activities (total and specific activities on a fresh weight and protein basis, respectively) of these cultivars at pre-exposure conditions are shown in Table 1. The GRase total activity and SOD specific activity were not different among all cultivars with cv Essex showing a slightly higher GRase total activity, cv Dare a slightly higher and cv Williams a lower SOD specific activity. However, cv Dare had the lowest GRase specific activity, cv Essex had the lowest APase total activity and cv Williams had the highest APase specific activity and the lowest SOD total activity.

Glutathione reductase activity, on a fresh weight basis, generally showed small, similar changes to either control or O_3 treatment in all cultivars (Tables 4, 5; Fig. 20). The cv Dare responded the most to O_3, with a 25% increase in activity at 2 hr exposure that decreased to near pre-exposure levels at the end of fumigation. In cvs Williams and Essex, activity essentially remained constant during the exposure period (Table 5; Fig. 20A). In contrast, cv Williams showed a 16% increase in activity in filtered air at 2 hr exposure, whereas activity in cvs Dare and Essex changed minimally throughout the treatment period (Fig. 20B). When compared to the control plants, GRase activity increased in cv Dare, and did not change in cvs Williams and Essex in response to O_3 (Fig. 26A). However, similar GRase activity and responses to O_3 were found in cvs Williams and Essex when specific activity, rather than total activity, was measured (Table 5; Fig. 21A). Both cultivars had similar enzyme activity during O_3 exposure with a minimal decrease at 4 hr, although cv Williams showed a higher activity at 0 and 2 hr in filtered air (Fig. 21B) and then a 19% reduction at 4 hr (Table 4). In cv Dare, GRase specific activity increased 28% at 2 hr and 10% at 4 hr of O_3 treatment compared to pre-exposure (Fig. 21A; Table 5). There were little changes in enzyme activity in filtered air (Fig. 21B; Table 4). Relative to the controls, GRase specific activity increased in response to O_3 in cvs Dare and Williams and did not change in cv Essex (Fig. 27A).

The APase activity in all cultivars responded positively to O_3 exposure, with a 19% increase in cv Essex (Table 5; Fig. 22A). Filtered air exposure imposed no effects on the enzyme activities of any cultivar (Fig. 22B). In cv Essex, enzyme activity was lowest in the O_3 treatment compared to that of plants exposed to filtered air or other cultivars under either treatment (Fig. 22).

Relative to the controls, O_3 exposure resulted in a stimulation of APase total activity in cv Essex, but not in the other cultivars (Fig. 26B).

The APase specific activity was not affected by O_3 exposure in cvs Dare and Williams, but increased in cv Essex compared to 0 time (Table 5; Fig. 23A). In filtered air, APase specific activity in cv Williams remained highest compared to the other cultivars (Table 4; Fig. 23B). Relative to the control plants, responses to O_3 were a slight increase in enzyme activity in all cultivars with cv Essex demonstrating the greatest increase (Fig. 26B).

A 4 hr O_3 exposure imposed a negative effect on SOD total activity in cvs Williams and Essex which was inhibited 20% and 35%, respectively, compared to pre-fumigation (Table 5; Fig. 24A). In cv Dare, O_3 exposure induced a 25% increase in enzyme activity. On the other hand, cv Dare responded to filtered air exposure with a 14% reduction in SOD activity, whereas cv Williams showed a 80% increase at 2 hr and a 37% increase at 4 hr in enzyme activity (Table 4; Fig. 24B). In cv Essex, no change in SOD activity was seen after 4 hr exposure in control plants. The effects of O_3 on SOD total activity, compared to the controls, showed a stimulatory effect in cv Dare and an inhibitory effect in cvs Williams and Essex (Fig. 26C).

The SOD specific activity was reduced 21% and 31% in cvs Williams and Essex by O_3, respectively, and increased 18% in cv Dare (Table 5; Fig. 25A). Filtered air exposure resulted in reductions of SOD activity by 18 and 22% in cvs Dare and Williams, respectively (Table 4; Fig. 25B). Relative to the controls, the effect of O_3 exposure resulted in a 27% decrease in cv Essex, a 44% increase in cv Dare and no change in cv Williams in SOD specific activity (Fig. 27C).

Specific SOD activity staining revealed several bands on the polyacrylamide gel that showed the same banding patterns and positions after O_3 exposure which indicated that there were no qualitative differences among cultivars or between treatments (Fig. 28A). The most mobile 5-6 bands apparently were copper-zinc SOD since they were sensitive to cyanide, while the least mobile bands were manganese SOD which were insensitive to cyanide (Fig. 28B).

Discussion

The endogenous physiological and biochemical characteristics of these soybean cultivars presented in Table 1 were means of plants in both treated and control chambers prior to pollutant exposure. The leaf texture of cv Williams was thicker and darker than the other cultivars, which might contribute some unknown barriers to O_3 uptake and result in the lower injury seen on the leaf surface. Although all cultivars showed a different degree of injury (similar between cvs Dare and Essex), symptoms could not be used as a sole indicator for sensitivity to O_3 because of a lack of consistency in response. Based on foliar injury, cv Essex would not be considered moderately tolerant as reported (Heagle and Letchworth, 1982) but sensitive to O_3 under our experimental conditions. It has been reported that O_3 exposure can cause reductions in growth and yield of plants showing no visible symptoms or *vice versa* (Heagle *et al.*, 1974)As an alternative to foliar injury, alteration of biochemical processes might be suitable for the role as sensitivity indicator(s) of O_3 stress.

The observed reduction of the physiological parameters at the end of 4 hr exposure of soybean cultivars in the control chambers (Table 2) could be a natural rhythm such as a diurnal cycle controlled by an endogenous circadian clock (Pemadasa, 1979; Gorton *et al.*, 1989). Plants in the fumigation chambers (Table 3) reflected the additional effects of O_3 which led to approximate reductions, relative to controls, of 16-29% in Pn, 45-55% in Cs, 5-16% in Ci and 35-45% in Ts. The environmental conditions inside the CSTR chambers were stable, with small variations throughout the exposure period, and did not appear to impose significant effect on the plant responses.

Different O_3 concentrations could affect Pn and Cs sequentially or simultaneously (Hill and Littlefield, 1969). As suggested by Temple (1986), a reduced Cs could be caused by increased Ci resulting from depressed Pn due to O_3 exposure. Changes in water use efficiency (WUE) may be used to explain the sequence of gas exchange events as explained by Reich *et al.* (1985). If O_3 inhibited Cs first, reduced Cs would inhibit Ts far more than CO_2 uptake, thus, WUE would increase. Conversely, if O_3 inhibited Pn first, the increased Ci would cause partial stomatal closure and WUE would remain relatively stable or decrease moderately. Under these circumstances, the slight increase in WUE (large increase in cv Williams) in the first 0.5 hr of O_3 exposure (Fig. 8A) could imply that O_3 affected Cs first in all cultivars, although stomatal limitation (σ_s) only contributed approximately 40% and non-stomatal limitation (σ_{ns}) 60% to change in limitation of Pn in cvs Dare and Essex during the same period.

The increase in WUE of all cultivars during the rest of exposure and recovery period also indicated that O_3 imposed more effects on Cs than on Pn which was extended into the 2 hr recovery period as demonstrated by the increased σ_s during the same period, particularly in cvs Williams and Essex (Fig. 7A). However, the suggestion made by Winner *et al.* (1988) indicated that gaseous pollutants could simultaneously affect Pn and Cs and then generate feedback regulations to each other. At 0.2 μl l^{-1}, O_3 appeared to affect Pn and Cs at 0.5 hr (Figs. 1A, 2A) and Ci also decreased during this time (Fig. 3A). However, the actual exposure duration was 50 min because of a 20 min interval required to reach the designated O_3 concentration in the CSTR. No definite conclusion could be made in terms of the sequential effect of O_3 on Pn or Cs. If gas exchange measurements were taken at 10 min intervals immediately after

addition of O_3, subtle changes in Pn, Cs and/or Ci might lead to conclusions about the path change associated with reduced gas exchange.

Wong *et al.* (1979) suggested a linear relationship between Pn and Cs, but also indicated that stomata might respond to a metabolite in the mesophyll other than CO_2 because of a lack of consistency in Ci and Cs changes. However, Radin *et al.* (1988) reported that no messenger to coordinate Cs with Pn was found at ambient CO_2 levels, but that abscisic acid (ABA) might be the messenger under a CO_2-enriched environment.

Intercellular CO_2 concentration is controlled by the photosynthetic rate and stomatal opening to optimize the WUE and has a feedback regulation to signal the movement of stomata or influence Pn. Although plants have the capability to maintain a constant ratio of internal to ambient CO_2, as demonstrated by Wong *et al.* (1979) using increased ambient CO_2 concentrations, under O_3 exposure, this capability might be inhibited to a certain extent since the Ci decreased slowly, but the ambient CO_2 did not change in the same proportion. The reduced inhibition of Ci in cv Dare by O_3 suggested that mesophyll processes associated with CO_2 fixation were inhibited greater in this cv than in cvs Williams and Essex. Transpiration rate is controlled by the Cs and the vapor pressure difference between the ambient air and the leaf surface and Ts followed the same response pattern of Cs to O_3 (Fig. 4A), but was less inhibited by O_3 (Table 3).

The effect of O_3 on Pn attained a point (3 hr) of stabilization in cvs Williams and Essex (Fig. 1A) indicating that a detoxification mechanism inside the plant cells was in an equilibrium state with O_3 flux as both cultivars could dispose of the absorbed O_3 dose without further suppressing their Pn. Of course,

O_3 flux was steadily decreasing during the exposure period which imposed a reduced threat to Pn. In addition, O_3 flux only decreased moderately after 2.5 hr of fumigation in all cultivars (Fig. 5A) with an average of 35 nmol m^{-2} s^{-1} ($\approx$ 0.1 μl l^{-1} O_3 in ambient air at Cs=1.4 cm s^{-1}) as Cs slowly decreased. It is possible that if cvs Williams and Essex were exposed to 0.1 μl l^{-1} O_3 for 4 hr, their Pn would not have been affected by the short-term O_3 fumigation. No recovery of Pn occurred among cultivars for 2 hr after ending O_3 exposure which suggests that a longer period was needed for Pn to recover toward its pre-fumigation level. The Pn was not measured on the fumigated plants sufficiently long after O_3 exposure ended to determine the period of time needed for recovery.

Because of the similarities in Cs, Ci and Ts among the cultivars in the control treatment (Figs. 2B, 3B, 4B), the higher Pn and lower Cs in cv Essex exposed to O_3 (Figs. 1A, 2A) demonstrated that this cv might have a more efficient photosynthesis system under O_3 stress, possibly partially accounted for by a higher and less affected residual conductance (Cr) to CO_2 (Fig. 6A). The higher Cs in cv Dare during O_3 fumigation (Fig. 2A) indicated a higher CO_2 entry, but actually a lower Cr (Fig. 6A); and a higher O_3 flux (Reich and Amundson, 1985) which affected Cr (Fig. 6A) and impaired the photosynthesis system to a greater degree, resulting in a higher Ci (Fig. 3A). Higher stomatal frequency per unit leaf area (Butler and Tibbitts, 1979b; Dean, 1972) or larger stomatal aperture could be factors responsible for the higher Cs in cv Dare under natural conditions. In addition, cv Dare's stomata also might be less sensitive to O_3. Obviously, 0.2 μl l^{-1} O_3 had a greater effect on Cs, which did not recover during the 2 hr post-fumigation period (Fig. 2A), suggesting either the stomata

were partially damaged by O_3 or a longer recovery was needed for Cs to reach its pre-fumigation level.

Lee *et al.* (1984) reported that O_3-resistant soybean and snapbean cultivars had higher endogenous AA levels than O_3-sensitive cultivars. After O_3 exposure, AA increased in the O_3-resistant cultivars, but decreased in the O_3-sensitive cultivars. On the contrary, Guri (1983) found that two O_3-sensitive and two O_3-insensitive *Phaseolus vulgaris* cultivars had the same AA and GSH levels before O_3 exposure; and that after fumigation, the AA levels were reduced slightly among all cultivars while GSH levels were reduced sharply in O_3-sensitive cultivars and declined only moderately in O_3-insensitive cultivars. There were no data presented in Guri's report about the change in metabolite levels of the control plants. However, GRase specific activity was almost twice as high in O_3-insensitive cultivars before and after O_3 exposure than in O_3-sensitive cultivars and GRase activity was not affected by O_3 (Guri, 1983). Tanaka *et al.* (1985) also suggested that the content of AA and GSH were related to the O_3 tolerance of higher plants.

The data from this present study (Table 1) showed that there were no consistent differences in the endogenous metabolite levels or enzyme activities among cultivars. Cultivar Essex had significantly higher endogenous GSH and AA concentrations, but significantly lower APase total and specific activity than cvs Dare and Williams. No difference in endogenous GRase activity was evident among cultivars. It is well known that GSH and AA can scavenge $O_2^{\cdot -}$, H_2O_2 and $\cdot OH$ produced during photosynthesis with the involvement of APase and GRase (Asada and Kanematsu, 1976; Foyer, 1984; Foyer and Halliwell, 1976; Gillham

and Dodge, 1987; Nishikimi, 1975) to protect plants from oxidant damage. Thus, there appears to be some degree of protection against oxidant stress in cv Essex relative to the other cultivars because of higher AA and GSH levels. However, cv Essex had a significantly lower APase activity, which catalyzes the H_2O_2-scavenging reaction by AA, and this might result in the accumulation of H_2O_2 in the chloroplasts and subsequent inhibition of other biochemical processes.

After O_3 exposure, GSH in cv Essex did not change while GSSG increased 15% (Fig. 13B-C); AA decreased 23% while dHAA increased 115% (Fig. 18B-C); and GRase activity did not change, APase activity increased about 17%, but SOD activity decreased approximately 27-40% (Figs. 26, 27). These results suggest that reduced SOD activity could result from inhibition by accumulated H_2O_2 (Foyer, 1984) thus, the enzyme could not dismutate $O_2^{\cdot -}$ efficiently; however, $O_2^{\cdot -}$ can dismutate spontaneously at a moderate rate to form H_2O_2 and O_2 under neutral or alkaline pH (Foyer, 1984) which occurs in the stroma under active photosynthesis. Addition of cyanide to purified, intact spinach chloroplasts to inhibit H_2O_2 scavengers (Nakano and Asada, 1980) resulted in O_2-uptake and H_2O_2 accumulation. The authors believed that even though cyanide can inhibit SOD activity, spontaneous disproportionation of $O_2^{\cdot -}$ is moderately fast ($\sim 10^5\ M^{-1}\ s^{-1}$ at pH 7); therefore, H_2O_2 accumulation should not be completely stopped by inhibited SOD activity. It is also possible that accumulation of H_2O_2 could be due to a slower reduction than production rate. However, the specific activity of SOD in cv Essex did not change until the end of O_3 exposure (a 27% reduction), while SOD total activity decreased 23% at 2 hr and 40% at 4 hr of fumigation. It is justified to postulate that H_2O_2 formation could occur through $O_2^{\cdot -}$ dismutation by SOD during O_3 fumigation and

accumulation of H_2O_2 at the end of the exposure inhibited SOD activity and stimulated APase activity to then reduce H_2O_2, since APase activity did not change until the end of fumigation (Figs. 26B, 27B). Obviously, it is necessary to monitor H_2O_2 concentrations during O_3 fumigations to substantiate the above hypotheses.

Results from Castillo and Greppin (1988), which showed that exposure to O_3 provoked a decrease in the AA and GSH concentration and imposed no effect on GRase in *Sedum album*, were confirmed in our study with cv Essex, except that no change in the GSH level was found. Apparently, 0.2 $\mu l\ l^{-1}$ O_3 had no effect on GRase activity and GSH levels in cv Essex even though Pn was decreased about 16% and the plants expressed slight O_3 injury.

Because GSSG represents only about 5% of the total glutathione, its concentration is much lower than GSH (approximately 1:25~35 in these soybean cvs) and a small change in GSSG would show a large percentage difference. The same condition also applies to dHAA which constitutes about 10% of the total ascorbate concentration. The observed increase of GSSG (15%) in cv Essex due to O_3 exposure might result from the reaction of some small amount of GSH with free radicals or with O_3 itself (Asada and Kanematsu, 1976; Meister and Anderson, 1983) with no apparent reduction in GSH. The increased APase activity might be induced either from substrate H_2O_2 accumulation (Tanaka *et al.*, 1985) or from O_3 exposure (Castillo and Greppin, 1986; Peters *et al.*, 1989). On the other hand, Curtis *et al.* (1976) found that O_3-tolerant soybean cultivars were biochemically less affected by O_3 than O_3-sensitive cultivars in terms of

peroxidase activity, although the authors did not state if the enzyme was ascorbate-specific peroxidase.

In response to O_3, SOD activity was not significantly increased in maize (Matters and Scandalios, 1987) and its activity had no relation to changes in susceptibility in snapbean (Chanway and Runeckles, 1984a, b), but Lee and Bennett (1982) have suggested otherwise. Scott *et al.* (1987) reported that SOD-rich *Escherichia coli* was more susceptible to oxidant stress, such as paraquat and 100% oxygen, accumulated more H_2O_2 and had a greater decline in GSH level compared to wild-type *E. coli*. These authors suggested that a balance among metabolites and/or enzymes might be more important to minimize the oxidant pressure rather than the dominance by one metabolite or enzyme. The 27-40% decline of SOD specific and total activity in cv Essex after O_3 exposure indicated that O_3 might have a direct inhibitory effect on SOD, or as mentioned above, that H_2O_2 accumulation could inhibit SOD activity, and that $O_2^{\cdot -}$ formed inside the tissue must be removed by the large pool of AA and GSH before further damage proceeded. Overall, the decrease in AA corresponded to the increase in dHAA (Fig. 18) and the ratio of AA/dHAA decreased (Fig. 17A), although some AA may have been metabolized to products other than dHAA (Loewus and Helsper, 1982).

Cultivars Dare and Williams had similar endogenous GSH and AA concentrations, with cv Dare showing somewhat lower metabolite levels (Table 1). Because cv Williams had less extractable protein than cv Dare (Fig. 19), the former cv had significantly higher specific activities of GRase and APase, although the total activities of both enzymes were similar (Table 1). However, the

total and specific SOD activities were higher in cv Dare than in cv Williams. No major effect of 0.2 μl l^{-1} O_3 was found on GSH, GSSG and AA levels, and on APase activity (Figs. 13B-C, 18B, 26B, 27B). However, a 47% reduction in dHAA and 19% and 45% increases in GRase and SOD, respectively, were observed in cv Dare after O_3 exposure. The reduction in dHAA by 50% at 2 hr corresponded to an increase in AA by 12% at 2 hr after O_3 fumigation (Fig. 18B-C), but no increase in AA was seen at 4 hr while dHAA still remained lower by 47%. The lack of correspondence between dHAA and AA at the end of O_3 exposure may be explained partially by either the turnover of AA to acid products other than dHAA or the hydrolyzation of dHAA to diketogulonate (DKG) under neutral and alkaline aqueous solutions (Tolbert and Ward, 1982). The increased SOD activity in O_3-treated plants could dismutate more $O_2^{\cdot -}$ to form H_2O_2, which would subsequently be reduced by AA and APase. In order to maintain the dHAA → AA reduction, the supply of electron donor must be sufficient, which should occur through the stimulated GRase activity. The ratio of AA/dHAA is a sensitive indication of cell physiology (Edgar, 1970) and may reflect the GSH/GSSG ratio, which is related to the NADP/NADPH ratio.

The 23% reduction in GSSG (Fig. 13C) and 15% increase in GRase specific activity (Fig. 27A) did not correspond to the unchanged GSH level of the O_3-fumigated cv Williams (Fig. 13B). However, GSH could be degraded to its constituent amino acids (Rennenberg, 1982) besides being oxidized to GSSG. Total activity of SOD decreased 41% (Fig. 26C) due to O_3 exposure but the specific activity was not affected (Fig. 27C) in cv Williams indicating possible normal function of SOD was not severely inhibited. The increase in dHAA (32%) (Fig. 18C) could be accounted for as a result of the small increase in APase

specific activity (Fig. 27B), although AA did not decrease correspondingly (Fig. 18B).

By comparing the effects of O_3 on the biochemical characteristics among the three soybean cultivars, it was concluded that the GSH level was not affected in any cultivars. Reduced ascorbate, dHAA and APase responded most (or may have an important role) in cv Essex against oxidant stress. The enzymes GRase and SOD were important in cv Dare, and cv Williams showed no distinct changes of metabolite levels or enzyme activities due to O_3 exposure that could impart oxidant tolerance. It appears likely that cv Essex has the greatest O_3 resistant potential considering its gas exchange responses and its biochemical characteristics. However, the O_3 injury produced on the leaves suggests that either 0.2 μl l^{-1} O_3 overwhelmed the antioxidant system to cause unavoidable cellular damages, or that lesion formation due to accumulated phenolic compounds in response to O_3 (Howell, 1970; Tingey *et al.*, 1975) was irrelevant to oxidant sensitivity, or that enhanced liberation of stress ethylene after O_3 fumigation was responsible for the injury (Mehlhorn and Wellburn, 1987).

In order to more thoroughly understand the antioxidant system in soybean cultivars, analyses based on a short time course following the beginning of exposure to different O_3 concentrations may reveal a more detailed trend of changes in enzyme activity and metabolite concentration. Similar short time intervals for gas exchange measurements may reveal coordinate changes in the antioxidant system, although a continuous monitoring system for gas exchange would be most suitable to investigate the response time of soybean cultivars to O_3.

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Table 1. Physiological and biochemical characteristics of three soybean (*Glycine max* (L.) Merr.) cultivars grown under glasshouse conditions and acclimated in CSTR chambers for 16 hr. (See text for details).

	Cultivar		
Variable	Dare	Williams	Essex
Pn ($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	22.2 ± 3.1^{aA} $(n=25)^{b}$	21.9 ± 2.6^{A} (n=26)	23.7 ± 3.5^{A} (n=27)
Cs ($cm\ s^{-1}$)	1.7 ± 0.5^{A} (n=24)	1.5 ± 0.4^{A} (n=26)	1.5 ± 0.4^{A} (n=23)
Ci (ppm)	309.2 ± 20.90^{A} (n=26)	302.6 ± 16.6^{A} (n=26)	298.7 ± 23.0^{A} (n=25)
Ts ($mmol\ m^{-2}\ s^{-1}$)	4.9 ± 1.2^{A} (n=26)	4.9 ± 1.5^{A} (n=26)	4.8 ± 1.3^{A} (n=27)
$Glutathione_{tot}$ ($\mu mol\ g^{-1}$ fw)	1.18 ± 0.07^{B} (n=8)	1.23 ± 0.14^{BA} (n=8)	1.36 ± 0.17^{A} (n=8)
$Glutathione_{ox}$ ($\mu mol\ g^{-1}$ fw)	0.05 ± 0.01^{A} (n=8)	0.05 ± 0.01^{A} (n=8)	0.04 ± 0.01^{A} (n=8)
$Glutathione_{red}$ ($\mu mol\ g^{-1}$ fw)	1.08 ± 0.07^{B} (n=8)	1.14 ± 0.14^{B} (n=8)	1.28 ± 0.18^{A} (n=8)
$Ascorbate_{tot}$ ($\mu g\ g^{-1}$ fw)	1048 ± 218^{B} (n=19)	1163 ± 241^{B} (n=19)	1356 ± 277^{A} (n=19)
$Ascorbate_{ox}$ ($\mu g\ g^{-1}$ fw)	155 ± 128^{A} (n=19)	161 ± 153^{A} (n=19)	109 ± 93^{A} (n=19)
$Ascorbate_{red}$ ($\mu g\ g^{-1}$ fw)	894 ± 161^{B} (n=19)	1017 ± 212^{B} (n=19)	1261 ± 330^{A} (n=19)

(Cont.)

(Table 1 continued)

Variable	Cultivar		
	Dare	Williams	Essex
Glutathione Reductase ($U\ g^{-1}$ fw)	0.74 ± 0.22^{A} (n=10)	0.76 ± 0.16^{A} (n=10)	0.86 ± 0.21^{A} (n=10)
Glutathione Reductase ($U\ mg^{-1}$ protein)	$.023 \pm .006^{B}$ (n=10)	$.030 \pm .005^{A}$ (n=10)	$.028 \pm .005^{A}$ (n=10)
Ascorbate Peroxidase ($U\ g^{-1}$ fw)	1.36 ± 0.19^{A} (n=10)	1.34 ± 0.22^{A} (n=10)	1.11 ± 0.25^{B} (n=10)
Ascorbate Peroxidase ($U\ mg^{-1}$ protein)	$.042 \pm .006^{B}$ (n=10)	$.053 \pm .007^{A}$ (n=10)	$.036 \pm .007^{B}$ (n=10)
SOD ($U\ g^{-1}$ fw)	33.7 ± 9.7^{A} (n=9)	13.8 ± 8.5^{B} (n=9)	28.9 ± 20.9^{A} (n=8)
SOD ($U\ mg^{-1}$ protein)	1.05 ± 0.26^{A} (n=9)	0.61 ± 0.32^{A} (n=9)	0.86 ± 0.67^{A} (n=10)

a: Mean ± sd.

b: Sample size.

A or B: Means across cultivars with the same letter are not statistically different ($\alpha = 0.05$).

Table 2. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the control (<0.025 $\mu l\ l^{-1}$ O_3) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	22.0±3.1[aA]	22.1±1.4[A]	24.5±1.7[A]
(mg CO_2 dm^{-2} hr^{-1})	4	20.6±2.8[A]	20.2±2.0[A]	21.8±2.0[B]
		(-6%)[b]	(-9%)	(-11%)
Cs	0	1.6±0.6[A]	1.4±0.4[A]	1.4±0.5[A]
(cm s^{-1})	4	1.2±0.5[B]	1.2±0.4[A]	1.1±0.4[A]
		(-25%)	(-15%)	(-20%)
Ci	0	305.5±14.1[A]	300.2±13.4[A]	296.2±13.4[A]
(ppm)	4	271.1±21.0[B]	272.5±24.8[B]	264.2±22.9[B]
		(-11%)	(-9%)	(-11%)
Ts	0	5.3±1.3[A]	5.4±1.6[A]	5.0±1.5[A]
(mmol H_2O m^{-2} s^{-1})	4	4.7±1.2[A]	4.8±1.3[A]	4.8±1.1[A]
		(-12%)	(-12%)	(-4%)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

Table 3. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the fumigated ($0.20\pm0.02\ \mu l\ l^{-1}\ O_3$) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	22.3 ± 3.2^{aA}	21.8 ± 3.3^{A}	23.0 ± 4.4^{A}
($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	4	14.6 ± 2.1^{B}	15.2 ± 2.8^{B}	16.8 ± 2.7^{B}
		$(-35\%)^{b}$	(-30%)	(-27%)
Cs	0	1.7 ± 0.4^{A}	1.6 ± 0.4^{A}	1.6 ± 0.3^{A}
($cm\ s^{-1}$)	4	0.5 ± 0.1^{B}	0.5 ± 0.1^{B}	0.5 ± 0.1^{B}
		(-71%)	(-69%)	(-69%)
Ci	0	312.3 ± 25.4^{A}	304.7 ± 19.2^{A}	301.1 ± 29.7^{A}
(ppm)	4	261.5 ± 40.5^{B}	228.4 ± 42.4^{B}	232.8 ± 36.3^{B}
		(-16%)	(-25%)	(-23%)
Ts	0	4.6 ± 1.1^{A}	4.6 ± 1.4^{A}	4.7 ± 1.2^{A}
($mmol\ H_2O\ m^{-2}\ s^{-1}$)	4	2.4 ± 0.8^{B}	2.4 ± 0.9^{B}	2.4 ± 0.9^{B}
		(-48%)	(-48%)	(-49%)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different ($\alpha=0.05$).

Table 4. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the control (<0.025 μl l^{-1} O_3) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$ (μmol g^{-1} fw)	0	1.17 ± 0.10^{aA}	1.22 ± 0.17^{A}	1.39 ± 0.11^{A}
	4	1.29 ± 0.11^{A} (10%)[b]	1.26 ± 0.14^{A} (3%)	1.43 ± 0.09^{A} (3%)
$Glutathione_{ox}$ (μmol g^{-1} fw)	0	$.045\pm.006^{A}$	$.042\pm.006^{A}$	$.047\pm.005^{A}$
	4	$.046\pm.008^{A}$ (2%)	$.043\pm.006^{A}$ (2%)	$.043\pm.013^{A}$ (-9%)
$Glutathione_{red}$ (μmol g^{-1} fw)	0	1.08 ± 0.09^{A}	1.13 ± 0.17^{A}	1.29 ± 0.11^{A}
	4	1.20 ± 0.10^{A} (11%)	1.17 ± 0.12^{A} (4%)	1.34 ± 0.07^{A} (4%)
$Ascorbate_{tot}$ (μg g^{-1} fw)	0	1074 ± 166^{A}	1217 ± 247^{A}	1325 ± 148^{A}
	4	1181 ± 214^{A} (10%)	1260 ± 205^{A} (4%)	1450 ± 203^{A} (9%)
$Ascorbate_{ox}$ (μg g^{-1} fw)	0	117 ± 106^{A}	203 ± 230^{A}	125 ± 116^{A}
	4	164 ± 118^{A} (40%)	124 ± 88^{A} (-39%)	58 ± 56^{A} (-54%)
$Ascorbate_{red}$ (μg g^{-1} fw)	0	958 ± 144^{A}	1051 ± 158^{A}	1214 ± 158^{A}
	4	1009 ± 140^{A} (5%)	1136 ± 182^{A} (8%)	1437 ± 229^{B} (18%)

(Cont.)

(Table 4 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	32.8±2.7[A]	25.3±5.1[A]	32.0±5.4[A]
(mg g^{-1} fw)	4	33.7±2.7[A]	27.7±3.8[A]	32.1±5.9[A]
		(3%)	(9%)	(0%)
Glutathione	0	0.80±0.21[A]	0.74±0.19[A]	0.85±0.26[A]
Reductase	4	0.73±0.08[A]	0.73±0.22[A]	0.87±0.25[A]
(U g^{-1} fw)		(-9%)	(-1%)	(2%)
Glutathione	0	.024±.005[A]	.032±.006[A]	.026±.005[A]
Reductase	4	.022±.003[A]	.026±.006[A]	.026±.004[A]
(U mg^{-1} protein)		(-8%)	(-19%)	(0%)
Ascorbate	0	1.31±0.18[A]	1.29±0.27[A]	1.22±0.19[A]
Peroxidase	4	1.36±0.13[A]	1.41±0.08[A]	1.23±0.22[A]
(U g^{-1} fw)		(4%)	(9%)	(1%)
Ascorbate	0	.040±.004[A]	.055±.010[A]	.039±.007[A]
Peroxidase	4	.041±.006[A]	.051±.007[A]	.038±.004[A]
(U mg^{-1} protein)		(3%)	(-7%)	(-3%)
SOD	0	37.6±10.6[A]	11.4±9.9[A]	28.2±14.0[A]
(U g^{-1} fw)	4	32.2±13.0[A]	15.6±6.7[A]	30.4±1.0[A]
		(-14%)	(37%)	(8%)
SOD	0	1.15±0.31[A]	0.60±0.41[A]	0.72±0.43[A]
(U mg^{-1} protein)	4	0.94±0.33[A]	0.47±0.33[A]	0.67±0.39[A]
		(-18%)	(-22%)	(-7%)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

Table 5. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the fumigated ($0.20\pm0.02\ \mu l\ l^{-1}\ O_3$) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$ ($\mu mol\ g^{-1}$ fw)	0	1.18 ± 0.05^{aA}	1.24 ± 0.14^{A}	1.34 ± 0.24^{A}
	4	1.25 ± 0.11^{A} (6%)	1.25 ± 0.13^{A} (1%)	1.40 ± 0.24^{A} (4%)
$Glutathione_{ox}$ ($\mu mol\ g^{-1}$ fw)	0	$.045\pm.008^{A}$	$.048\pm.012^{A}$	$.036\pm.016^{A}$
	4	$.046\pm.008^{A}$ (2%)	$.038\pm.002^{A}$ (-21%)	$.038\pm.006^{A}$ (6%)
$Glutathione_{red}$ ($\mu mol\ g^{-1}$ fw)	0	1.09 ± 0.05^{A}	1.14 ± 0.14^{A}	1.27 ± 0.25^{A}
	4	1.16 ± 0.09^{A} (6%)	1.17 ± 0.13^{A} (3%)	1.32 ± 0.25^{A} (4%)
$Ascorbate_{tot}$ ($\mu g\ g^{-1}$ fw)	0	1029 ± 255^{A}	1123 ± 241^{A}	1378 ± 348^{A}
	4	1078 ± 193^{A} (5%)	1146 ± 140^{A} (2%)	1278 ± 256^{A} (-7%)
$Ascorbate_{ox}$ ($\mu g\ g^{-1}$ fw)	0	182 ± 140^{A}	130 ± 49^{A}	96 ± 75^{A}
	4	135 ± 47^{A} (-26%)	104 ± 61^{A} (-20%)	97 ± 97^{A} (1%)
$Ascorbate_{red}$ ($\mu g\ g^{-1}$ fw)	0	847 ± 163^{A}	993 ± 249^{A}	1296 ± 419^{A}
	4	950 ± 209^{A} (12%)	1069 ± 178^{A} (8%)	1191 ± 298^{A} (-8%)

(Cont.)

(Table 5 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	31.6±3.8[A]	27.2±1.5[A]	30.0±2.3[A]
(mg g^{-1} fw)	4	32.4±3.0[A]	28.0±2.8[A]	30.0±4.6[A]
		(3%)	(3%)	(0%)
Glutathione	0	0.67±0.23[A]	0.78±0.14[A]	0.87±0.18[A]
Reductase	4	0.73±0.24[A]	0.74±0.14[A]	0.85±0.12[A]
(U g^{-1} fw)		(9%)	(-5%)	(-2%)
Glutathione	0	.021±.007[A]	.028±.004[A]	.029±.004[A]
Reductase	4	.023±.007[A]	.027±.007[A]	.028±.004[A]
(U mg^{-1} protein)		(10%)	(-4%)	(-3%)
Ascorbate	0	1.40±0.20[A]	1.38±0.17[A]	1.00±0.27[A]
Peroxidase	4	1.53±0.20[A]	1.43±0.17[A]	1.19±0.32[A]
(U g^{-1} fw)		(9%)	(4%)	(19%)
Ascorbate	0	.044±.007[A]	.051±.004[A]	.033±.007[A]
Peroxidase	4	.047±.004[A]	.051±.006[A]	.040±.006[A]
(U mg^{-1} protein)		(7%)	(0%)	(21%)
SOD	0	28.8±6.4[A]	16.8±6.6[A]	29.3±25.8[A]
(U g^{-1} fw)	4	35.9±13.9[A]	13.4±8.0[A]	19.0±1.6[A]
		(25%)	(-20%)	(-35%)
SOD	0	0.93±0.10[A]	0.63±0.22[A]	0.99±0.88[A]
(U mg^{-1} protein)	4	1.10±0.32[A]	0.50±0.32[A]	0.68±0.02[A]
		(18%)	(-21%)	(-31%)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

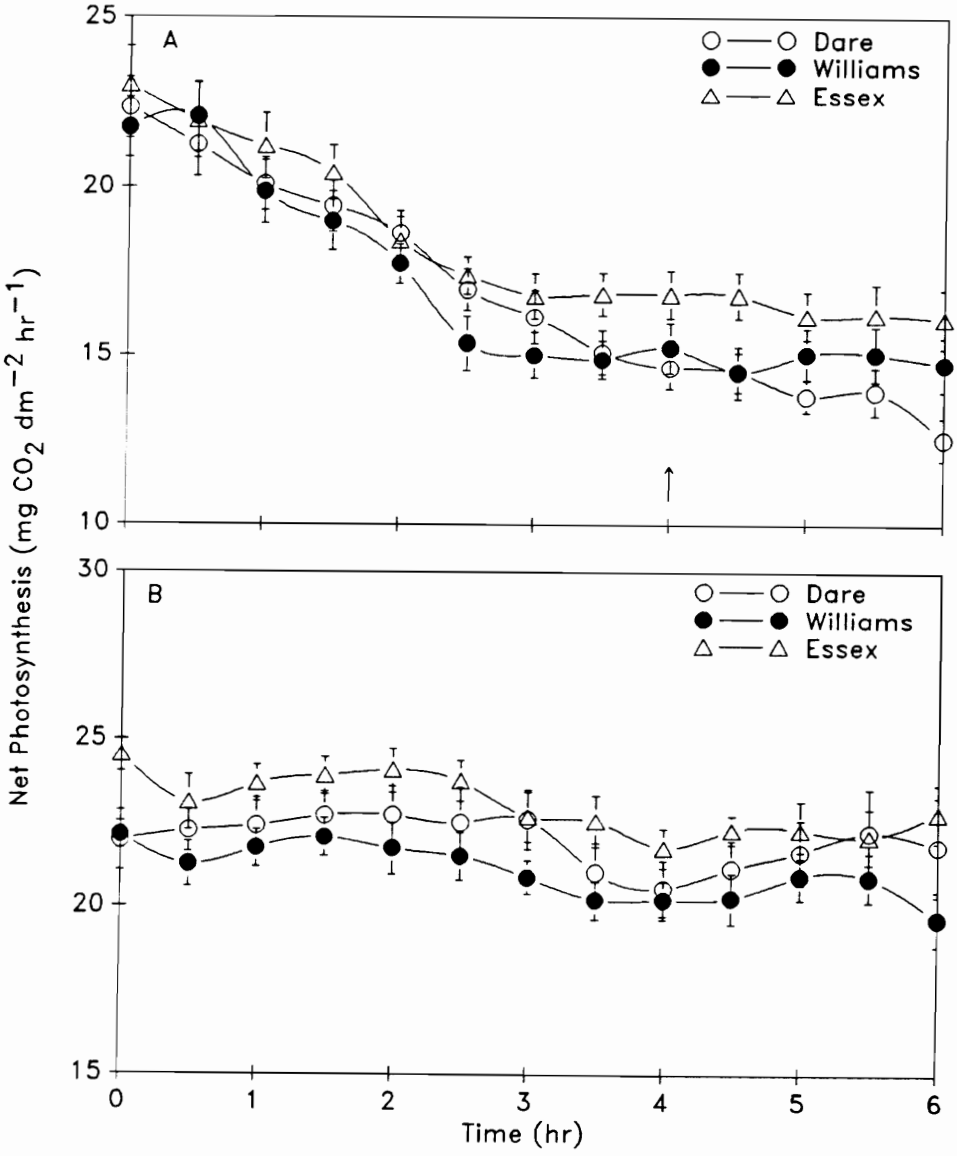


Fig. 1. Net photosynthetic rate of soybean cultivars exposed to A) 0.2 $\mu l\ l^{-1}$ O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 15 samples. Where error bars are not shown, they are contained within the symbols. ↑ indicates O_3 fumigation terminated.

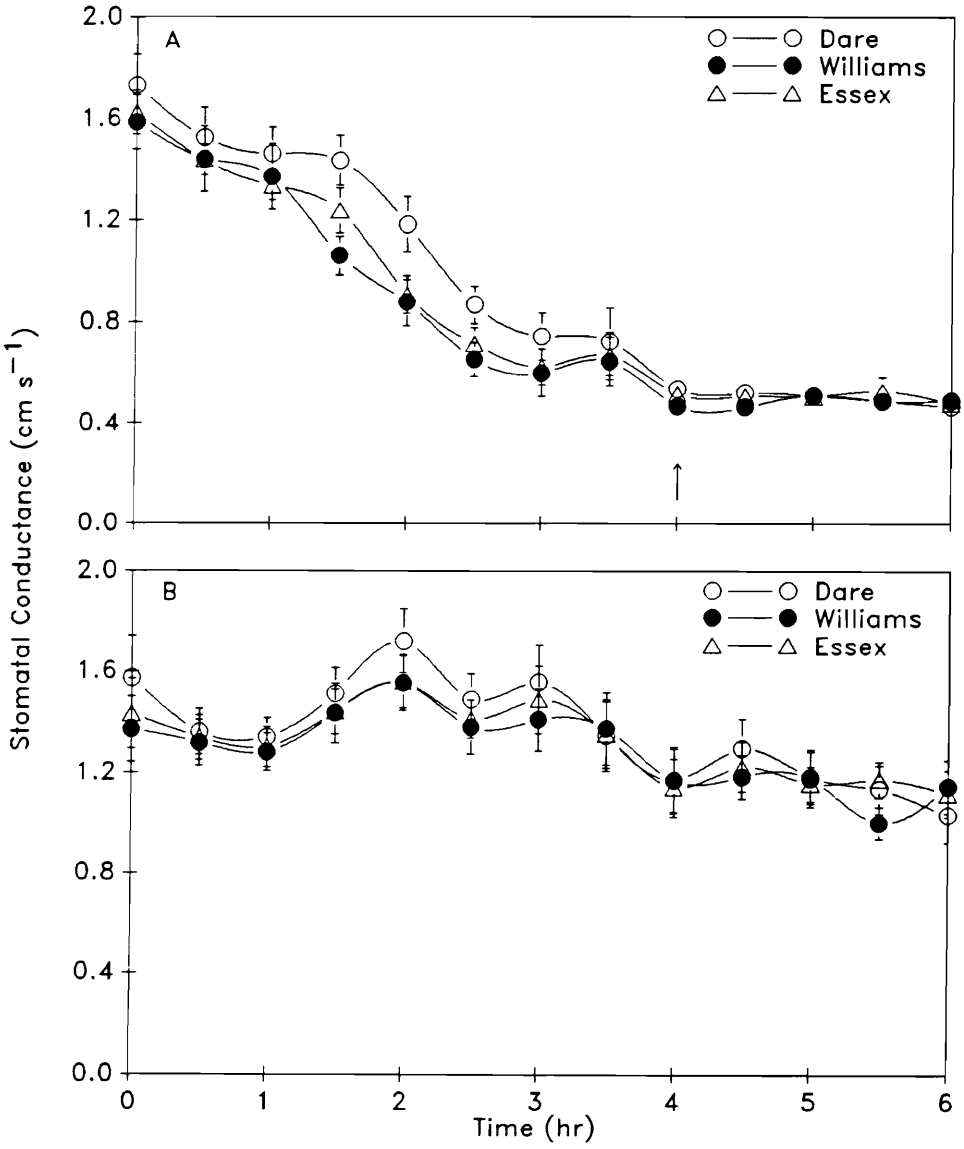


Fig. 2. Stomatal conductance of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 15 samples. Where error bars are not shown, they are contained within the symbols. † indicates O_3 fumigation terminated.

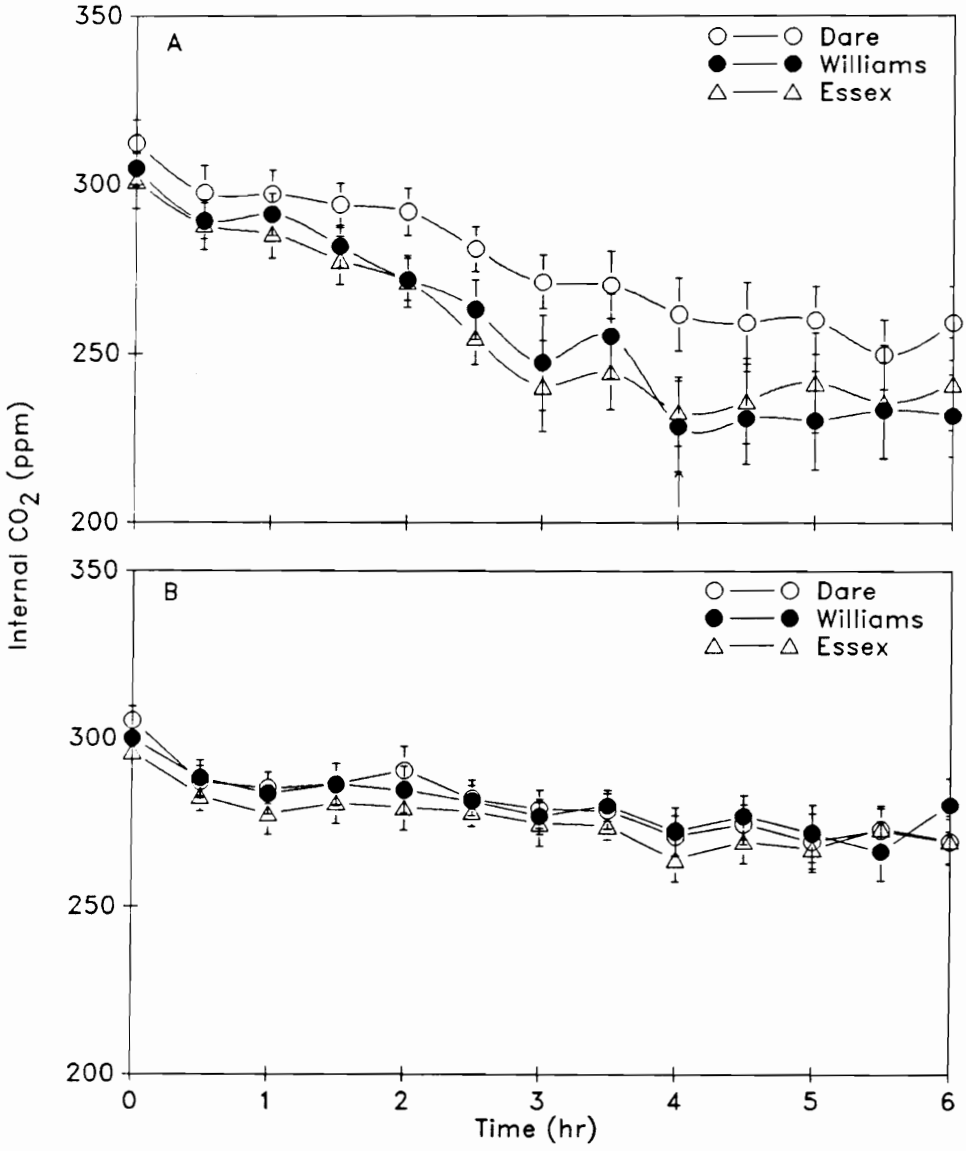


Fig. 3. Intercellular CO_2 concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 15 samples. Where error bars are not shown, they are contained within the symbols. † indicates O_3 fumigation terminated.

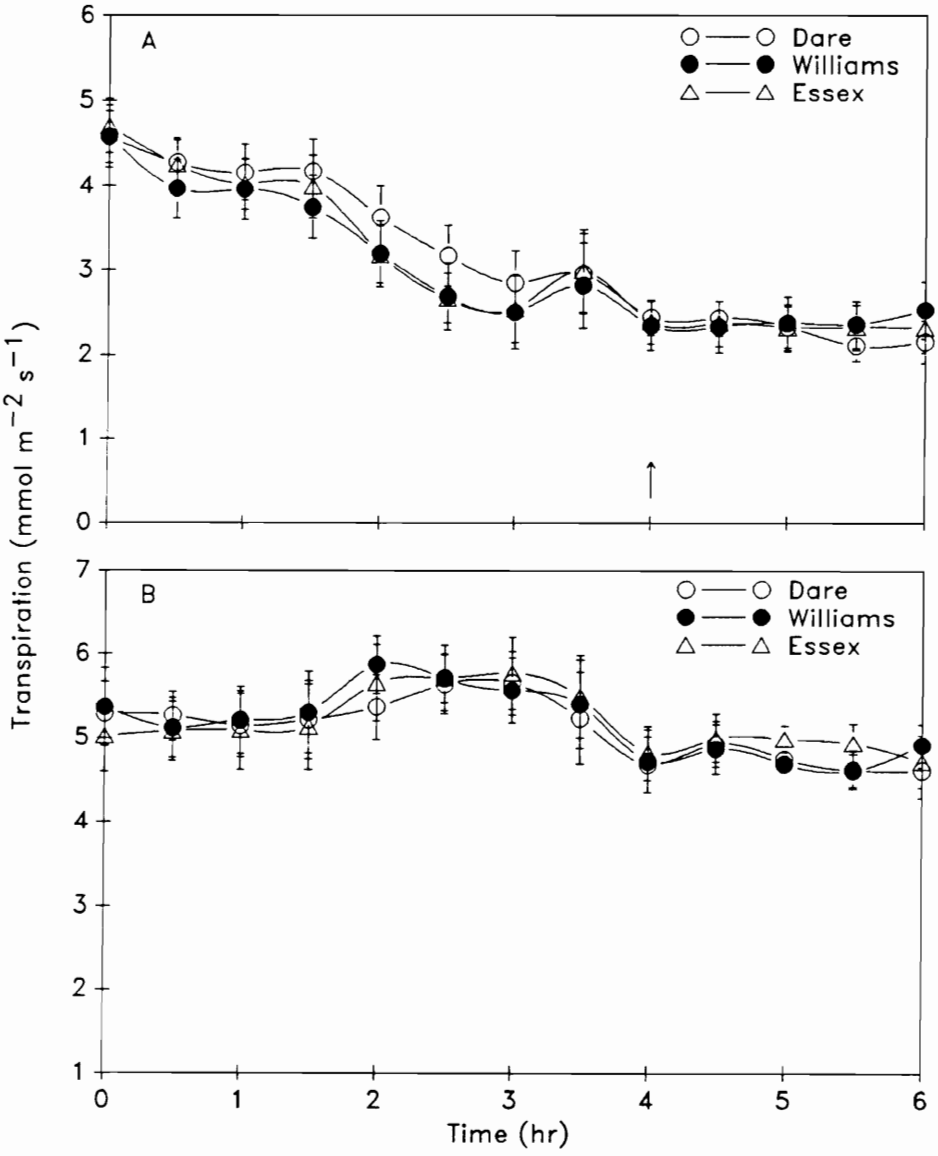


Fig. 4. Transpiration rate of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 15 samples. Where error bars are not shown, they are contained within the symbols. † indicates O_3 fumigation terminated.

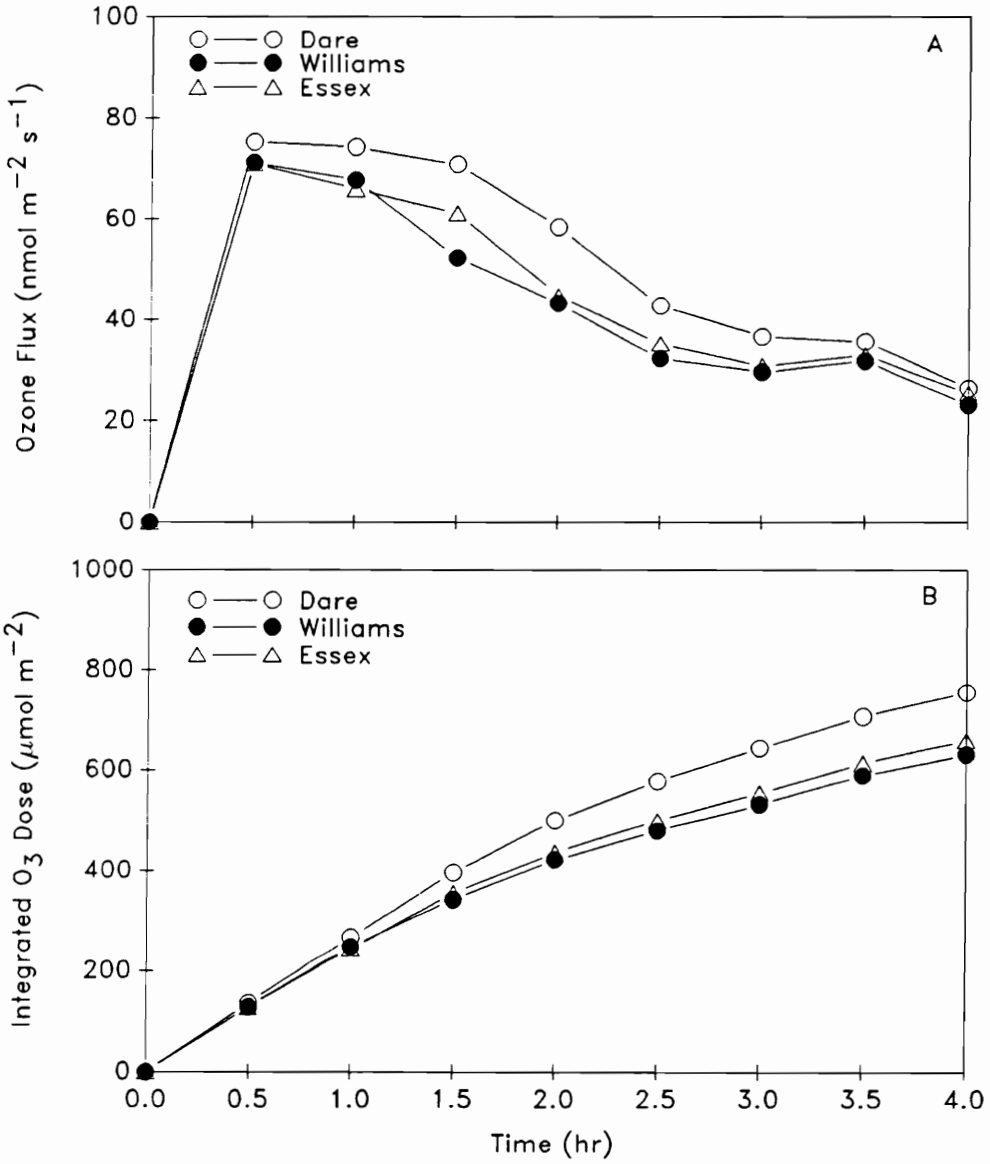


Fig. 5. Estimated ozone flux A) and integrated O_3 dose B) of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr. Each point is calculated from the mean of 15 samples.

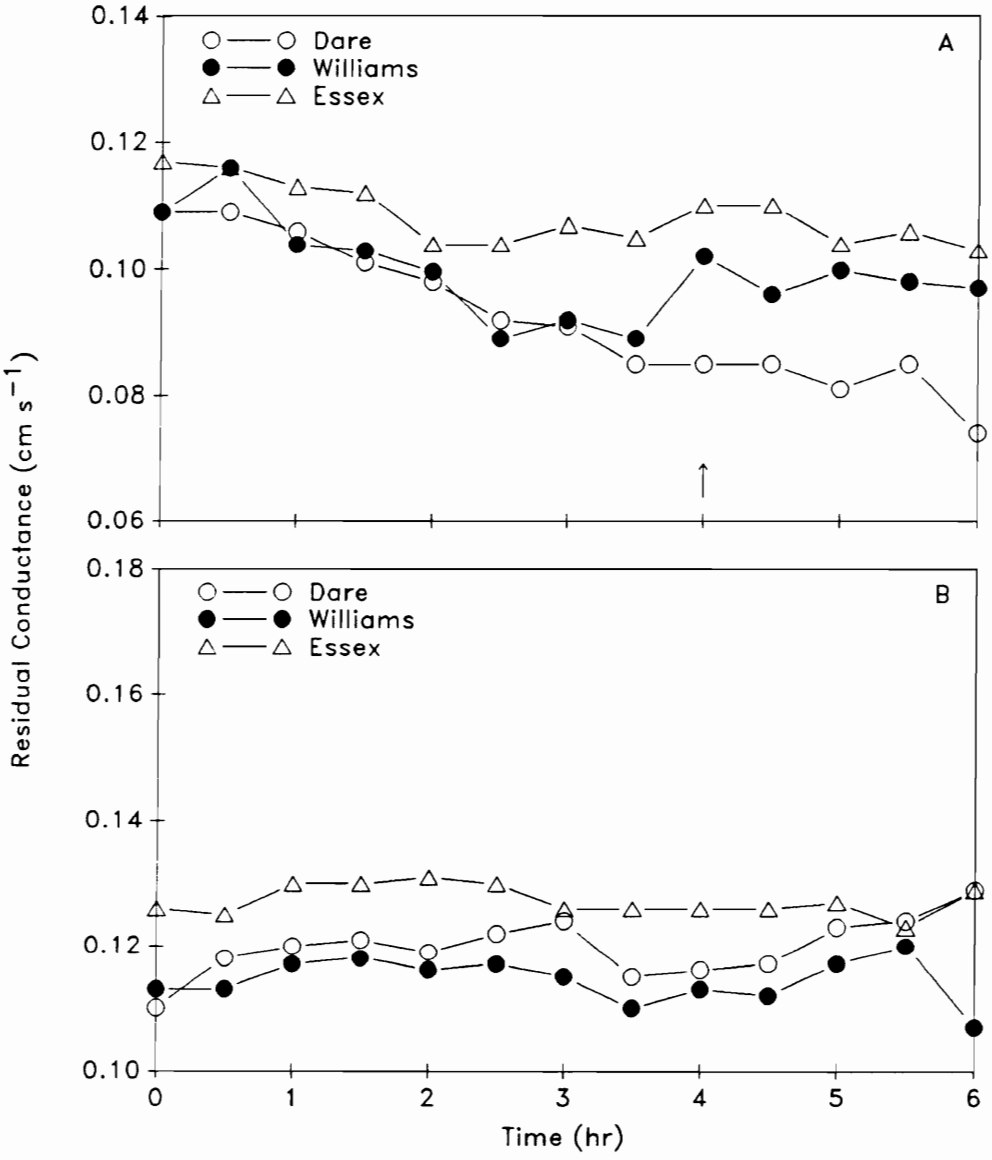


Fig. 6. Residual conductance to CO_2 of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point is calculated from the mean of 15 samples. ↑ indicates O_3 fumigation terminated.

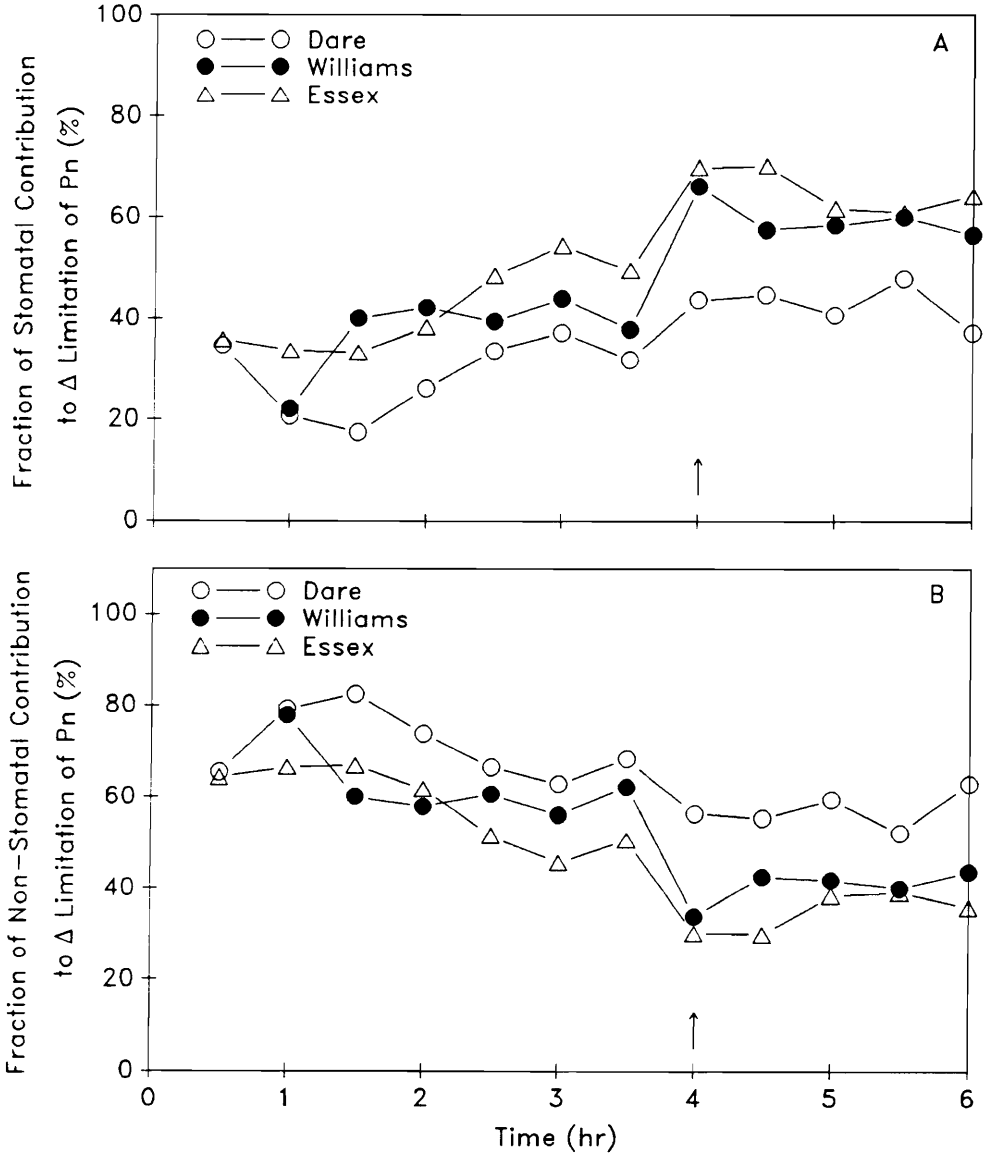


Fig. 7. Fraction of A) stomatal and B) non-stomatal contribution to change in limitation to Pn of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr and allowed to recover for 2 hr. Relative to 0 time, each point is calculated from the mean of 15 samples. † indicates O_3 fumigation terminated.

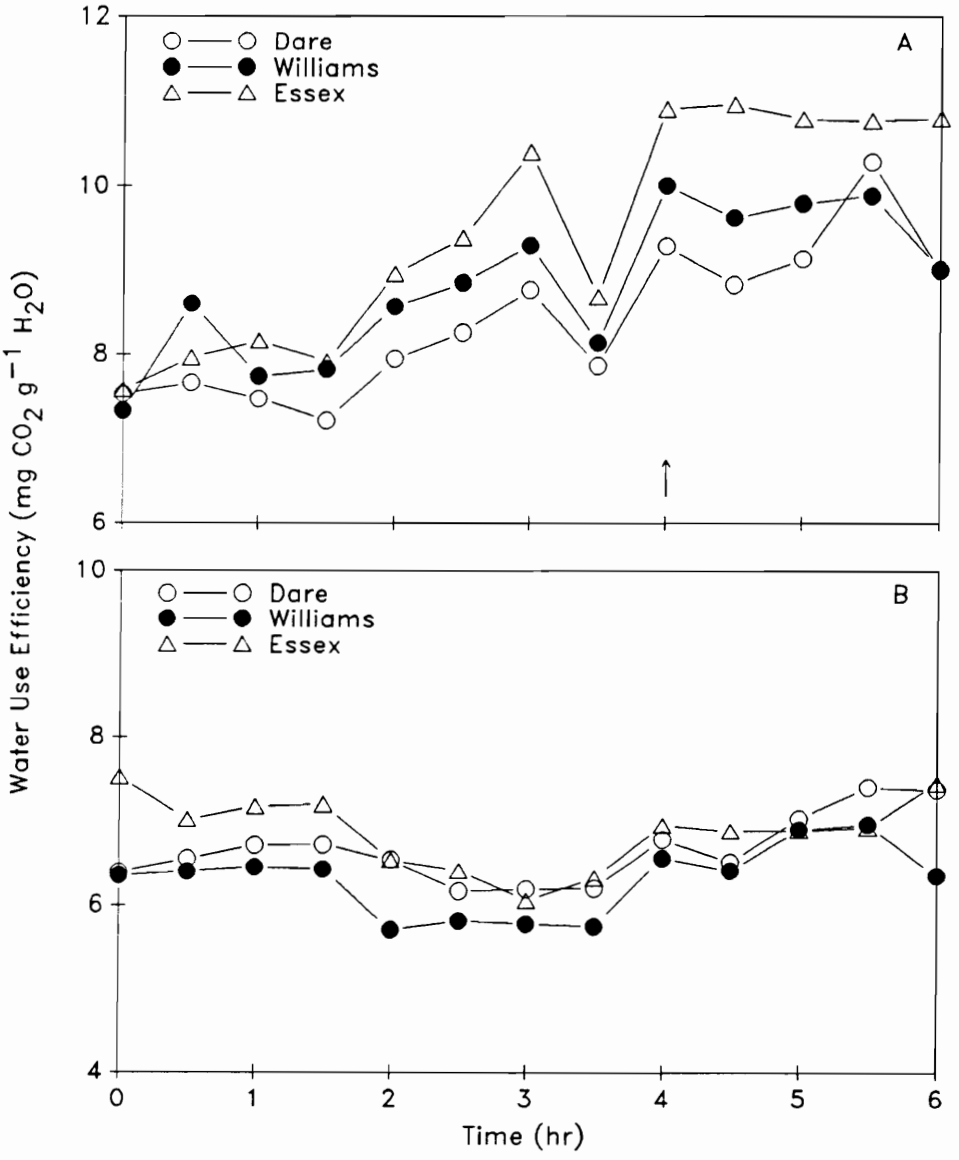


Fig. 8. Water use efficiency of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point is calculated from the mean of 15 samples. ↑ indicates O_3 fumigation terminated.

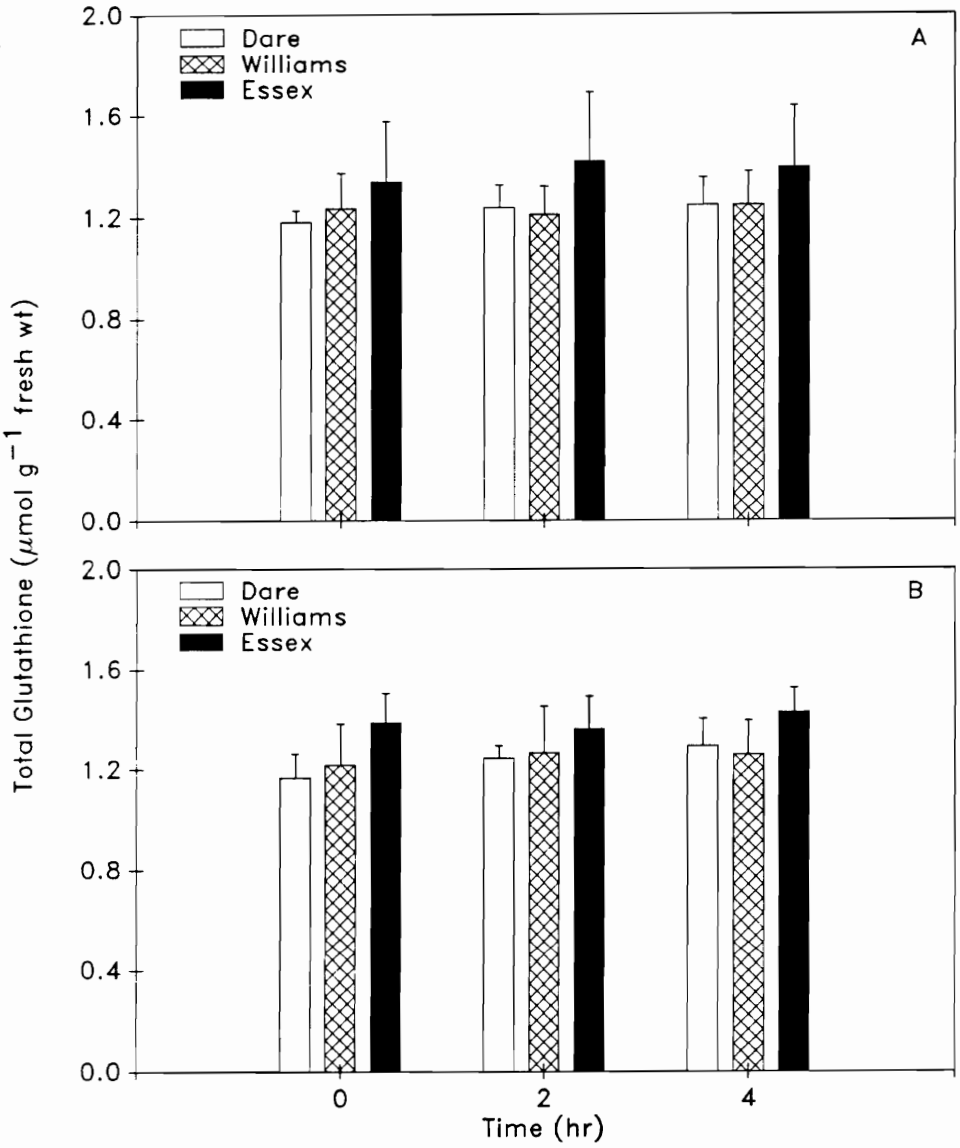


Fig. 9. Total glutathione concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 4 samples.

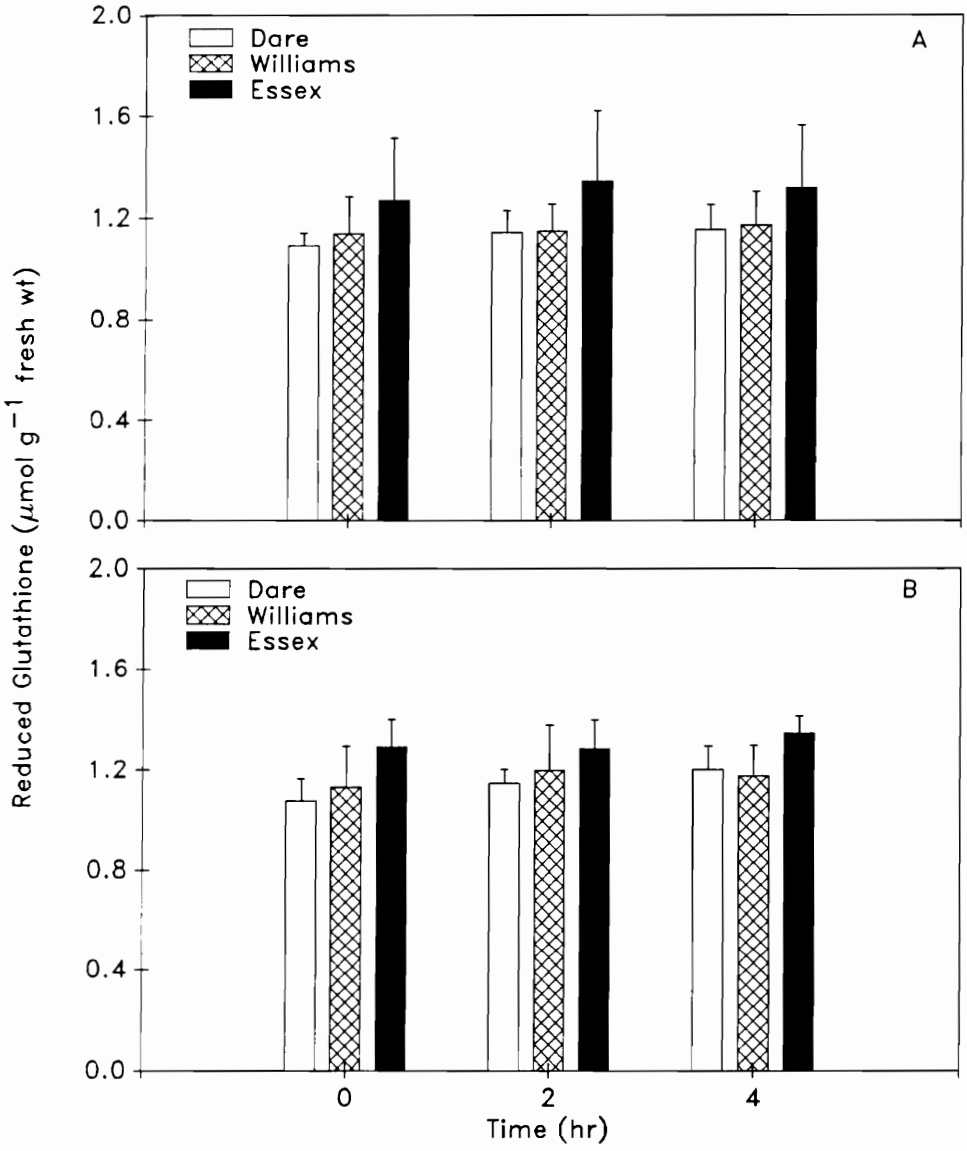


Fig. 10. Reduced glutathione concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 4 samples.

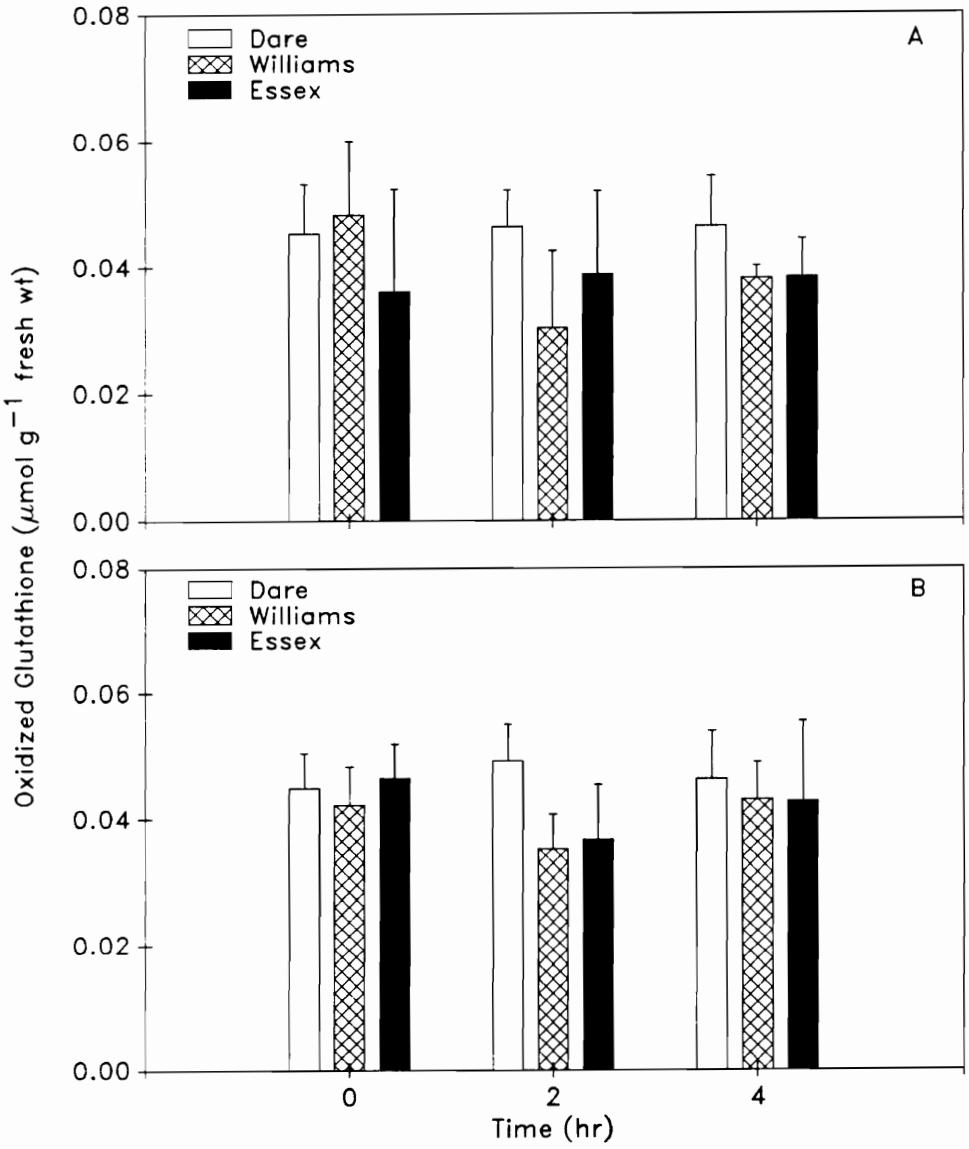


Fig. 11. Oxidized glutathione concentration of soybean cultivars exposed to A) 0.2 $\mu l\ l^{-1}$ O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 4 samples.

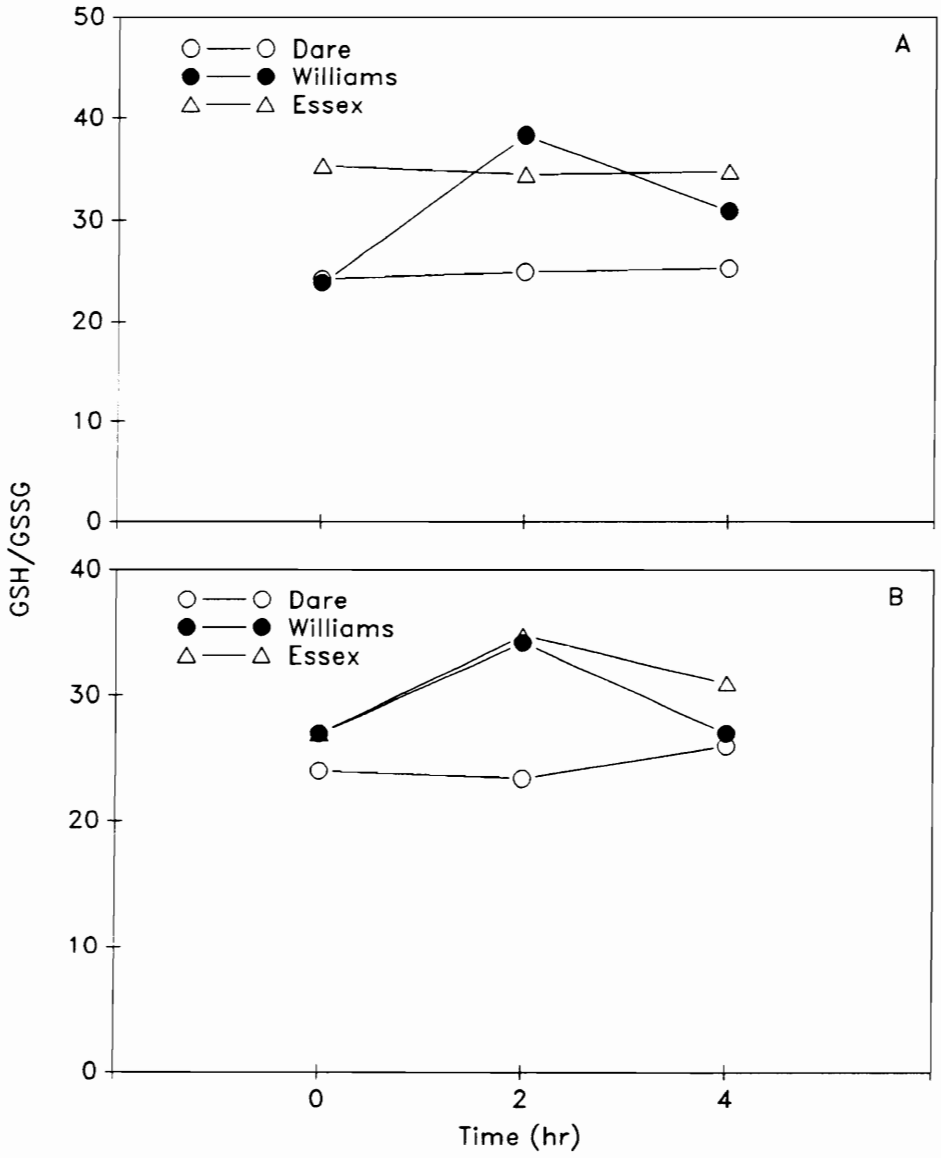


Fig. 12. Mean ratio of reduced (GSH) to oxidized (GSSG) glutathione in soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr.

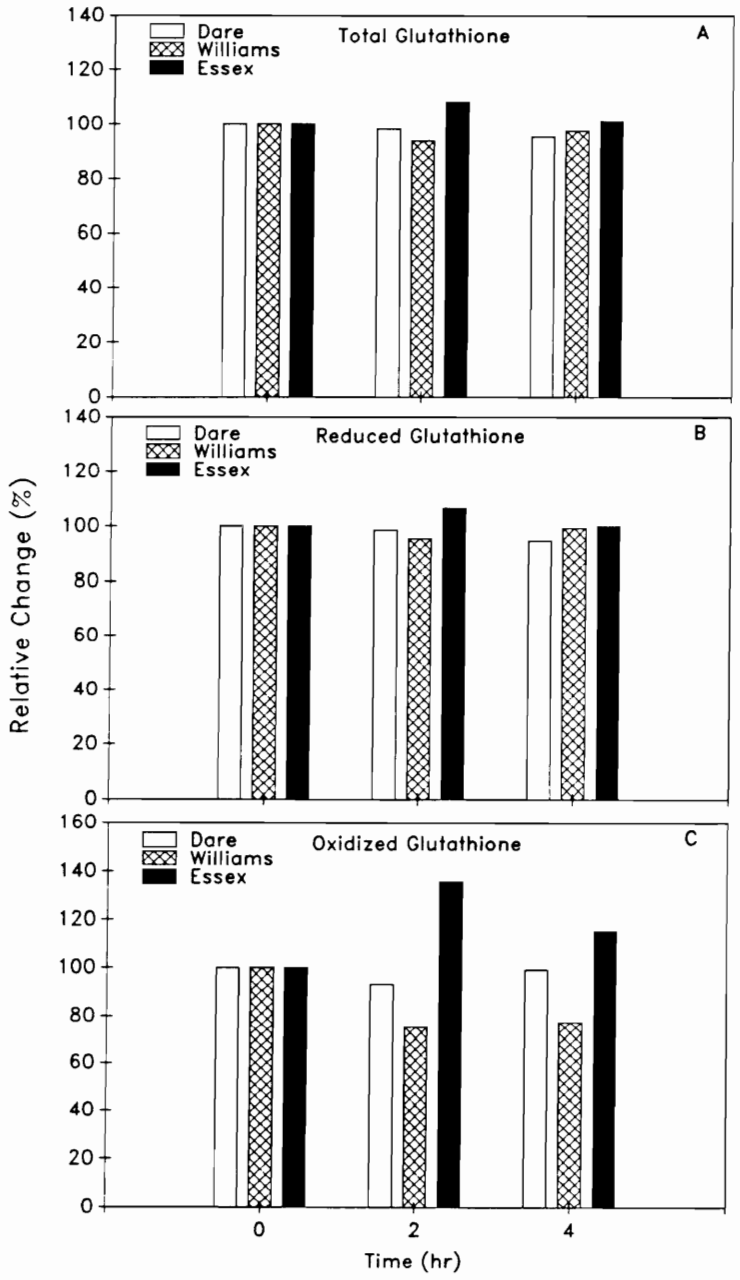


Fig. 13. Changes of A) total glutathione, B) reduced glutathione and C) oxidized glutathione concentrations of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 4 samples.

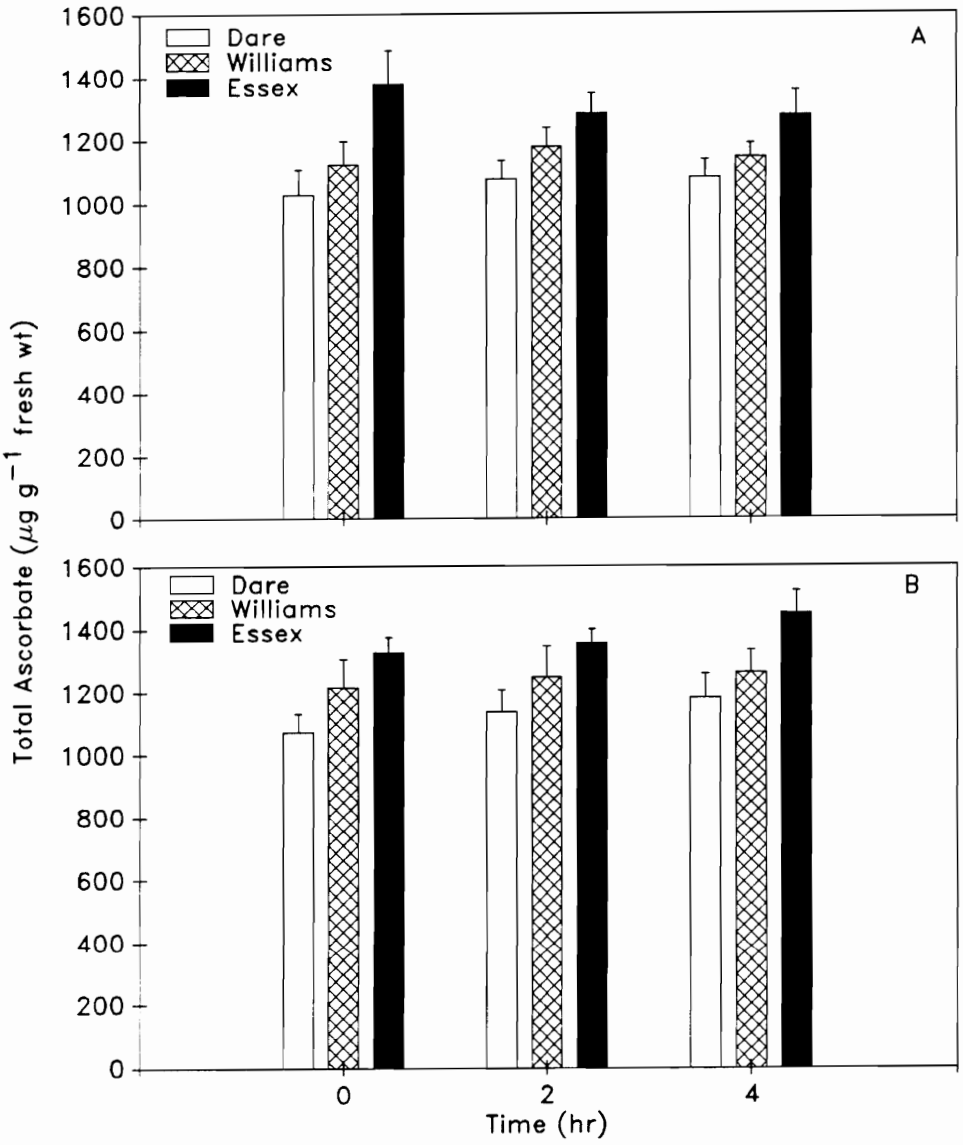


Fig. 14. Total ascorbate concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 11 samples.

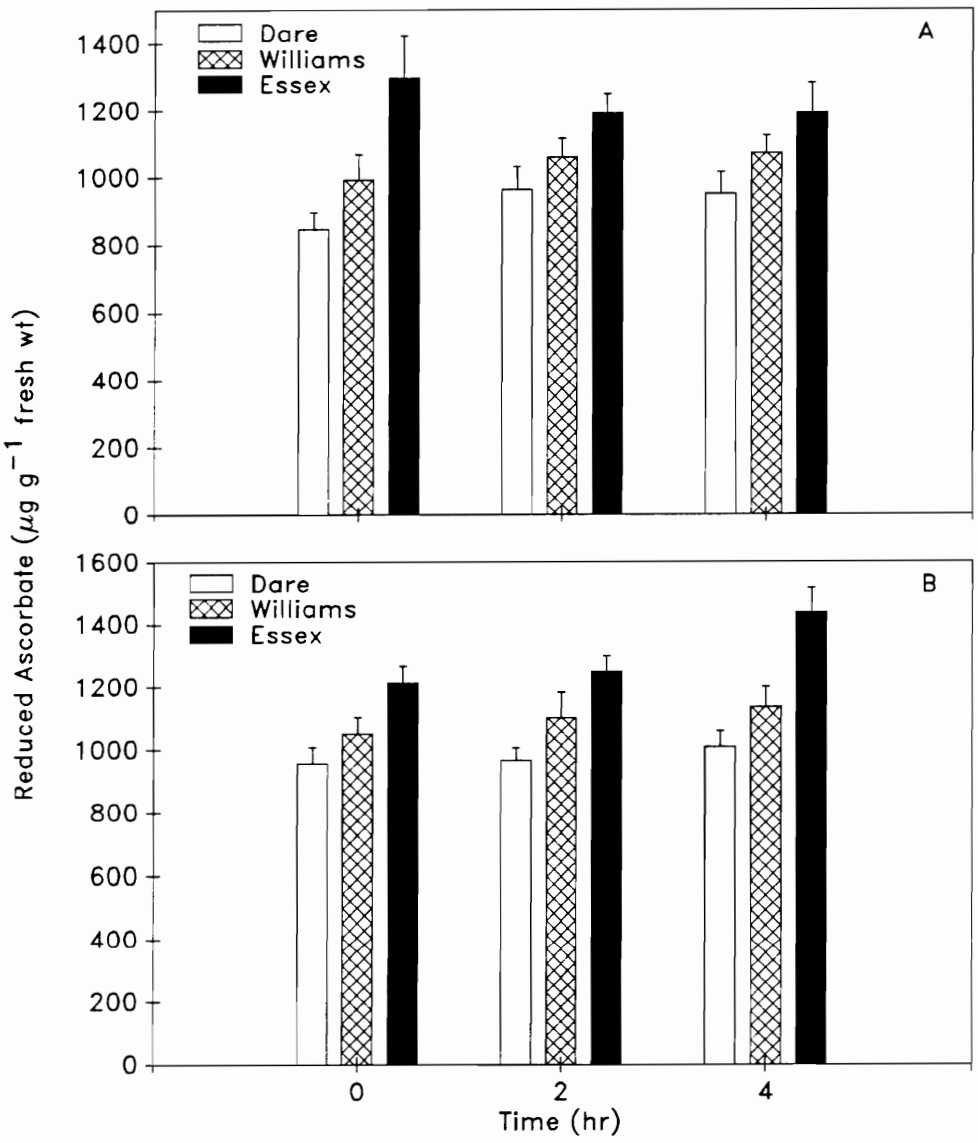


Fig. 15. Reduced ascorbate concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 11 samples.

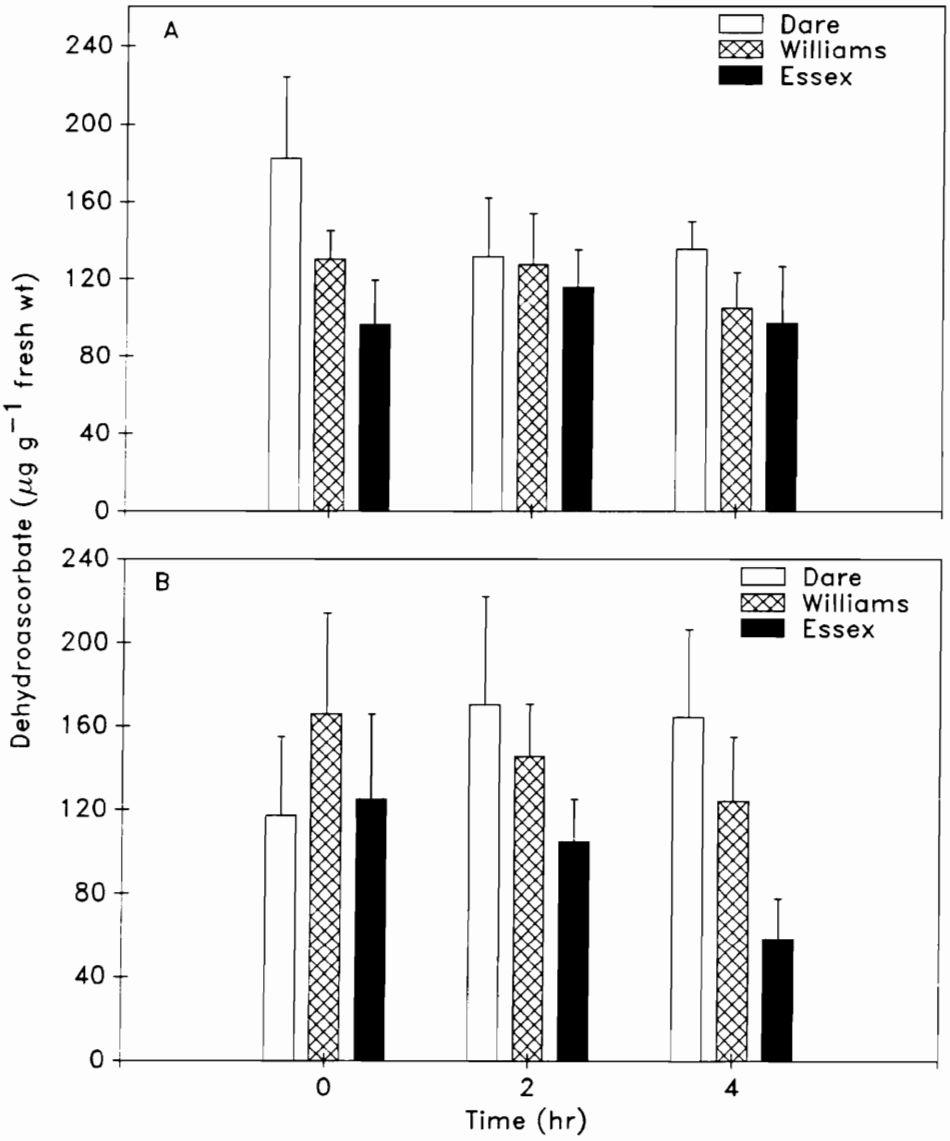


Fig. 16. Dehydroascorbate concentration of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 11 samples.

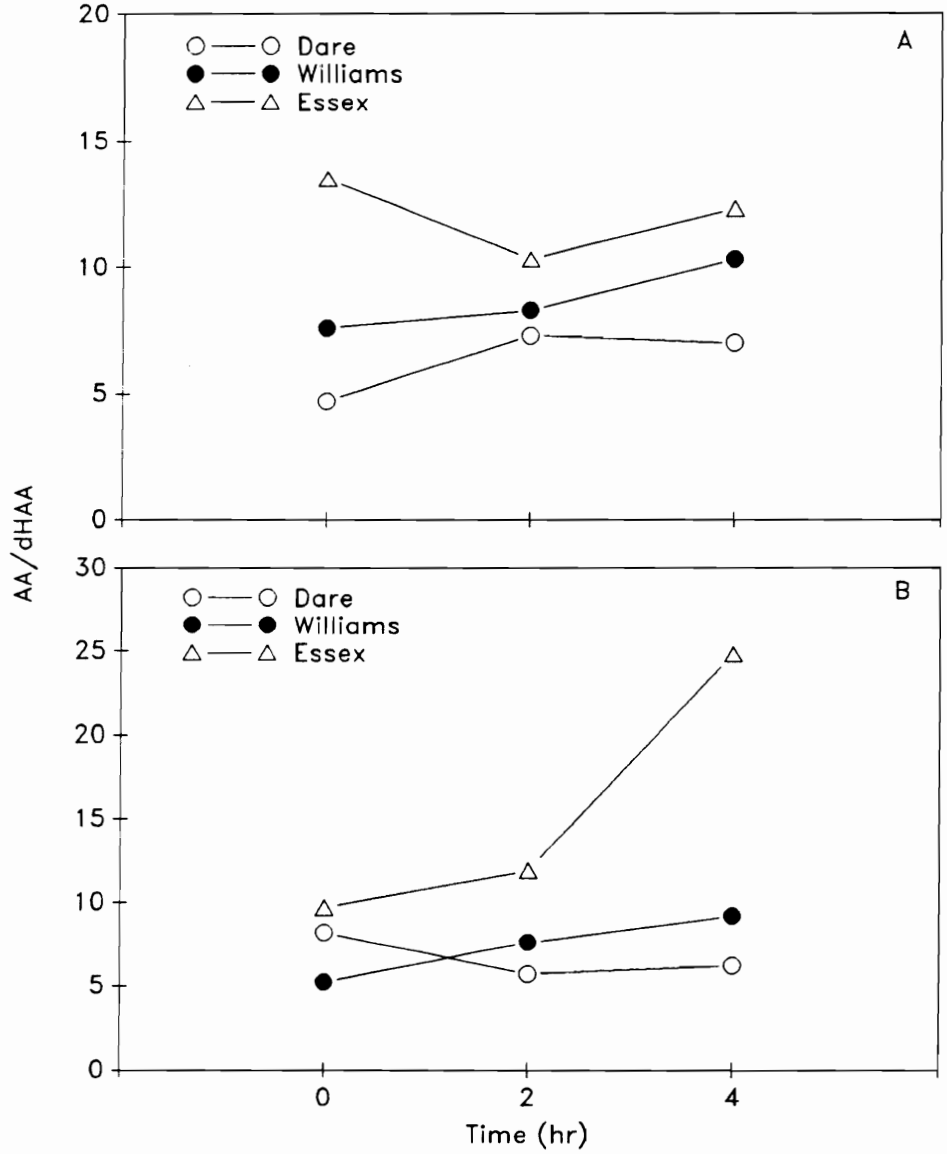


Fig. 17. Mean ratio of reduced (AA) to oxidized (dHAA) ascorbate in soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr.

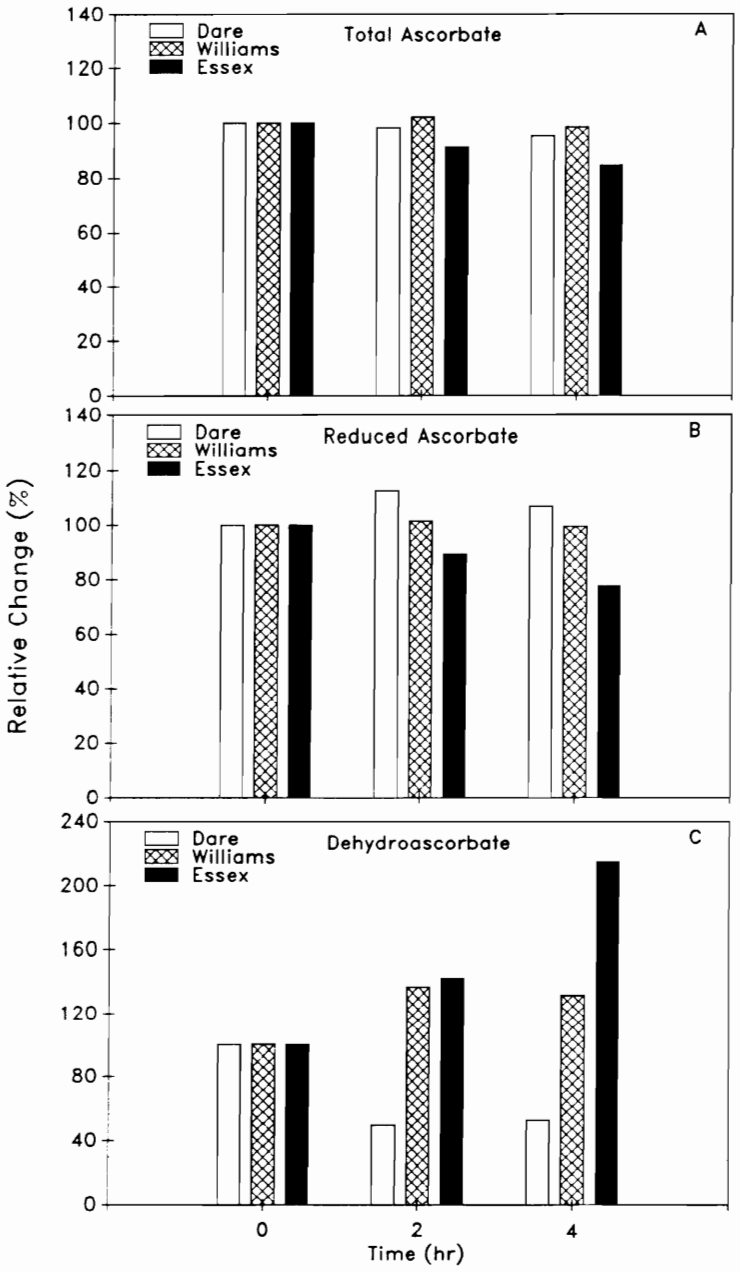


Fig. 18. Changes of A) total ascorbate, B) reduced ascorbate and C) dehydroascorbate concentrations of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 11 samples.

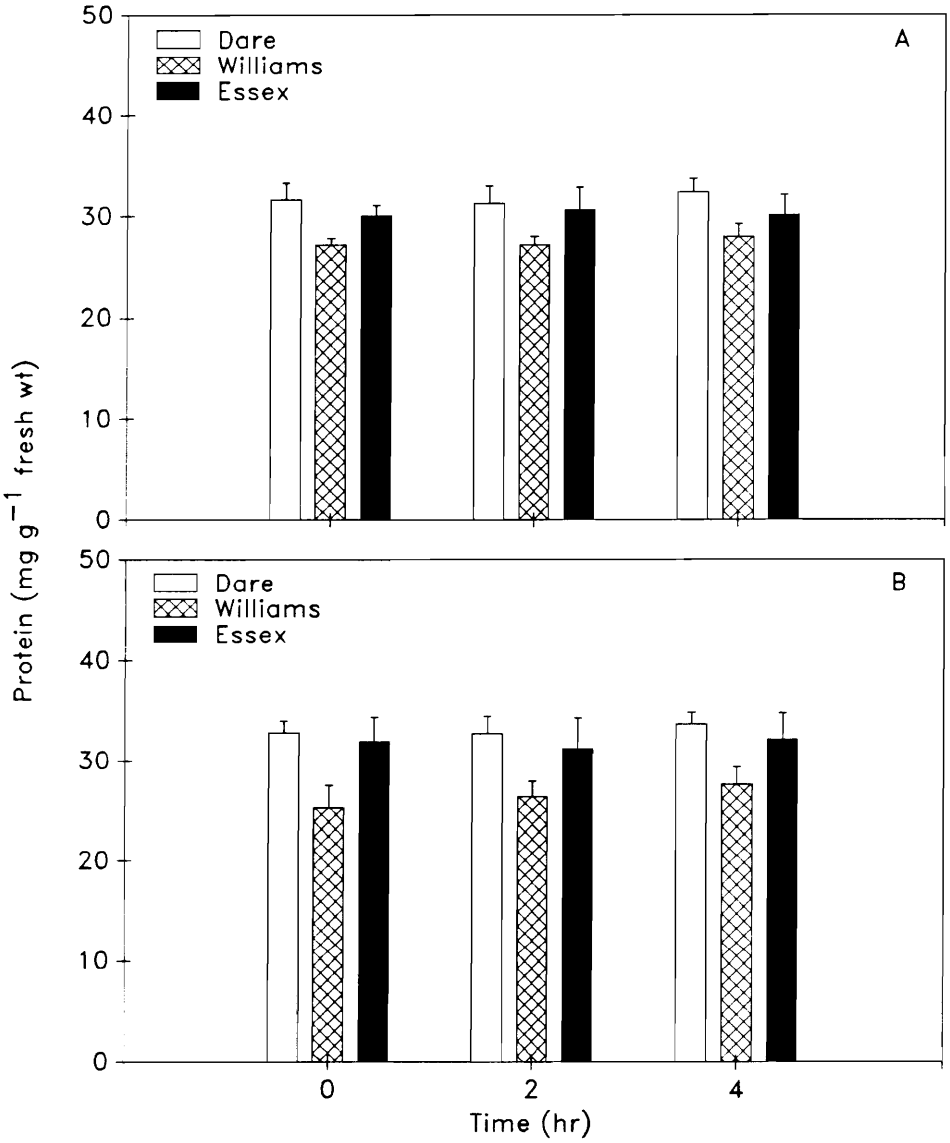


Fig. 19. Protein concentrations of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

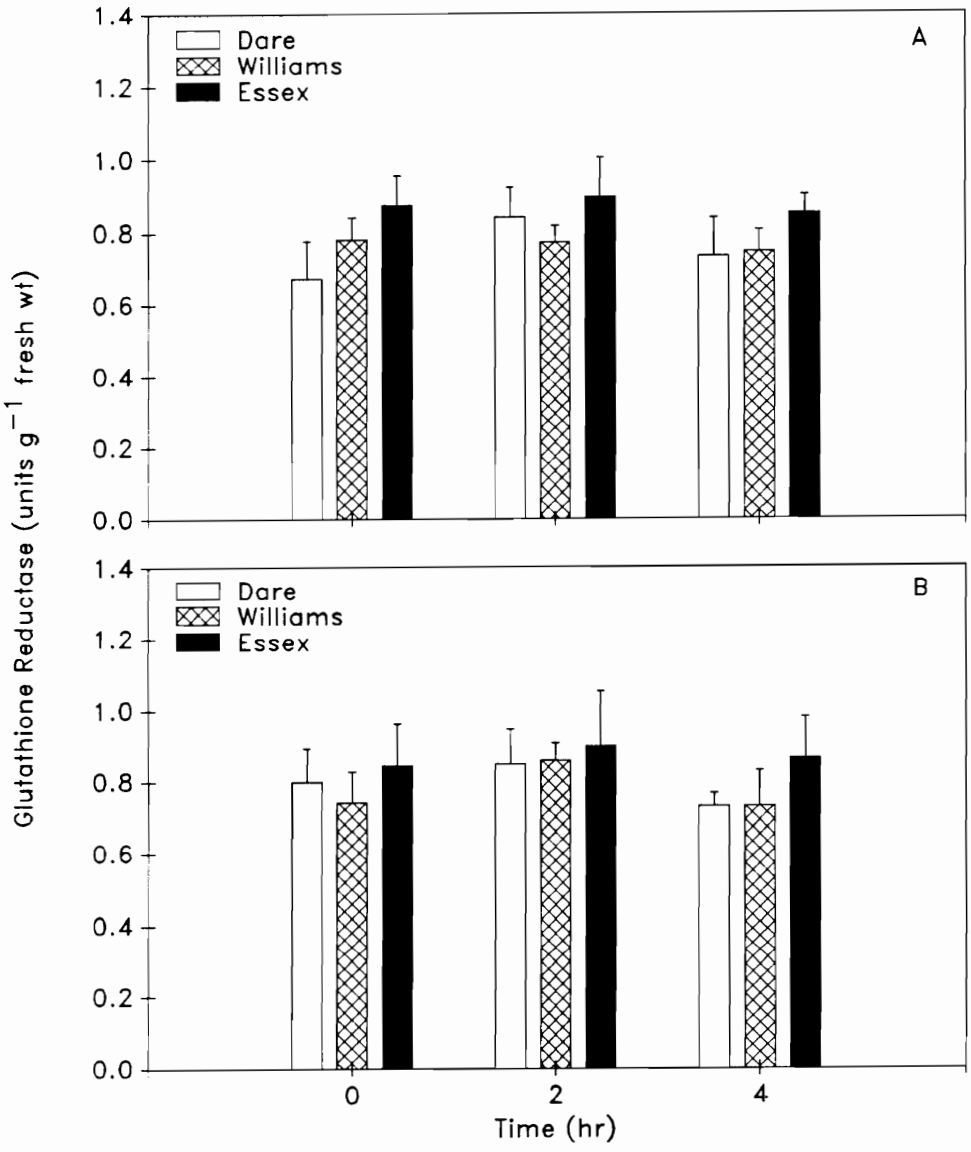


Fig. 20. Changes in glutathione reductase total activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

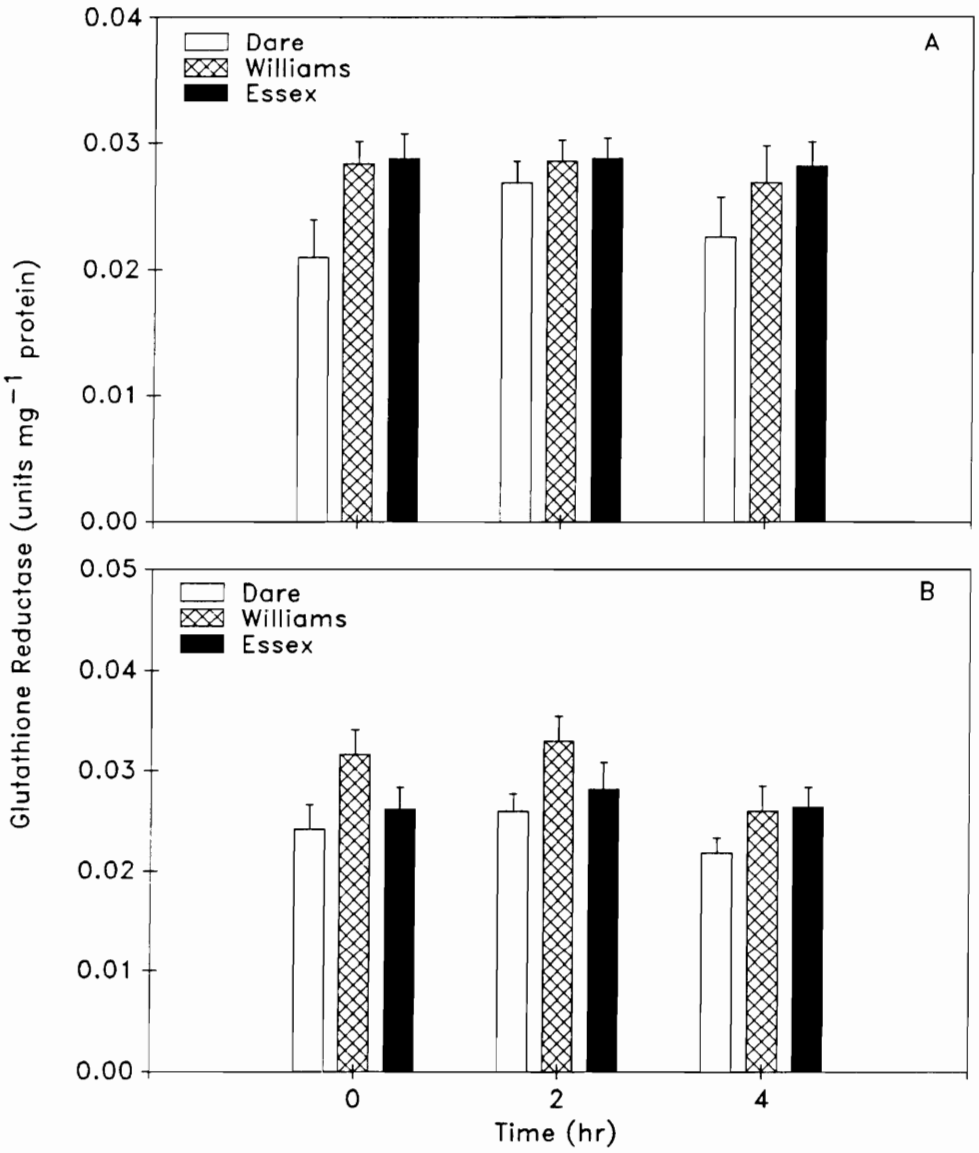


Fig. 21. Changes in glutathione reductase specific activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

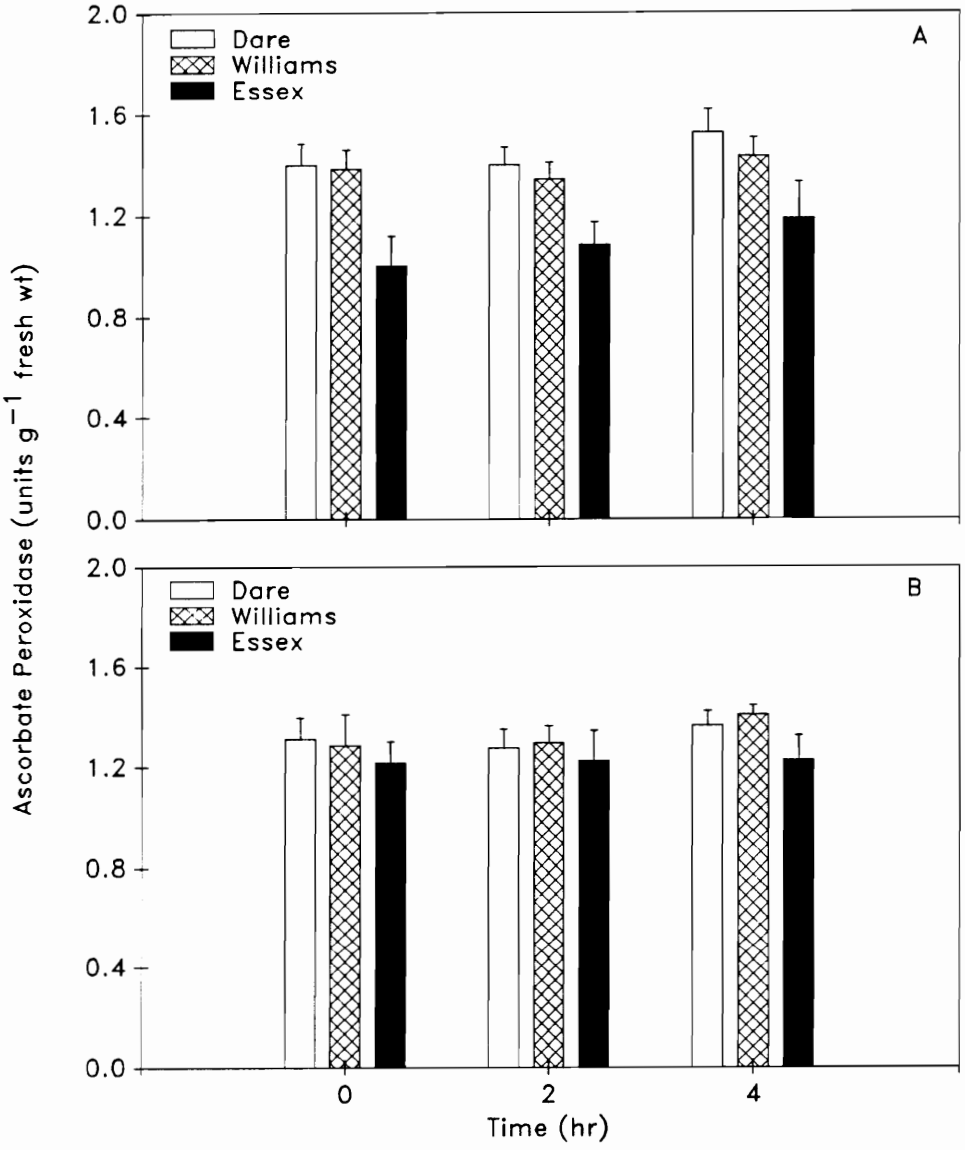


Fig. 22. Changes in ascorbate peroxidase total activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

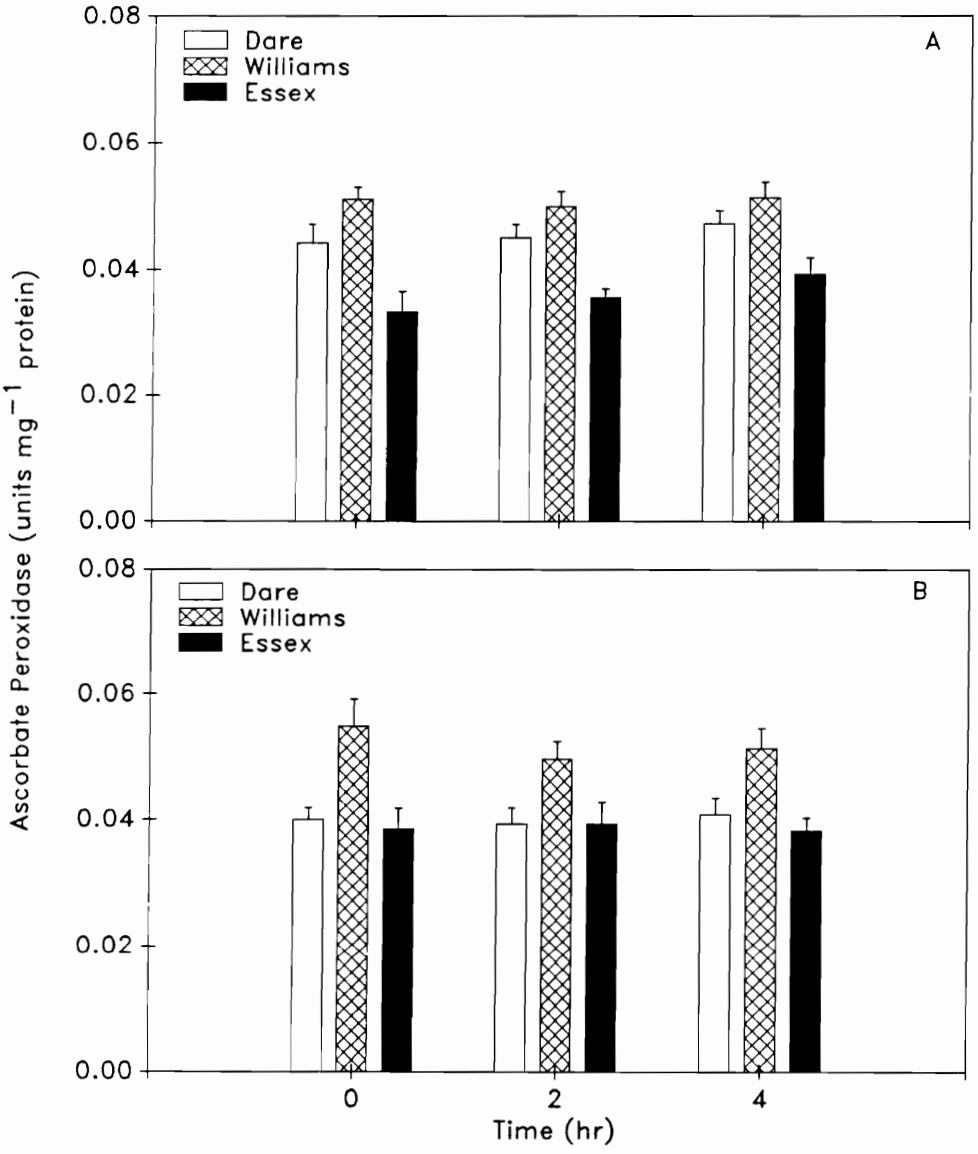


Fig. 23. Changes in ascorbate peroxidase specific activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

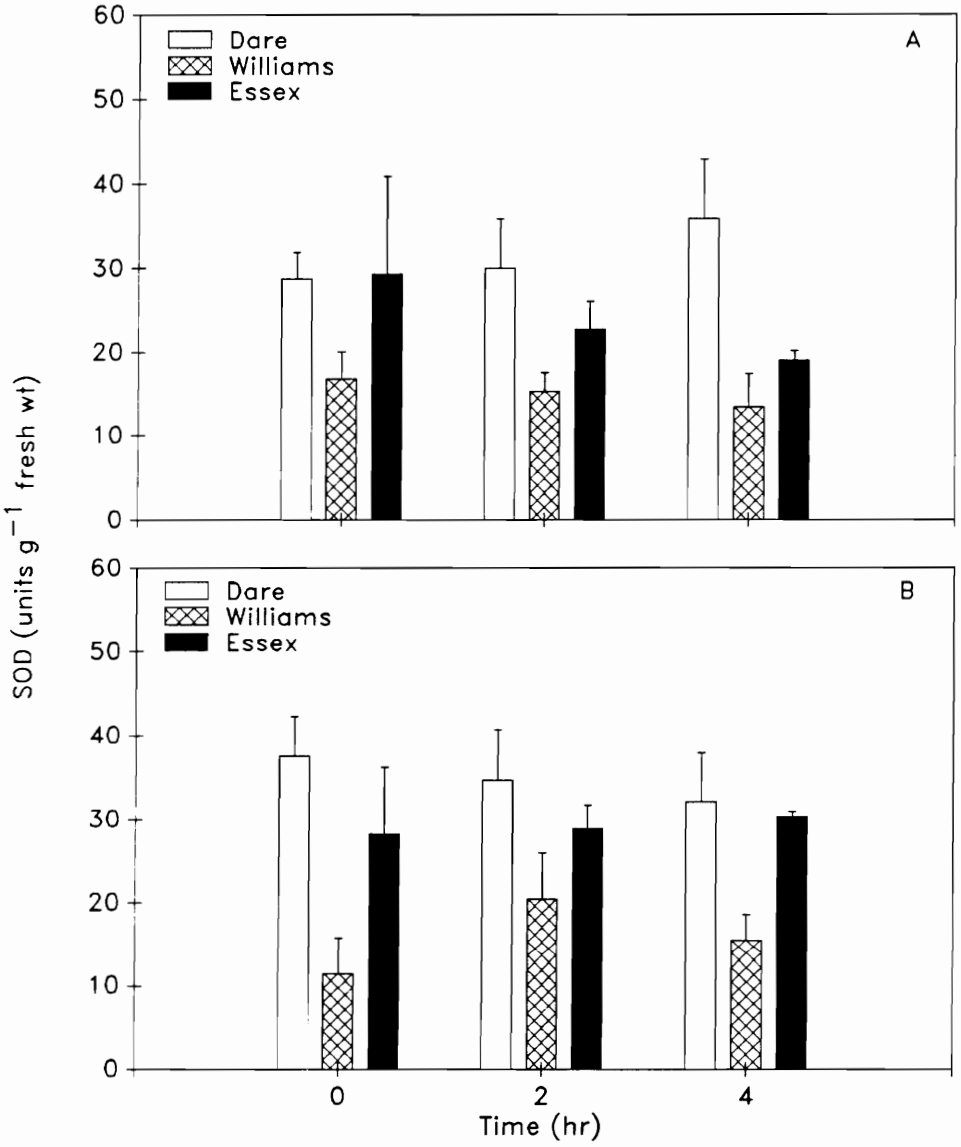


Fig. 24. Changes in superoxide dismutase total activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

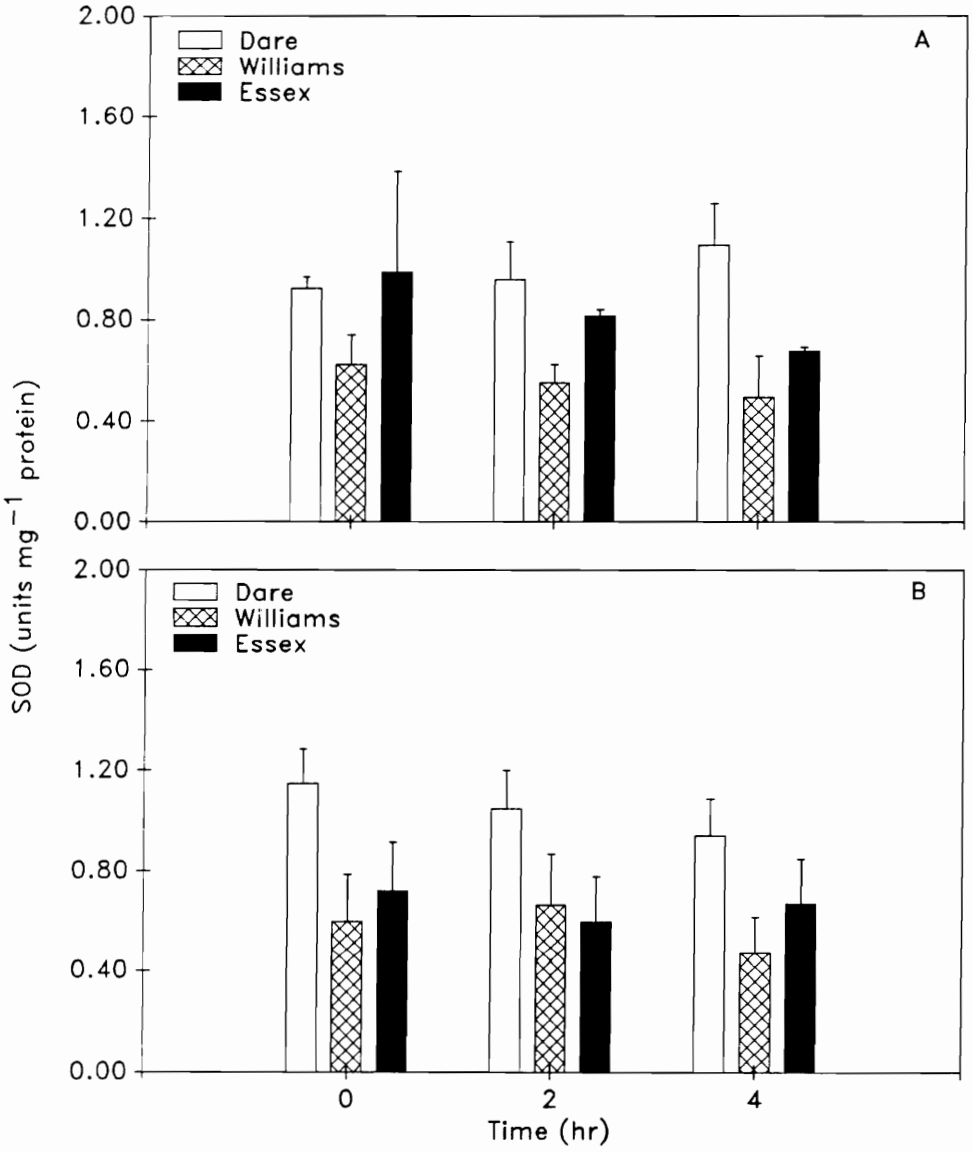


Fig. 25. Changes in superoxide dismutase specific activity of soybean cultivars exposed to A) 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

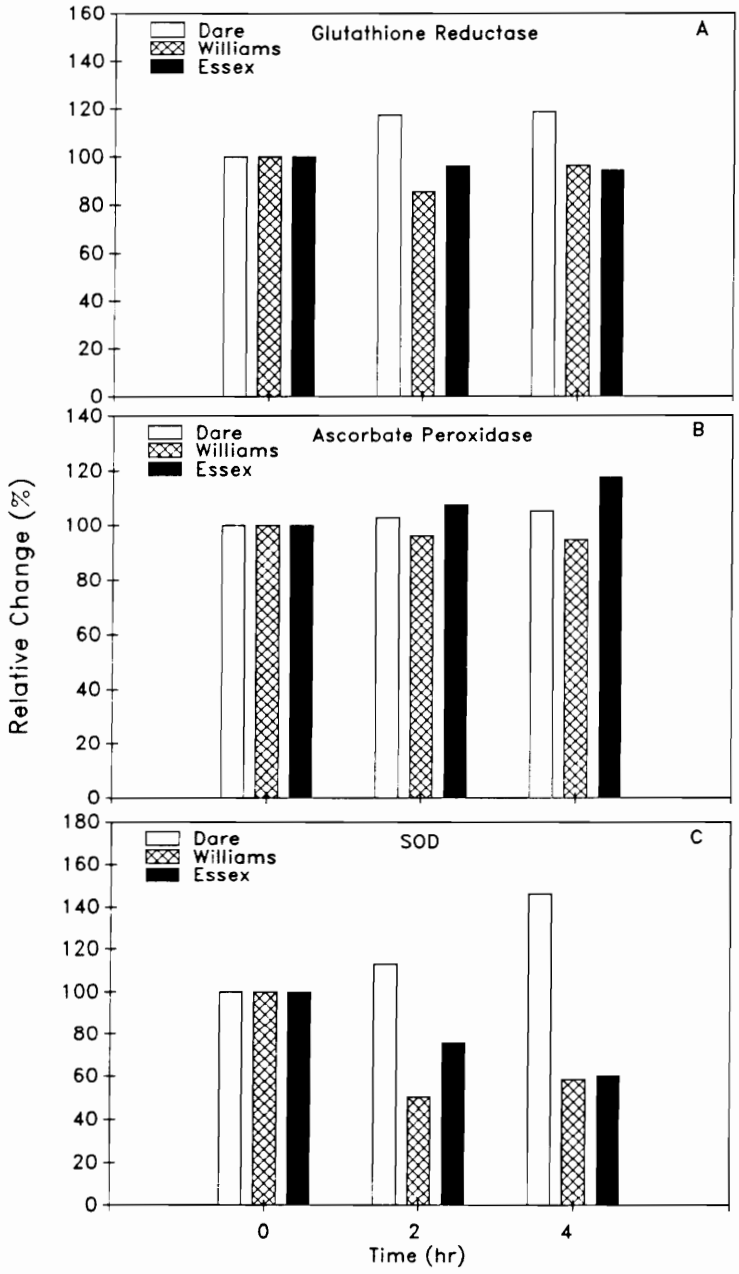


Fig. 26. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD total activities of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 5 samples.

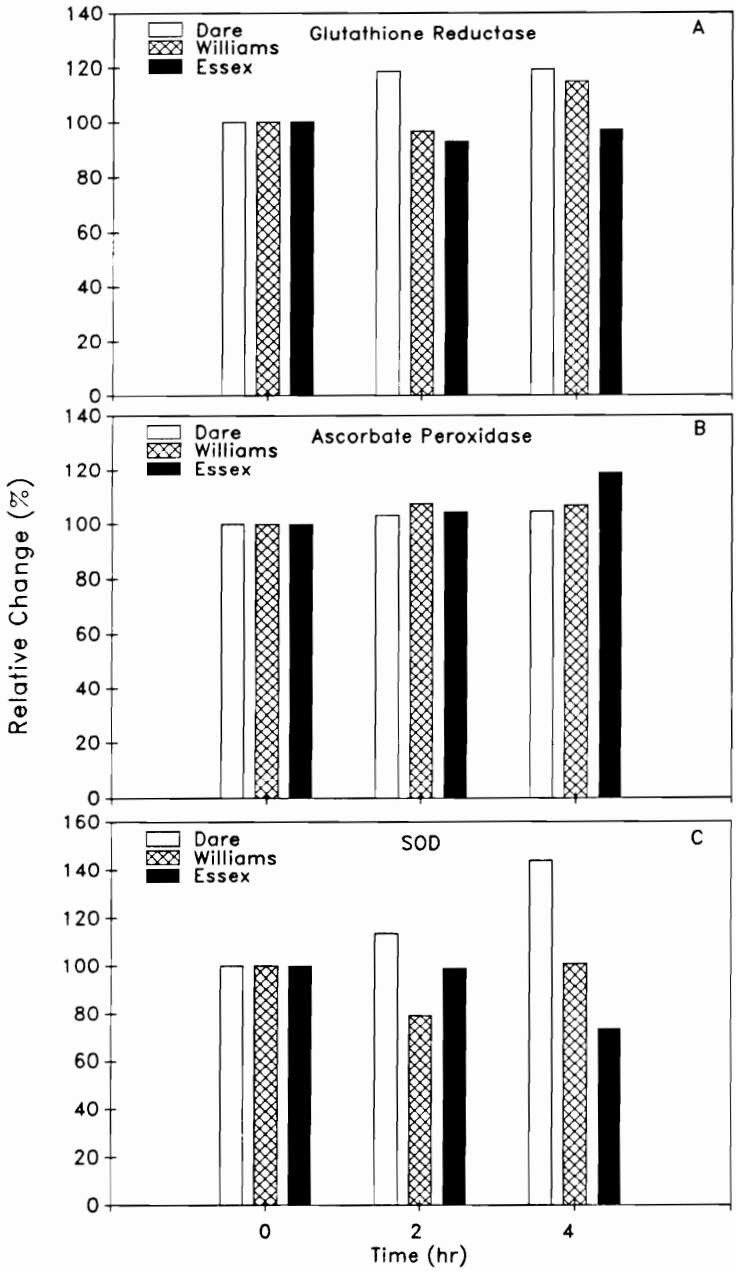
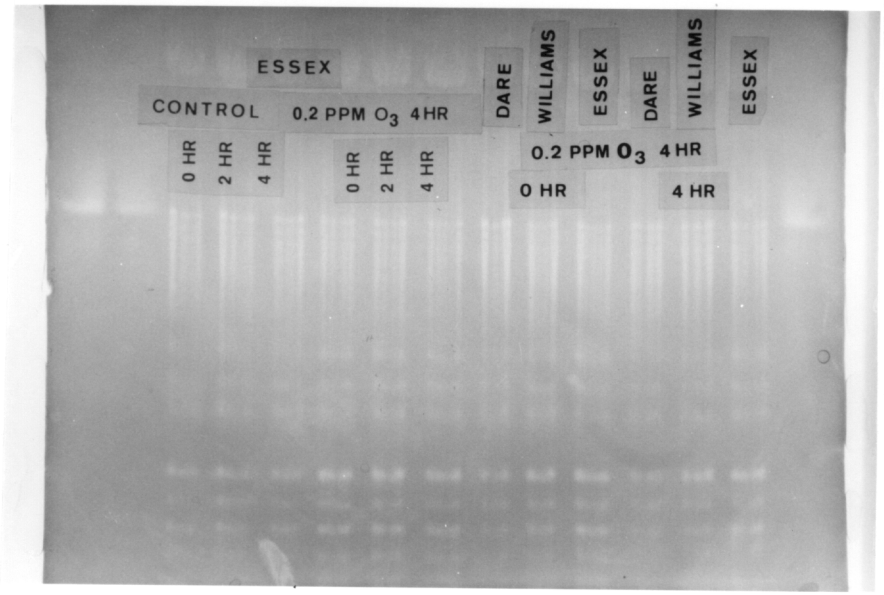


Fig. 27. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD specific activities of soybean cultivars exposed to 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 5 samples.

A.



B.

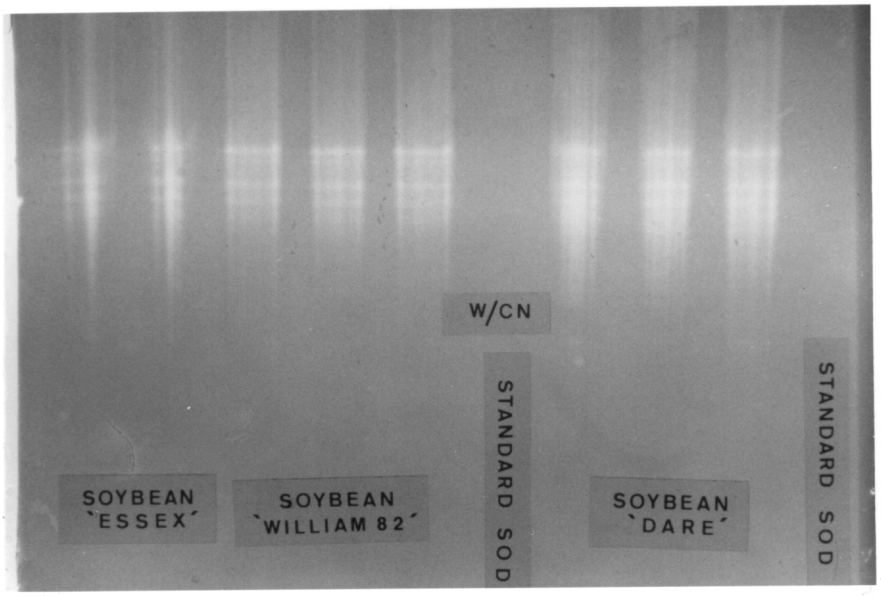


Fig. 28. Banding patterns of superoxide dismutase on native polyacrylamide gel of soybean cultivars. Gel was stained A) without or B) with cyanide in the staining solution. Standard SOD was on the left first 2 lanes and the far right lane in A. Bands in B correspond to the bands on the upper portion gel in A.

CHAPTER 3. RESPONSES OF GAS EXCHANGE AND THE ANTIOXIDANT SYSTEM OF SOYBEAN (*Glycine max* (L.) Merr.) CULTIVARS TO SULFUR DIOXIDE

Introduction

Sulfur dioxide (SO_2) is emitted to the atmosphere by the combustion of the fossil fuels, coal and oil (Mudd, 1975). Household burning of wood during the winter also contributes a small portion of SO_2 emissions to the environment. Annual emission in the U.S. of SO_2 in 1990 is estimated to be 30 million tons. In October 1990 the Clean Air Act was rewritten from the 1977 version to reduce emissions of SO_2 by 12 to 14 million tons annually by the year 2000. Half of the total SO_2 reductions would occur by Jan. 1, 1995, when the sulfur-emitting plants will be required to meet stricter standards for sulfur emission.

The effects of SO_2 on stomatal conductance and photosynthesis of plants are well documented (Unsworth *et al.*, 1972; Sij & Swanson, 1974; Black & Unsworth, 1979; Hällgren, 1984). Various SO_2 concentrations, exposure regimes and environmental conditions have been applied to many different plant species. Low SO_2 concentrations may either increase or decrease stomatal conductance (Majernik & Mansfield, 1970; Mansfield & Majernik, 1970; Unsworth *et al.*, 1972; Black & Unsworth, 1980), depending on plant species and environmental conditions during the exposure period. However, high SO_2 concentrations cause stomatal closure (Black, 1982). Photosynthesis is generally inhibited by SO_2

exposure (Black & Unsworth, 1979; Winner & Mooney, 1980a; Carlson, 1983; Darrall, 1986; Alscher *et al.*, 1987). However, SO_2 may enhance photosynthesis (Muller *et al.*, 1979; Winner & Mooney, 1980b) or result in a lack of response at lower concentrations (Darrall, 1986).

Formation of sulfite (SO_3^{2-}), bisulphite (HSO_3^-) and sulfate (SO_4^{2-}) ions occur after SO_2 absorption through stomata (Silvius *et al.*, 1975). Either SO_4^{2-} or SO_3^{2-} can be reduced to sulfide (S^{2-}) (Trebst & Schmidt, 1969). Using labelled sulfur dioxide ($^{35}SO_2$), Silvius *et al.* (1976) detected labelled sulfides in spinach leaf blades following fumigations in light indicating the occurrence of photoreduction of SO_2. Possibly, the photoreduction of absorbed SO_2 to sulfide was via the sulphite ion (Tamura & Itoh, 1974).

The sulphite ion has numerous effects on plant physiological processes, such as photosynthetic oxygen evolution (Silvius *et al.*, 1975; Soldatini *et al.*, 1978), CO_2 incorporation (Libera *et al.*, 1973; Paul & Bassham, 1978), and activities of Rubisco (Ziegler, 1972) and PEP carboxylase (Ziegler, 1973). Silvius *et al.* (1975) reported that HSO_3^- had a greater inhibitory effect on O_2 evolution than SO_3^{2-}. Since, at physiological pH, dissolved SO_2 produces HSO_3^- and SO_3^{2-} in a ratio of less than 1:5, SO_3^{2-} may be primarily responsible for SO_2 toxicity.

Tanaka and Sugahara (1980) have suggested, however, that SO_2 toxicity is partly due to the superoxide radical ($O_2^{\cdot -}$) because young poplar leaves, having five times the superoxide dismutase (SOD) activity of the old leaves, were more resistant to SO_2. In addition, spraying diethyldithiocarbamate onto spinach leaves caused a reduced SOD activity which resulted in a decrease of resistance to SO_2. Asada and Kiso (1973) and Asada *et al.* (1974) have provided evidence that

SO_3^{2-} and/or HSO_3^- would induce $O_2^{\cdot-}$ formation through a free radical chain reaction initiated by $O_2^{\cdot-}$ from illuminated chloroplasts.

Superoxide dismutase catalyzes the dismutation of 2 $O_2^{\cdot-}$ to form O_2 and H_2O_2. The H_2O_2 can accumulate in the chloroplasts and inactivate the Calvin-cycle SH enzymes as demonstrated by Tanaka *et al.* (1982a, b) using spinach leaves fumigated with SO_2. The immediate inhibition of photosynthesis at the beginning of SO_2 fumigation was due to inhibition of chloroplast SH enzymes (e.g. glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase) by H_2O_2 (Tanaka *et al.*, 1982b). Accumulation of H_2O_2 in chloroplasts of SO_2-fumigated spinach leaves could have resulted from inactivation of the chloroplastic H_2O_2-scavenging system and stimulation of H_2O_2 production (Tanaka *et al.*, 1982a). Catalase, ascorbate peroxidase and glutathione reductase were inactivated by SO_2 fumigation, but superoxide dismutase and dehydroascorbate reductase were not affected (Tanaka *et al.*, 1982a).

Nakano and Asada (1980) reported that intact spinach chloroplasts can scavenge H_2O_2 at a high rate with an ascorbate (AB)-specific peroxidase (Groden & Beck, 1979; Nakano & Asada, 1981) using ascorbate (AB) as the electron donor. When glutathione (GSH) and dehydroascorbate (DHA) were added, the photoreductions of H_2O_2 and DHA with the evolution of O_2 occurred in the ruptured spinach chloroplasts (Nakano & Asada, 1981). These observations confirmed the proposed system for the scavenging of H_2O_2 by Foyer and Halliwell (1976): photosystem I → ferridoxin → NADP → glutathione → ascorbate system.

Ascorbate can act as a scavenger of $O_2^{\cdot -}$ (Allen & Hall, 1973, 1974) or H_2O_2 (Foyer & Halliwell, 1976; Groden & Beck, 1979) using ascorbate peroxidase to catalyze this reaction, although the amount of ascorbate peroxidase activity and ascorbate content change with season (Gillham and Dodge, 1987) and with leaf age. Glutathione, a major low molecular weight thiol compound in plant cells, functions to scavenge free radicals and peroxides (Foyer, 1984) and to maintain SH enzymes in their metabolically active, reduced form. It also may function as a storage and transport form of reduced sulphur in plants [Bergmann and Rennenberg, 1978 (summary); Rennenberg and Bergmann, 1979 (summary)]. Glutathione may conjugate with many pesticide as a detoxification mechanism (Rennenberg, 1982). Reaction of $O_2^{\cdot -}$ or H_2O_2 with ascorbate produces DHA (oxidized form of ascorbic acid) which is reduced back to ascorbate by GSH using DHA reductase to catalyze the reaction. Oxidation of GSH generates the oxidized form of glutathione (GSSG) which is converted back to GSH by NADPH, obtained from the photosynthetic electron transport chain, with glutathione reductase involved in the reaction (Foyer & Halliwell, 1976; Foyer, 1984). As reported by Law *et al.* (1983) the ratios of GSH/GSSG and AB/DHA in isolated chloroplasts were high under both light and dark conditions. This cycle can provide an effective mechanism for detoxification of $O_2^{\cdot -}$ and H_2O_2 in plants.

Increase of ascorbate due to SO_2 exposure was found only in 2-year-old spruce needles (Mehlhorn *et al.*, 1986). Increased GSH levels in leaves of *Picea*, *Pinus*, *Larix* and *Betula* fumigated with SO_2 was observed by Grill *et al.* (1979). Glutathione levels varied with SO_2 concentrations used to fumigate soybean plants (Chiment *et al.*, 1986). An increase in GSH levels for all SO_2 exposures

was found with both higher and lower SO_2 concentrations inducing smaller increase in GSH. Using two cultivars of pea with differential sensitivities to SO_2, Alscher *et al.* (1987) suggested that increased GSH in the insensitive pea cultivar might contribute to the protection of the photosynthetic apparatus against SO_2 when compared with that of the sensitive pea cultivar.

The objectives of these experiments were to investigate the gas exchange characteristics and measure metabolite levels and enzyme activities involved in the antioxidant system among soybean cultivars fumigated with SO_2. Cultivars with differential foliar sensitivities to gaseous pollutants were used in these studies to explore potential physiological or biochemical mechanisms associated with pollutant tolerance.

Materials and methods

Plant Material

Three soybean cultivars: Dare, Williams and Essex were grown and selected for fumigation as previously described in Chapter 2.

Pollutant Application

Prior to SO_2 exposure, plants were acclimated overnight in the CSTR (Heck *et al.*, 1978) chambers. Environmental conditions within the CSTRs have been described in Chapter 2.

Sulfur dioxide was supplied from a gas cylinder with 1.7% SO_2 in balanced nitrogen (Industrial Gas & Supply Company, Radford, VA). Sulfur dioxide concentration was $0.70 \pm 0.05\ \mu l\ l^{-1}$ and $< 0.03\ \mu l\ l^{-1}$ for fumigated and control chambers, respectively. The total fumigation time was 4 hr and the plants were allowed to recover in charcoal filtered air ($< 0.03\ \mu l\ l^{-1}\ SO_2$) for 2 hr inside the CSTRs after SO_2 fumigation was terminated. Less than 5 min was required for SO_2 depletion to $< 0.03\ \mu l\ l^{-1}$ within the chamber. Control plants were exposed to charcoal filtered air ($< 0.03\ \mu l\ l^{-1}\ SO_2$) for 6 hr. The concentrations of SO_2 were controlled by a SO_2 mass flow controller (Sierra Instruments, Inc., Carmel Valley, CA) through a data acquisition system (Keithley Data Acquisition & Control, Cleveland, OH) and monitored by a pulsed fluorescent SO_2 analyzer (series 43, Thermo Electron Corp., Hopkinton, MA) which was calibrated by a SO_2 calibrator (model CS 10-2, Meloy Laboratories, Inc., Springfield, VA) on a monthly basis according to EPA standard procedures. After fumigation was

terminated, plants were returned immediately to the greenhouse for injury observation, 24 hr later.

Gas Exchange Measurements

A Li-Cor 6000 Portable Photosynthesis System (Li-Cor, Inc., Lincoln, NE) used for gas exchange measurements of leaf samples, the environmental conditions during measurements and the statistical analysis design have been described in Chapter 2.

Metabolite and Enzyme Analyses

Handling and collecting of leaf samples and the preparation processes for metabolite and enzyme analyses have been described in Chapter 2.

Ascorbate Analysis

Ascorbate was analyzed by the methods of Watada (1982) and Lee *et al.* (1984) as described in Chapter 2.

Glutathione Analysis

Glutathione was analyzed by the methods of Brehe and Burch (1976) and Griffith (1980) as described in Chapter 2.

Protein Analysis

Protein was analyzed according to the method of Bradford (1976) as described in Chapter 2.

Glutathione Reductase Analysis

Glutathione reductase was analyzed by the method of Schaedle and Bassham (1977) as described in Chapter 2.

Ascorbate Peroxidase Analysis

Ascorbate peroxidase was analyzed by the method of Peters *et al.* (1989) as described in Chapter 2.

Superoxide Dismutase Analysis

Superoxide dismutase was analyzed spectrophotometrically based on the method of Beauchamp and Fridovich (1971) but modified by Dhindsa *et al.* (1981). The following reagents were used: 0.05 M phosphate buffer, pH 7.8 containing 13 mM methionine, 75 μM NBT (nitrobluetetrazolium) and 0.1 mM EDTA; and 100 μM riboflavin in buffer only. A total of 3.0 ml mixture of buffer, riboflavin and desalted sample extract was illuminated for 10 min. A mixture containing H_2O as the sample extract was used as a control under either dark or light conditions. Absorbance at 560 nm of the dark control was measured before the light control. Automated calculations and adjustments were applied to the samples to obtain a valid linear regression with a r value > 0.95 from varying amounts of sample extract (10-50 μl) in the mixture. One unit of SOD activity was the amount of enzyme required to inhibit 50% of the reaction. Units of SOD activity were calculated from the linear regression.

Results

Foliar Response to SO_2

Typical SO_2 injury appeared as intercostal bleaching 24 hr after fumigation in cvs Dare and Williams (≈ 20%). Slightly more injury was seen in cv Dare than in cv Williams. Occasionally, water soaking symptoms would appear during fumigation in these cultivars. The cv Essex showed no SO_2 injury at all. Control plants had no injury on their leaves.

Gas Exchange

At pre-exposure conditions (plants in both treated and control chambers), physiological characteristics of the three soybean cultivars are shown in Table 1. No differences among cultivars in net photosynthesis (Pn), stomatal conductance (Cs), internal CO_2 concentration (Ci) or transpiration (Ts) were found.

There were slight changes in physiological variables of control plants after 4 hr exposure to filtered air (Table 2). The exceptions were a significant increase of Pn in cv Dare and significant reductions of Ts ranging from 17 to 31% among cultivars. Generally, these physiological variables were relatively stable throughout the 6 hr period (Figs. 1B-4B) and no differences were seen among cultivars.

Sulfur dioxide inhibited gas exchange in soybean cultivars greatly after the 4 hr exposure period (Table 3). Reduction of Pn was 31-46% with cv Williams being suppressed the most. Stomatal conductance of all cultivars was decreased by SO_2 about 46-64% and again, cv Williams was affected the most. Internal CO_2

showed very little response to SO_2 and yet, there were significant reductions in cvs Williams and Essex. Similar to the Pn response, Ts was reduced 37-54% by SO_2 among cultivars.

The effect of SO_2 on Pn was observed immediately after the initiation of fumigation (Fig. 1A). After SO_2 reached the designated concentration of 0.7 μl l^{-1} ($\approx$ 35 min into the fumigation schedule), Pn of cvs Dare and Essex attained an equilibrium state which continued throughout the fumigation and recovery period. In cv Williams, Pn declined gradually to the end of recovery period. A different response of Pn to SO_2 among cultivars was seen at 1 hr after fumigation: cv Essex had the highest Pn during the exposure period while cv Williams had the lowest (Fig. 1A). No recovery of Pn was noted during the 2 hr period after terminating of SO_2 except in cv Dare.

Stomatal conductance responded more slowly than Pn to SO_2 at the beginning of fumigation except in cv Williams (Fig. 2A). However, a substantial and continuous decrease of Cs during the period of fumigation with 0.7 μl l^{-1} SO_2 occurred. The cv Williams had a greater inhibition of Cs than cvs Dare and Essex at 2.5 hr after fumigation (Fig. 2A). No equilibrium state of Cs was seen until the recovery period and no recovery of Cs was observed during this same time until the end of 2 hr period in cvs Dare and Williams.

When SO_2 reached 0.7 μl l^{-1}, Ci of all cultivars only had decreased approximately 10 ppm (Fig. 3A). Then, an abrupt increase in Ci in cvs Dare and Williams was evident, followed by a gradual decrease to near pre-exposure levels. The cv Essex maintained a lower and less variable Ci than cvs Dare and Williams during the exposure period (Fig. 3A). During the recovery period, Ci varied

depending upon cultivar. A continuous decline of Ci for 1.5 hr and then an increase occurred in cv Dare, a slow increase of Ci occurred in cv Williams and no change was evident in cv Essex. In none of the cultivars, did Ci return to pre-exposure concentrations.

An increase in Ts of all cultivars in response to SO_2 in the first 30 min of fumigation was evident (Fig. 4A). A substantial reduction then occurred at 1 hr after SO_2 exposure followed by a gradual and slow decline of Ts in all cultivars during the remainder of the fumigation period. The cv Williams showed a lower Ts than cvs Dare and Essex. No recovery, but a near equilibrium state of Ts of all cultivars was seen for 2 hr after ending SO_2 treatment (Fig. 4A).

Sulfur dioxide mean flux to the leaf interior was estimated similarly to the method of Laisk *et al.* (1989) for O_3 flux with a SO_2 to H_2O diffusivity ratio of 1.89. When SO_2 reached 0.7 $\mu l\ l^{-1}$, peak SO_2 flux also occurred at about 180 nmol $m^{-2}\ s^{-1}$ in cvs Dare and Essex and 150 in cv Williams (Fig. 5A). As fumigation proceeded, SO_2 flux gradually declined in all cultivars and cv Williams had a lower SO_2 flux than cvs Dare and Essex from 2.5 hr to the end of exposure. The difference among cultivars was approximately 30 nmol $m^{-2}\ s^{-1}$ (75 in cv Williams vs. 105 in cvs Dare and Essex) at the end of 4 hr fumigation period.

Estimated integrated SO_2 dose, calculated according to Olszyk and Tingey (1986), accumulated steadily in an almost linear rate in all cultivars (Fig. 5B). By the end of fumigation, cv Williams ($\approx$ 1950 μmol m^{-2}) received 13% less in total integrated SO_2 dose than cvs Dare and Essex ($\approx$ 2250 μmol m^{-2}).

Response of the mean residual conductance to CO_2 (Cr) to SO_2 fumigation is shown in Figure 6A. The Cr responded similarly to Pn under SO_2 stress: a substantial decrease in Cr in the first 35 min when SO_2 reached 0.7 $\mu l\ l^{-1}$, followed by a near equilibrium state to the end of fumigation. The cv Essex maintained a higher Cr than cvs Dare and Williams (Fig. 6A). There was no recovery of Cr for 2 hr after SO_2 exposure ended except in cv Dare. A relatively stable Cr was seen in all cultivars under control conditions throughout the 6 hr treatment period (Fig. 6B).

Fractions of stomatal and non-stomatal contributions (σ_s and σ_{ns}, respectively) to the change in limitation of Pn showed that σ_s was low (< 10%) and σ_{ns} was high (> 90%) in the beginning of SO_2 fumigation (Fig. 7A-B) reflecting an inhibition of Pn. As fumigation proceeded, σ_s increased and σ_{ns} decreased gradually to about 30% and 70%, respectively, in all cultivars at the end of SO_2 exposure. The σ_s increased in cvs Dare and Essex and did not change in cv Williams during the recovery period.

Water use efficiency (WUE) was greatly affected by SO_2 immediately after fumigation was initiated (Fig. 8A). Approximately a 40% reduction in WUE was observed when SO_2 reached the 0.7 $\mu l\ l^{-1}$ designated concentration. However, as fumigation proceeded, WUE gradually increased and returned to a level slightly higher than at pre-exposure when SO_2 treatment was terminated, and continued to increase through the 2 hr recovery period in cvs Dare and Essex but not in cv Williams (Fig. 8A). For a 2 hr period (between 1 and 3 hr of SO_2 exposure), cv Essex had a higher WUE than cvs Dare and Williams. Control plants showed an increase in WUE for 1.5 hr followed by a decrease and then a

rise to a near equilibrium state which was 20-50% higher than the pre-fumigation level (Fig. 8B).

Metabolites

The endogenous antioxidant metabolites levels prior to exposure (mean values from the combination of control and treated plants) are shown in Table 1. Only a significant difference between cvs Essex and Dare in total (1.06 vs. 0.94 μmol g^{-1} fw) and reduced (1.02 vs. 0.89 μmol g^{-1} fw) glutathione (GSH) was found.

For control plants, there was no change in either total glutathione or GSH after 4 hr exposure to filtered air among cultivars (Table 4; Figs. 9B, 10B). However, reductions of 15%, 39% and 74% in oxidized glutathione (GSSG) of cvs Dare, Williams and Essex, respectively, after 4 hr were seen (Table 4; Fig. 11B), which resulted in an increased GSH/GSSG ratio (Fig. 12B).

After SO_2 fumigation, total glutathione and GSH increased greatly ($\approx$ 35%) in all cultivars (Table 5; Figs. 9A, 10A). The increases were proportional to the fumigation time, i.e. a similar rate of increase at the 2 hr and 4 hr sampling period (Figs. 9A, 10A). Concurrently, GSSG increased substantially ($\approx$ 130% in cvs Dare and Williams, $\approx$ 170% in cv Essex) (Table 5) and the increase was much more dramatic at the 2nd 2 hr period of SO_2 fumigation (Fig. 11A). As a result, the GSH/GSSG ratio declined sharply (Fig. 12A). Relative to control plants, total and reduced glutathione of all cultivars increased approximately 15% for every 2 hr SO_2 fumigation interval and rose 30% at the end of the 4 hr exposure period (Figs. 13A-B). Oxidized glutathione showed increases of approximately 60% in all

cultivars at 2 hr and, 170% in cv Dare and 260% in cvs Williams and Essex at the end of 4 hr of fumigation (Fig. 13C).

Total and reduced ascorbate of control plants did not change during the 4 hr treatment period (Table 4; Figs. 14B, 15B). The cv Essex had a slightly higher ascorbate than cvs Dare and Williams. A considerable variation in dehydroascorbate (dHAA) was observed in all cultivars. Due to possible analysis errors, such as measuring samples in non-paired sequences and no periodic calibrations of the HPLC with a series of standard ascorbate solutions, the dHAA data and the AA/dHAA ratio were excluded from discussion.

Similar to control plants, total and reduced ascorbate of SO_2-stressed plants showed no change during the fumigation period (Table 5; Figs. 14A, 15A). No change in total ascorbate (Fig. 16A) and a 15% and a 20% rise of reduced ascorbate in cvs Dare and Williams, respectively, relative to control plants was found (Fig. 16B).

Protein

The amount of extractable protein either in SO_2-treated or in control plants did not change significantly during the 4 hr exposure period (Fig. 17A-B). In cv Essex control plants, protein increased at each sampling interval. The cv Williams had a lower protein concentration than cvs Dare and Essex.

Enzymes

Prior to fumigation, the endogenous enzyme activities of all cultivars are shown in Table 1. Only a significant difference in superoxide dismutase (SOD) total activity between cv Essex and cvs Dare and Williams was found.

After 4 hr, cv Dare control plants had an increase of 21% in glutathione reductase (GRase) total activity; no changes were observed in GRase activity (specific or total) of any cultivar (Table 4; Figs. 18B, 19B). An increase in GRase activity of SO_2-stressed plants was seen only in cv Williams (16% and 13% in total and specific activity, respectively) (Table 5; Figs. 18A, 19A). Relative to the control plants, there was a decrease of approximately 16% in GRase total activity in cv Dare and no change in other cultivars (Figs. 24A, 25A).

A slight reduction (11%) of ascorbate peroxidase (APase) specific activity in cv Dare of control plant was noted; no other change was detected in APase total activity of all cultivars (Table 4; Figs. 20B, 21B). However, a rise of APase activity was seen at 2 hr in cvs Dare and Essex ($\approx$ 30% in total and $\approx$ 20% in specific activity). As for SO_2-treated plants, only cv Williams showed a substantial but insignificant reduction in APase activity (Table 5; Figs. 20A, 21A), which was inhibited approximately 20% at 2 hr and 27% at the end of fumigation. Relative to control plants, an approximate 25% reduction in APase total activity of all cultivars was found at 2 hr; then, at 4 hr, cv Williams sustained the same inhibition while cv Dare fully recovered and cv Essex recovered to a 11% inhibition (Fig. 24B). Similar changes of APase specific activity occurred in cvs Williams and Essex and, a slight decrease (11%) at 2 hr and an increase (21%) at 4 hr in cv Dare were observed (Fig. 25B).

Reductions of SOD activity in all cultivars of control plants were observed at 4 hr with cv Dare showing a substantial but statistically insignificant decrease (Table 4; Figs. 22B, 23B). Under SO_2 stress, SOD total activity in cv Essex significantly decreased by 35% at 4 hr compared to pre-fumigation; SOD specific activity was inhibited 15% in cvs Dare and Williams and 46% in cv Essex, although these changes were not statistically significant due to large sampling variation (Table 5; Figs. 22A, 23A). Relative to the controls, SOD total activity increased in cv Dare (50%), but decreased in cv Essex (27%) (Fig. 24C); SOD specific activity increased in cvs Dare (43%) and Williams (17%) but decreased in cv Essex (27%) (Fig. 25C).

Discussion

Tissue damage in cvs Dare and Williams may have resulted from the toxic byproducts of SO_2 (HSO_3^-, SO_3^{2-}) which were not removed fast enough by the mesophyll and caused chlorophyll breakdown. As water soaking symptoms appeared occasionally during SO_2 fumigation, it was likely that cellular membranes and plastid membranes were disrupted. Swelling of chloroplasts and thylakoids proceeded before the collapse of plastid membranes in plant tissue exposed to SO_2 or SO_3^{2-} (Wellburn *et al.*, 1972; Ziegler, 1975). No injury occurred in cv Essex which could indicate a better or more efficient disposal system for the byproducts of SO_2 in the mesophyll compared to cvs Dare and Williams.

The rapid inhibition of Pn by SO_2 and the subsequent attainment of a near equilibrium state (Fig. 1A) suggested that chloroplastic functions were suppressed to a point where electron supply/demand or energy production/utilization was at a balance with the effect of toxic byproducts from SO_2 (Kropff, 1987). A similar stable rate of Pn of several plant species obtained during SO_2 exposure have been reported (Darrall, 1986; Kropff, 1987; Sisson *et al.*, 1981). Tanaka *et al.* (1982a) suggested that suppression of Pn at the beginning of SO_2 fumigation was caused by the inhibition of chloroplast SH enzymes with H_2O_2.

As chloroplasts are capable of oxidizing and reducing SO_2 in light-dependent reactions (Asada and Kiso, 1973; Schwenn *et al.*, 1976), it is the reduction that is considered to be a detoxifying process as demonstrated in SO_2-tolerant spinach plants with a high capacity of sulfite reduction (Ghisi *et al.*,

1990). However, Carlson (1983) reported that reduction in Pn was highly correlated with the total amount of SO_2 absorbed into the leaves. Thus, the higher Pn in cv Essex than in cvs Dare and Williams (Fig. 1A) was possibly due to a more efficient photosynthetic capacity, a genetic capacity for SO_2 resistance, a lesser amount of total SO_2 absorbed and/or a better detoxifying system for SO_2 byproducts.

No recovery of Pn within 2 hr was found after SO_2 fumigation was terminated except in cv Dare (Fig. 1A). However, Darrall (1986) reported that recovery of Pn was obtained within 2 hr after termination of SO_2 exposure if inhibition of Pn was less than 20%. Partial Pn recovery within 2 hr and full recovery within 12 hr dark period has been reported (Sisson *et al.*, 1981). It is possible that a longer period was needed for Pn of cv Essex to recover partially or fully. As for cvs Dare and Williams, which showed SO_2 injury, recovery of Pn would not be expected, but nevertheless, occurred in cv Dare. Apparently, Pn of different plant species responds differently to SO_2 under different environmental conditions.

Steady inhibition of stomatal conductance (Cs) by SO_2 fumigation (Fig. 2A) indicated that stomatal closure did not play a major role in initial Pn reduction which appeared to be mainly caused by mesophyll changes due to SO_2 (Fig. 6A). Exposure of soybean cv Wayne under variable ambient CO_2 concentrations to 0.2-1 $\mu l\ l^{-1}$ SO_2 led to the conclusion that SO_2 did not cause stomatal reduction in Pn at 300 and 450 ppm CO_2 (Carlson, 1983). Kropff (1987), using *Vicia faba* exposed to 0.3 $\mu l\ l^{-1}$ SO_2, demonstrated that stomatal behavior was not altered by SO_2 directly, but by a feedback reaction from increased Ci

resulting from the suppression of Pn caused by a SO_2-induced increase in mesophyll resistance to CO_2.

However, high SO_2 concentrations could damage stomatal guard cells (Unsworth and Black, 1981) leading to stomatal closure. Comparison between photosynthetic and stomatal responses to SO_2 (Figs. 1A, 2A) gave a bold suggestion that effects on Cs were preceded by Pn which was affected by SO_2 directly. Similar sequence changes in Pn and then Cs by SO_2 were also reported (Alscher *et al.*, 1987; Sisson *et al.*, 1981). However, a model was suggested by Winner *et al.* (1988) that possibly a simultaneous mutual influence between Pn then Cs and Cs then Pn by air pollutants could exist.

Stomatal closure would result in a reduced SO_2 flux (Fig. 5A) into the leaves which would not necessarily correspond to SO_2 resistance or sensitivity at the cellular level. A SO_2-resistant cultivar could actually absorb more SO_2 than a sensitive cultivar (Bell and Mudd, 1976). The more suppressed Cs in cv Williams led to a lower (13%) amount of total SO_2 absorbed (Fig. 5B), but not a higher Pn than in cvs Dare and Essex. Of course it is arguable that the different amount of absorbed SO_2 among cultivars contributed insignificantly to any effects on leaf antioxidant metabolism, nevertheless CO_2 reduction was substantially impaired. Although SO_2 effects on gas exchange have been reported to be reversible, no substantial recovery of Cs in all cultivars was observed (Fig. 2A) which indicated either the changes were irreversible due to high SO_2 or required a longer recovery time.

Changes in Ci (Fig. 3A) showed a slight lag period compared to responses of Pn to SO_2. As Pn reached a steady state of inhibition rate (Fig. 1A) and Cs was

not greatly inhibited [except in cv Williams (Fig. 2A)], Ci did not reach peak concentrations until 30 min later. Throughout the period, Pn maintained a stable low rate, indicating a stable CO_2 uptake, and the feedback effect of Ci on Cs proceeded. There were no changes in Ci at the end of 4 hr fumigation (Table 3).

The small increase of Ts at the beginning of SO_2 fumigation (Fig. 4A), which did not reflect the Cs response, could be due to an increased vapor pressure deficit since input of SO_2 into CSTR chamber tended to reduce the relative humidity in the first 35 min of treatment. An increase in Ts followed by a decline with SO_2 fumigation (high concentrations) has been reported (cited from Ziegler, 1975). As expected, Ts declined (Fig. 4A) as stomatal closure continued (Fig. 2A).

The estimated SO_2 flux oversimplifies the pathway of SO_2 entering into the leaves due to differences in water solubility (from CO_2), boundary layer resistance, magnitude of the transpirational stream, and leaf surface absorption. However, our interests were to compare the flux among cultivars and differences in the above variables were presumed to be minimal. The lower SO_2 flux in cv Williams (Fig. 5A), due to a lower Cs than in cvs Dare and Essex, did not seem to have any positive impact on its Pn which was inhibited most among cultivars (Fig. 1A). Similarly, the integrated SO_2 dose in cv Williams was also the lowest among cultivars (Fig. 5B). No correlation between the estimated total absorbed SO_2 dose and Pn reduction was found in our studies as was suggested by Carlson (1983). It appears to be more important to have an efficient metabolic process to remove SO_2 and/or its byproducts effectively than to have a lesser SO_2 flux or total amount of SO_2 absorbed to limit Pn inhibition. On the other hand, the

difference in integrated SO_2 dose among cultivars was not large enough to provide a positive correlation with a reduction in Pn.

Residual conductance to CO_2 (Cr) declined with SO_2 fumigation (Fig. 6A) (Carlson, 1983; Kropff, 1987). Using the time course study of Pn versus Ci at ambient CO_2, Kropff (1987) found a strong reduction in Pn occurred in the first 10 min of SO_2 fumigation indicating that the reduction was entirely due to an increasing mesophyll resistance (or declining Cr). Because Cr in cv Essex was inhibited less (Fig. 6A) by SO_2, Pn was higher than in cvs Dare and Williams (Fig. 1A). Besides the change of Cr, the CO_2 compensation point increased after SO_2 fumigation (Kropff, 1987). The competitive mechanism of SO_2 inhibition on Pn for CO_2 uptake (Ziegler, 1975) was modified to explain that the increased CO_2 compensation point indicated SO_2 had a stronger effect on the affinity of rubisco for CO_2 than for O_2 (Kropff, 1987).

No differences in σ_s and σ_{ns} among cultivars were found when the designated SO_2 concentration was reached (Fig. 7). The high σ_{ns} fully accounted for the immediate suppression of Pn after initiation of SO_2 fumigation. Although Pn attained an equilibrium rate after 1 hr of SO_2 exposure, σ_s increased and σ_{ns} decreased gradually. At the end of SO_2 fumigation, σ_{ns} contributed approximately 70-80% to Pn reduction. The dramatic decrease of water use efficiency (WUE) in the first 35 min (Fig. 8A), due to the large decline of Pn (Fig. 1A) and the slight increase of Ts (Fig. 4A), was followed by a gradual rise throughout the fumigation and recovery periods. The increase in WUE was due to the stable rate of Pn and a continued decline of Ts such that at the end of SO_2 fumigation, WUE was slightly higher than pre-fumigation levels. However, Kropff

(1987) found no change in WUE as both Pn and Ts were simultaneously reduced by SO_2.

Glutathione serves as one part in H_2O_2-removing system involving ascorbate, GRase and APase (Foyer and Halliwell, 1976). The non-enzymatic reaction between GSH and $O_2^{\cdot -}$ which generates singlet oxygen (Wefers and Sies, 1983) was considered unlikely to occur when SOD was present (Smith *et al.*, 1990). The increase of glutathione (total and reduced; Figs. 9A, 10A) by SO_2 exposure has been reported previously (Alscher *et al.*, 1987; Chiment *et al.*, 1986; Grill *et al.*, 1982), although De Kok *et al.* (1985) did not find a rapid accumulation of glutathione in spinach shoots fumigated with 0.25 μl l^{-1} SO_2 for 24 hr and neither did Tanaka *et al.* (1982b) observed any change of glutathione in spinach leaves exposed to 2 μl l^{-1} SO_2. Nonetheless, Mehlhorn *et al.* (1986) suggested that glutathione is a sensitive marker for plant response to air pollutant. Chiment *et al.* (1986) showed that soybean cv Essex, fumigated with 0.05 ~ 0.70 μl l^{-1} SO_2, had a smaller increase in GSH at both higher and lower SO_2 levels and a maximum increase in GSH at 0.30 μl l^{-1} SO_2. According to Grill *et al.* (1982), the SO_2-fumigated plant has a reduced capacity for the biosynthesis of GSH and a close relationship between sulfate content and GSH level exists.

The increase of glutathione in all cultivars could be due to either the altered sulfur metabolism as absorbed SO_2 dose increased over fumigation period or the reduced turnover and/or the increased biosynthesis of glutathione as a defense mechanism against the accumulation of H_2O_2 resulting from SO_2 exposure (Tanaka *et al.*, 1982b). The increase in GSH during SO_2 fumigation that occurred in SO_2-insensitive pea cv Progress, but not in SO_2-sensitive cv Nugget,

was regarded important for protecting the photosynthetic apparatus against SO_2 (Alscher *et al.*, 1987). On the other hand, Grill *et al.* (1979) suggested that increase in GSH was partly responsible for the phytotoxicity of SO_2 and, Schindlbeck (1977, cited from Grill *et al.*, 1979) reported that SO_2 exposure increased GSH in SO_2-sensitive *Picea abies*, but did not affect GSH in SO_2-resistant trees. In our studies, since all cultivars showed an increase in GSH and yet cv Essex exhibited a higher Pn than cvs Dare and Williams during SO_2 fumigation, no conclusive remarks could be made in terms of SO_2 sensitivity and the role of GSH in protecting physiological function.

Oxidized glutathione (GSSG) can inhibit protein synthesis and was suggested to be involved in dark deactivation of some Calvin cycle enzymes (cited from Rennenberg, 1982). Law *et al.* (1983) concluded that GSSG is not involved *in vivo* in dark deactivation of fructose bisphosphatase. The great increase in GSSG of all cultivars during SO_2 fumigation, especially the burst in the 2-4 hr period (Fig. 11A) could result from an accumulation of H_2O_2 which has been proposed to directly or indirectly oxidize GSH to GSSG and thus, to prevent feedback inhibition and allow additional GSH synthesis (Smith, 1985; Smith *et al.*, 1985). Barley, tobacco and soybean treated with a catalase inhibitor (aminotriazole) under illumination showed an increase in leaf glutathione that was mainly accounted for as GSSG; and corn, treated with paraquat, also showed an increase in GSSG, but not in total glutathione (Smith, 1985). The GSH/GSSG ratio in the control plants was approximately 20:1-35:1 (Fig. 12B) and the decreased GSH/GSSG ratio (15:1) in the soybean cultivars exposed to SO_2 (Fig. 12A) might indicate a disrupted cellular process.

Ascorbate of all cultivars showed no change under SO_2 stress (Figs. 14A, 15A; Tanaka *et al.*, 1982b) but increased in cvs Dare and Williams, relative to the controls (Fig. 16B). It has been considered as a less responsive antioxidant (Mehlhorn *et al.*, 1986), however, some reports have shown changes in ascorbate levels in response to air pollutants (Lee *et al.*, 1984; Tanaka *et al.*, 1985).

Glutathione reductase activity has been reported to be inhibited by SO_2 fumigation (Tanaka *et al.*, 1982b) and stimulated by O_3 fumigation (Tanaka *et al.*, 1988). In our study, no effects of SO_2 on GRase were found except for a slight increase in cv Williams (Table 5; Figs. 18A, 19A) and a slight decline in cv Dare relative to control plants (Fig. 24A). As multiple forms of GRase occur in plants (Bielawski and Joy, 1986), it has been suggested that changes in the total pool of GRase may be less significant than changes in individual isozymes (Smith *et al.*, 1989; Smith *et al.*, 1990). Since GRase is the rate limiting enzyme in the H_2O_2 scavenging pathway (Joblonski and Anderson, 1981), no change in GRase activity imposed by SO_2 in our study would indicate that there was no rate limitation by GRase in scavenging H_2O_2.

Ascorbate peroxidase activity was reported to be inactivated in spinach (Tanaka *et al.*, 1982b) and extracellular APase activity was unaffected in pinto bean (Peters *et al.*, 1989) by SO_2. Two isozymes of APase were found in tea leaves (Chen and Asada, 1989) and again, it may be more significant to investigate response of the individual isozymes, rather than the total pool of APase, to SO_2. In our experiments, only cv Williams showed an APase activity inhibited by SO_2 fumigation (Table 5; Figs. 20A, 21A), although, relative to

control plants, APase activity of all cultivars was inhibited at 2 hr and cv Dare had a 21% increase in APase activity at 4 hr (Figs. 24B, 25B).

The inhibition of APase activity, which lessens the capacity to scavenge H_2O_2 formed during SO_2 fumigation, might be caused by accumulated H_2O_2 concentration since removal of SO_2 resulted in H_2O_2 disappearance and a rapid recovery of APase activity (Tanaka *et al.*, 1982b). The SO_2 flux and total integrated dose were the lowest in cv Williams (Fig. 5A-B), but not significantly different from other cultivars, and yet the APase activity in cv Williams was inhibited. It was possible that APase in cv Williams was either more sensitive to SO_2 exposure or less efficient in removing H_2O_2, and thus was inhibited by H_2O_2 to a greater extent than those in cvs Dare and Essex.

The endogenous SOD total activity was significantly higher in cv Essex than cvs Dare and Williams and the SOD specific activity was also higher (but not significantly) in cv Essex (Table 1). After SO_2 fumigation, SOD activity (both total and specific) was reduced in cv Essex and, in cvs Dare and Williams only SOD specific activity was reduced (Table 5; Figs. 22A, 23A). Tanaka and Sugahara (1980) suggested that $O_2^{\cdot -}$ participated in SO_2 toxicity and SO_2 fumigation enhanced the production of $O_2^{\cdot -}$ in leaves. By using a SOD inhibitor the authors found that $O_2^{\cdot -}$ concentration increased and the inactivation of SOD lowered the resistance of spinach leaves to SO_2 exposure. Apparently SOD plays an important role in the defense mechanism against SO_2 toxicity (Tanaka and Sugahara, 1980; Tanaka *et al.*, 1988).

Our data indicated that the high endogenous SOD activity in cv Essex could function to actively dismutate $O_2^{\cdot -}$ formed during SO_2 fumigation to

H_2O_2. The H_2O_2 if was not removed rapid enough, could inhibit SOD activity. This might be the reason why SOD activity declined in cv Essex exposed to SO_2. Scott *et al.* (1987) found that a plasmid-transformed SOD-rich *E. coli* strain produced more H_2O_2 and was killed more readily after paraquat treatment than the parental or the plasmid-transformed control strains. The conclusion was made that an effective defense system against toxic oxygen species may require a balance among antioxidant enzymes (Scott *et al.*, 1987). As mentioned above, since estimated SO_2 flux and total integrated dose were lower in cv Williams than in cvs Dare and Essex, conceivably less $O_2^{\cdot -}$ formation would occur in cv Williams. However, SO_2 tolerance might inversely correlate with the total amount of SO_2 absorbed. Paraquat-tolerant tobacco plants showed a tolerance to SO_2 and exhibited a higher transpiration rate than the controls both in the presence and absence of SO_2, suggesting more SO_2 absorption by paraquat-tolerant tobacco plants (Tanaka *et al.*, 1988). Thus, cv Williams might be more susceptible to SO_2.

The Cu-Zn and Mn-SOD present in chloroplasts and mitochondria, respectively, might respond differently to any oxidant stress. Analyzing the pool of SOD activity would overlook any changes of SOD in these organelles under pollutant stress.

Under SO_2 stress, the responses of these three soybean cultivars in our studies were different. The greater inhibition of Pn, Cs and APase activity, slight stimulation of GRase and the lesser SO_2 flux and integrated dose during SO_2 fumigation in cv Williams compared to cvs Dare and Essex indicated that cv Williams might be most sensitive to SO_2. On the other hand, the lesser

suppression of Pn and Cr and the greater inhibition of SOD in cv Essex may indicate that this cultivar is less sensitive to SO_2 than cvs Dare and Williams. The sensitivity to SO_2 of cv Dare might be between cvs Williams and Essex.

Despite different responses to SO_2 seen among cultivars, however, ascorbate and GRase were more or less insensitive to SO_2 stress. The considerable increase of GSSG seen at 2 hr after SO_2 fumigation suggested a dramatic change in metabolism, although GSH increase was steady during this period. It is not known how fast H_2O_2 accumulated under our fumigation regime, but the immediate inhibition of Pn at the beginning of SO_2 exposure suggested a rapid formation of H_2O_2 if this was responsible for suppression of Pn.

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Table 1. Physiological and biochemical characteristics of three soybean (*Glycine max* (L.) Merr.) cultivars grown under glasshouse conditions and acclimated in CSTR chambers for 16 hr. (See text for details).

	Cultivar		
Variable	Dare	Williams	Essex
Pn ($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	23.4 ± 2.9^{aA} $(n=22)^{b}$	24.0 ± 2.6^{A} (n=24)	24.7 ± 2.6^{A} (n=24)
Cs ($cm\ s^{-1}$)	1.28 ± 0.27^{A} (n=22)	1.28 ± 0.31^{A} (n=24)	1.28 ± 0.35^{A} (n=24)
Ci (ppm)	288.4 ± 19.5^{A} (n=22)	286.3 ± 15.0^{A} (n=24)	284.4 ± 15.5^{A} (n=24)
Ts ($mmol\ m^{-2}\ s^{-1}$)	4.2 ± 0.6^{A} (n=20)	4.0 ± 0.6^{A} (n=22)	4.0 ± 0.8^{A} (n=22)
$Glutathione_{tot}$ ($\mu mol\ g^{-1}$ fw)	0.94 ± 0.16^{B} (n=12)	1.01 ± 0.13^{AB} (n=12)	1.06 ± 0.12^{A} (n=12)
$Glutathione_{ox}$ ($\mu mol\ g^{-1}$ fw)	0.04 ± 0.01^{A} (n=12)	0.04 ± 0.01^{A} (n=12)	0.04 ± 0.01^{A} (n=12)
$Glutathione_{red}$ ($\mu mol\ g^{-1}$ fw)	0.89 ± 0.16^{B} (n=12)	0.96 ± 0.13^{AB} (n=12)	1.02 ± 0.12^{A} (n=12)
$Ascorbate_{tot}$ ($\mu g\ g^{-1}$ fw)	1173 ± 254^{A} (n=12)	1223 ± 301^{A} (n=12)	1265 ± 342^{A} (n=12)
$Ascorbate_{ox}$ ($\mu g\ g^{-1}$ fw)	39 ± 36^{A} (n=12)	74 ± 108^{A} (n=12)	68 ± 124^{A} (n=12)
$Ascorbate_{red}$ ($\mu g\ g^{-1}$ fw)	1177 ± 323^{A} (n=12)	1187 ± 275^{A} (n=12)	1272 ± 365^{A} (n=12)

(Cont.)

(Table 1 continued)

Variable	Cultivar		
	Dare	Williams	Essex
Glutathione Reductase ($U\ g^{-1}$ fw)	0.88 ± 0.22^{A} (n=18)	0.92 ± 0.19^{A} (n=18)	0.97 ± 0.29^{A} (n=18)
Glutathione Reductase ($U\ mg^{-1}$ protein)	$.029 \pm .010^{A}$ (n=18)	$.031 \pm .006^{A}$ (n=18)	$.032 \pm .006^{A}$ (n=18)
Ascorbate Peroxidase ($U\ g^{-1}$ fw)	3.07 ± 0.93^{A} (n=10)	3.21 ± 0.59^{A} (n=10)	3.16 ± 0.92^{A} (n=10)
Ascorbate Peroxidase ($U\ mg^{-1}$ protein)	$.101 \pm .026^{A}$ (n=10)	$.107 \pm .020^{A}$ (n=10)	$.096 \pm .024^{A}$ (n=10)
SOD ($U\ g^{-1}$ fw)	353 ± 125^{B} (n=9)	332 ± 78^{B} (n=10)	509 ± 219^{A} (n=10)
SOD ($U\ mg^{-1}$ protein)	50.2 ± 23.9^{A} (n=9)	51.9 ± 26.0^{A} (n=10)	80.7 ± 56.9^{A} (n=10)

a: Mean ± sd.

b: Sample size.

A or B: Means across cultivars with the same letter are not statistically different (α=0.05).

Table 2. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the control (<0.03 μl l^{-1} SO_2) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	20.8 ± 2.2^{aA}	23.2 ± 0.8^{A}	22.4 ± 2.4^{A}
(mg CO_2 dm^{-2} hr^{-1})	4	23.2 ± 1.0^{B}	23.6 ± 1.2^{A}	22.3 ± 0.6^{A}
		(11 %)[b]	(1 %)	(0 %)
Cs	0	1.2 ± 0.4^{A}	1.0 ± 0.2^{A}	1.1 ± 0.4^{A}
(cm s^{-1})	4	1.1 ± 0.3^{A}	0.9 ± 0.1^{A}	1.1 ± 0.3^{A}
		(-8 %)	(-10 %)	(0 %)
Ci	0	297.2 ± 20.0^{A}	283.3 ± 9.7^{A}	287.1 ± 16.0^{A}
(ppm)	4	284.4 ± 16.1^{A}	274.4 ± 5.7^{A}	284.1 ± 12.9^{A}
		(-4 %)	(-3 %)	(-1 %)
Ts	0	4.2 ± 0.2^{A}	3.6 ± 0.3^{A}	3.8 ± 0.5^{A}
(mmol H_2O m^{-2} s^{-1})	4	2.9 ± 0.4^{B}	3.0 ± 0.2^{B}	2.9 ± 0.3^{B}
		(-31 %)	(-17 %)	(-24 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

Table 3. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the fumigated ($0.70\pm0.05\ \mu l\ l^{-1}\ SO_2$) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	24.4 ± 2.6^{aA}	24.3 ± 2.9^{A}	25.5 ± 2.3^{A}
($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	4	15.1 ± 5.4^{B}	13.2 ± 6.0^{B}	17.5 ± 3.3^{B}
		(-38 %)[b]	(-46 %)	(-31 %)
Cs	0	1.3 ± 0.2^{A}	1.4 ± 0.3^{A}	1.4 ± 0.3^{A}
($cm\ s^{-1}$)	4	0.7 ± 0.2^{B}	0.5 ± 0.2^{B}	0.7 ± 0.2^{B}
		(-46 %)	(-64 %)	(-50 %)
Ci	0	285.2 ± 18.9^{A}	287.3 ± 16.5^{A}	283.5 ± 15.7^{A}
(ppm)	4	278.4 ± 18.6^{A}	267.8 ± 22.0^{B}	266.9 ± 15.4^{B}
		(-2 %)	(-7 %)	(-6 %)
Ts	0	4.2 ± 0.7^{A}	4.1 ± 0.7^{A}	4.1 ± 0.9^{A}
$mmol\ H_2O\ m^{-2}\ s^{-1}$)	4	2.4 ± 0.5^{B}	1.9 ± 0.8^{B}	2.6 ± 0.4^{B}
		(-43 %)	(-54 %)	(-37 %)

a: Means ± sd.

b: Percentage reduction from time 0.

A or B: Means within cultivars with the same letter are not statistically different ($\alpha=0.05$).

Table 4. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the control (<0.030 μl l^{-1} SO_2) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$	0	0.89 ± 0.13^{aA}	0.98 ± 0.11^{A}	1.08 ± 0.12^{A}
(μmol g^{-1} fw)	4	0.95 ± 0.05^{A}	0.93 ± 0.08^{A}	1.12 ± 0.18^{A}
		(7 %)[b]	(-5 %)	(4 %)
$Glutathione_{ox}$	0	$.041\pm.017^{A}$	$.044\pm.017^{A}$	$.042\pm.017^{A}$
(μmol g^{-1} fw)	4	$.035\pm.014^{A}$	$.027\pm.017^{A}$	$.031\pm.018^{A}$
		(-15 %)	(-39 %)	(-26 %)
$Glutathione_{red}$	0	0.84 ± 0.13^{A}	0.92 ± 0.10^{A}	1.02 ± 0.11^{A}
(μmol g^{-1} fw)	4	0.90 ± 0.05^{A}	0.90 ± 0.06^{A}	1.09 ± 0.19^{A}
		(7 %)	(-2 %)	(7 %)
$Ascorbate_{tot}$	0	1109 ± 212^{A}	1197 ± 233^{A}	1301 ± 377^{A}
(μg g^{-1} fw)	4	1154 ± 237^{A}	1186 ± 207^{A}	1333 ± 466^{A}
		(4 %)	(-1 %)	(2 %)
$Ascorbate_{red}$	0	1132 ± 288^{A}	1240 ± 293^{A}	1255 ± 339^{A}
(μg g^{-1} fw)	4	1038 ± 130^{A}	1141 ± 253^{A}	1255 ± 483^{A}
		(-8 %)	(-8 %)	(0 %)

(Cont.)

(Table 4 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	30.7±6.3[A]	30.3±2.9[A]	32.2±2.1[A]
(mg g^{-1} fw)	4	32.8±5.1[A]	31.2±2.8[A]	35.9±3.0[A]
		(7%)	(3%)	(11%)
Glutathione	0	0.78±0.18[A]	0.89±0.17[A]	0.92±0.27[A]
Reductase	4	0.94±0.20[A]	0.97±0.15[A]	0.99±0.31[A]
(U g^{-1} fw)		(21 %)	(9 %)	(8 %)
Glutathione	0	.027±.012[A]	.030±.006[A]	.031±.005[A]
Reductase	4	.029±.008[A]	.031±.004[A]	.030±.006[A]
(U mg^{-1} protein)		(7 %)	(3 %)	(-3 %)
Ascorbate	0	2.97±0.62[A]	3.24±0.39[A]	2.82±0.67[A]
Peroxidase	4	3.01±1.24[A]	3.29±1.00[A]	2.89±0.78[A]
(U g^{-1} fw)		(1 %)	(2 %)	(2 %)
Ascorbate	0	.108±.027[A]	.109±.014[A]	.087±.025[A]
Peroxidase	4	.096±.027[A]	.102±.028[A]	.082±.021[A]
(U mg^{-1} protein)		(-11 %)	(-6 %)	(-6 %)
SOD	0	419±148[A]	316±86[A]	451±150[A]
(U g^{-1} fw)	4	255±59[A]	297±96[A]	402±217[A]
		(-39 %)	(-6 %)	(-6 %)
SOD	0	62±31[A]	52±31[A]	70±38[A]
(U mg^{-1} protein)	4	37±20[A]	38±18[A]	53±51[A]
		(-40 %)	(-27 %)	(-24 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

Table 5. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the fumigated (0.70 ± 0.05 $\mu l\ l^{-1}$ SO_2) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$ ($\mu mol\ g^{-1}$ fw)	0	0.98 ± 0.18^{aA}	1.04 ± 0.16^{A}	1.05 ± 0.13^{A}
	4	1.38 ± 0.16^{B} (-41 %)[b]	1.40 ± 0.13^{B} (-35 %)	1.42 ± 0.11^{B} (-35 %)
$Glutathione_{ox}$ ($\mu mol\ g^{-1}$ fw)	0	$.036\pm.009^{A}$	$.035\pm.012^{A}$	$.031\pm.008^{A}$
	4	$.084\pm.041^{B}$ (133 %)	$.078\pm.040^{B}$ (123 %)	$.083\pm.037^{B}$ (168 %)
$Glutathione_{red}$ ($\mu mol\ g^{-1}$ fw)	0	0.94 ± 0.18^{A}	0.99 ± 0.16^{A}	1.01 ± 0.13^{A}
	4	1.28 ± 0.14^{B} (36 %)	1.31 ± 0.15^{B} (32 %)	1.33 ± 0.10^{B} (32 %)
$Ascorbate_{tot}$ ($\mu g\ g^{-1}$ fw)	0	1237 ± 296^{A}	1249 ± 379^{A}	1230 ± 335^{A}
	4	1315 ± 131^{A} (6 %)	1190 ± 287^{A} (-5 %)	1233 ± 298^{A} (0 %)
$Ascorbate_{red}$ ($\mu g\ g^{-1}$ fw)	0	1222 ± 376^{A}	1134 ± 272^{A}	1288 ± 421^{A}
	4	1295 ± 173^{A} (6 %)	1234 ± 381^{A} (9 %)	1235 ± 343^{A} (-4 %)

(Cont.)

(Table 5 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	33.0±5.5[A]	29.8±1.6[A]	34.5±3.3[A]
(mg g^{-1} fw)	4	33.2±3.0[A]	30.8±4.3[A]	34.5±3.0[A]
		(0%)	(3%)	(0%)
Glutathione	0	0.97±0.23[A]	0.94±0.21[A]	1.02±0.32[A]
Reductase	4	1.03±0.18[A]	1.09±0.19[A]	1.05±0.37[A]
(U g^{-1} fw)		(6 %)	(16 %)	(3 %)
Glutathione	0	.030±.010[A]	.032±.007[A]	.033±.008[A]
Reductase	4	.031±.006[A]	.036±.007[A]	.034±.008[A]
(U mg^{-1} protein)		(3 %)	(13 %)	(3 %)
Ascorbate	0	3.17±1.24[A]	3.19±0.80[A]	3.50±1.08[A]
Peroxidase	4	3.39±0.56[A]	2.37±1.22[A]	3.20±1.21[A]
(U g^{-1} fw)		(7 %)	(-26 %)	(-9%)
Ascorbate	0	.094±.027[A]	.106±.027[A]	.104±.023[A]
Peroxidase	4	.101±.019[A]	.076±.034[A]	.094±.029[A]
(U mg^{-1} protein)		(7 %)	(-28 %)	(-10 %)
SOD	0	301±83[A]	349±76[A]	567±278[A]
(U g^{-1} fw)	4	273±111[A]	330±115[A]	370±87[B]
		(-9 %)	(-5 %)	(-35 %)
SOD	0	41±14[A]	52±23[A]	92±75[A]
(U mg^{-1} protein)	4	35±23[A]	44±25[A]	50±27[A]
		(-15 %)	(-15 %)	(-46 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivars with the same letter are not statistically different (α=0.05).

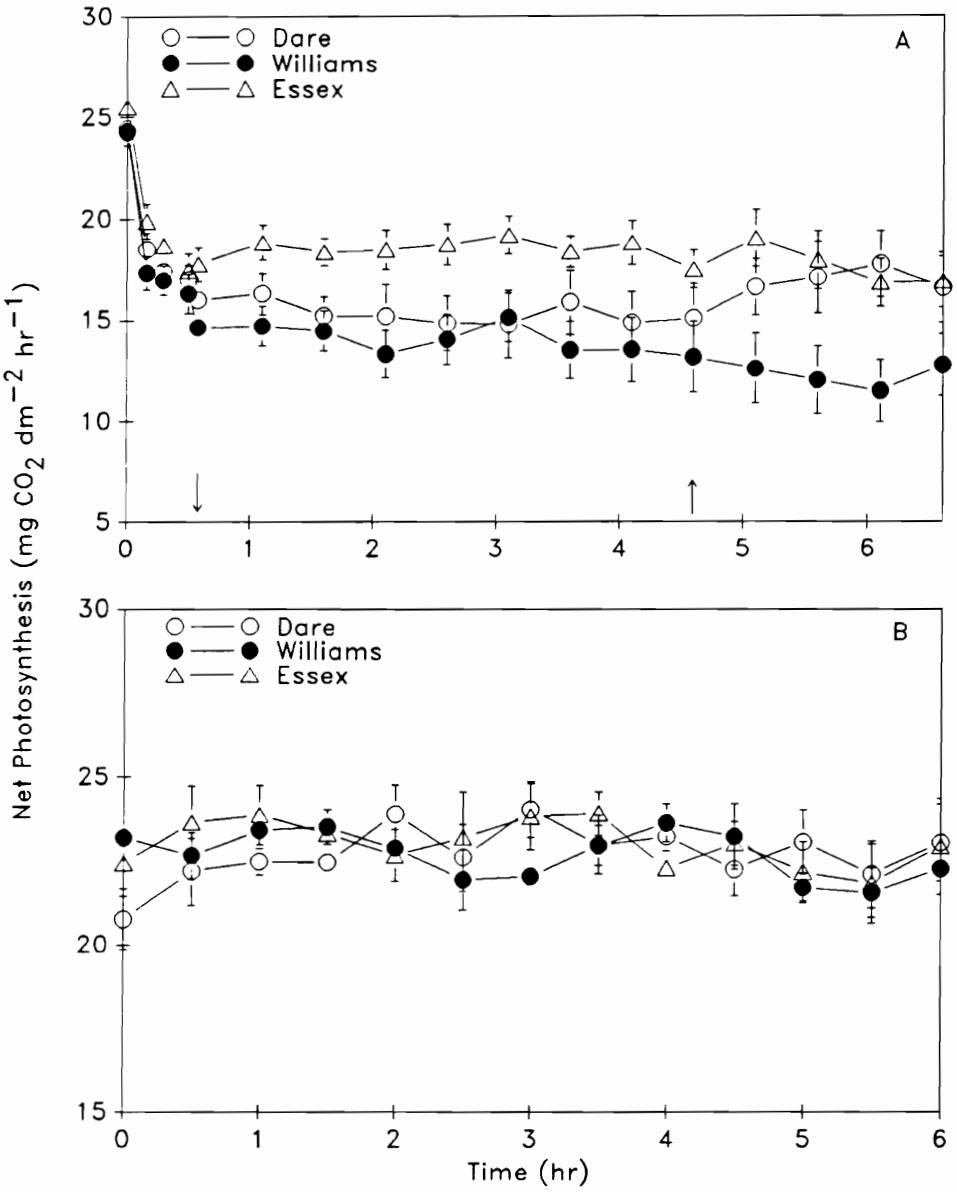

Fig. 1. Net photosynthetic rate of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 3-18 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

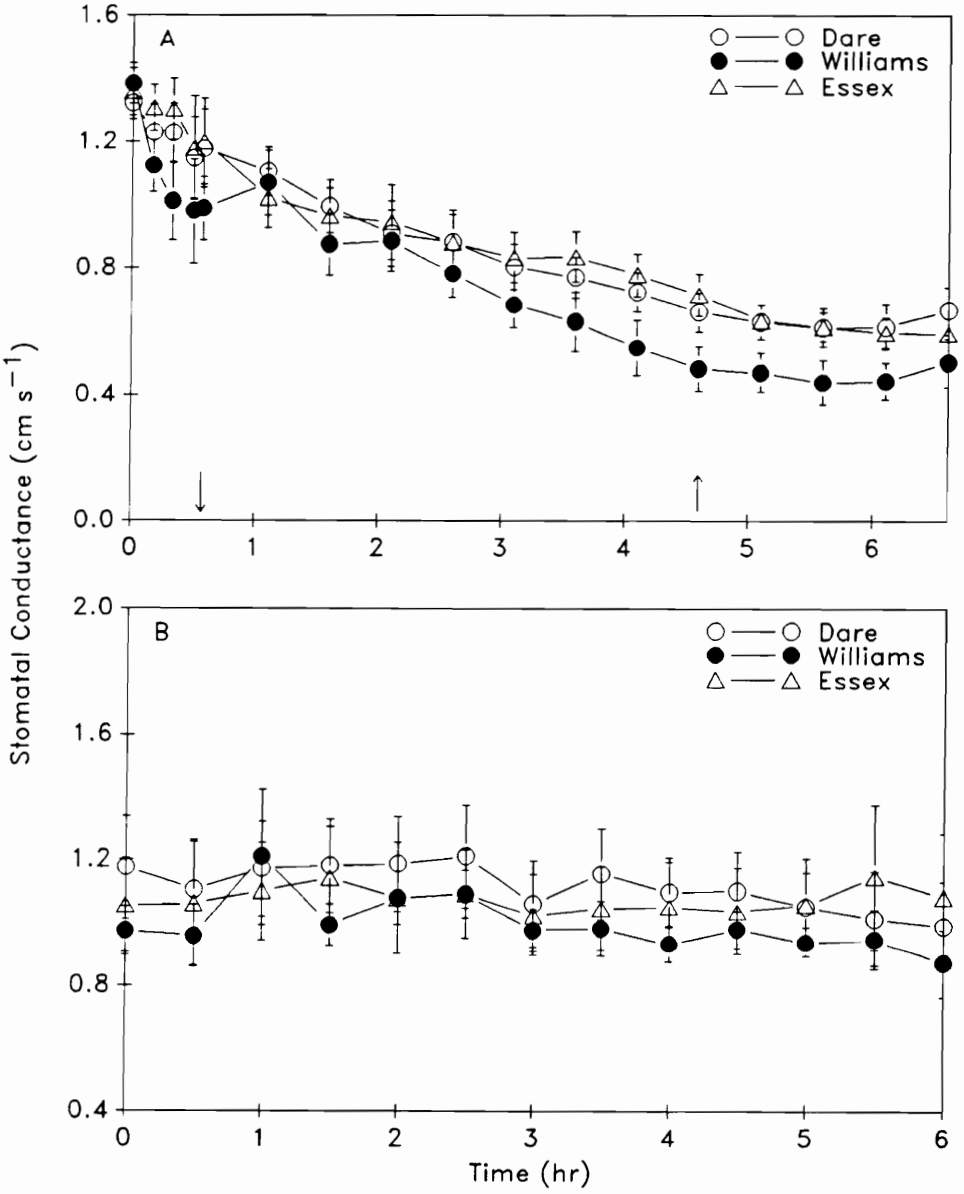

Fig. 2. Stomatal conductance of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 3-18 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

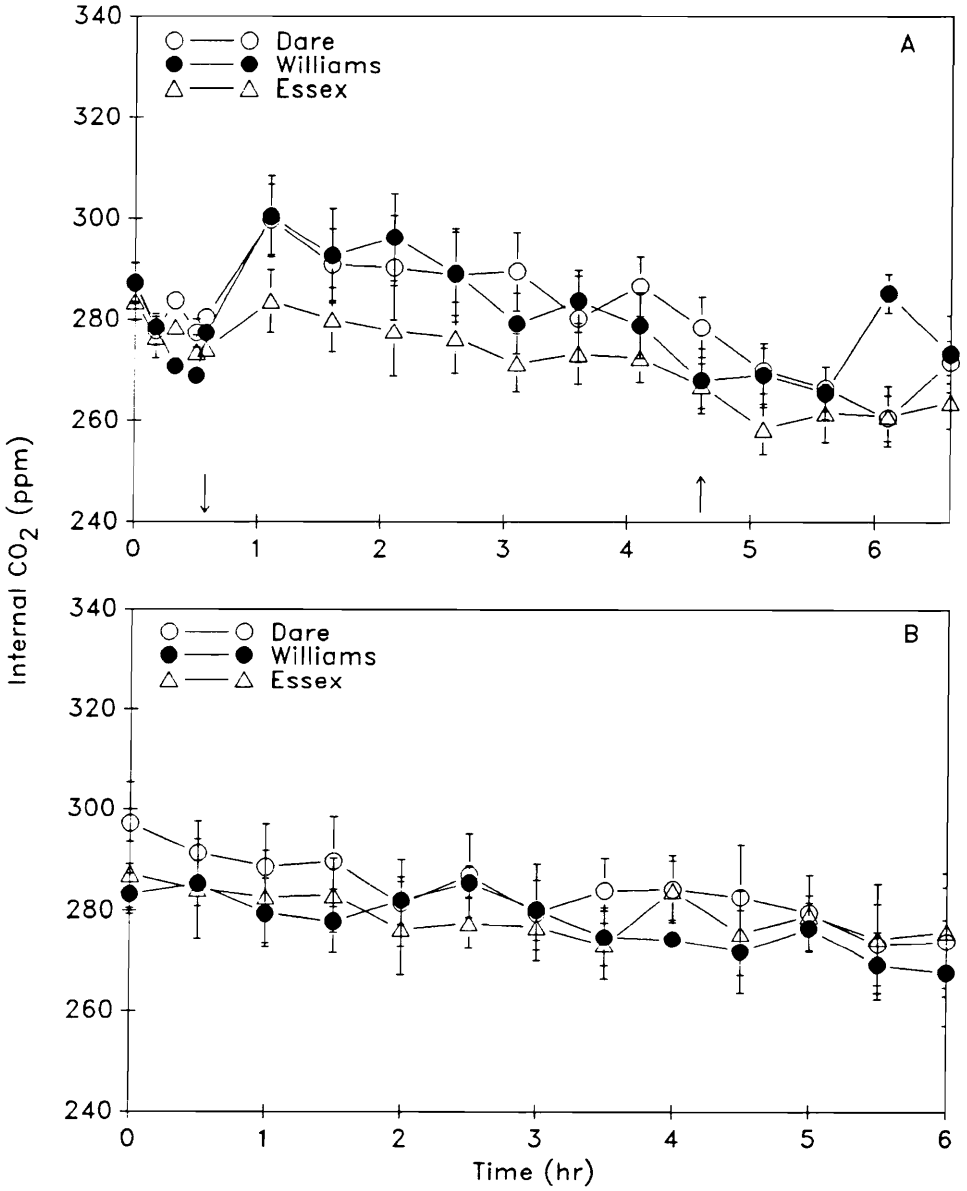

Fig. 3. Intercellular CO_2 concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 3-18 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

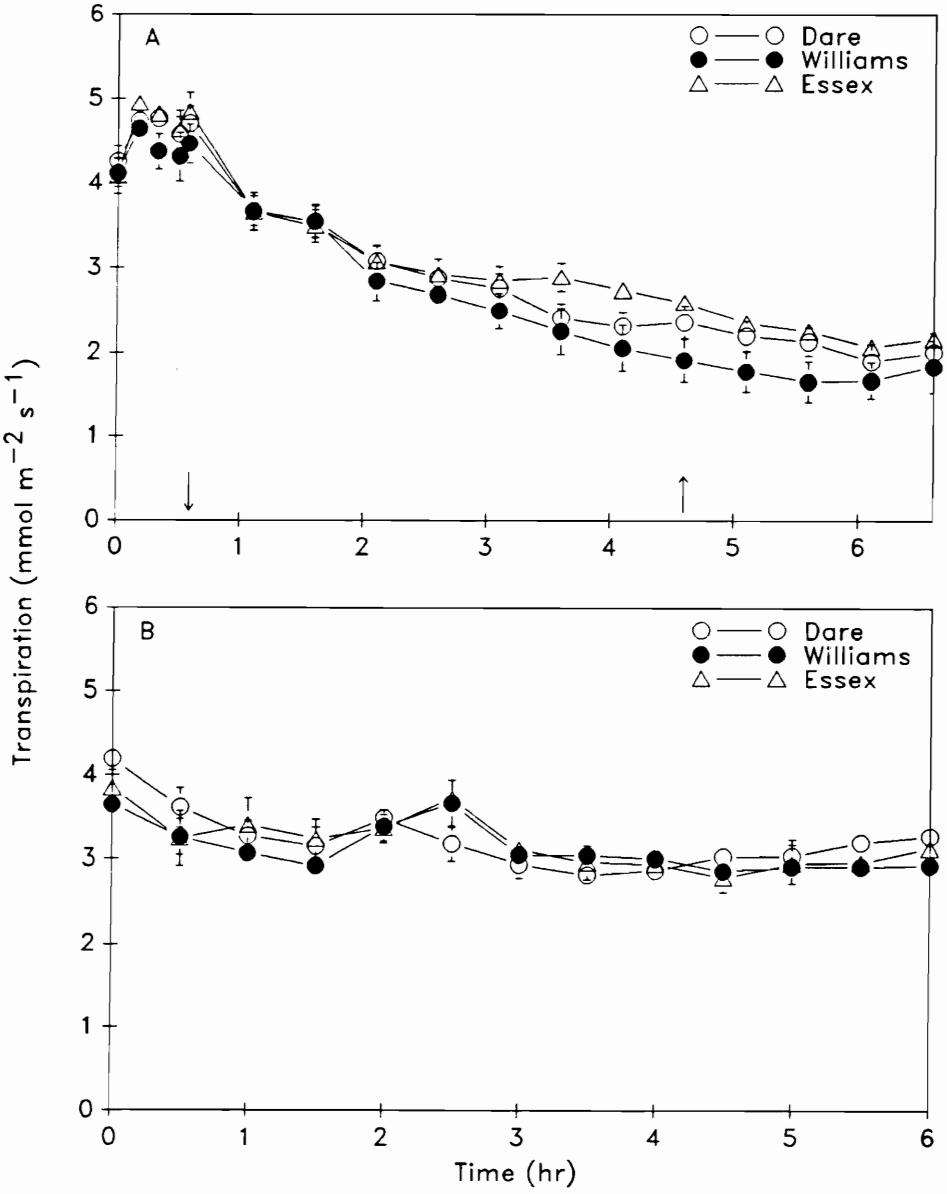

Fig. 4. Transpiration rate of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point represents the mean ± SE of 3-18 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

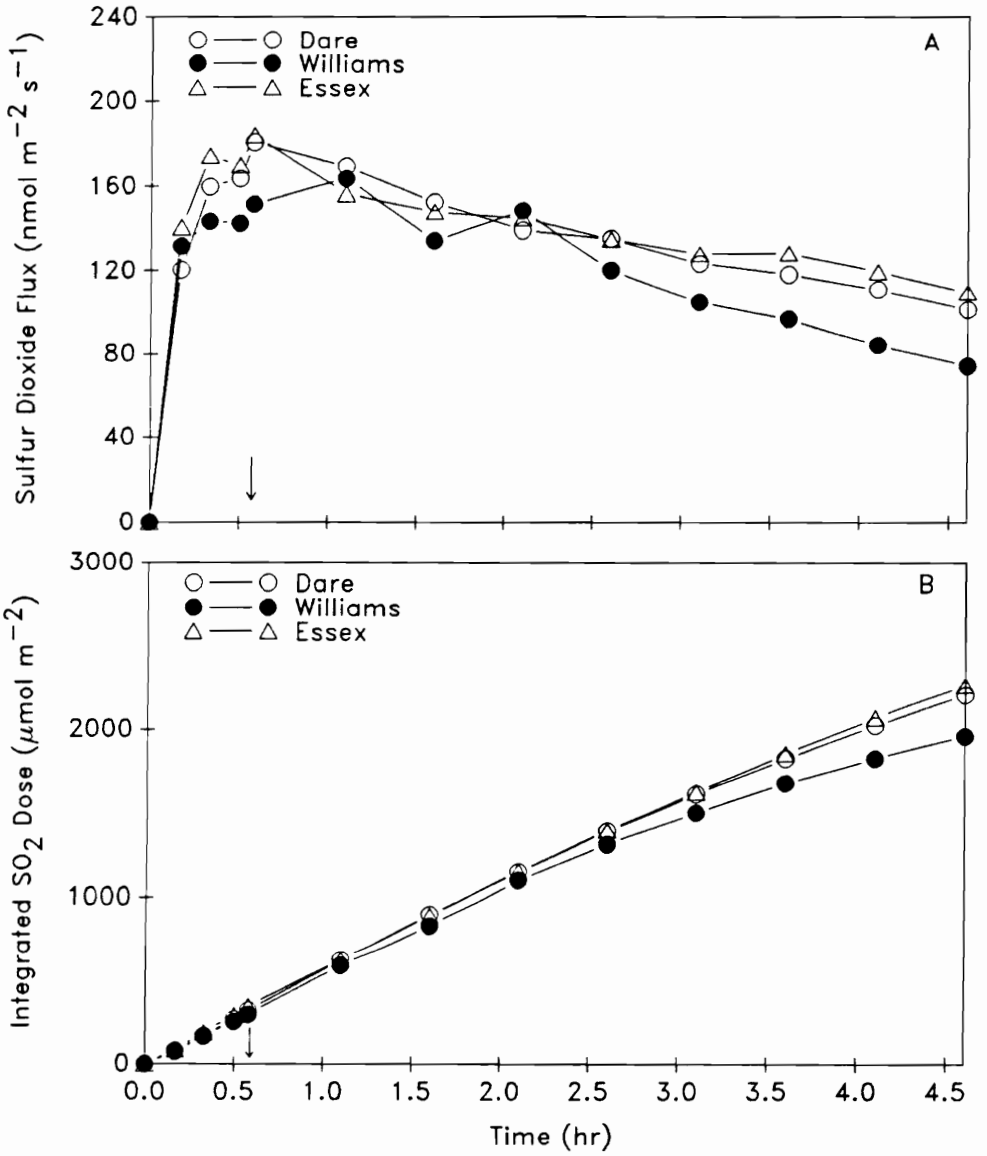

Fig. 5. Estimated sulfur dioxide flux A) and integrated SO_2 dose B) of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr. Each point is calculated from the mean of 3-18 samples. ↓ indicates target SO_2 concentrations reached.

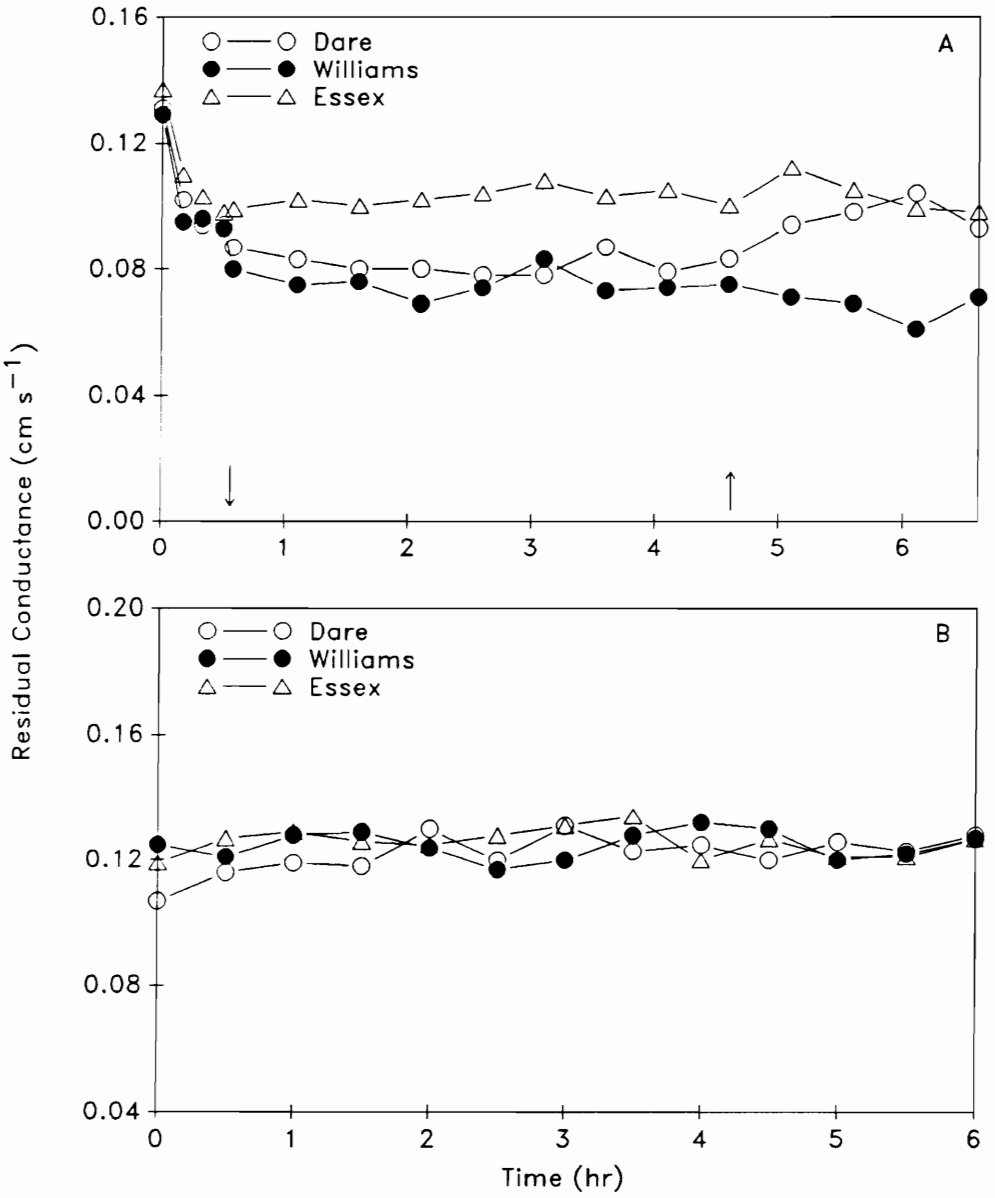

Fig. 6. Residual conductance to CO_2 of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point is calculated from the mean of 3-18 samples. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

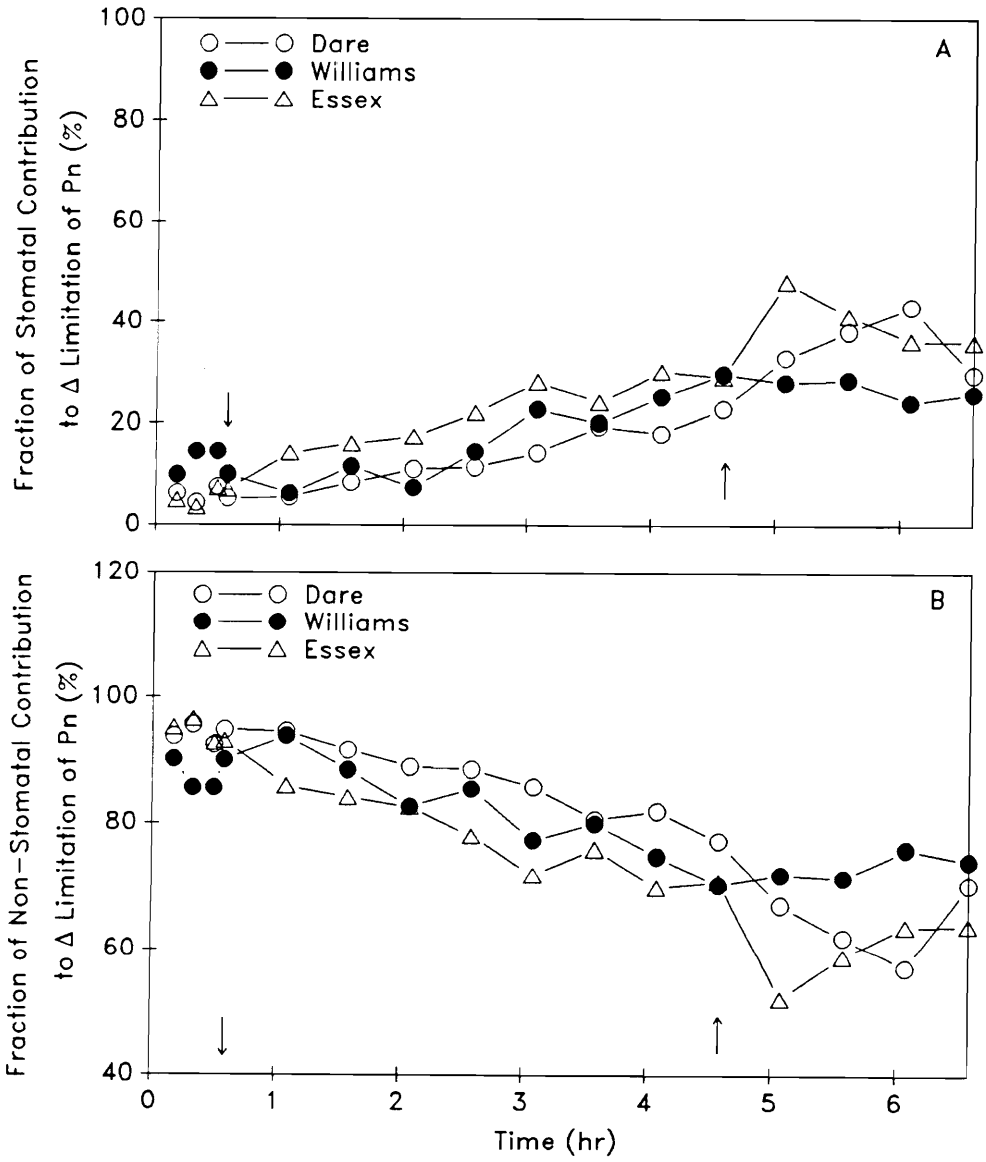

Fig. 7. Fraction of A) stomatal and B) non-stomatal contribution to change in limitation to Pn of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr and allowed to recover for 2 hr. Relative to 0 time, each point is calculated from the mean of 3-18 samples. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

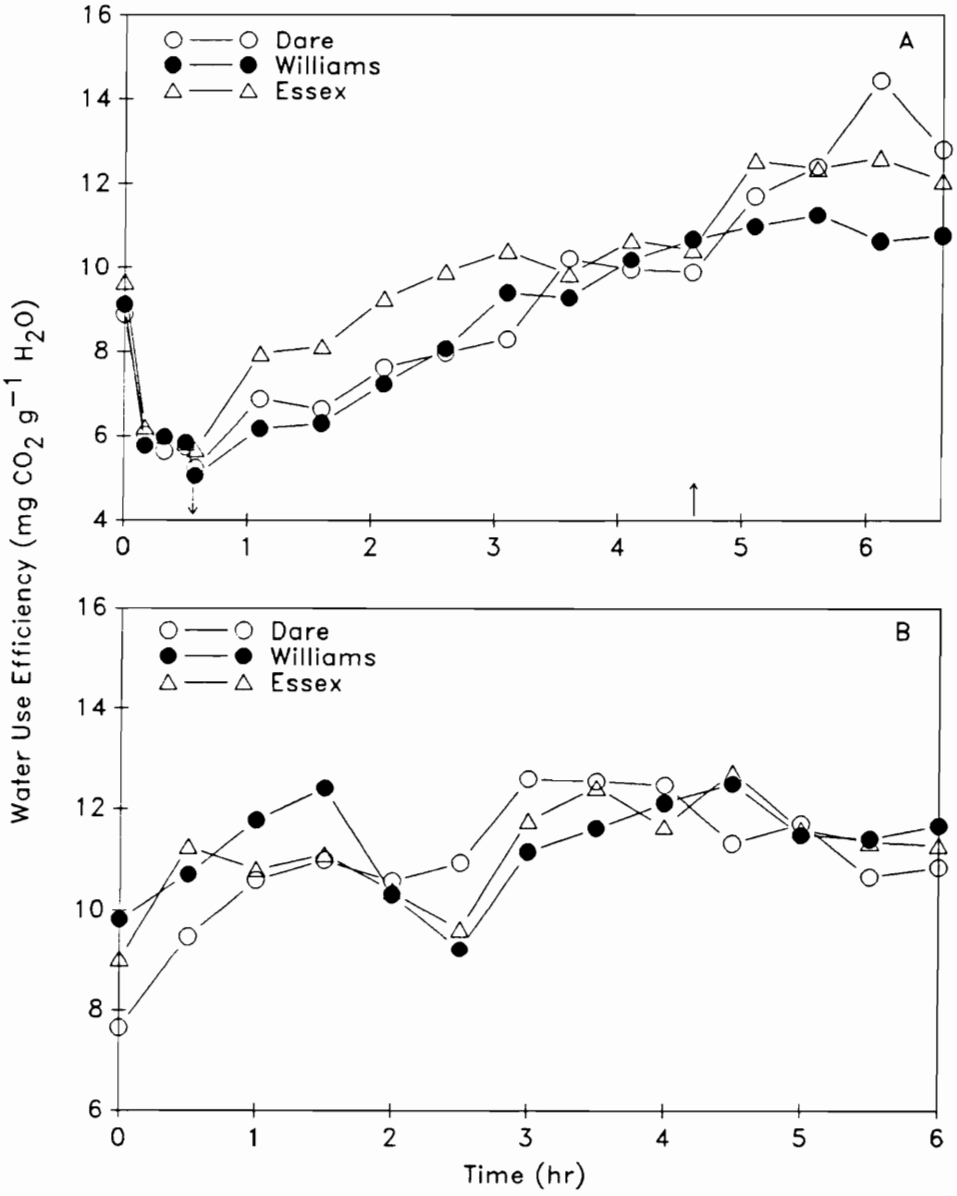

Fig. 8. Water use efficiency of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr and allowed to recover for 2 hr. Each point is calculated from the mean of 3-18 samples. ↓ indicates target SO_2 concentrations reached and ↑ indicates SO_2 fumigation terminated.

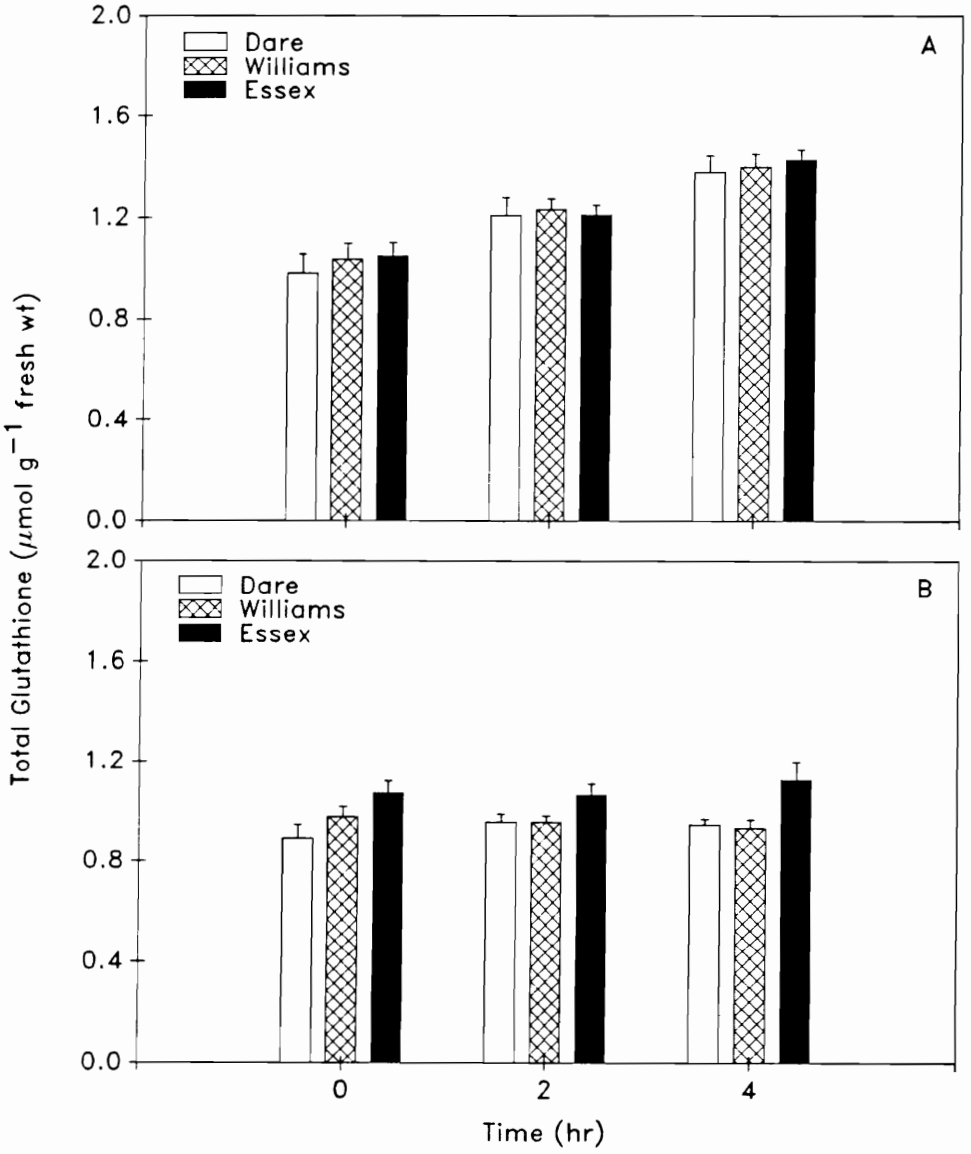

Fig. 9. Total glutathione concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 6 samples.

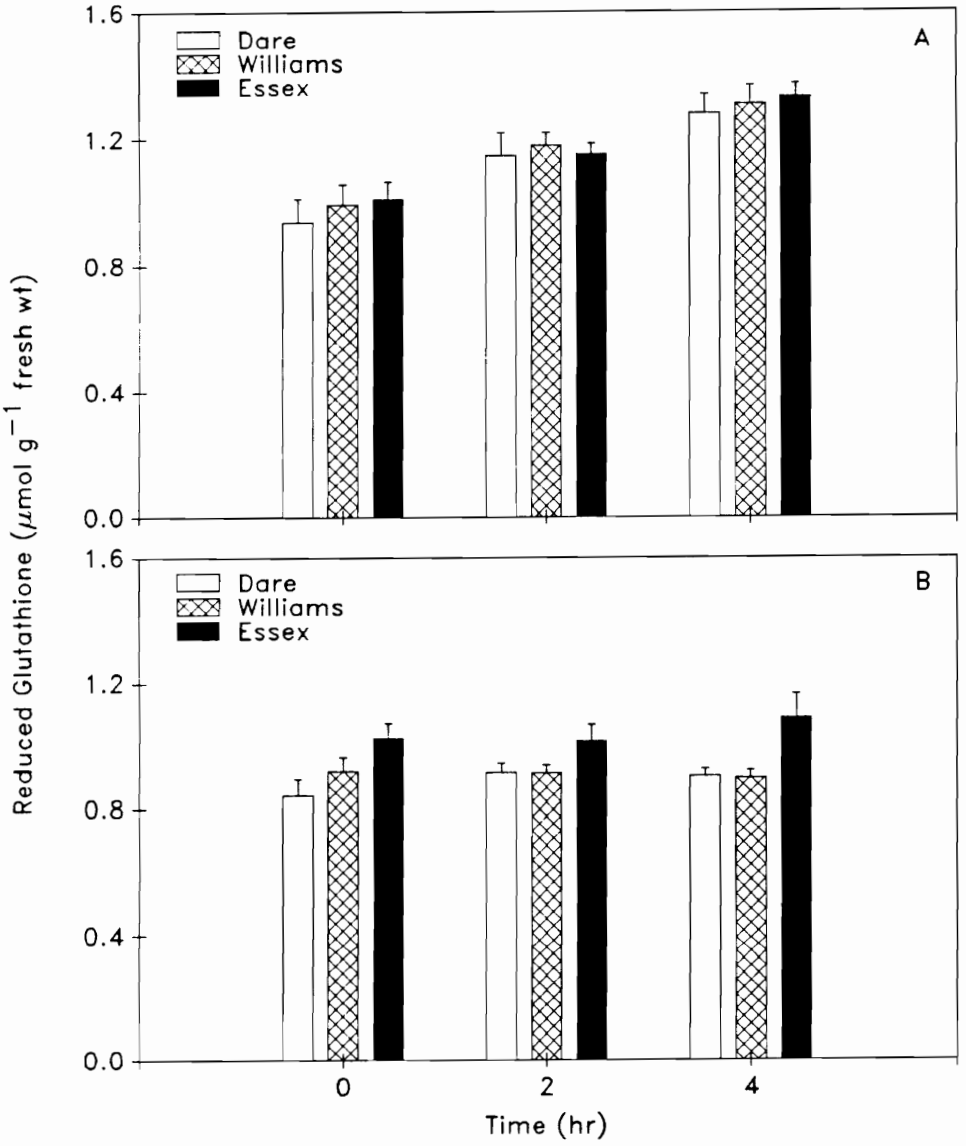

Fig. 10. Reduced glutathione concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 6 samples.

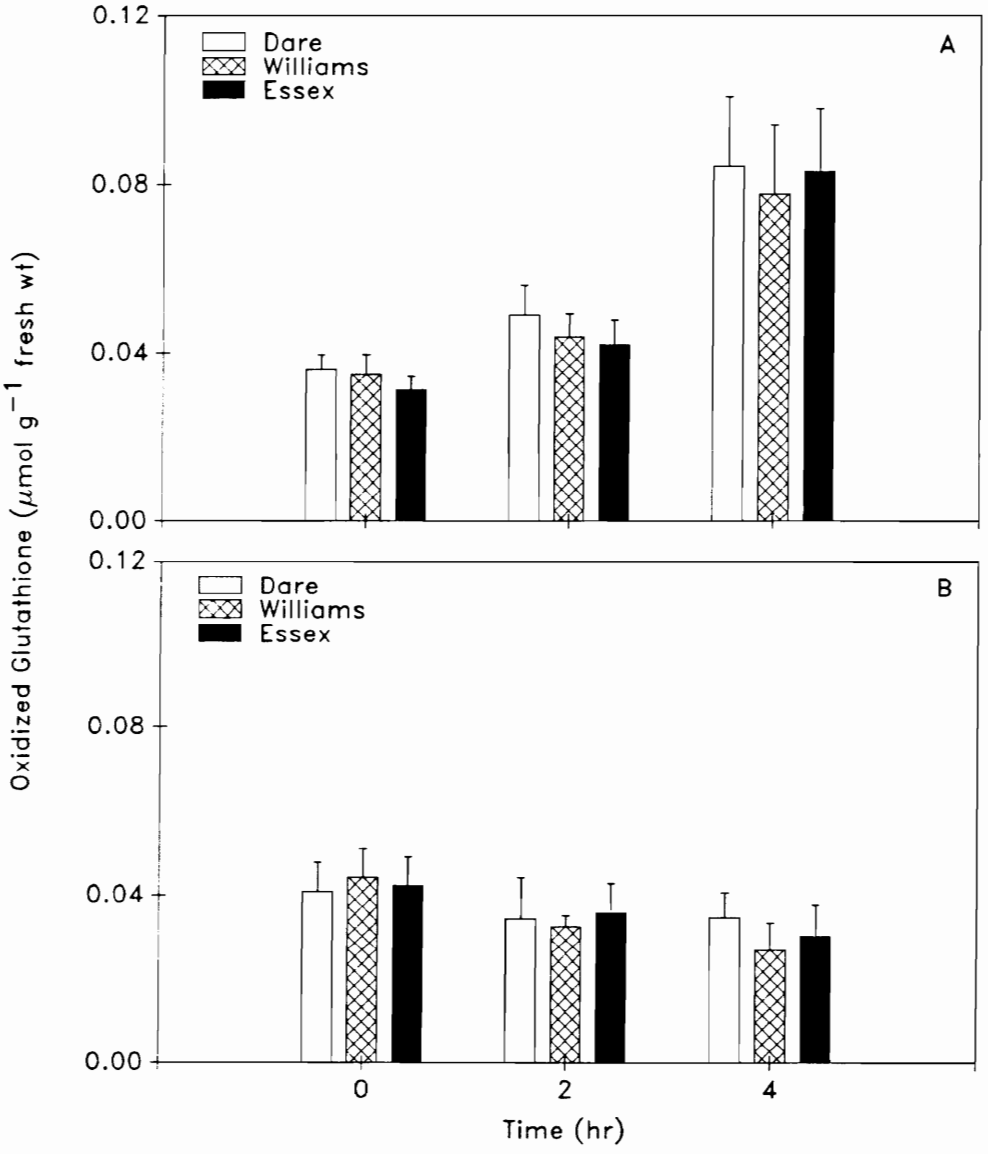

Fig. 11. Oxidized glutathione concentration of soybean cultivars exposed to A) 0.7 $\mu l\ l^{-1}$ SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 6 samples.

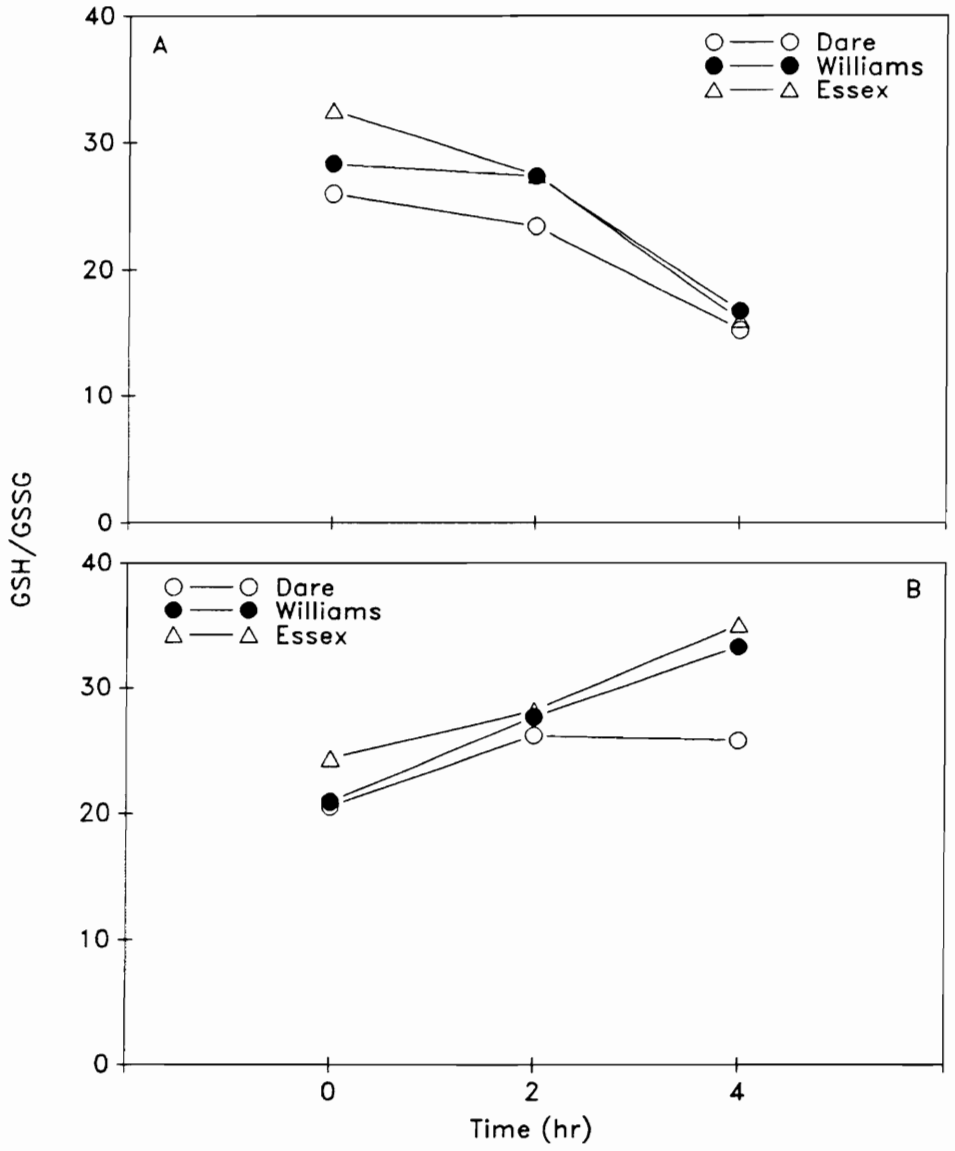

Fig. 12. Mean ratio of reduced (GSH) to oxidized (GSSG) glutathione in soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr.

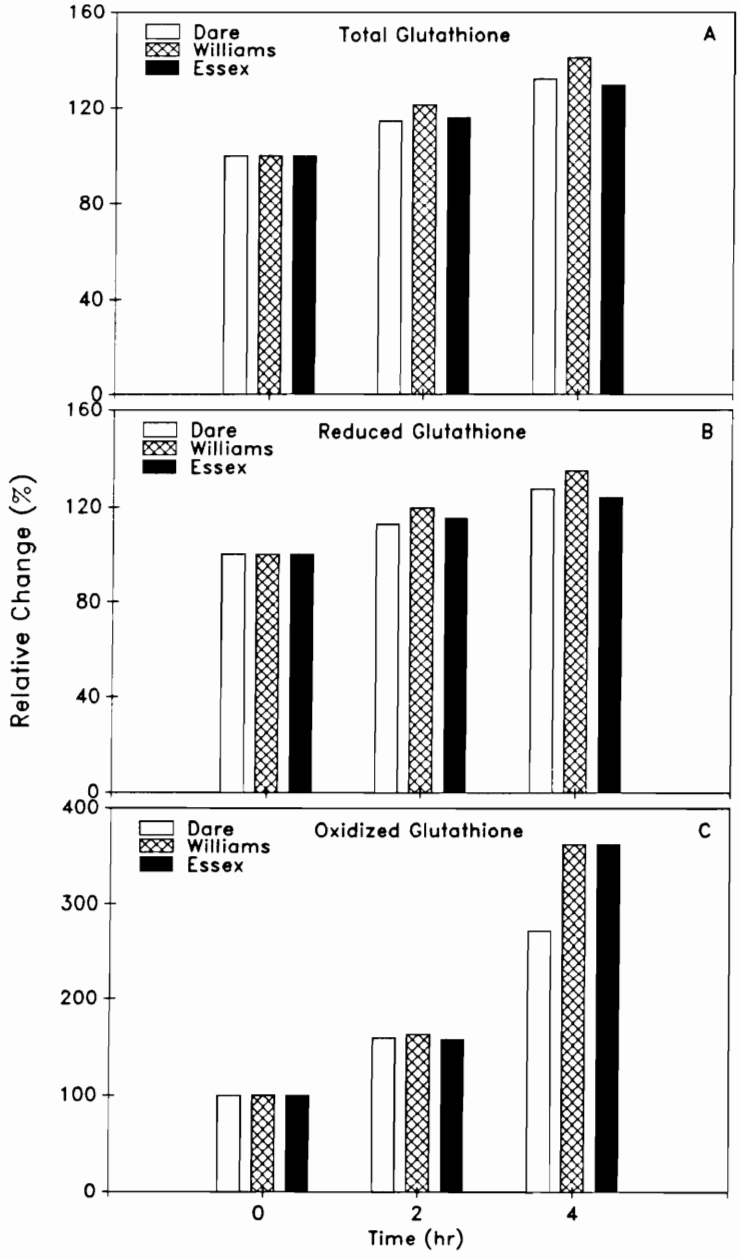

Fig. 13. Changes of A) total glutathione, B) reduced glutathione and C) oxidized glutathione concentrations of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr relative to control plants. Relative changes represent mean differences of 6 samples.

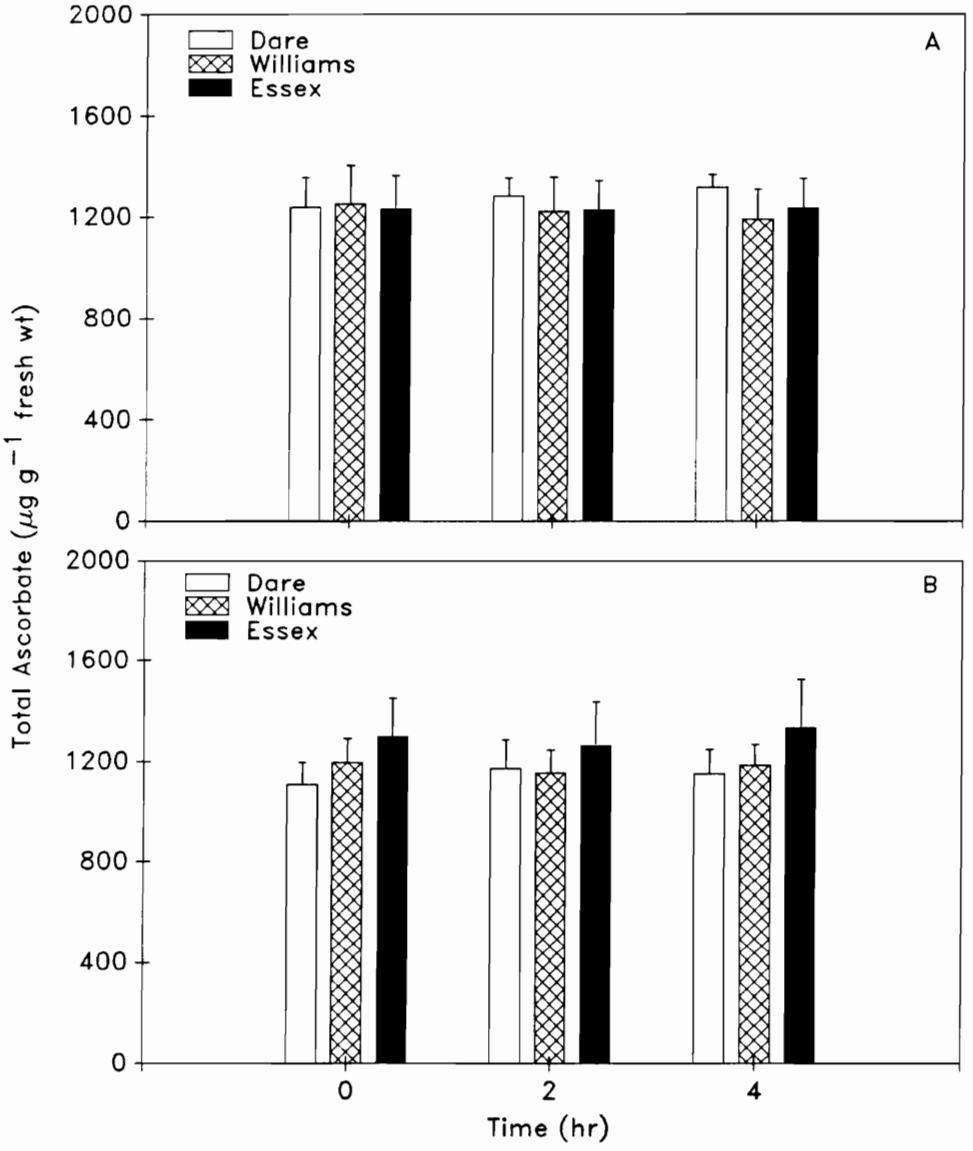

Fig. 14. Total ascorbate concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 6 samples.

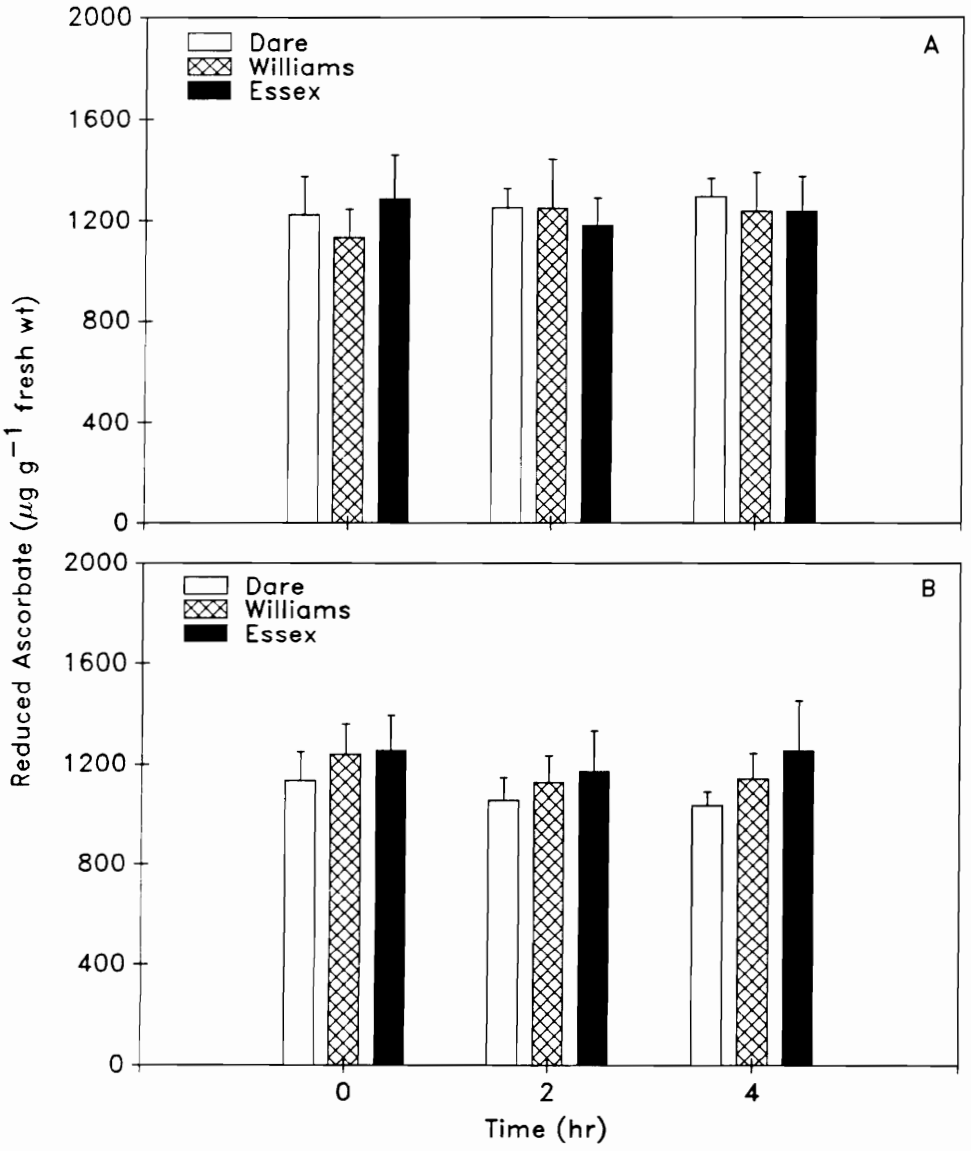

Fig. 15. Reduced ascorbate concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 6 samples.

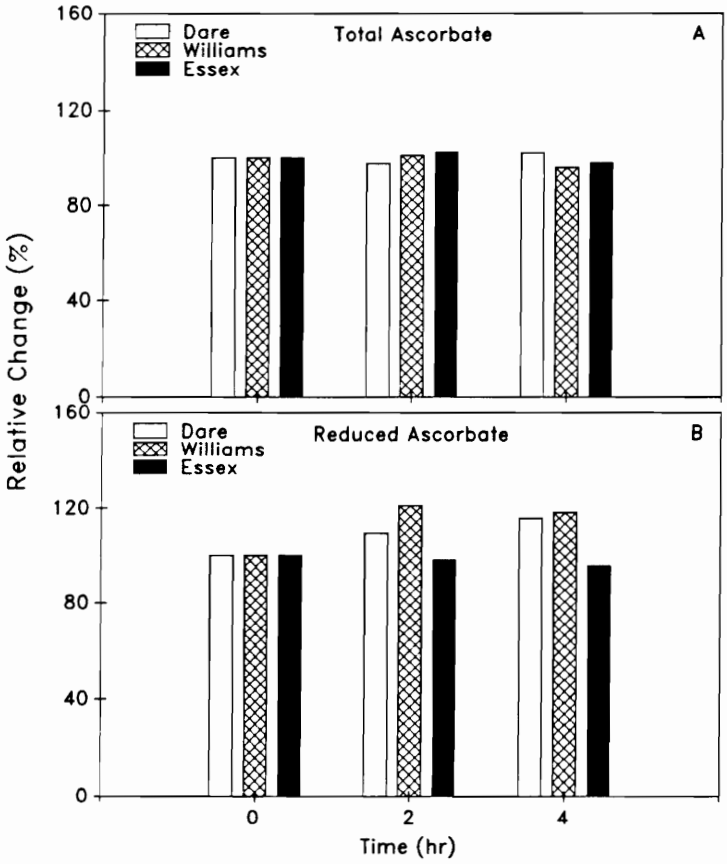

Fig. 16. Changes of A) total ascorbate and B) reduced ascorbate concentrations of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr relative to control plants. Relative changes represent mean differences of 6 samples.

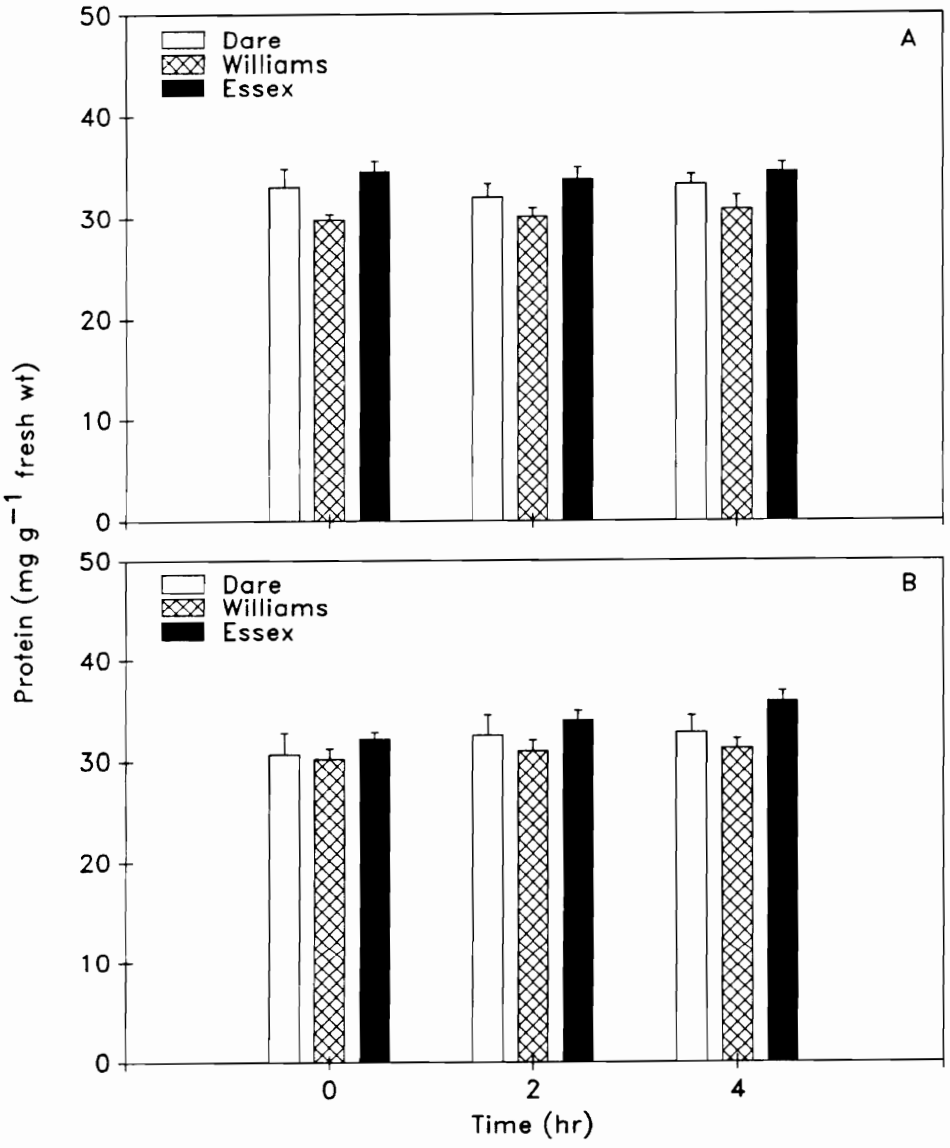

Fig. 17. Protein concentrations of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9 samples.

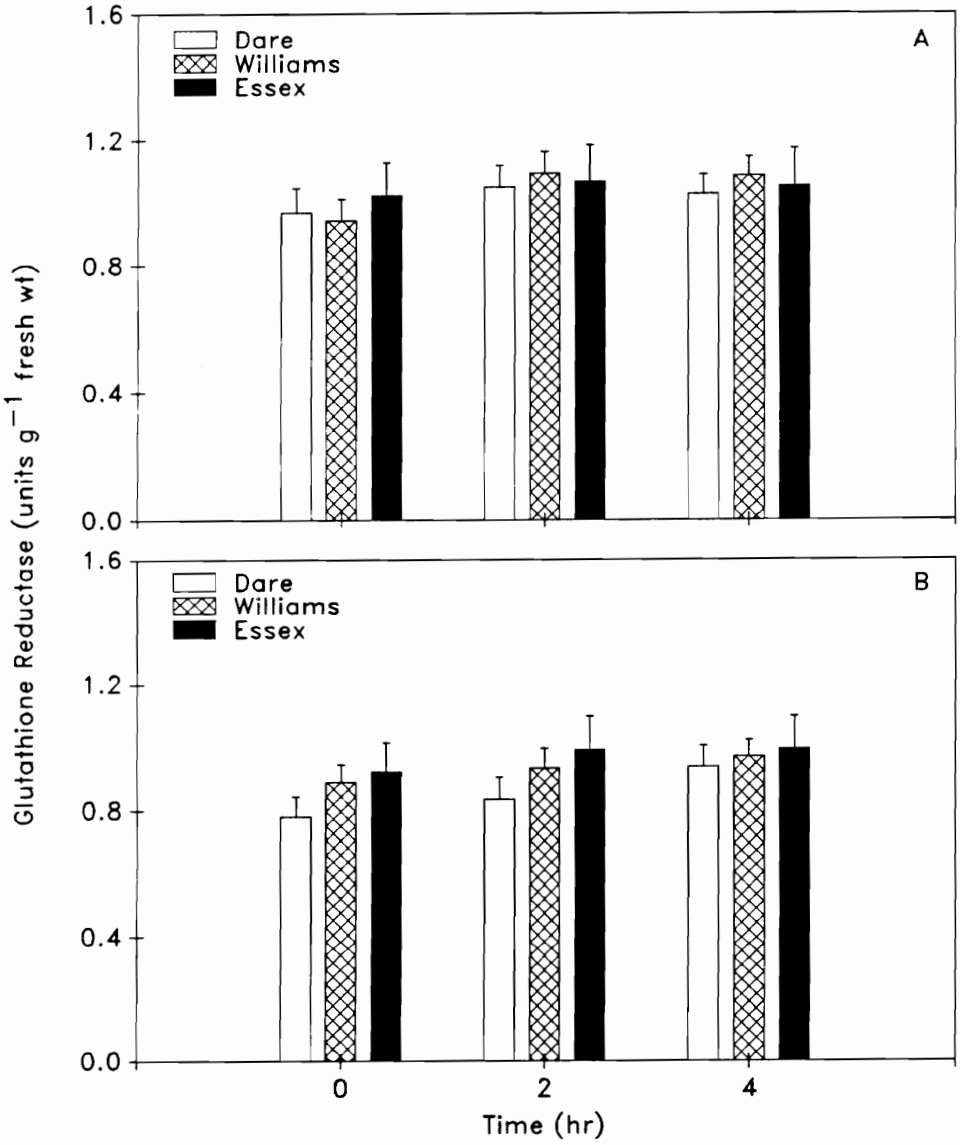

Fig. 18. Changes in glutathione reductase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9 samples.

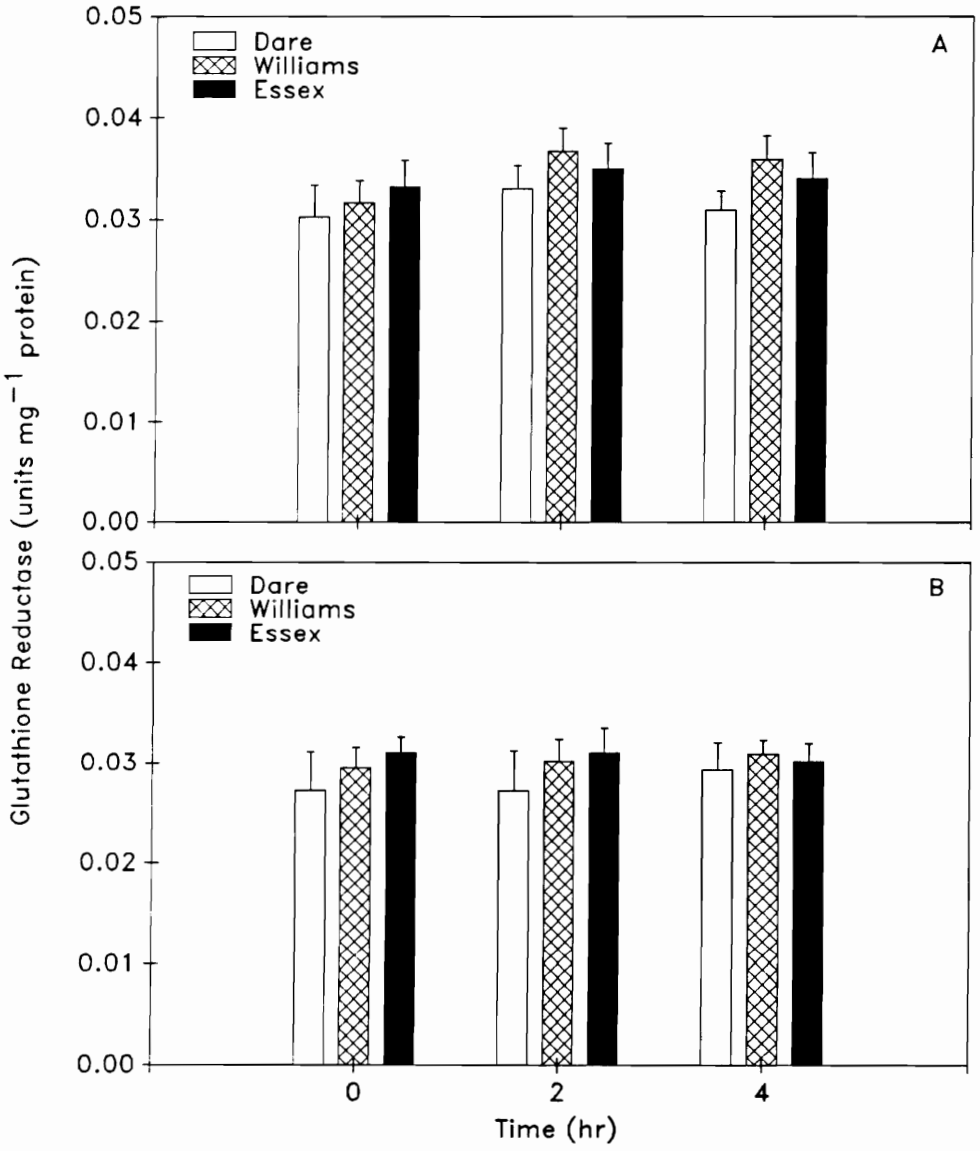

Fig. 19. Changes in glutathione reductase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9 samples.

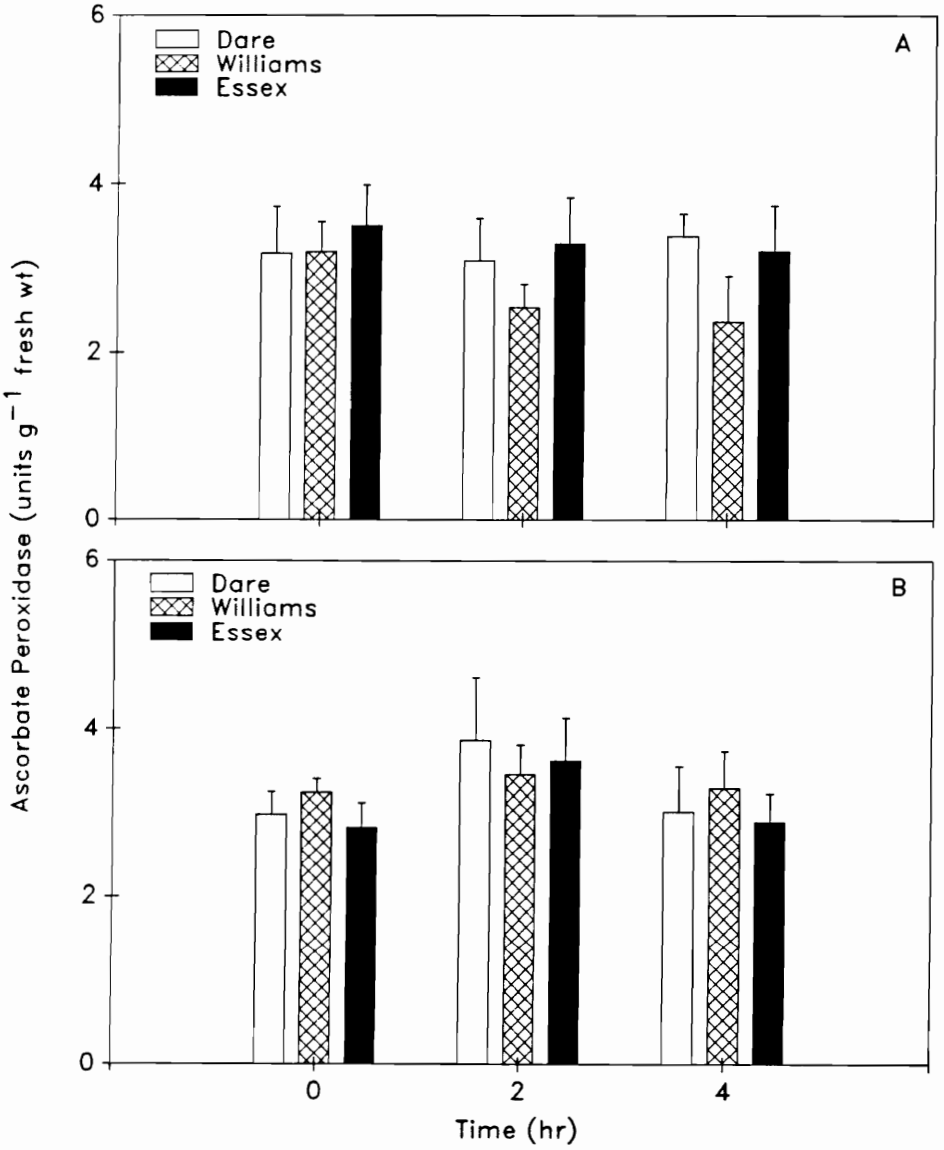

Fig. 20. Changes in ascorbate peroxidase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

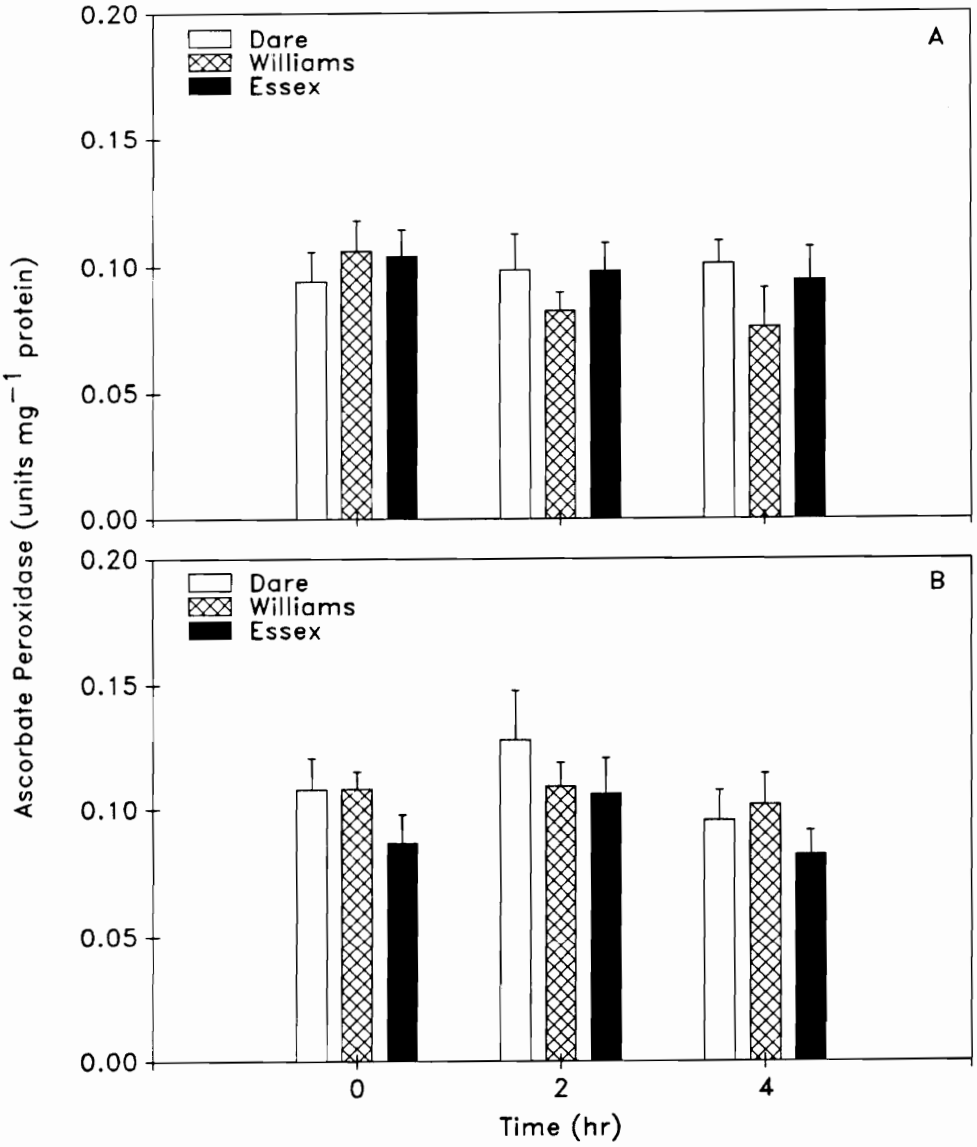

Fig. 21. Changes in ascorbate peroxidase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

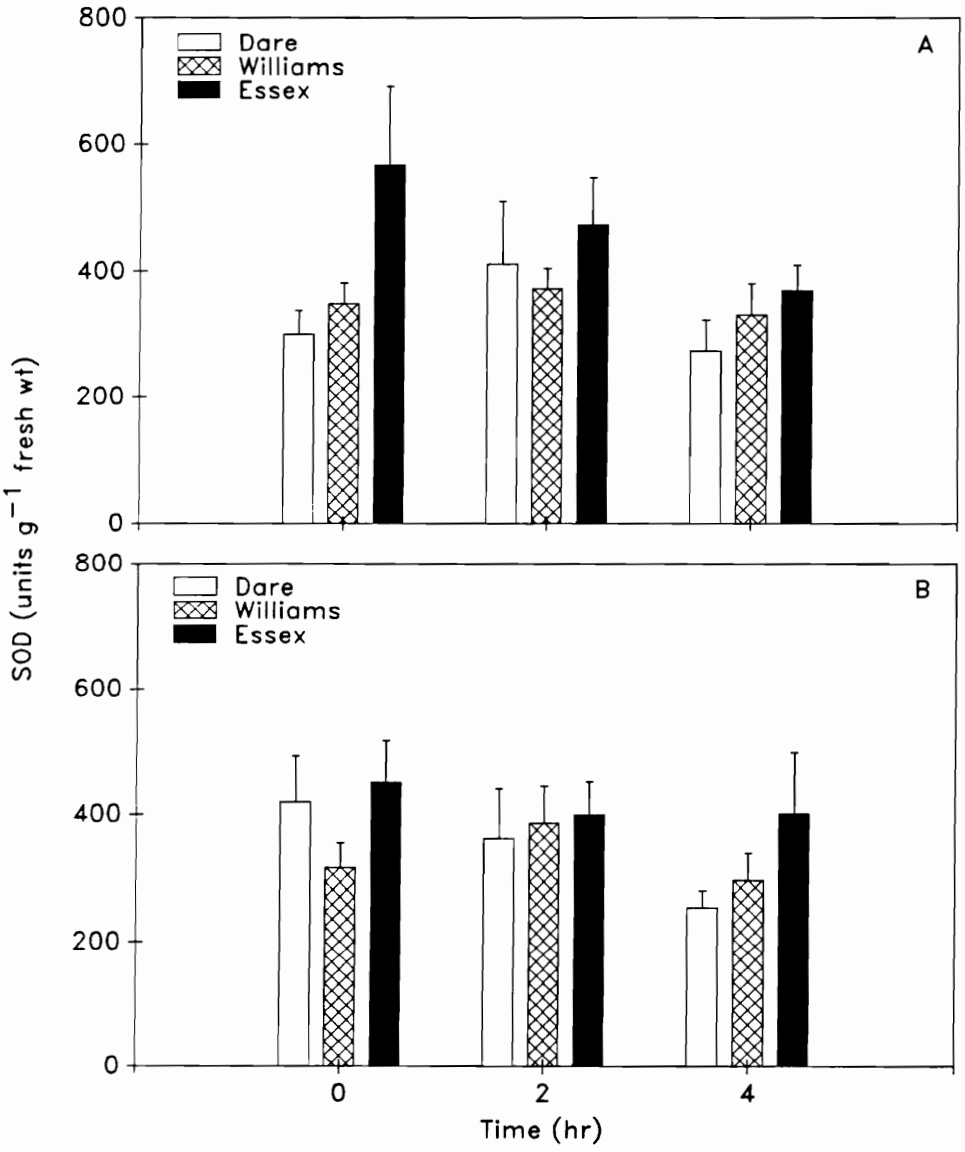

Fig. 22. Changes in superoxide dismutase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 4-5 samples.

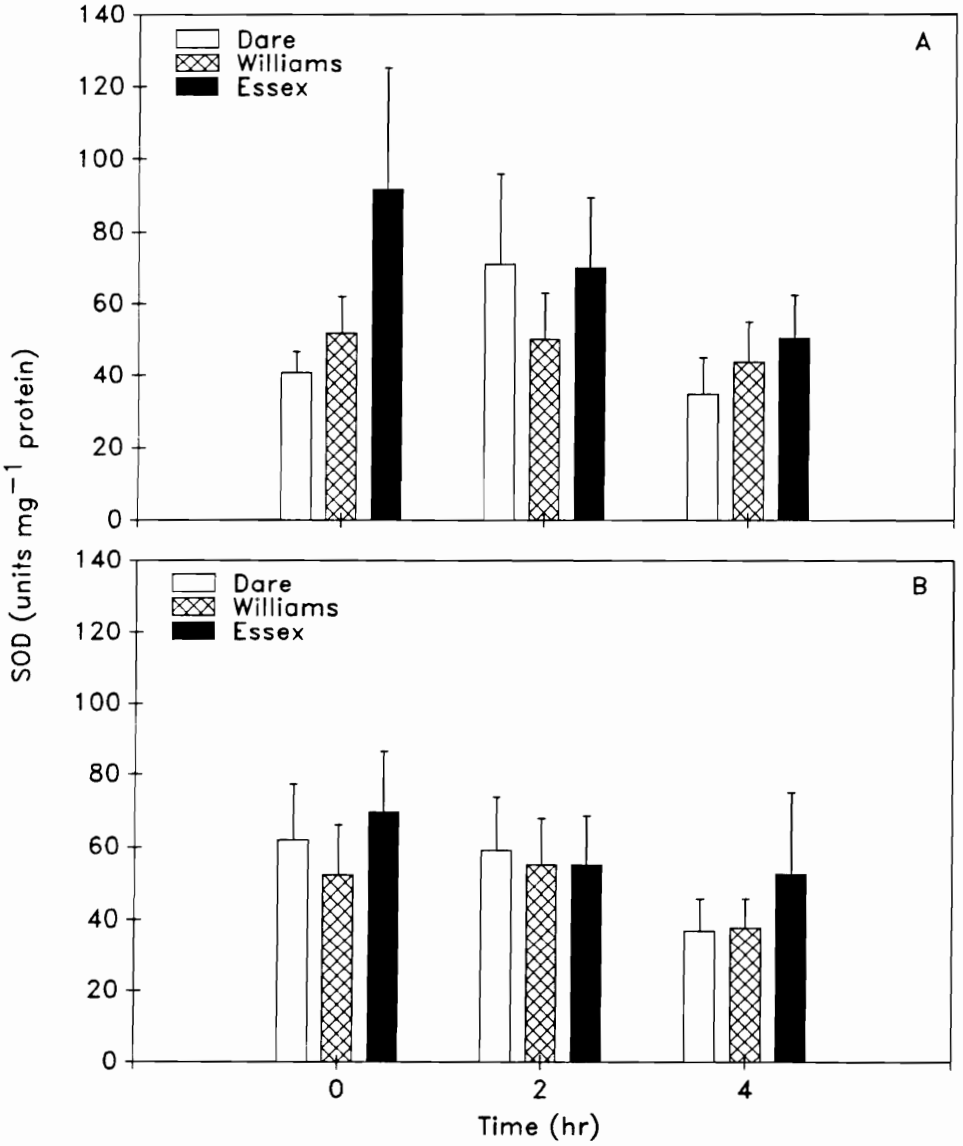

Fig. 23. Changes in superoxide dismutase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 or B) filtered air for 4 hr. Bars represent the mean ± SE of 4-5 samples.

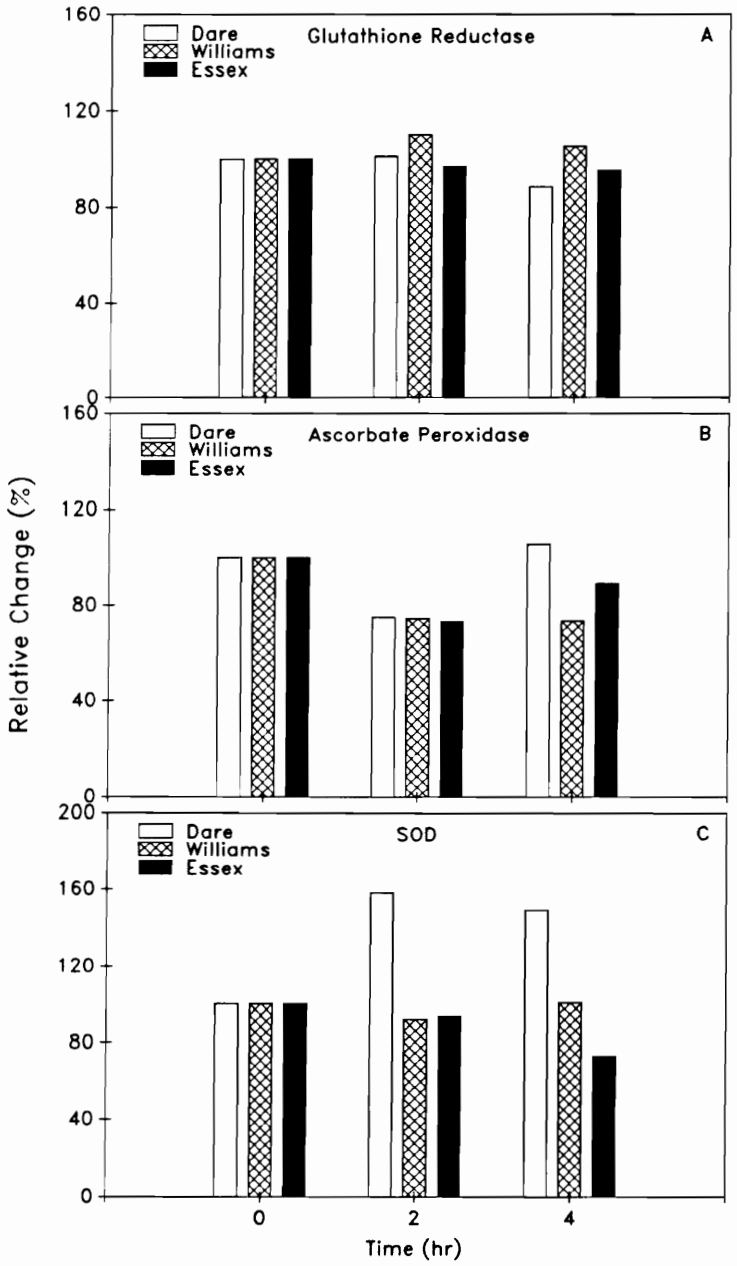

Fig. 24. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD total activities of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr relative to control plants. Relative changes represent mean differences of 4-9 samples.

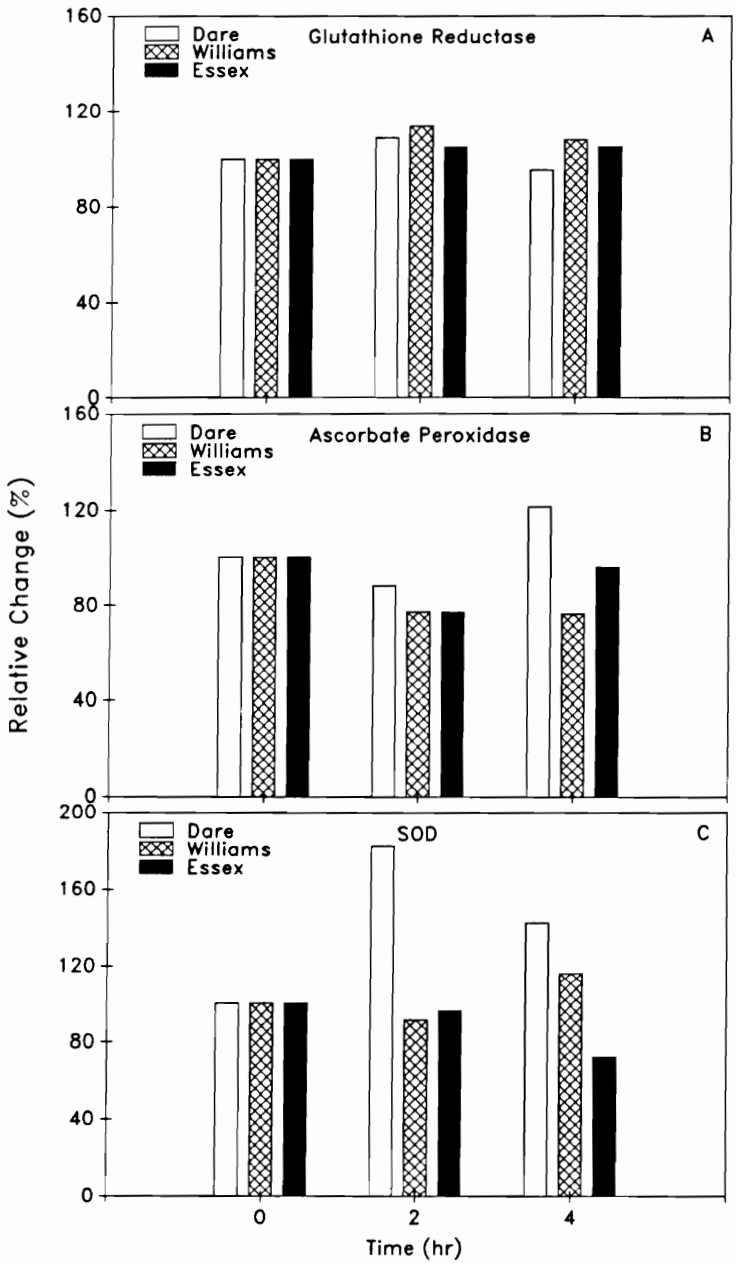

Fig. 25. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD specific activities of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 for 4 hr relative to control plants. Relative changes represent mean differences of 4-9 samples.

CHAPTER 4. RESPONSES OF GAS EXCHANGE AND THE ANTIOXIDANT SYSTEM OF SOYBEAN (*Glycine max* (L.) Merr.) CULTIVARS TO OZONE AND SULFUR DIOXIDE

Introduction

Formation of ozone through photochemical reactions and the generation of sulfur dioxide through industrial activities occur in our daily life. Each air pollutant has inhibitory effects on net photosynthesis, growth rate and productivity of many plants (Freer-Smith and Dobson, 1989; Reich *et al.*, 1986; Roper and Williams, 1989; Rowland-Bamford *et al.*, 1989; Saxe and Murali, 1989; Scherzer and McClenahen, 1989) when the concentrations reach a harmful threshold and environmental conditions are favorable for the uptake of the pollutants. It is estimated that the annual crop loss due to these pollutants is about 3 billion dollars (Agricultural Research, USDA, July 1989) and O_3 alone accounts for 15% of total crop losses a year. Because O_3 and SO_2 have different toxicity mechanisms to the plant tissues, it is conceivable that there would be synergistic effects to the tissues when both pollutants are present simultaneously. However, the severity of injury is dependent upon plant species, exposure regime and the concentrations of each pollutant. An antagonistic, additive or less than additive effect of O_3 plus SO_2 is possible (Heagle and Johnston, 1979).

Damage of tissue by O_3 is believed to be through formation of the superoxide radical ($O_2^{-\cdot}$) and/or hydroxyl radical ($\cdot OH$) after O_3 enters the leaf interior through open stomates. The $O_2^{-\cdot}$ and $\cdot OH$ could quickly damage cell membranes by inducing lipid peroxidation as well as oxidize sulfhydryl groups of many enzymes and thereby impair plant metabolism. On the other hand, sulfite (SO_3^{2-}), bisulfite (HSO_3^-) or sulfate (SO_4^{2-}) can be formed after SO_2 is taken up into the tissues. The acidic byproducts of SO_2 were believed earlier to be responsible for tissue damage (Asada *et al.*, 1968; Silvius *et al.*, 1975; Ziegler, 1975); however, the involvement of superoxide radical and active oxygen ($^1O_2$) in SO_2 toxicity is believed now to be the primary mode of action according to the latest evidence (Shimazaki *et al.*, 1980; Tanaka and Sugahara, 1980; Tanaka *et al.*, 1982; Tanaka *et al.*, 1988).

It is evident that gaseous pollutants can do extensive damage to plants. However, there are defense mechanisms existing in plants to minimize the harmful effects of foreign substances. Enzymes and metabolites form these defense systems: e.g. superoxide radicals are dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD); H_2O_2 is reduced to water (H_2O) and oxygen (O_2) by ascorbate and ascorbate peroxidase or by catalase; and SO_3^{2-} or HSO_3^- are metabolized into sulfur-containing amino acids, or other sulfur metabolites.

It is reasonable to believe that some plant species have higher activities or levels of these antioxidant enzymes and metabolites than others and, therefore, possess a greater defense capability against the gaseous pollutants (Guri, 1983; Lee *et al.*, 1984). This variation in defense systems might contribute to the

differential sensitivity existing among plant species, cultivars or individuals. Plants with high stomatal conductance are likely to be more sensitive to gaseous pollutants because of high influx (Dean, 1972; Engle and Gabelman, 1966; Winner and Mooney, 1980), although pollutant uptake might not totally account for differential sensitivity. Photosynthetic capacity of plants under a stress condition could be an indicator suggesting the presence of effective detoxifying mechanisms that maintain, or depress minimally, the photosynthetic process without causing a chain of inhibitory reactions. Researchers have found that the antioxidant system of enzymes and metabolites was stimulated by air pollutants and that the enzyme activities or metabolite concentrations increased in the resistant cultivars, but not in the sensitive ones (Alscher *et al.*, 1987; Guri, 1983; Lee *et al.*, 1984; Tanaka *et al.*, 1985).

When different cultivars of a plant species are stressed and show different degrees of injury, there must be some explanation genetically, physiologically or biochemically. At present, genetic factors are linked to the physical structure of the leaves, e.g. stomatal frequency, aperture or location, and are not included in the objectives of this present research. Previous research indicated that soybean cv Dare was highly sensitive (Howell *et al.*, 1979; Heagle, 1979), cv Williams moderately sensitive and cv Essex was moderately tolerant (Heagle and Letchworth, 1982) to O_3 based on visible injuries. No test for these cultivars was done to characterize the sensitivity to SO_2. When crops grow near industrial areas or under favorable environmental conditions, they might experience high concentrations of pollutants which could exceed the National Air Quality standards for short periods before dilution into the atmosphere.

The objectives of this research were to investigate how soybean cultivars with differential pollutant sensitivity responded to the short-term acute doses of O_3 and SO_2 physiologically and biochemically. These objectives were met by measuring the gas exchange rates of the leaves and by assaying the antioxidant enzyme activities and metabolite concentrations of the leaves collected during pollutant exposure.

Materials and Methods

Plant Material

Three soybean cultivars: Dare, Williams and Essex were used in these studies. Plants were grown and selected for fumigation as previously described in Chapter 2.

Pollutant Application

Prior to exposure, plants were acclimated overnight in the CSTR (Heck *et al.*, 1978) chamber and the environmental conditions were as described in Chapter 2.

Ozone was generated from pure O_2 (Industrial Gas & Supply Company, Radford, VA) by UV light using a Welsbach ozonator (Model T-408, Welsbach Ozone System Corp., Philadelphia, PA). Sulfur dioxide was supplied from a cylinder containing 1.7% SO_2 balanced in N_2 (Industrial Gas & Supply Company, Radford, VA). Both O_3 and SO_2 concentrations within a CSTR were controlled by O_3 and SO_2 mass flow controllers (Sierra Instruments, Inc., Carmel Valley, CA) and monitored with a UV photometric O_3 analyzer (Model 49, Thermo Electron Instruments, Hopkinton, MA) and a pulsed fluorescent SO_2 analyzer (Series 43, Thermo Electron Corp., Hopkinton, MA) which were calibrated every 2 weeks with a photocal 3000 ozone calibrator (Columbia Scientific Industries, Austin, TX) and a SO_2 calibrator (Model CS10-2, Meloy Labs, Springfield, VA), respectively.

Gas Exchange Measurements

A Li-Cor 6000 Portable Photosynthesis System (Li-Cor, Inc., Lincoln, NE) was used for gas exchange measurements. The procedures were described in Chapter 2. No further measurements were taken after the 4 hr fumigation. The data were analyzed using statistical methods presented in Chapter 2.

Metabolite and Enzyme Analyses

Collection of leaf samples prior to and during fumigation and the preparations for metabolite and enzyme analysis were conducted as described in Chapter 2.

Ascorbate Analysis

Ascorbate was analyzed by the methods of Watada (1982) and Lee *et al.* (1984) as described in Chapter 2.

Glutathione Analysis

Glutathione was analyzed by the methods of Brehe and Burch (1976) and Griffith (1980) as described in Chapter 2.

Protein Analysis

Protein was analyzed according to the method of Bradford (1976) as described in Chapter 2.

Glutathione Reductase Analysis

Glutathione reductase was analyzed by the method of Schaedle and Bassham (1977) as described in Chapter 2.

Ascorbate Peroxidase analysis

Ascorbate peroxidase activity was analyzed by the method of Peters *et al.* (1989) as described in Chapter 2.

Superoxide Dismutase Analysis

Superoxide dismutase activity was analyzed by the method of McCord and Fridovich (1969) as described in Chapter 2 under spectrophotometric assay.

Results

Foliar Response to O_3 and SO_2

All the cultivars exposed to O_3/SO_2 expressed injury on both side of the leaf surface; this was in contrast to O_3 injury which occurred only on the upper leaf surface. Intercostal lesions induced by SO_2 were seen occasionally in cvs Dare and Williams. The cv Essex had less SO_2-induced injury compared to cvs Dare and Williams. Approximately a 20% injury caused by O_3/SO_2 was found on all cultivars.

Gas Exchange

The physiological and biochemical characteristics of all cultivars prior to fumigation are shown in Table 1. These data were the average of plants from control and treated chambers. All of the physiological characteristics (Pn, Cs, Ci and Ts) were similar among cultivars except cv Essex had a significantly higher Pn (22.2 mg CO_2 dm^{-2} hr^{-1}) than cvs Dare and Williams (19.2 to 19.8 mg CO_2 dm^{-2} hr^{-1}). Because the computer-controlled O_3 and SO_2 concentrations required 30 min to reach the target levels, gas exchange measurements were first taken at 15 min after the initial addition of the pollutants.

The combination of O_3/SO_2 had an immediate effect on Pn of all cultivars even before reaching the designated concentrations (Fig. 1A). After a 2 hr fumigation with target levels, Pn declined to an equilibrium state which was not further inhibited by O_3/SO_2 as fumigation proceeded for another 2 hr. The Pn was similar among cultivars during the later 2 hr exposure (Fig. 1A). The cv Williams had a lower Pn while cv Essex had a higher Pn than cv Dare during the

first 2 hr exposure. However, by the end of the fumigation, Pn was reduced approximately 55% in all cultivars (Table 3). Control plants showed a relatively stable Pn throughout the 4 hr exposure (Fig. 1B) with a slight reduction of approximately 10% in all cultivars at the end of the treatment period (Table 2). The cv Essex had a consistently higher Pn than cvs Dare and Williams (Fig. 1B).

Inhibition of Cs by O_3/SO_2 was dramatic during the first hr of fumigation (Fig. 2A). The cv Williams exhibited the greatest response of Cs to O_3/SO_2, and cv Essex the least, in the first 15 min of treatment. The decline in Cs continued for 1 hr when an equilibrium rate was reached that persisted during the rest of the exposure period (Fig. 2A). Reduction of Cs by O_3/SO_2 at the end of 4 hr fumigation was approximately 80% in all cultivars (Table 3). The lower response of Cs in cv Essex, during the first hr, to O_3/SO_2 indicates a higher influx of both pollutants to the leaf interior. All cultivars showed a similar and steady Cs in response to filtered air (Fig. 2B) with approximate reductions of 20% in cvs Dare and Essex and of 30% in cv Williams (Table 2) at 4 hr.

In response to O_3/SO_2, Ci of all cultivars decreased immediately at the beginning of fumigation and continued to decline for about 3 hr until a steady state condition was attained (Fig. 3A). A slight, cyclic oscillation response of Ci in cvs Williams and Essex was observed during the fumigation. Throughout the 4 hr exposure to O_3/SO_2, Ci was reduced approximately 35% in cvs Dare and Essex and 30% in cv Williams (Table 3). All of the control plants showed a constant Ci during the 4 hr period (Fig. 3B) with a small reduction of approximately 10% in all cultivars (Table 2).

Reduction of Ts by O_3/SO_2 in all cvs, which followed closely the response of Cs, is shown in Figure 4A. The cv Essex had the least inhibited Ts in the first 0.5 hr of fumigation since it had the highest Cs during the same period (Fig. 2A). The approximate inhibition of Ts resulting from fumigation was 70% in all cultivars at the end of 4 hr period (Table 3). No major changes of Ts in any cultivars exposed to filtered air were observed (Fig. 4B), only a slight reduction of approximately 10% was noted after 4 hr (Table 2).

Flux and integrated dose of O_3 and SO_2, calculated according to Laisk *et al.* (1989) using the diffusivity ratio of 1.68 for H_2O/O_3 and Olszyk and Tingey (1986) using the diffusivity ratio of 1.89 for H_2O/SO_2, respectively, in all cultivars are shown in Figures 5 and 6. Lower and higher O_3 and SO_2 fluxes in cvs Williams and Essex, respectively, for the first 1 hr of fumigation (Figs. 5A, 6A) and lower and higher integrated O_3 and SO_2 doses in cvs Williams and Essex, respectively, during the exposure period than cv Dare (Figs. 5B, 6B) were observed. Because Cs of all cultivars was inhibited 80% by O_3/SO_2 after 1.5 hr of fumigation, both pollutant fluxes were reduced to an equilibrium rate of $\approx$ 10 and $\approx$ 22 nmol m^{-2} s^{-1} of O_3 and SO_2, respectively, at this time. In addition, the slopes of integrated pollutant doses after 0.5 hr, the time at which the designated concentrations and the peaks of flux of O_3 and SO_2 were reached, also decreased (Figs 5B, 6B). At the end of fumigation, cv Essex absorbed 9% and 25% more O_3 and SO_2 than cvs Dare (252 vs. 231 μmol m^{-2}; and 776 vs. 712 μmol m^{-2}) and Williams (252 vs. 201 μmol m^{-2}; and 776 vs. 619 μmol m^{-2}), respectively.

As Pn and Cs of all cultivars was inhibited by O_3/SO_2, residual conductance (Cr) to CO_2, calculated by the method of Jones (1985), was reduced

gradually for 3 hr and remained relatively constant afterwards, with the exception of a slight increase in cv Essex (Fig. 7A). The cv Essex maintained a higher Cr than cvs Dare and Williams during the exposure period. The control plants of all cultivars showed a constant Cr for the 4 hr period with cv Essex showing the higher Cr than cvs Dare and Williams (Fig. 7B).

Fractions of stomatal and non-stomatal contributions (σ_s and σ_{ns}, respectively) to the change in limitation of Pn showed that σ_s increased while σ_{ns} decreased for 1.5 hr after initial O_3/SO_2 exposure and then remained relatively constant throughout the fumigation period in cvs Dare and Essex (Fig. 8A-B). However, a dramatic, fluctuating change in σ_s and σ_{ns} of cv Williams was observed for the initial 3 hr of fumigation after which σ_s and σ_{ns} stabilized.

Water use efficiency (WUE) of all cultivars increased very slightly before the designated O_3 and SO_2 concentrations were attained, except for a substantial increase in cv Williams at 15 min (Fig. 9A). This increase was due to a much greater inhibition of Ts than Pn after beginning pollutant application. One hr after initiating fumigation, WUE increased dramatically in all cultivars and remained relatively constant to the end of the exposure, although cv Williams showed a greater fluctuation in WUE between 1 and 2.5 hr of fumigation than the other cultivars. The WUE was also lowest in cv Williams. The control plants showed a similar and constant WUE during the exposure period among all cultivars (Fig. 9B).

Metabolites

Endogenous enzyme activities and metabolite concentrations were generally consistent among cultivars, although some differences did exist. As shown in Table 1, cv Dare had a significantly higher GSSG content and SOD total activity and lower APase specific activity than cv Williams, and a lower AA content than cv Essex prior to fumigation. Although not statistically significant, cv Essex had a higher GSH concentration, GRase and APase total activities than cvs Dare and Williams.

After O_3/SO_2 fumigation, total glutathione, GSH and GSSG concentrations increased in all cultivars (Figs. 10A, 11A, 12A) with significant increases of total glutathione in cvs Dare (43%) and Williams (25%), of GSH in cv Dare (44%) and of GSSG in cvs Williams (77%) and Essex (59%) (Table 5). Control plants showed small increases (not statistically significant) of total glutathione, GSH and GSSG in the three cultivars except for a decrease of GSSG in cv Dare (Table 4; Figs. 10B, 11B, 12B). The ratio of GSH/GSSG decreased as a result of O_3/SO_2 exposure in all cultivars, except cv Dare which showed no change (Fig. 13A). In control plants, cv Dare showed an increase of GSH/GSSG (Fig. 13B). Relative to the control plants, total glutathione, GSH and GSSG all showed increases in all cultivars (Fig. 14A-C). This increase was especially dramatic in GSSG where concentration nearly doubled in cvs Dare and Williams.

The concentrations of total and reduced ascorbate increased slightly during O_3/SO_2 fumigation in all cultivars (Figs. 15A, 16A) with a higher increase of 16% AA in cv Essex (Table 5). Dehydroascorbate (dHAA) content was reduced significantly in cvs Williams (49%) and Essex (34%) due to fumigation,

with basically no change in cv Dare (Table 5; Fig. 17A). The ratio of AA/dHAA increased most at 2 hr and was slightly higher than the pre-fumigation ratio at the end of the pollutant exposure period in all cultivars (Fig. 18A). The control plants of all cultivars showed a slight increase in their total and reduced ascorbate concentrations (Table 4; Figs. 15B, 16B). In cv Essex, a decrease of dHAA level was observed (Table 4; Fig. 17B). The ratio of AA/dHAA showed no change in cvs Dare and Williams but increased dramatically in cv Essex at the end of 4 hr period (Fig. 18B). The O_3/SO_2 exposure resulted in small relative reductions of total and reduced ascorbate in cvs Williams and Essex. However, dHAA in cv Williams decreased about 60% compared to the control plants (Fig. 19A-C).

Protein

The protein concentration remained unchanged in cvs Dare and Williams but decreased somewhat in cv Essex after O_3/SO_2 treatment (Fig. 20A). There were very slight increases of protein concentration found in the three cultivars exposed to filtered air (Fig. 20B).

Enzymes

Total and specific GRase activities increased substantially in cv Essex while cv Williams showed a small increase in total GRase activity after O_3/SO_2 fumigation (Table 5; Figs. 21A, 22A). The control plants showed no change in GRase activity, except for an 18% decrease in specific activity in cv Williams (Table 4; Fig. 22B), due to a small increase in protein concentration (Fig. 20B). As shown in Figures 27A and 28A, GRase activities of all cultivars were stimulated by O_3/SO_2 relative to the control plants.

Ascorbate peroxidase activity of all cultivars showed little change resulting from O_3/SO_2 exposure, except in cv Essex, which had a 23% increase in APase specific activity (Table 5; Figs. 23A, 24A). There was an increase of APase activity in cv Dare and a small decrease of APase specific activity in cv Williams exposed to filtered air (Table 4; Figs. 23B, 24B). Relative to the control plants, APase activity was not affected by O_3/SO_2 exposure, except for a 30% increase in APase specific activity in cv Essex (Figs. 28B, 29B).

Stimulation of SOD activities by O_3/SO_2 fumigation was observed in all cultivars with a higher increase (44% vs. 25~33% in total activity; 80% vs. 23~32% in specific activity) in cv Essex compared to cvs Williams and Dare (Table 5; Figs. 25A, 26A). However, control plants of all cultivars showed an even greater increase of SOD activities at the end of exposure with a higher response (100% vs. 32~58% in total activity; 74% vs. 24~49% in specific activity) in cv Williams (Table 4; Figs. 25B, 26B). As compared to control plants, cv Dare showed no change in SOD activity, cv Williams showed a 36% and 24% reduction in SOD total and specific activity, respectively, and cv Essex showed a 20% increase in SOD specific activity (Figs. 27C, 28C).

Discussion

Even though SO_2 did not result in typical injury on the soybean cultivars all the time, it certainly enhanced the injury caused by O_3. The damage on the upper leaf surface was typical of O_3 injury (stippling) while the injury on the lower surface could be due to O_3 with SO_2 facilitation. Hofstra and Ormrod (1977) observed that SO_2 modified the O_3 injury in white bean cv Sanilac, and explained that a delayed injury development identical to O_3 injury in soybean cv Harsoy exposed to 0.15 $\mu l\ l^{-1}$ O_3 and 0.075~0.6 $\mu l\ l^{-1}$ SO_2, was due to SO_2 protection of the leaves. Beckerson and Hofstra (1979) also observed the protective action of SO_2 against O_3 injury. However, Applegate and Durrant (1969), Menser and Heggestad (1966) and Macdowall and Cole (1971) reported a synergistic action of O_3 and SO_2 on foliar injury of peanuts and tobacco plants. The ratio of O_3/SO_2 might determine the symptom expressed (Middleton *et al.*, 1958). The injury in all cultivars caused by O_3/SO_2 was less than the additive injury of the single gases (20% vs 25% by O_3 or 20% by SO_2). Apparently, injury development caused by single or mixed gases was dependent on pollutant concentrations and plant species, some showed more injury from mixed gases than the additive response of single gases, and some showed otherwise (Tingey *et al.*, 1973). These results could be due to different effects of each gases on either stomatal function or physiological or metabolic processes (Jacobson and Colavito, 1976).

Although cv Essex had a significantly higher net photosynthetic rate (Pn) than cvs Dare and Williams prior to O_3/SO_2 exposure, the inhibition of Pn in the first 15 min was similar among cultivars with cv Essex showing the least

suppression (Fig. 1A). However, after 1.5 hr of fumigation, cv Essex did not maintain the highest Pn among cvs, rather Pn was inhibited to the same level as cv Dare, which resulted in a greater reduction of Pn in cv Essex relative to its pre-fumigation rate. At the end of 4 hr fumigation, however, Pn of all cultivars was suppressed to a similar level of $\approx$ 55%.

To compare the effects of O_3 and SO_2 on Pn of all cultivars, it is recognized that in combination, O_3/SO_2 inhibited Pn greater than each pollutant alone, but less than additive inhibition (refer to Chapters 2 and 3). Jensen and Noble (1984) found that 0.5 μl l^{-1} SO_2 and 0.25 μl l^{-1} O_3 fumigation reduced Pn in hybrid poplar greater than 0.5 μl l^{-1} SO_2 treatment. There was no synergistic effects of O_3 and SO_2 found in our studies. Ormrod *et al.* (1982) reported a maximum reduction of Pn (20-30%) of field bean (*Vicia faba*, cv Dylan) by 0.3 μl l^{-1} O_3 and 0.04 μl l^{-1} SO_2 exposure for 4 hr which was less than additive.

Sulfur dioxide and O_3 together exerted a greater impact on stomatal conductance (Cs) than either O_3 or SO_2 alone, which is in agreement with the results of Beckerson and Hofstra (1979). This consequence affected not only pollutant fluxes, but also H_2O and CO_2 exchange rates, which in turn affected Pn and transpiration (Ts) and thus, water use efficiency (WUE). The higher Cs (Fig. 2A) and higher O_3 and SO_2 fluxes (Figs. 5A, 6A) in cv Essex in the first 0.5 hr of exposure might have contributed to the greater reduction in Pn at 1.5 hr after fumigation (Fig. 1A). Black *et al.* (1982) found that epidermal and guard cells of *Vicia faba* cv Dylan were damaged by SO_2. Stomatal opening induced by low SO_2 concentration results from loss of turgor in the epidermal cells, while stomatal closure by higher SO_2 concentration is due to damaged guard cells. Atkinson and

Winner (1989) reported that 0.08 μl l^{-1} SO_2 exposure, resulted in changes in Cs first followed by changes in Pn of alder (*Alnus serrulata*), can affect guard cells directly, although the authors only observed 1 out of 4 replicates that showed the preceding changes in Cs. However, it is not known how rapidly SO_2 can injure the guard cells, and stomatal closure does not necessarily indicate guard cell damage.

The intercellular CO_2 concentration (Ci) appeared to respond correspondingly with Cs in the first hr of fumigation (Figs. 3A, 2A) and with Pn thereafter (Figs. 3A, 1A). The ability to maintain a constant ratio of Ci to ambient CO_2 concentration in plant cells (Wong *et al.*, 1979) was evidently impaired to some degree by O_3/SO_2 fumigation since Ci decreased at constant ambient CO_2 during the fumigation period. Transpiration (Ts) showed a similar response to O_3/SO_2 (Fig. 4A) as Cs and the greater reduction in Ts (due to substantial inhibition of Cs) than Pn led to the increase in WUE of all cultivars by O_3/SO_2 (Fig. 9A).

Both O_3 and SO_2 fluxes and integrated doses in all cultivars (Figs. 5, 6) were much less in the combined fumigation compared to fumigation with each pollutant alone (Chapters 2 and 3: Fig. 5). This difference in doses could contribute to the less than additive effects of O_3/SO_2 on gas exchange which were nevertheless greater than the effects of each individual pollutant. However, Olszyk and Tingey (1986) reported that pea plants (*Pisum sativum* cv Alsweet) exposed to O_3 and SO_2 simultaneously had lower O_3 and higher SO_2 fluxes and a greater total dose than with the single pollutants. The authors used a much lower concentrations of O_3 (0.11 μl l^{-1}) and SO_2 (0.12 μl l^{-1}) compared to our study. Because O_3/SO_2 inhibited Cs of all cultivars so quickly, the ambient

concentrations of O_3 and SO_2 at their peak flux were equivalent to ≈ 0.095-0.14 and 0.33-0.48 $\mu l\ l^{-1}$, respectively, at a Cs of 1 cm s^{-1}. It is more meaningful to express both pollutant concentrations and fluxes, even if estimated, imposed on plants for comparison among fumigation regimes and plant responses.

At the equilibrium conditions which occurred 2.5 hr after fumigation, plant tissues could endure and metabolize the products of O_3/SO_2 produced in the leaves even if some physiological and biochemical changes already had been induced during the initial 2.5 hr fumigation period. Under this circumstance, when the plants maintained their physiological and biochemical functions at equilibrium rates, there should be no additional effects on gas exchange properties by the pollutants. This condition would be satisfied if plants were exposed to 0.04 $\mu l\ l^{-1}$ O_3 (flux = 7.1~7.4 nmol $m^{-2}\ s^{-1}$) and 0.1 $\mu l\ l^{-1}$ SO_2 (flux = 22~22.9 nmol $m^{-2}\ s^{-1}$), which would be the ambient O_3 and SO_2 concentrations at steady state if Cs = 1 cm s^{-1}.

The calculated, integrated dose of O_3 and SO_2 experienced by the plant tissues increased sharply in the first 0.5 hr and more slowly during the rest of fumigation period (Figs. 5B, 6B) coinciding with the sharp decrease of pollutant flux that occurred after 0.5 hr and the gradual decline to a very low and steady flux throughout the remainder of the exposure period (Figs. 5A, 6A). Apparently, the dose of O_3 and SO_2 absorbed in the first 0.5 hr of fumigation is most responsible for changes observed in the physiological and biochemical processes.

Residual conductance (Cr) to CO_2 (Fig. 7A) mainly reflects the net photosynthetic rate since an efficient CO_2 fixation system can generate a large driving force for CO_2 diffusion. The decline in Cr through the fumigation period

indicates that the CO_2 fixation process or electron transport was inhibited by O_3/SO_2. More definitive studies on Pn/Ci and Pn/light curves would be necessary to determine pollutant effects on electron transport, rubisco activity and ribulose bisphosphate regeneration.

It is interesting to see changes in the fraction of stomatal (σ_s) and non-stomatal (σ_{ns}) contributions to the changes in limitation of Pn in response to O_3/SO_2 of all cultivars (Fig. 8). Differences in σ_s and σ_{ns} among cvs, observed when the designated O_3 and SO_2 concentrations were reached, might reflect early differential responses to the pollutants. In this instance, mesophyll function responded rapidly in cv Williams (Fig. 8B), reflecting the decrease in residual conductance (Fig. 7A). In spite of the fluctuations during fumigation, σ_s and σ_{ns} were relatively constant among cultivars indicating an altered, but stabilized and balanced, physiological reaction as a result of O_3/SO_2 exposure.

The increases of glutathione, either total (Fig. 10A), reduced (Fig. 11A) or oxidized (Fig. 12A) form, in all cultivars could suggest that the biosynthesis of the metabolite was increased which might be triggered by the oxidation of GSH to GSSG (Smith, 1985; Smith *et al.*, 1985) and/or the enzymes involved in the oxidation-reduction cycle were partially inhibited by the O_3/SO_2 fumigation. The increase of GSSG was due to oxidation of GSH which would suggest H_2O_2 production during O_3/SO_2 fumigation. However, the large increase of GSSG occurred only at 2 hr then slightly declined at 4 hr period (Fig. 14C). This indicated that the biochemical process(es) being affected greatly by O_3/SO_2 at 2 hr period of fumigation was possibly a delay response since gas exchange (Figs. 1A, 2A) and pollutant flux (Figs. 5A, 6A) were at their minimum and equilibrium

rate at 2 hr. The decline of GSSG in cvs Williams and Essex (Fig. 14C) at 4 hr would suggest a partial repair mechanism of the biochemical process.

As Pn is inhibited, the generation of $O_2^{\cdot -}$ would be inevitable during the influx of O_3 and SO_2. A non-enzymatic reaction between $O_2^{\cdot -}$ and GSH could occur as could the reaction of H_2O_2 and/or other peroxides produced from substantial light-stimulated oxidation (Bielawski and Joy, 1986a) [although a reaction between GSH and H_2O_2 in spinach catalyzed by a component similar to glutathione peroxidase was reported (Flohé and Menzel, 1971)]. Presumably SOD would dismutate $O_2^{\cdot -}$ to form H_2O_2 and H_2O_2 would be removed through the scavenging system of GSH/AA.

The effect of O_3/SO_2 exposure on glutathione was similar to that of SO_2 alone (Chapter 3: Figs. 9A, 10A, 11A), namely, an increase in GSH and GSSG while 0.2 μl l^{-1} O_3 had no effect on glutathione. However, the effects on total glutathione or GSH resulting from O_3/SO_2 exposure were less than SO_2 alone in cvs Williams and Essex, but similar in cv Dare. Oxidized glutathione (GSSG) response of all cultivars to either SO_2 alone or O_3/SO_2 in combination was similar at 2 hr after fumigation was initiated. However, a much greater increase of GSSG in response to SO_2 alone than to O_3/SO_2 was found at the end of the 4 hr exposure (approximately 270~360% vs 150% relative to the control). Tanaka *et al.* (1985) reported that ascorbate and GSH of spinach plants were reduced by 0.3 μl l^{-1} O_3 but not by 1 μl l^{-1} SO_2 for 20 hr. It is possible that ascorbate and GSH will respond differently under the combined stress of 0.3 μl l^{-1} O_3 and 1 μl l^{-1} SO_2. Fir and spruce exposed to SO_2 showed a higher increase of glutathione and vitamin E than those exposed either to O_3 or filtered air, and a combination of

O_3/SO_2 gave the maximum response of these antioxidant metabolites (Mehlhorn *et al.*, 1986). Apparently, the damaging mechanisms to plant tissue were different between O_3 and SO_2 to induce different glutathione responses; the different effective dosage of O_3 or SO_2 also could have contributed to the response differences (Mehlhorn *et al.*, 1986). In the O_3/SO_2 combined exposure of soybean cultivars, the response to SO_2 dominated which indicated that either the SO_2 effects were sufficient to mask any O_3 effects or that O_3 was actually enhancing the response caused by SO_2.

The greater increase of GSSG than GSH (Fig. 14B, C) resulted in a decreased GSH/GSSG ratio in O_3/SO_2 treated plants (Fig. 13A) except in cv Dare. The GSH/GSSG ratio might indicate the physiological status of plants under any variety of oxidative stresses. With catalase inhibitors, e.g. aminotriazole, glutathione synthesis in photorespiring plants was stimulated and most of the increase was observed as GSSG (Smith, 1985). Obviously, O_3/SO_2 exposure did not impose an inhibitory effect on catalase activity since the increase of GSSG was a relatively small portion of the total glutathione increase.

The observed increases of total and reduced ascorbate in control plants (Table 4; Figs. 15B, 16B) would suggest a new biosynthesis of AA, although no change in ascorbate was found in control plants in the O_3 (Chapter 2: Table 4; Figs. 14B, 15B; except an increase of AA in cv Essex) or SO_2 studies (Chapter 3: Table 4; Figs 14B, 15B). The decrease of dehydroascorbate (dHAA) in cv Essex (Table 4; Fig. 17B) could occur through decomposition of dHAA to form oxalic acid (Foyer *et al.*, 1983). Using isolated spinach chloroplasts under illumination, the ascorbate pool was found to decline slowly with time and this decline did not

relate to free radical production even under a low or zero CO_2 condition, which favors $O_2^{\cdot -}$ and H_2O_2 production (Foyer *et al.*, 1983). However, the illumination time in this experiment was only 6 min which may reflect only the early response of ascorbate to oxidative conditions. Our observations showed that relative to the control plants, there was approximately a 17% reduction of both total and reduced ascorbate in cv Essex exposed to O_3/SO_2 (Fig. 19A-B). This could be due to an increased formation of H_2O_2 from dismutation of $O_2^{\cdot -}$ by increased SOD specific activity (Fig. 28C).

The decline of dHAA in cvs Williams and Essex exposed to O_3/SO_2 (Table 5; Fig. 17A) might have resulted not from the reduction by GSH to AA, but from decomposition to oxalic acid, since the actual quantity of dHAA was too small to be accounted for by the increase in AA (Fig. 16A). Relative to the control plants, however, the decline of dHAA was observed among all cultivars at 2 hr and recovery of dHAA level was found in cvs Dare and Essex but not in cv Williams (Fig. 19C) at the end of the 4 hr fumigation. It was possible that the turnover rate of dHAA was enhanced by O_3/SO_2 at 2 hr and the sensitivity of that process to O_3/SO_2 among cultivars was different.

There is a lack of reports concerning antioxidant metabolite changes in plants exposed to O_3/SO_2 in combination, the exception being the work of Mehlhorn *et al.*,(1986) with spruce. However, changes were found in GSH or AA of plants exposed to either O_3 or SO_2 singly (Alscher *et al.*, 1987; Chiment *et al.*, 1986; Grill *et al.*, 1979; Guri, 1983; Lee *et al.*, 1984; McKersie *et al.*, 1982; Tanaka *et al.*, 1985). Due to different plant species, fumigation regimes and environmental conditions used, these experimental results were not consistent

with each other. Nevertheless, the authors believed that either GSH and/or ascorbate play important roles in protecting plants against oxidant damages.

Ozone or SO_2 were reported to affect glutathione reductase (Tanaka *et al.*, 1982; Tanaka *et al.*, 1988), ascorbate peroxidase (Castillo and Greppin, 1986; Tanaka *et al.*, 1982; Tanaka *et al.*, 1985) and superoxide dismutase (Tanaka and Sugahara, 1980) activities. Guri (1983) and Castillo and Greppin (1988) reported that O_3 did not affect glutathione reductase and Matters and Scandalios (1987) demonstrated that neither O_3 nor SO_2 significantly increased superoxide dismutase activity. However, sensitivity of plant cultivar and concentrations of pollutant might alter the response of enzymes. There are no reports of combined O_3 and SO_2 effects on these antioxidant enzymes.

Significant increase of total or specific activity of glutathione reductase (GRase) in cv Essex in response to O_3/SO_2 fumigation (Table 5; Figs. 21A, 22A, 27A, 28A) could facilitate the AA-GSH system to sustain a reduced state of AA and GSH under oxidant stress. Isozymes of GRase has been demonstrated in pea roots (Bielawski and Joy, 1986b) and in spinach (Guy and Carter, 1984 cited in Smith *et al.*, 1989), but no substantial information on which isozyme is important to the plant under oxidant stress is provided. The increase of GRase activity could be stimulated either specifically by its substrate, GSSG, or by a general stimulation of protein synthesis (Tanaka *et al.*, 1988). However, protein concentration was reduced somewhat in cv Essex, and essentially unchanged in the other cultivars, after O_3/SO_2 exposure (Fig. 20A). Apparently, induction of GRase activity in all cultivars occurred through substrate stimulation or through some other mechanism responding to oxidative pressure.

Hydrogen peroxide can be generated in different organelles like chloroplasts (in the light), peroxisomes and mitochondria and also in the cytoplasm. Either catalase or the GSH-AA system can decompose and scavenge H_2O_2 to non-toxic products. In animals, glutathione peroxidase (GPase) functions as a H_2O_2 or other peroxides scavenger, but the presence of GPase in plants is uncertain and reports of finding GPase or GPase-like activity in algae or higher plants are scattered (Flohé and Menzel, 1971; Jablonski and Anderson, 1984; Overbaugh and Fall, 1985; Yokota *et al.*, 1988). Nevertheless, GRase is the rate limiting enzyme in GSH-AA system to scavenge H_2O_2 (Jablonski and Anderson, 1981) and is required to regenerate GSH (Smith *et al.*, 1989).

Ascorbate peroxidase (APase) activity was not affected by O_3/SO_2 fumigation except in cv Essex (Figs. 27B, 28B). Exposure of *Sedum album* to 0.4 $\mu l\ l^{-1}$ O_3 for 2 hr showed a greater increase of extracellular peroxidase than in other compartments and the increase was linked to new synthesis of protein (Castillo *et al.*, 1984). Castillo and Greppin (1986) separated the extracellular peroxidases into anionic and cationic forms and demonstrated that the cationic form increased in response to O_3. Ascorbate peroxidase activity was stimulated after 0.4 $\mu l\ l^{-1}$ O_3 exposure for 2 hr, but returned to near control level 24 hr after O_3 fumigation. It is possible that due to varied plant responses, APase of soybean cultivars might show post-fumigation responses. However, O_3 and O_3/SO_2 induced APase activity in cv Essex (Chapter 2: Figs. 22A, 23A, 26B, 27B), whereas SO_2 inhibited APase activity in cv Williams (Chapter 3: Figs. 20A, 21A, 24B, 25B).

Superoxide dismutase (SOD) activity increased in both control and treated plants (Tables 4-5; Figs. 25-26), however, relative changes were a decrease in cv Williams, increase in cv Essex and no change in cv Dare (Figs. 27C, 28C). According to Gillham and Dodge (1987), changes in light intensity had no effect on SOD activity of pea plants. However, the pea plants were grown either in low (100 μE m^{-2} s^{-1} PAR) or high (400 μE m^{-2} s^{-1} PAR) light intensity continuously until analyzed. Soybean cultivars were grown in a glasshouse with a PAR of 900 ± 100 μE m^{-2} s^{-1} for 3 weeks and were moved to CSTR chamber with a PAR of 550 ± 100 μE m^{-2} s^{-1}. Given the time for light adaptation, SOD activity might display some changes during the exposure period. Not all the control plants of O_3, SO_2 or O_3 plus SO_2 fumigation showed similar responses (Chapter 2: Tables 4-5; Figs. 24-25; Chapter 3: Tables 4-5; Figs. 22-23). One possible reason is that the SOD activity assay was quite variable when using desalted plant materials due to interfering substances. A more reliable analysis would be native gel electrophoresis and subsequent densitometer scans.

Production of $O_2^{\cdot -}$ radicals from O_3/SO_2 fumigation or through the Mehler reaction could stimulate SOD activity. Lee and Bennett (1982) found that EDU [N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea] also induced SOD activity in snapbeans to prevent O_3 injury , but Chanway and Runeckles (1984a) claimed that EDU protected bush bean against acute O_3 injury with a mechanism not involving SOD. The increased SOD activity was correlated with the appearance of visible symptoms caused by either cumulative chronic or acute O_3 exposure (Chanway and Runeckles, 1984b).

The activity of SOD decreased from a high level in young leaves showing no O_3 injury to a lower level in mature leaves which showed increased O_3 damage (Lee and Bennett, 1982). On the contrary, an O_3-sensitive cultivar of *Phaseolus vulgaris*, 'Seafarer', exposed to 0.4 μl l^{-1} O_3 for 4 hr displayed an increased tolerance to O_3, a decreased visual injury and a declined SOD activity in the primary leaves between 8 and 18 days of age (McKersie *et al.*, 1982). The same authors also analyzed SOD activity in several *Phaseolus vulgaris* cultivars with differential O_3 sensitivity and found no significant, positive correlation between O_3 tolerance and SOD activity (McKersie *et al.*, 1982). Bennett *et al.* (1984) regarded the findings as skeptical and possibly due to chemical interferences resulting from the crude enzyme extracts used.

Obviously a great variation of SOD activity exists among different plant species and cultivars in response to air pollutants which has resulted in some conflicting findings. In our results, relative to control plants, SOD activity declined in cv Williams (Figs. 27C, 28C). This could result from inhibition directly or indirectly, through increased H_2O_2, by O_3/SO_2 exposure. The increase of SOD specific activity in cv Essex (Fig. 28C), again, could be due to a decreased protein concentration.

In conclusion, the soybean cultivars used in these experiments responded differently at the biochemical and physiological level to O_3/SO_2 fumigation despite the similarity of symptoms that occurred on the leaves. It appeared, that in combination, 0.2 μl l^{-1} O_3 and 0.7 μl l^{-1} SO_2 quickly and powerfully inhibited the gas exchange system and stimulated the GSH and GSSG levels in all cultivars. Although all cultivars showed an increased GRase activity relative to control

plants, the greatest response was in cv Essex. Such a response could lead to better protection against oxidation of SH-compounds like sulfhydryl-containing enzymes, and peroxidation of lipids. However, cv Essex did not demonstrate any better tolerance towards O_3 and SO_2 exposure, compared to cvs Williams and Dare, judging from injury, Pn and metabolites of the antioxidant system. Perhaps the concentrations of O_3 and SO_2 used in these experiments were sufficiently high to overwhelm the oxidative defense system even in a tolerant cultivar.

Due to the same sampling time for antioxidant metabolites and enzymes there was lack of information to establish the response sequence to pollutant stress. As reported by Schmidt and Kunert (1986), increased antioxidant levels was a primary event followed by an increased activity of the antioxidant recycling enzymes. It was suggested that GRase activity was determined by NADPH and GSSG (Mata *et al.*, 1985). The decline in AA in cv Essex, SOD activity in cv Williams and the increased specific activity of APase and SOD in cv Essex suggested that these metabolites or enzymes might be important besides glutathione and GRase to this cultivar. Other metabolites or enzymes which showed no changes to O_3/SO_2 exposure could be less sensitive and capable of defending against oxidative stress or the absorbed O_3 and SO_2 doses were not high enough to trigger effects. Additionally, these metabolites and enzymes may simply play a less important and secondary role in the detoxification of O_3 and SO_2 than glutathione and GRase. It is also possible that O_3/SO_2 fumigation inhibited the synthesis or turnover processes for these metabolites and enzymes to result in a no net change in concentration or activity.

To establish a further understanding of gas exchange and antioxidant responses to O_3 plus SO_2 in soybeans, more research is needed in conducting different fumigation regimes in terms of lower pollutant concentrations, and decreased time intervals of consecutive sampling periods for analysis. Such studies can reveal threshold concentrations and exposure periods for inducing responses and how gas exchange processes are coupled to the changes of antioxidant metabolites and enzymes. Furthermore, longer term exposures at lower pollutant concentrations are needed to understand the effects of chronic pollutant exposure.

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Table 1. Physiological and biochemical characteristics of three soybean (*Glycine max* (L.) Merr.) cultivars grown under glasshouse conditions and acclimated in CSTR chambers for 16 hr. (See text for details).

	Cultivar		
Variable	Dare	Williams	Essex
Pn ($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	19.8 ± 1.8^{aB} $(n=12)^{b}$	19.2 ± 2.2^{B} $(n=12)$	22.2 ± 1.8^{A} $(n=12)$
Cs ($cm\ s^{-1}$)	0.97 ± 0.24^{A} $(n=12)$	$1.00 \pm .35^{A}$ $(n=12)$	0.98 ± 0.22^{A} $(n=12)$
Ci (ppm)	269.3 ± 34.3^{A} $(n=12)$	271.4 ± 40.9^{A} $(n=12)$	262.6 ± 37.4^{A} $(n=12)$
Ts ($mmol\ m^{-2}\ s^{-1}$)	3.8 ± 0.5^{A} $(n=12)$	3.9 ± 0.6^{A} $(n=12)$	3.9 ± 0.4^{A} $(n=12)$
$Glutathione_{tot}$ ($\mu mol\ g^{-1}\ fw$)	1.07 ± 0.34^{A} $(n=19)$	1.10 ± 0.28^{A} $(n=18)$	1.16 ± 0.33^{A} $(n=19)$
$Glutathione_{ox}$ ($\mu mol\ g^{-1}\ fw$)	0.06 ± 0.04^{A} $(n=19)$	0.04 ± 0.01^{B} $(n=18)$	0.05 ± 0.01^{AB} $(n=19)$
$Glutathione_{red}$ ($\mu mol\ g^{-1}\ fw$)	0.95 ± 0.33^{A} $(n=19)$	1.02 ± 0.27^{A} $(n=18)$	1.07 ± 0.32^{A} $(n=19)$
$Ascorbate_{tot}$ ($\mu g\ g^{-1}\ fw$)	1082 ± 192^{B} $(n=13)$	1217 ± 150^{A} $(n=12)$	1326 ± 150^{A} $(n=13)$
$Ascorbate_{ox}$ ($\mu g\ g^{-1}\ fw$)	108 ± 58^{A} $(n=13)$	124 ± 45^{A} $(n=12)$	114 ± 68^{A} $(n=13)$
$Ascorbate_{red}$ ($\mu g\ g^{-1}\ fw$)	975 ± 223^{B} $(n=13)$	1093 ± 162^{AB} $(n=12)$	1212 ± 157^{A} $(n=13)$

(Cont.)

(Table 1 continued)

Variable	Cultivar		
	Dare	Williams	Essex
Glutathione Reductase ($U\ g^{-1}$ fw)	0.75 ± 0.15^{A} (n=10)	0.71 ± 0.10^{A} (n=10)	0.79 ± 0.20^{A} (n=10)
Glutathione Reductase ($U\ mg^{-1}$ protein)	$.024 \pm .004^{A}$ (n=10)	$.028 \pm .003^{A}$ (n=10)	$.028 \pm .004^{A}$ (n=10)
Ascorbate Peroxidase ($U\ g^{-1}$ fw)	1.15 ± 0.11^{A} (n=10)	1.15 ± 0.15^{A} (n=10)	1.18 ± 0.22^{A} (n=10)
Ascorbate Peroxidase ($U\ mg^{-1}$ protein)	$.038 \pm .004^{B}$ (n=10)	$.045 \pm .004^{A}$ (n=10)	$.043 \pm .011^{AB}$ (n=10)
SOD ($U\ g^{-1}$ fw)	33.1 ± 14.3^{A} (n=10)	19.5 ± 10.2^{B} (n=10)	24.0 ± 7.9^{AB} (n=10)
SOD ($U\ mg^{-1}$ protein)	1.07 ± 0.40^{A} (n=10)	0.77 ± 0.40^{A} (n=10)	0.87 ± 0.30^{A} (n=10)

a: Mean ± sd.

b: Sample size.

A or B: Means across cultivars with the same letter are not statistically different ($\alpha = 0.05$).

Table 2. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the control (<0.030 μl l^{-1} SO_2 & 0.025 μl l^{-1} O_3) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	20.1±2.2[aA]	18.9±2.9[A]	22.6±2.3[A]
(mg CO_2 dm^{-2} hr^{-1})	4	17.7±1.7[A]	16.9±1.5[A]	19.7±2.4[B]
		(-12 %)[b]	(-11 %)	(-13 %)
Cs	0	0.99±0.32[A]	1.03±0.49[A]	0.97±0.31[A]
(cm s^{-1})	4	0.76±0.15[A]	0.70±0.27[A]	0.78±0.15[A]
		(-23 %)	(-32 %)	(-20 %)
Ci	0	263.8±39.0[A]	267.1±49.7[A]	254.4±45.9[A]
(ppm)	4	236.9±30.0[A]	233.6±38.7[A]	228.0±33.9[A]
		(-10 %)	(-13 %)	(-10 %)
Ts	0	3.8±0.6[A]	3.9±0.7[A]	3.8±0.5[A]
(mmol H_2O m^{-2} s^{-1})	4	3.5±0.4[A]	3.3±0.8[A]	3.4±0.5[A]
		(-8 %)	(-15 %)	(-11 %)

a: Mean ± sd.

b: Percentage change from time 0.

A or B: Means within cultivar with the same letter are not statistically different (α=0.05).

Table 3. Changes in physiological variables in three soybean cultivars during a 4 hr treatment in the fumigated ($0.70\pm0.05\ \mu l\ l^{-1}\ SO_2 + 0.20\pm0.02\ \mu l\ l^{-1}\ O_3$) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Pn	0	19.4 ± 1.3^{aA}	19.5 ± 1.4^{A}	21.8 ± 1.2^{A}
($mg\ CO_2\ dm^{-2}\ hr^{-1}$)	4	8.5 ± 1.1^{B}	8.7 ± 1.7^{B}	10.1 ± 2.2^{B}
		(-56 %)[b]	(-55 %)	(-54 %)
Cs	0	0.96 ± 0.17^{A}	0.97 ± 0.21^{A}	1.00 ± 0.09^{A}
($cm\ s^{-1}$)	4	0.16 ± 0.05^{B}	0.18 ± 0.04^{B}	0.19 ± 0.06^{B}
		(-83 %)	(-81 %)	(-81 %)
Ci	0	274.8 ± 31.5^{A}	275.7 ± 34.3^{A}	270.7 ± 28.6^{A}
(ppm)	4	175.7 ± 18.1^{B}	196.4 ± 13.2^{B}	176.0 ± 21.5^{B}
		(-36 %)	(-29 %)	(-35 %)
Ts	0	3.77 ± 0.45^{A}	3.90 ± 0.57^{A}	3.88 ± 0.24^{A}
($mmol\ H_2O\ m^{-2}\ s^{-1}$)	4	0.96 ± 0.32^{B}	1.11 ± 0.34^{B}	1.13 ± 0.39^{B}
		(-75 %)	(-72 %)	(-71 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivar with the same letter are not statistically different ($\alpha=0.05$).

Table 4. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the control (<0.030 μl l^{-1} SO_2 & 0.025 μl l^{-1} O_3) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$	0	1.13 ± 0.38^{aA}	1.17 ± 0.29^{A}	1.18 ± 0.41^{A}
(μmol g^{-1} fw)	4	1.25 ± 0.34^{A}	1.26 ± 0.30^{A}	1.25 ± 0.31^{A}
		(11 %)[b]	(8 %)	(6 %)
$Glutathione_{ox}$	0	$.067\pm.049^{A}$	$.042\pm.015^{A}$	$.044\pm.011^{A}$
(μmol g^{-1} fw)	4	$.056\pm.019^{A}$	$.047\pm.017^{A}$	$.049\pm.013^{A}$
		(-16 %)	(12 %)	(11 %)
$Glutathione_{red}$	0	1.00 ± 0.38^{A}	1.09 ± 0.28^{A}	1.09 ± 0.40^{A}
(μmol g^{-1} fw)	4	1.14 ± 0.31^{A}	1.17 ± 0.29^{A}	1.15 ± 0.31^{A}
		(14 %)	(7 %)	(6 %)
$Ascorbate_{tot}$	0	1072 ± 222^{A}	1219 ± 110^{A}	1292 ± 90^{A}
(μg g^{-1} fw)	4	1194 ± 113^{A}	1456 ± 59^{B}	1713 ± 105^{B}
		(11 %)	(19 %)	(33 %)
$Ascorbate_{ox}$	0	115 ± 44^{A}	119 ± 59^{A}	120 ± 81^{A}
(μg g^{-1} fw)	4	111 ± 22^{A}	131 ± 97^{A}	63 ± 51^{A}
		(-3 %)	(10 %)	(-47 %)
$Ascorbate_{red}$	0	957 ± 234^{A}	1100 ± 104^{A}	1172 ± 107^{A}
(μg g^{-1} fw)	4	1083 ± 111^{A}	1336 ± 1272^{B}	1654 ± 125^{B}
		(13 %)	(21 %)	(41 %)

(Cont.)

(Table 4 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	32.0±2.4[A]	26.3±1.6[A]	29.4±6.6[A]
(mg g^{-1} fw)	4	34.2±3.5[A]	30.8±2.3[A]	30.9±5.0[A]
		(7 %)	(17 %)	(5 %)
Glutathione	0	0.76±0.14[A]	0.74±0.12[A]	0.83±0.24[A]
Reductase	4	0.75±0.16[A]	0.71±0.09[A]	0.85±0.20[A]
(U g^{-1} fw)		(-1 %)	(-4 %)	(2 %)
Glutathione	0	.024±.004[A]	.028±.004[A]	.028±.003[A]
Reductase	4	.022±.005[A]	.023±.004[A]	.027±.004[A]
(U mg^{-1} protein)		(-8 %)	(-18 %)	(-4 %)
Ascorbate	0	1.13±0.15[A]	1.21±0.12[A]	1.21±0.29[A]
Peroxidase	4	1.35±0.09[A]	1.26±0.16[A]	1.21±0.22[A]
(U g^{-1} fw)		(19 %)	(4 %)	(0 %)
Ascorbate	0	.035±.004[A]	.046±.003[A]	.043±.007[A]
Peroxidase	4	.040±.005[A]	.041±.005[A]	.041±.011[A]
(U mg^{-1} protein)		(14 %)	(-11 %)	(-5 %)
SOD	0	35.9±20.3[A]	17.6±9.4[A]	25.0±8.1[A]
(U g^{-1} fw)	4	47.5±28.2[A]	36.4±22.2[A]	39.4±16.0[A]
		(32 %)	(107 %)	(58 %)
SOD	0	1.10±0.56[A]	0.67±0.35[A]	0.87±0.27[A]
(U mg^{-1} protein)	4	1.37±0.76[A]	1.17±0.71[A]	1.29±0.54[A]
		(25 %)	(75 %)	(48 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivar with the same letter are not statistically different (α=0.05).

Table 5. Changes in antioxidant components in three soybean cultivars during a 4 hr treatment in the fumigated (0.70±0.05 μl l^{-1} SO_2 + 0.20±0.02 μl l^{-1} O_3) CSTR chambers.

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
$Glutathione_{tot}$	0	1.02±0.31[aA]	1.05±0.28[A]	1.15±0.25[A]
(μmol g^{-1} fw)	4	1.46±0.38[B]	1.31±0.17[B]	1.40±0.18[A]
		(43 %)[a]	(25 %)	(22 %)
$Glutathione_{ox}$	0	.055±.016[A]	.044±.012[A]	.051±.014[A]
(μmol g^{-1} fw)	4	.078±.023[A]	.078±.033[B]	.081±.039[B]
		(42 %)	(77 %)	(59 %)
$Glutathione_{red}$	0	0.91±0.29[A]	0.96±0.27[A]	1.05±0.25[A]
μmol g^{-1} fw)	4	1.31±0.35[B]	1.16±0.22[A]	1.23±0.13[A]
		(44 %)	(21 %)	(17 %)
$Ascorbate_{tot}$	0	1091±180[A]	1215±182[A]	1355±190[A]
(μg g^{-1} fw)	4	1164±183[A]	1273±152[A]	1493±202[A]
		(7 %)	(5 %)	(10 %)
$Ascorbate_{ox}$	0	101±71[A]	128±38[A]	109±61[A]
(μg g^{-1} fw)	4	109±44[A]	65±30[B]	72±46[A]
		(8 %)	(-49 %)	(-34 %)
$Ascorbate_{red}$	0	991±231[A]	1088±202[A]	1246±193[A]
(μg g^{-1} fw)	4	1055±187[A]	1207±161[A]	1444±156[A]
		(6 %)	(11 %)	(16 %)

(Cont.)

(Table 5 continued)

Variable	Time (hr)	Cultivar		
		Dare	Williams	Essex
Protein	0	29.6±1.7[A]	25.0±3.1[A]	27.6±5.8[A]
(mg g^{-1} fw)	4	30.2±3.2[A]	26.4±3.8[A]	23.0±8.2[A]
		(2 %)	(6 %)	(-17 %)
Glutathione	0	0.75±0.17[A]	0.68±0.07[A]	0.75±0.15[A]
Reductase	4	0.82±0.10[A]	0.78±0.13[A]	1.02±0.37[B]
(U g^{-1} fw)		(9 %)	(15 %)	(36 %)
Glutathione	0	.025±.005[A]	.027±.002[A]	.027±.006[A]
Reductase	4	.027±.002[A]	.029±.001[A]	.046±.012[B]
(U mg^{-1} protein)		(8 %)	(7 %)	(70 %)
Ascorbate	0	1.17±0.07[A]	1.08±0.17[A]	1.14±0.15[A]
Peroxidase	4	1.28±0.09[A]	1.10±0.13[A]	1.09±0.04[A]
(U g^{-1} fw)		(9 %)	(2 %)	(-4 %)
Ascorbate	0	.040±.003[A]	.044±.004[A]	.043±.014[A]
Peroxidase	4	.043±.006[A]	.042±.008[A]	.053±.019[A]
(U mg^{-1} protein)		(8 %)	(-5 %)	(23 %)
SOD	0	30.3±5.4[A]	21.4±11.6[A]	23.0±8.5[A]
(U g^{-1} fw)	4	38.0±16.3[A]	28.3±17.3[A]	33.0±16.5[A]
		(25 %)	(32 %)	(43 %)
SOD	0	1.03±0.21[A]	0.87±0.46[A]	0.87±0.35[A]
(U mg^{-1} protein)	4	1.27±0.52[A]	1.16±0.83[A]	1.56±0.70[A]
		(23 %)	(33 %)	(79 %)

a: Means ± sd.

b: Percentage change from time 0.

A or B: Means within cultivar with the same letter are not statistically different (α=0.05).

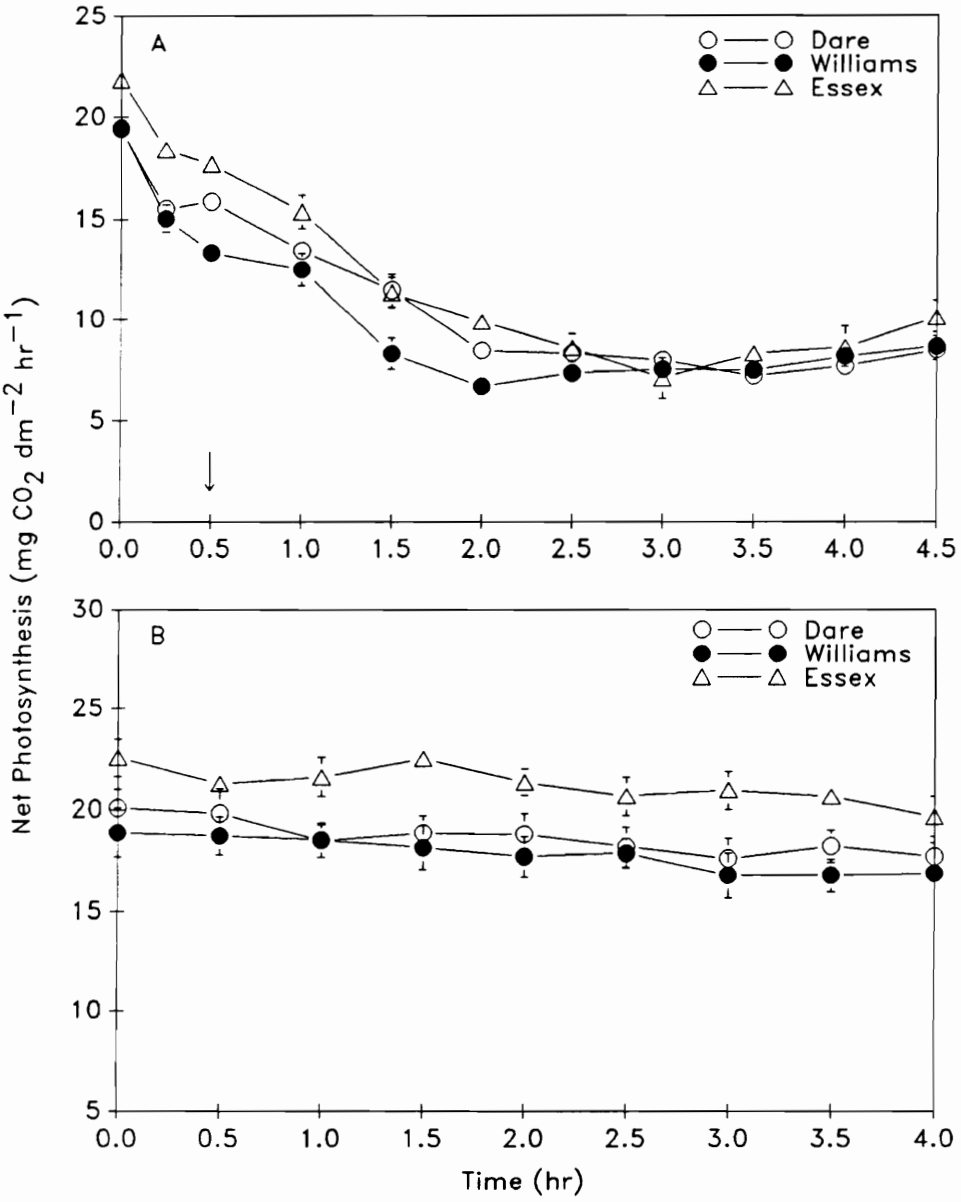


Fig. 1. Net photosynthetic rate of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point represents the mean ± SE of 6 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicating target pollutant concentrations reached.

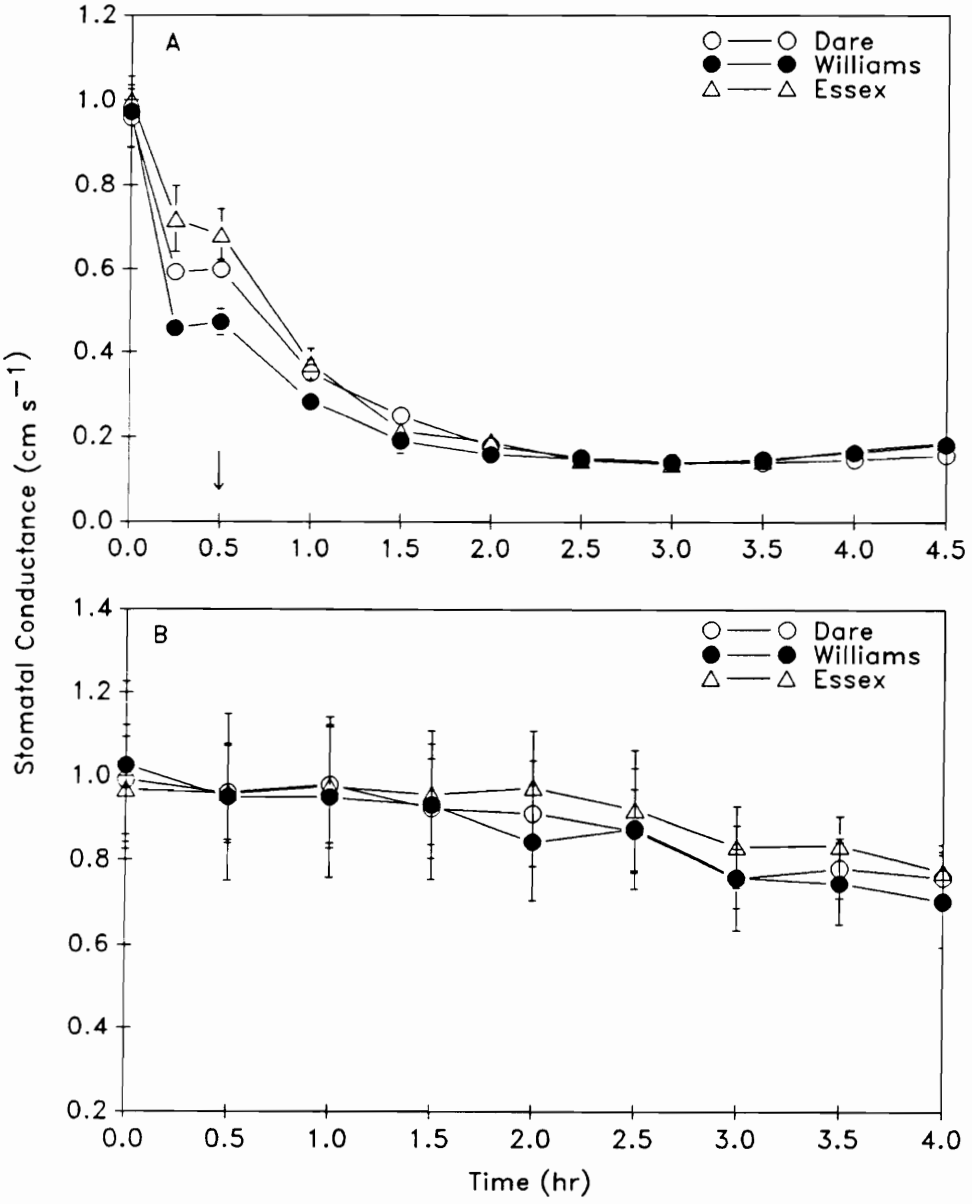


Fig. 2. Stomatal conductance of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point represents the mean ± SE of 6 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicating target pollutant concentrations reached.

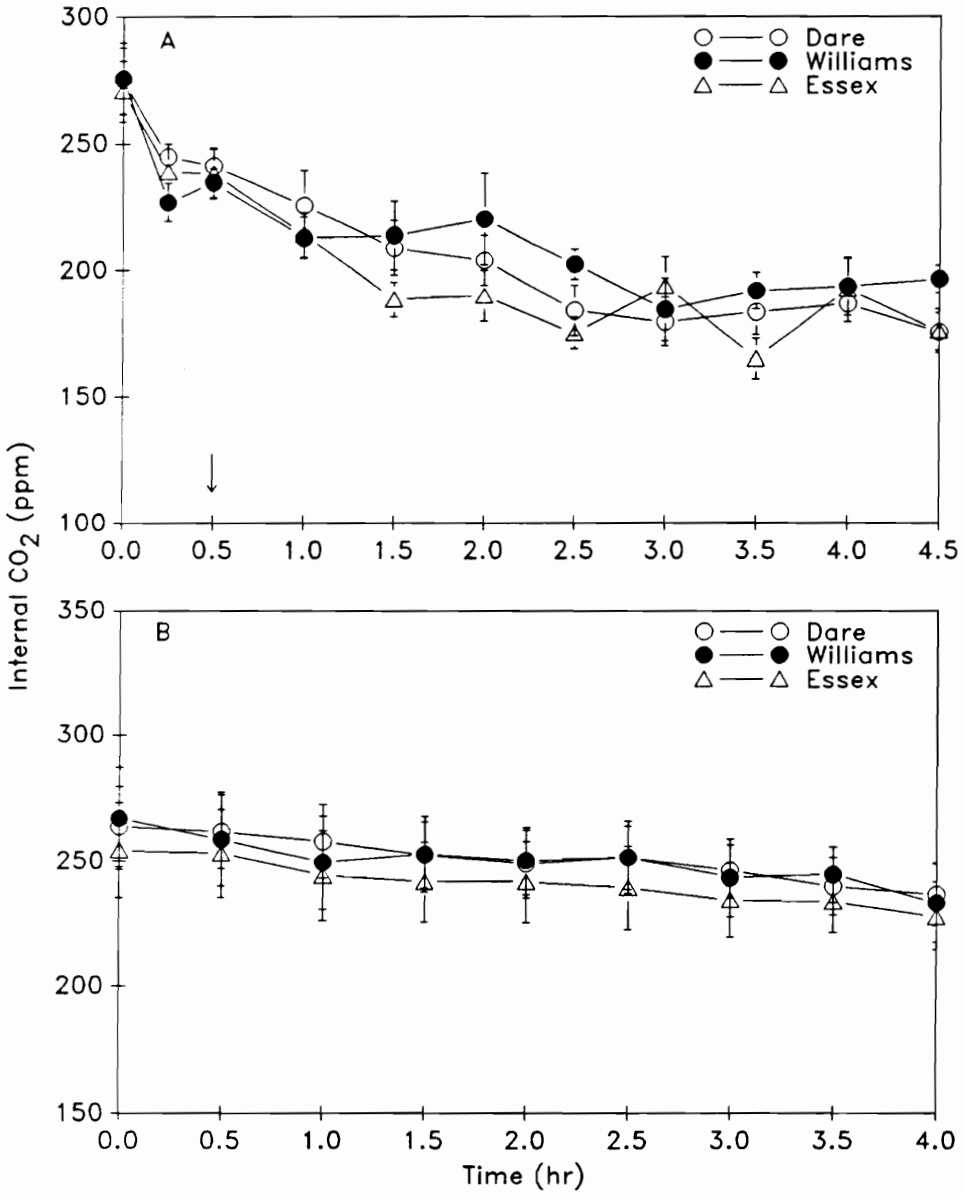


Fig. 3. Intercellular CO_2 concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point represents the mean ± SE of 6 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicating target pollutant concentrations reached.

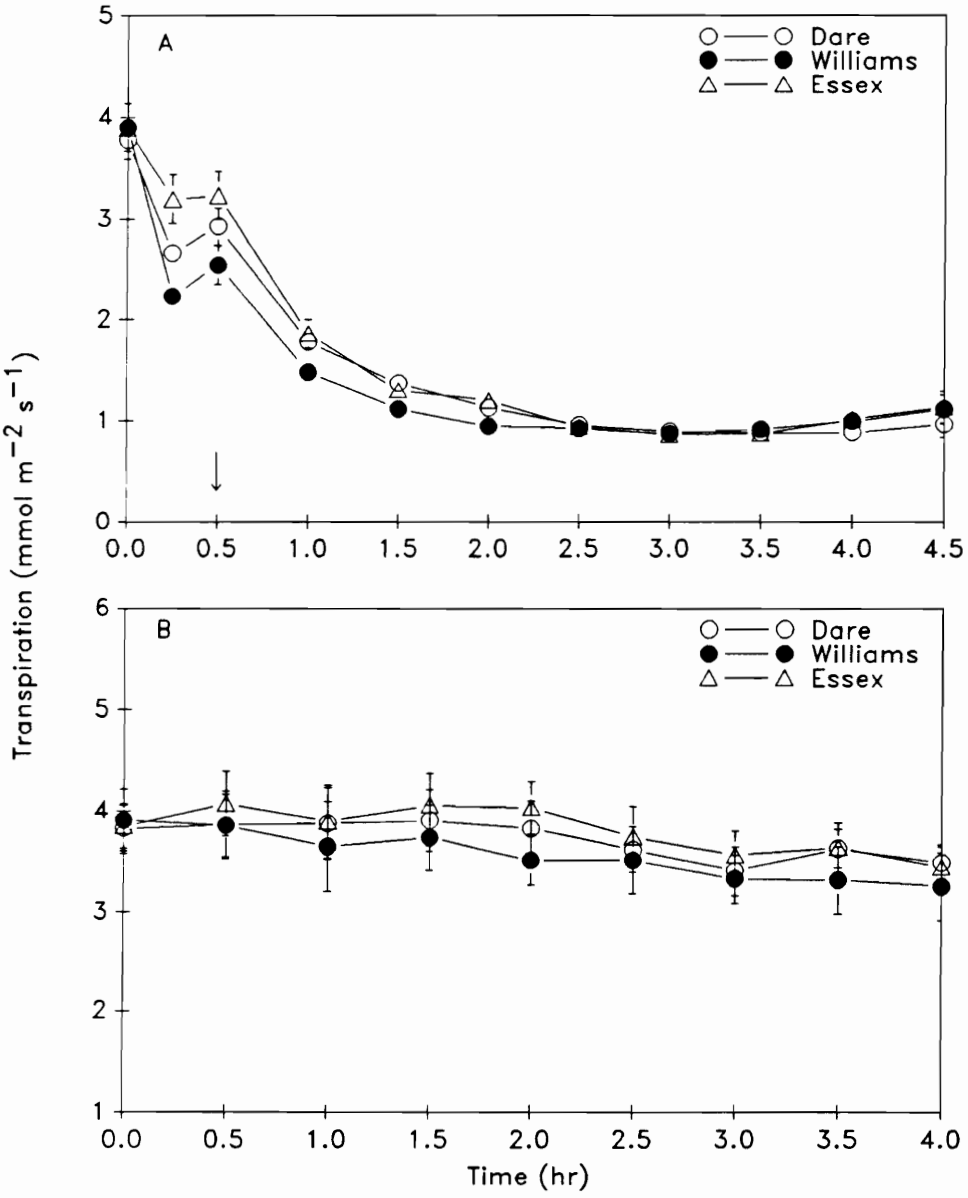


Fig. 4. Transpiration rate of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point represents the mean ± SE of 6 samples. Where error bars are not shown, they are contained within the symbols. ↓ indicating target pollutant concentrations reached.

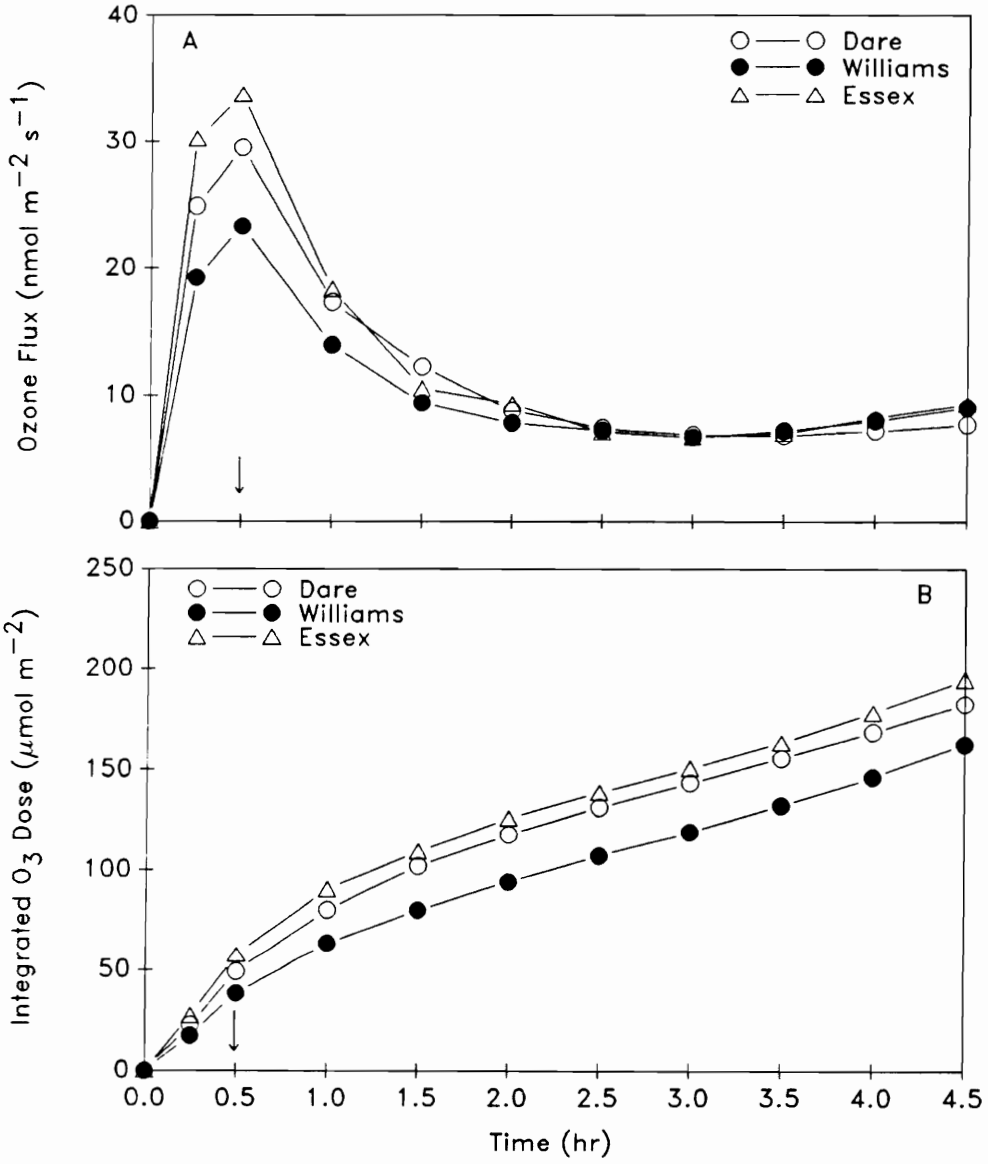


Fig. 5. Estimated ozone flux A) and integrated O_3 dose B) of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr. Each point is calculated from the mean of 6 samples. ↓ indicating target pollutant concentrations reached.

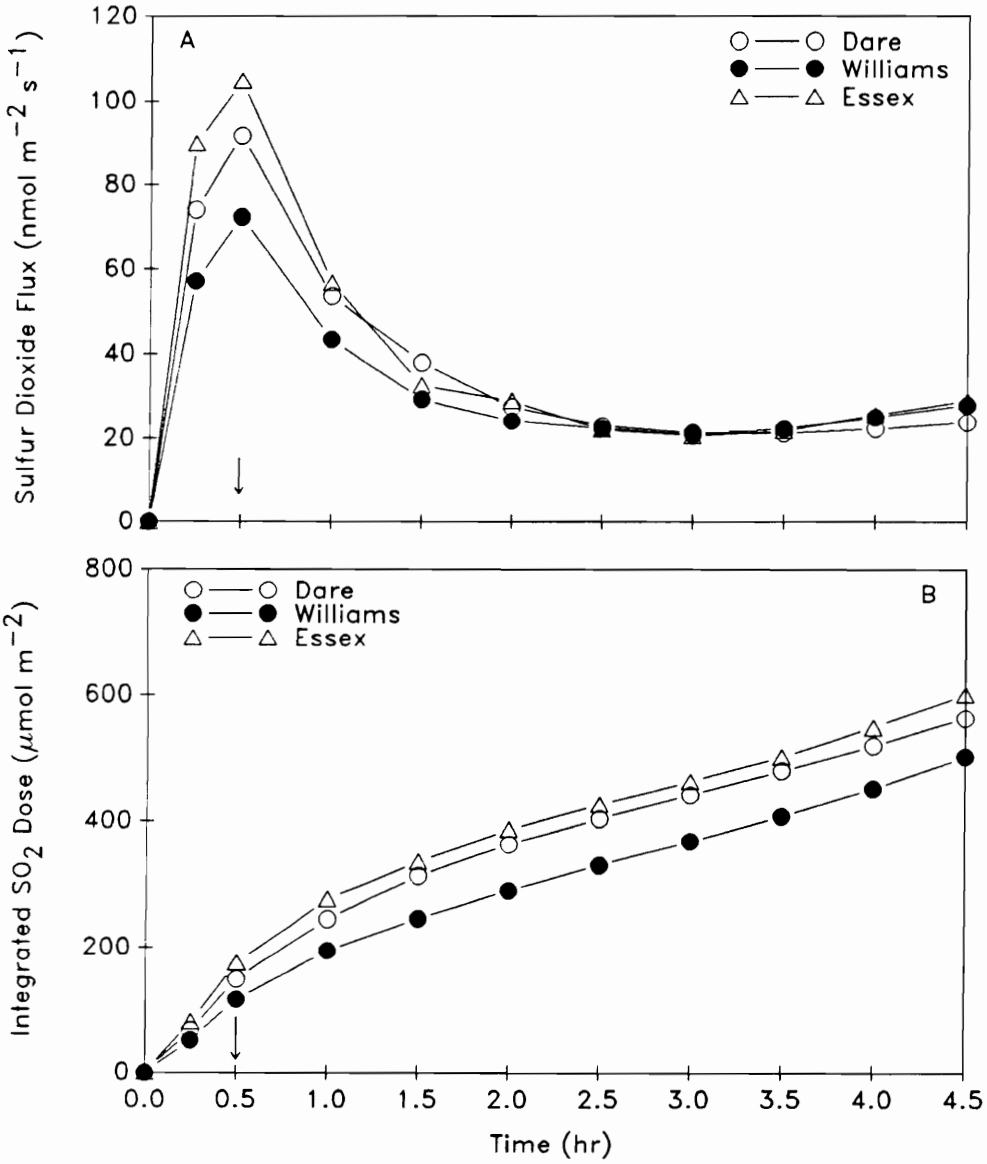


Fig. 6. Estimated sulfur dioxide flux A) and integrated SO_2 dose B) of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr. Each point is calculated from the mean of 6 samples. ↓ indicating target pollutant concentrations reached.

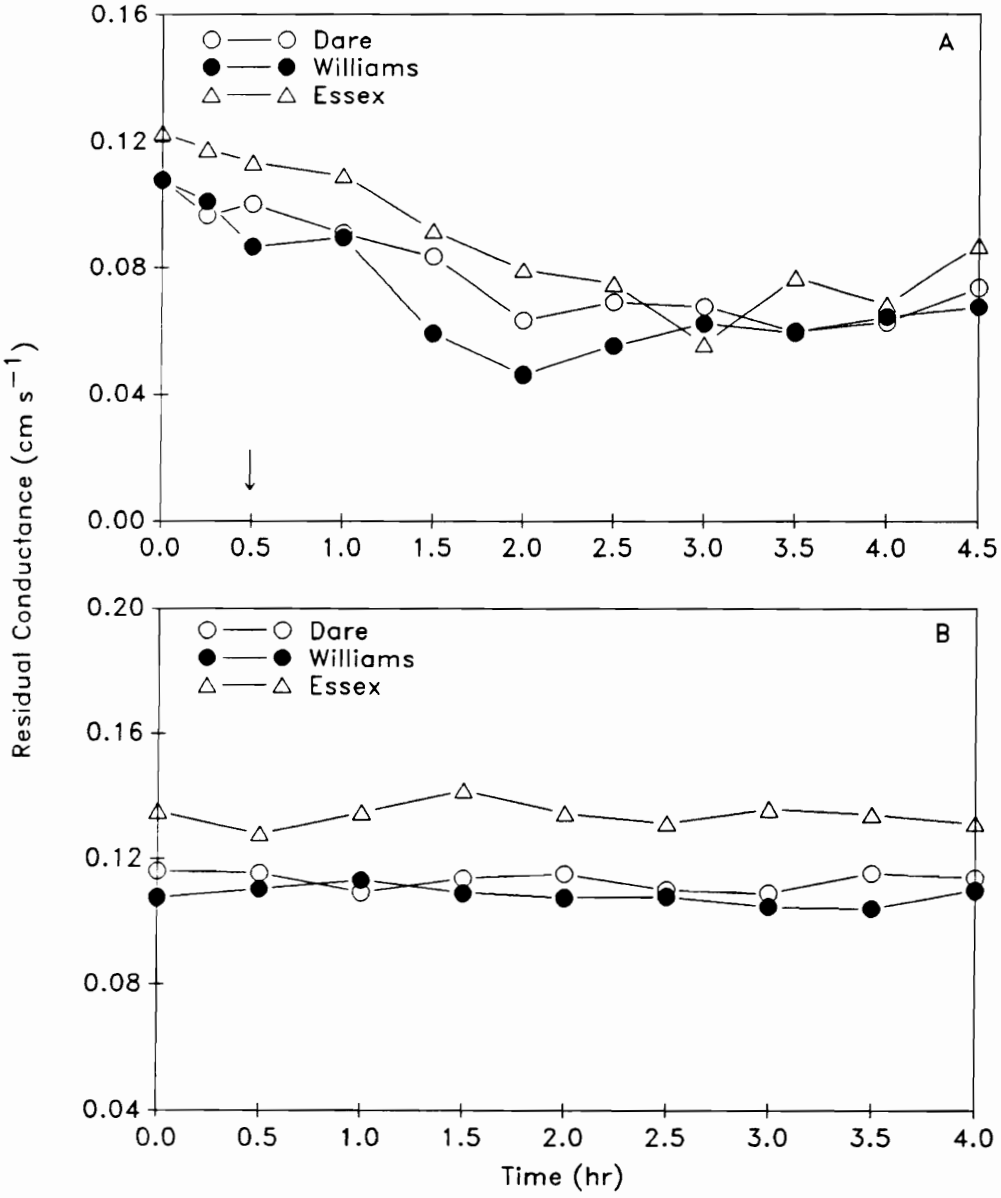


Fig. 7. Residual conductance to CO_2 of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point is calculated from the mean of 6 samples. ↓ indicating target pollutant concentrations reached.

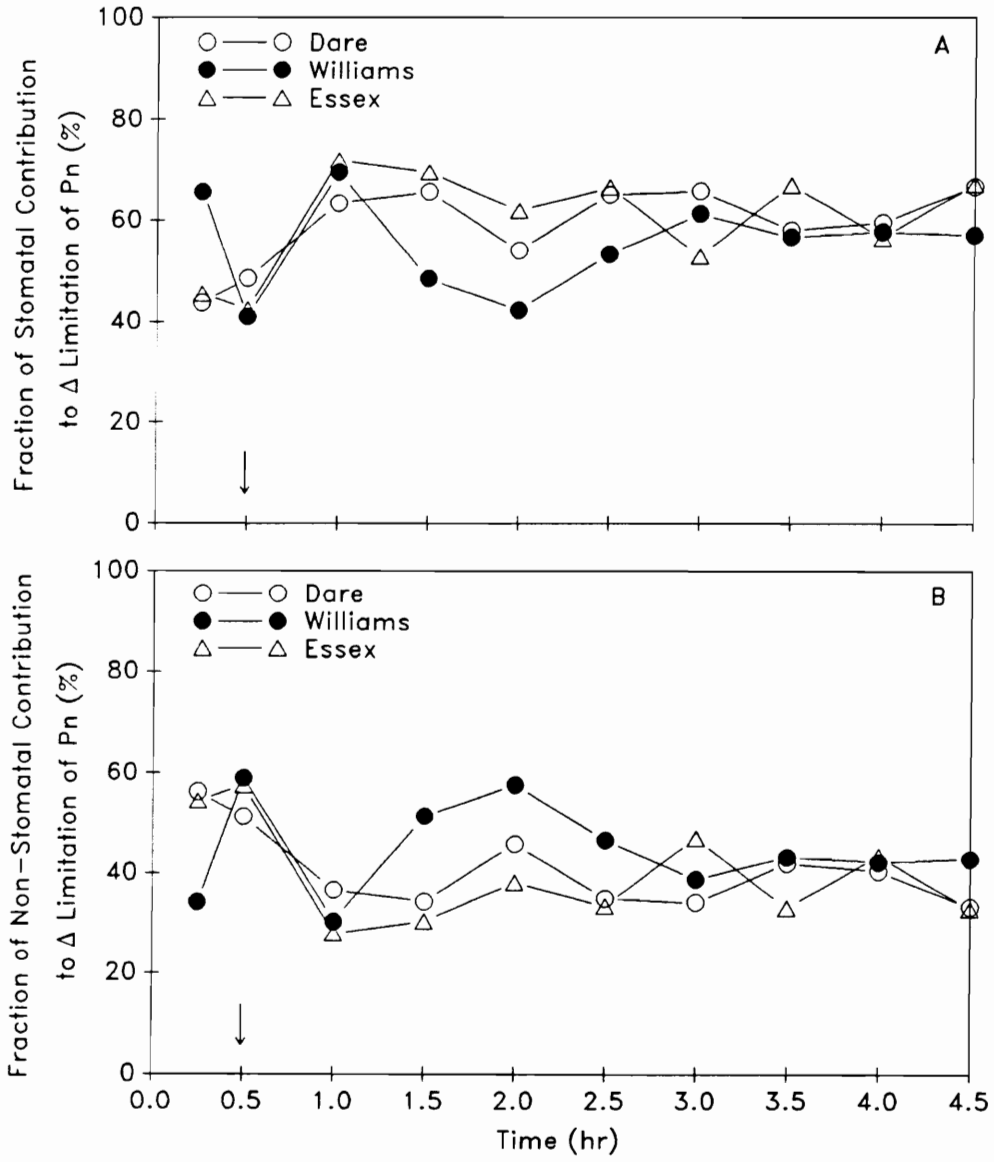


Fig. 8. Fraction of A) stomatal and B) non-stomatal contribution to change in limitation of Pn of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr. Relative to 0 time, each point is calculated from the mean of 6 samples. ↓ indicating target pollutant concentrations reached.

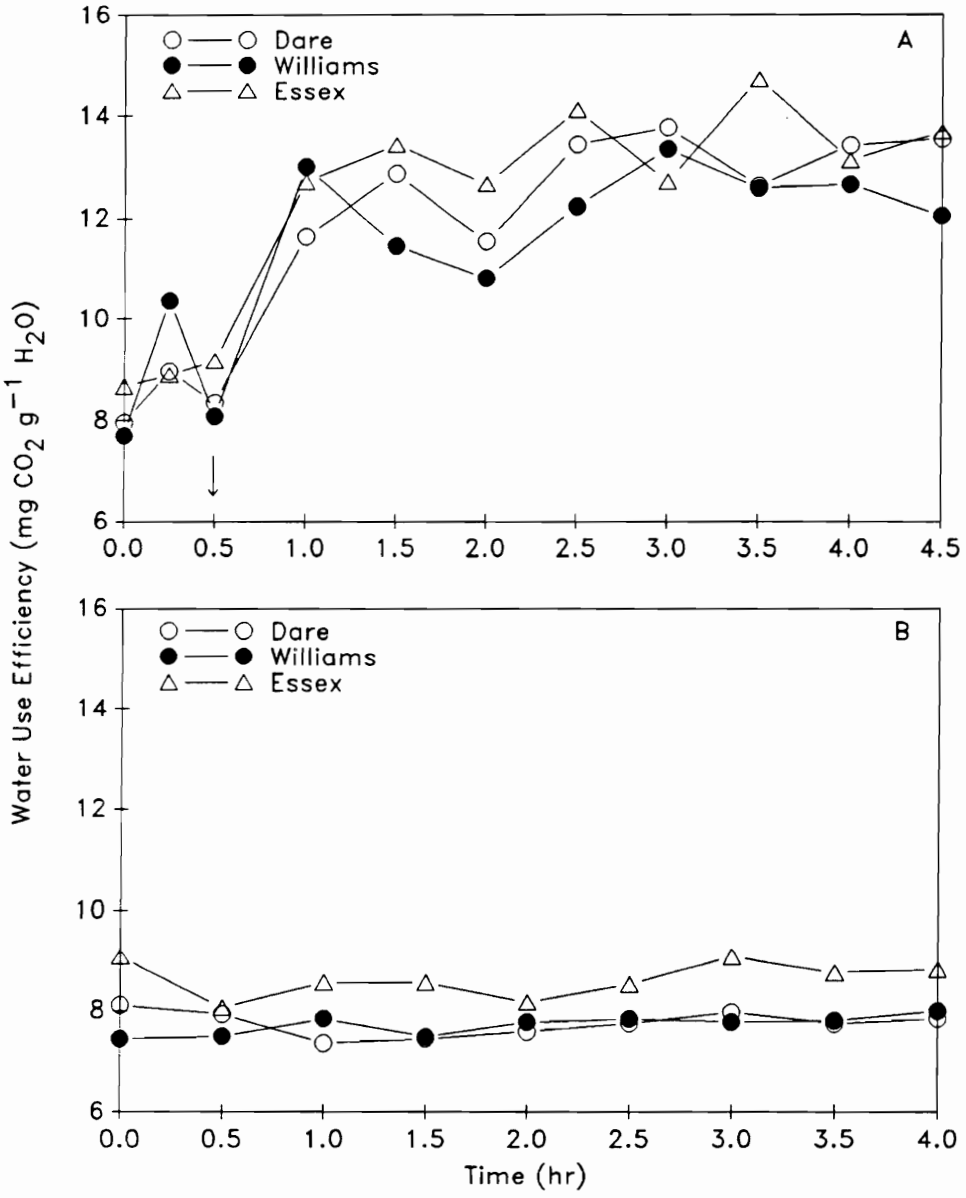


Fig. 9. Water use efficiency of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Each point is calculated from the mean of 6 samples. ↓ indicating target pollutant concentrations reached.

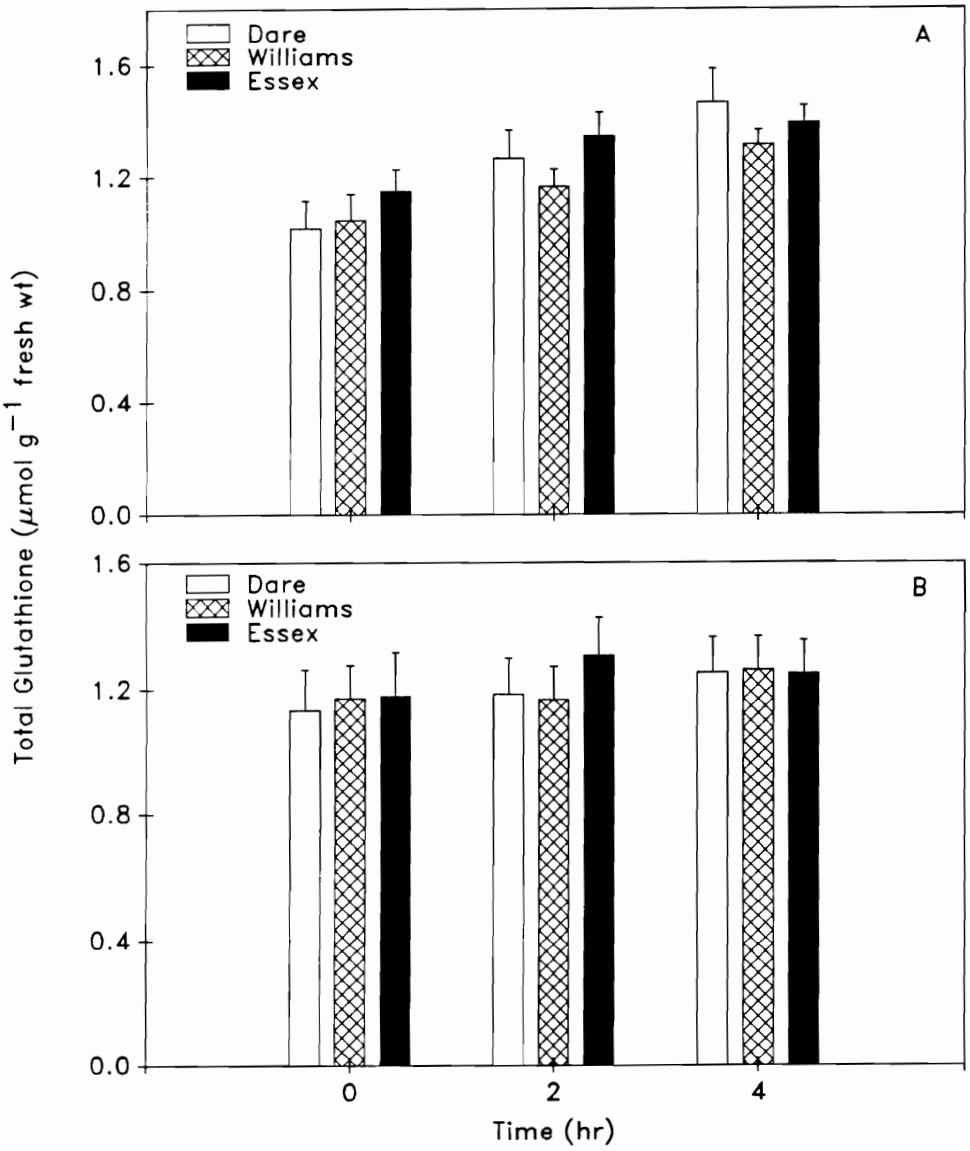


Fig. 10. Total glutathione concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

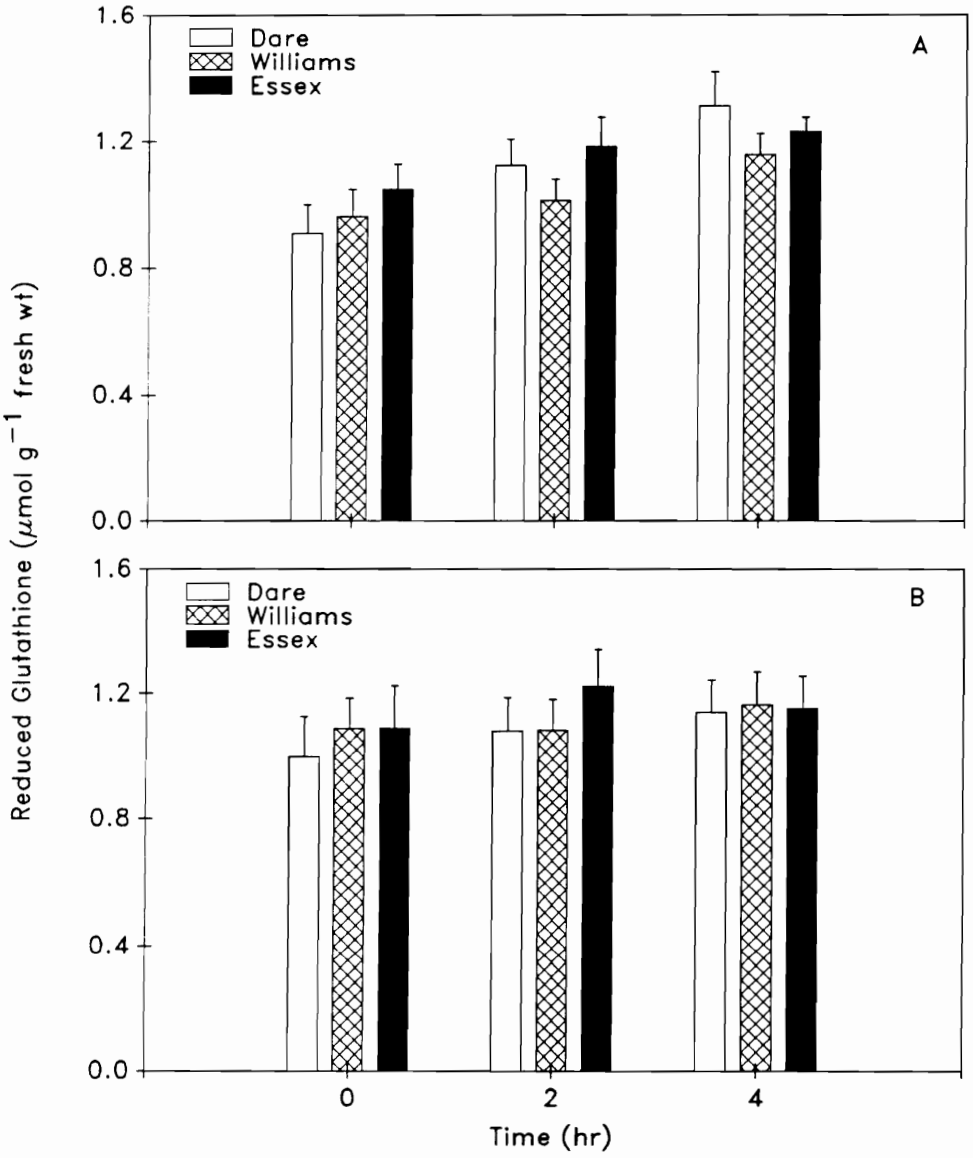


Fig. 11. Reduced glutathione concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

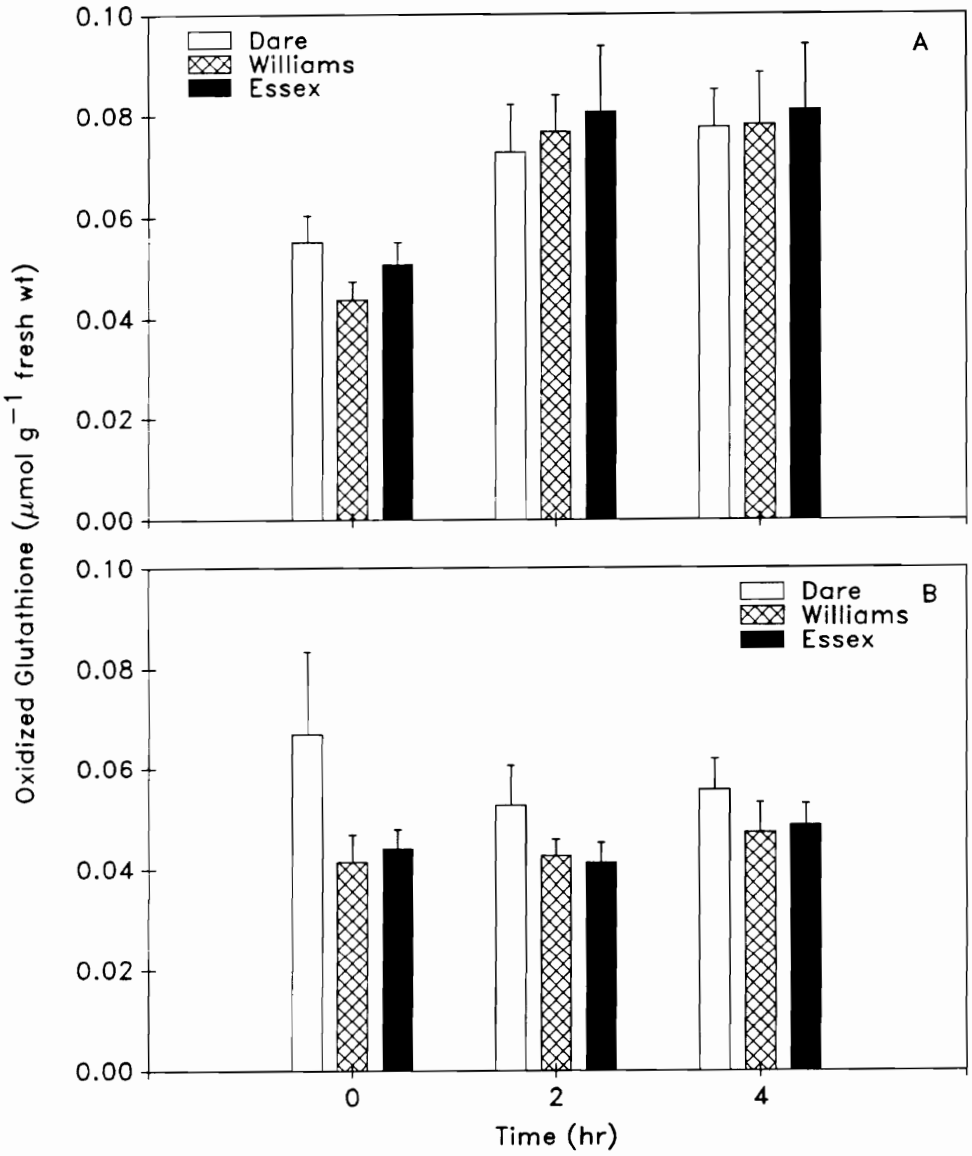


Fig. 12. Oxidized glutathione concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

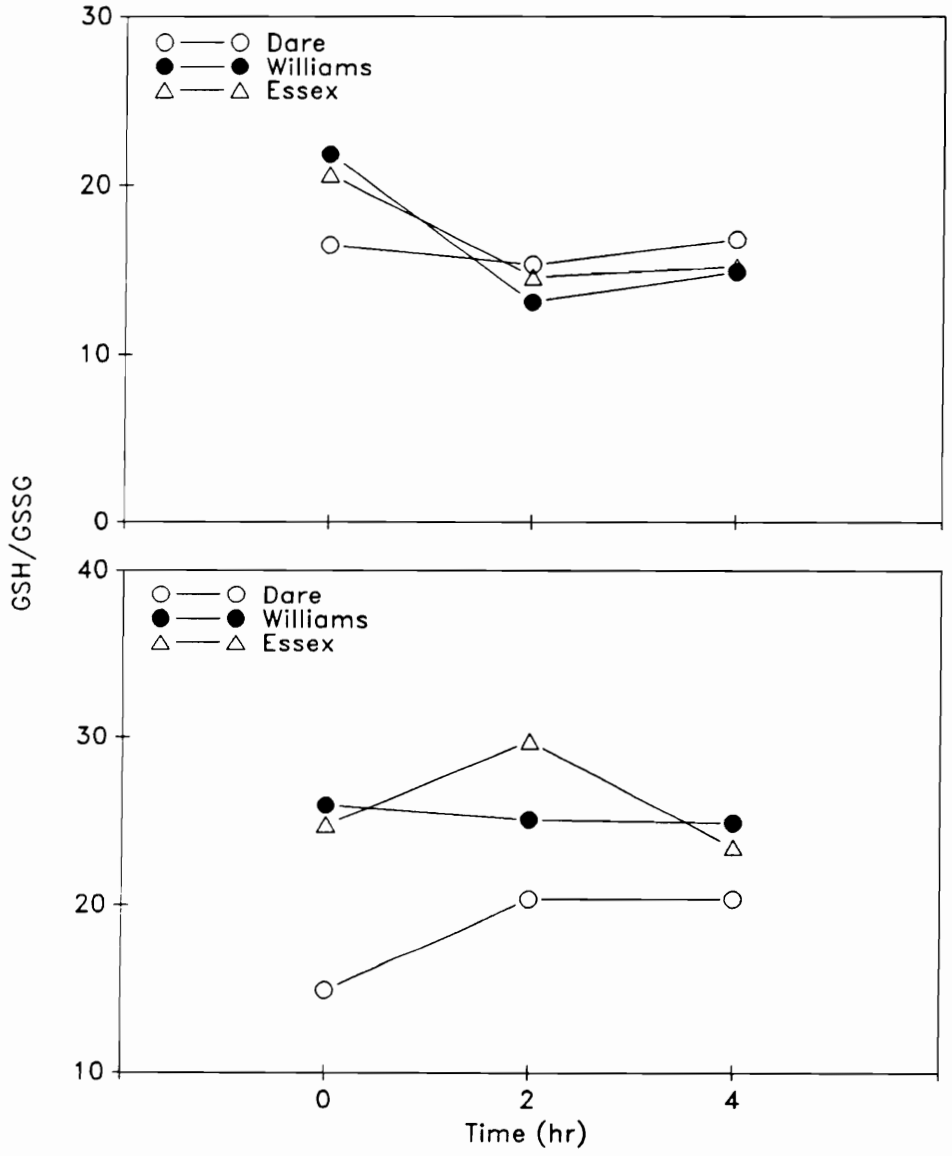


Fig. 13. Mean ratio of reduced (GSH) to oxidized (GSSG) glutathione in soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr.

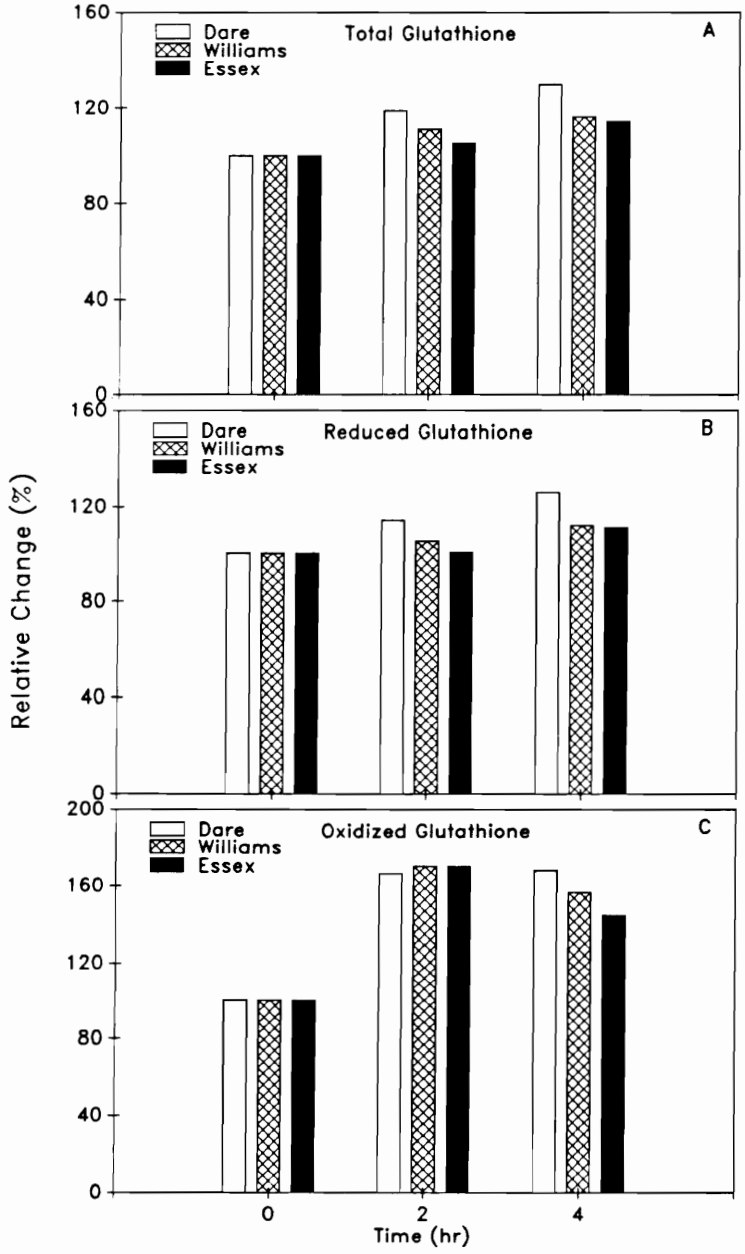


Fig. 14. Changes of A) total glutathione, B) reduced glutathione and C) oxidized glutathione concentrations of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 9-10 samples.

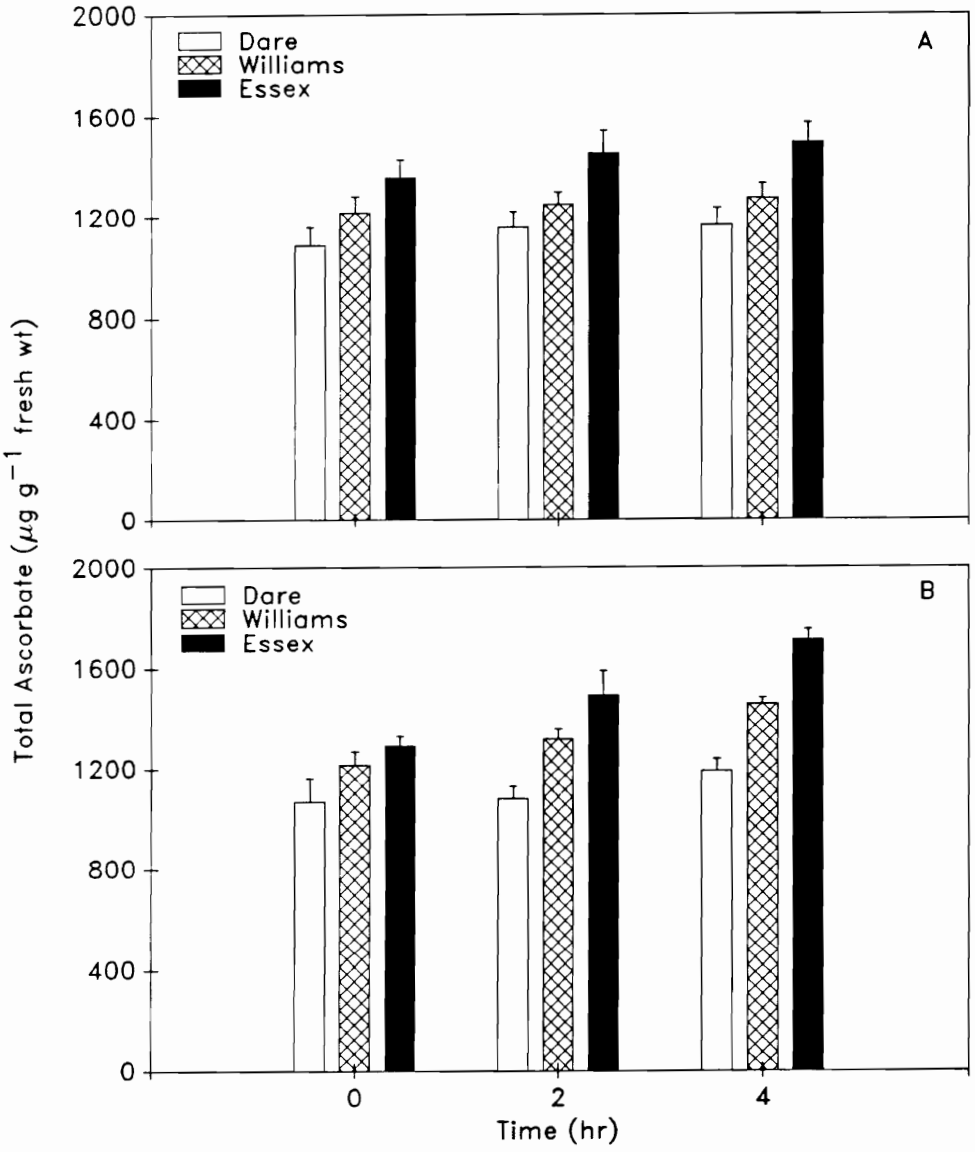


Fig. 15. Total ascorbate concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

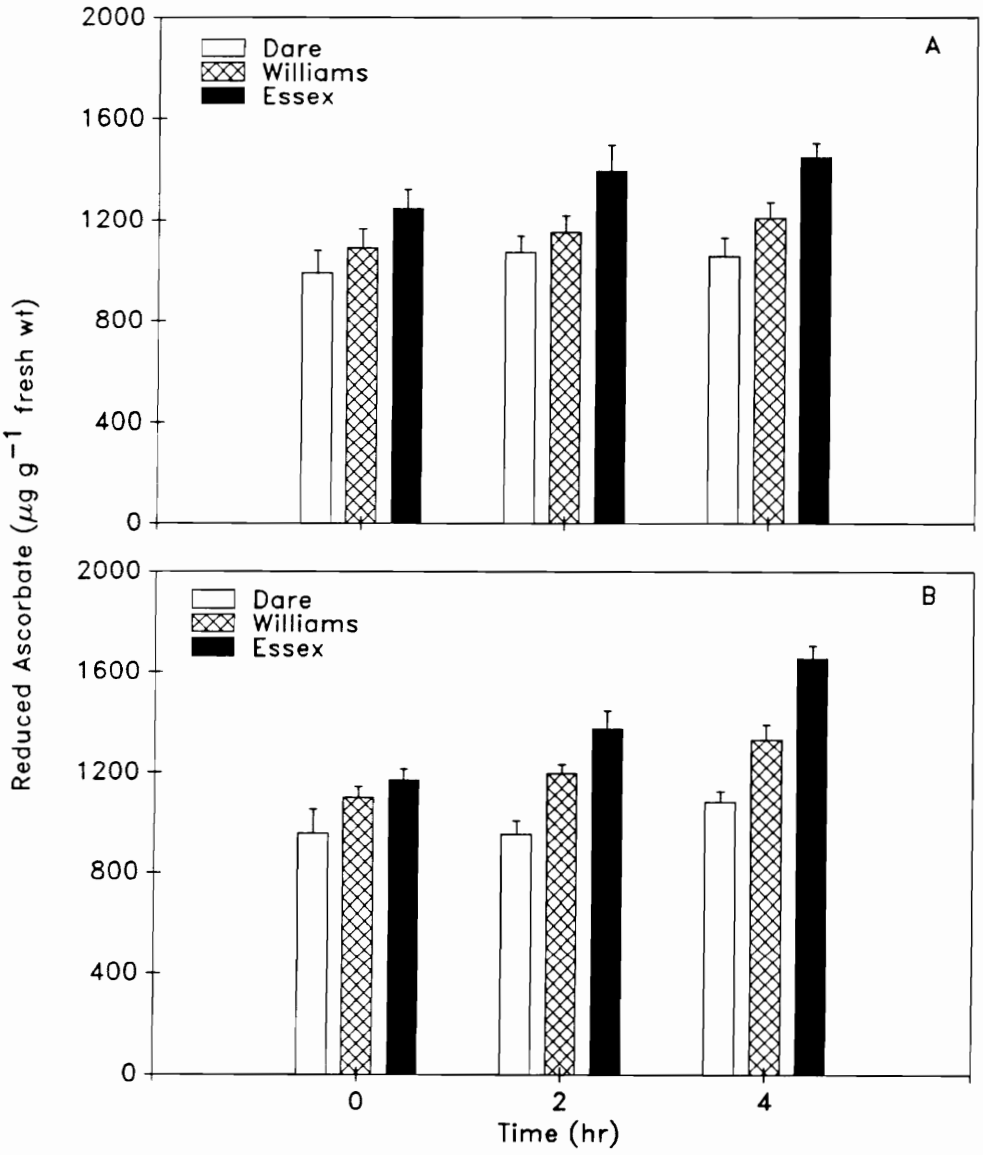


Fig. 16. Reduced ascorbate concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

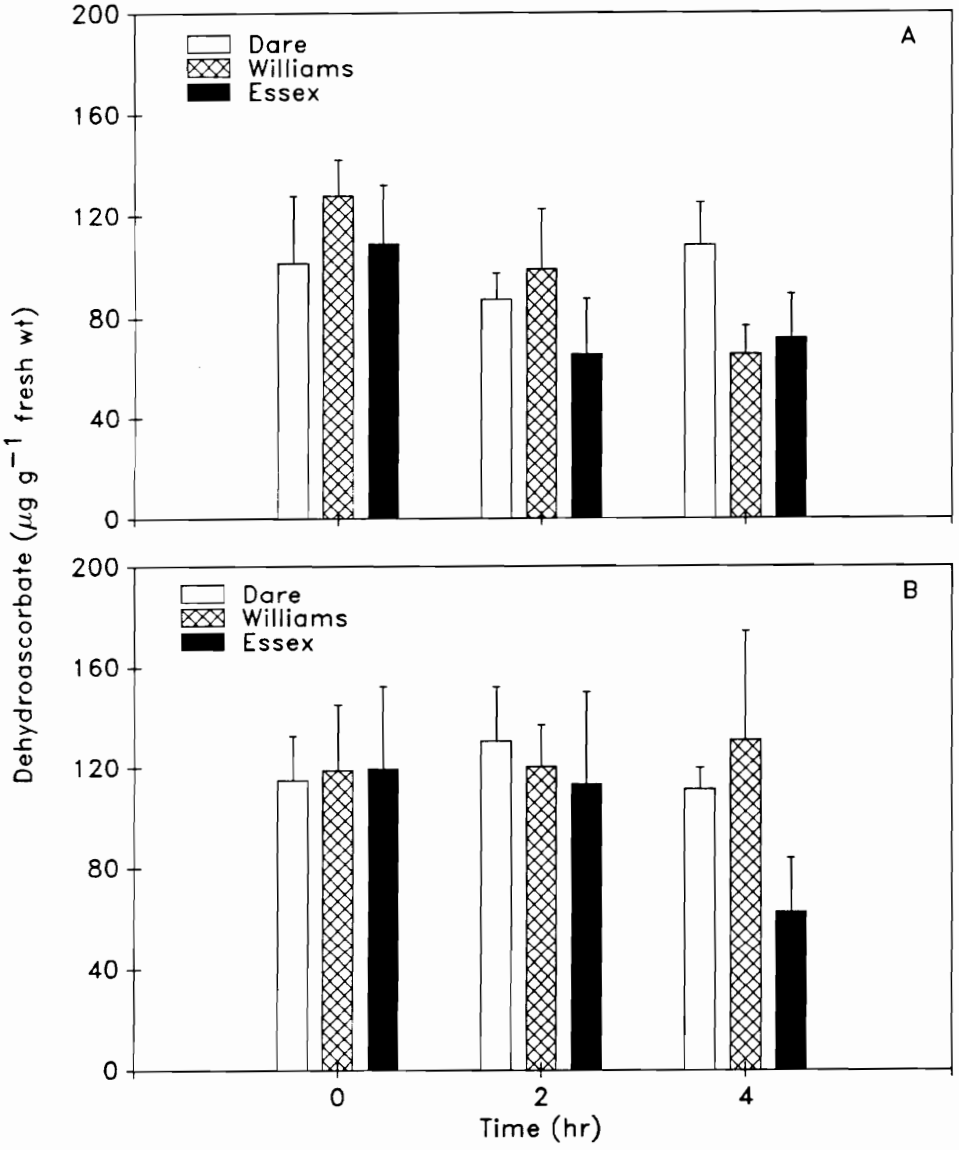


Fig. 17. Dehydroascorbate concentration of soybean cultivars exposed to A) 0.7 $\mu l\ l^{-1}$ SO_2 and 0.2 $\mu l\ l^{-1}$ O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

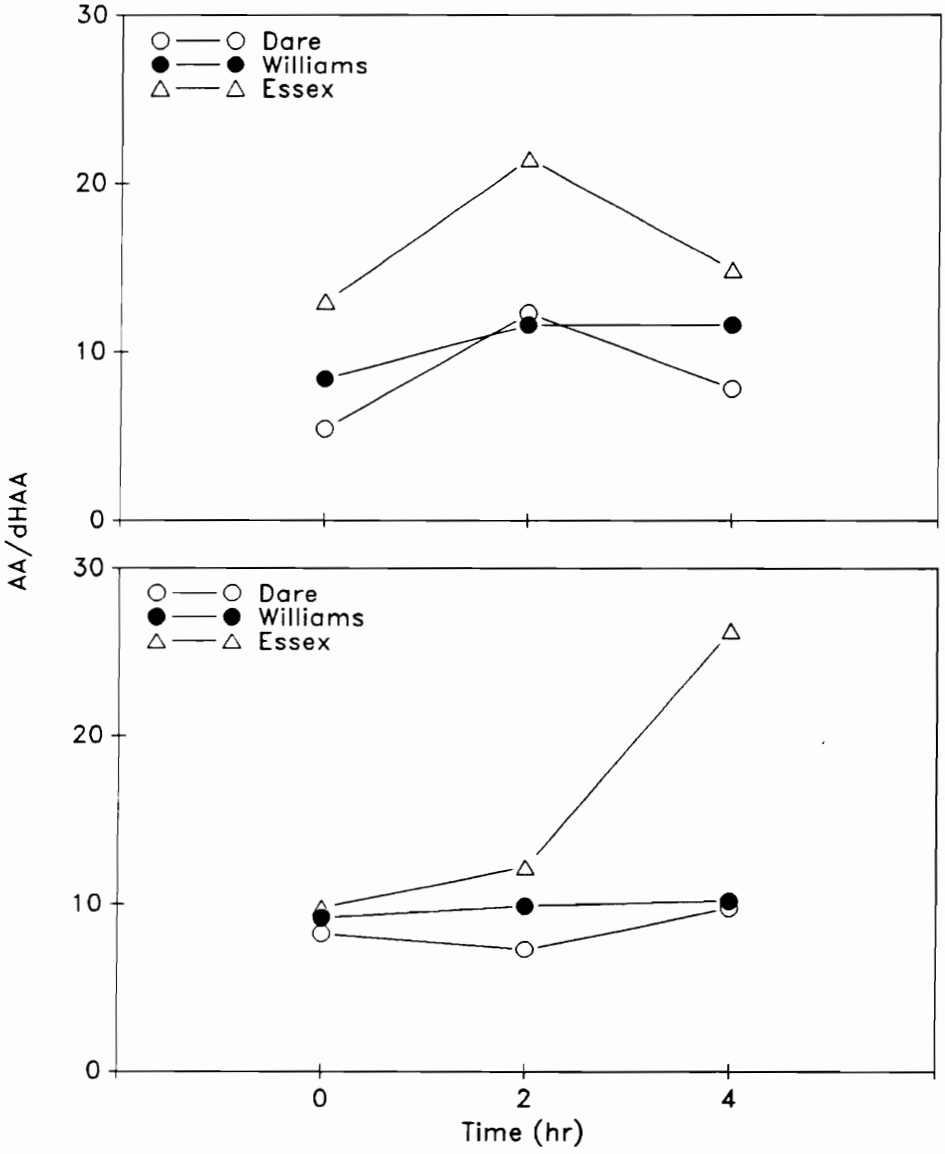


Fig. 18. Mean ratio of reduced (AA) to oxidized (dHAA) ascorbate in soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr.

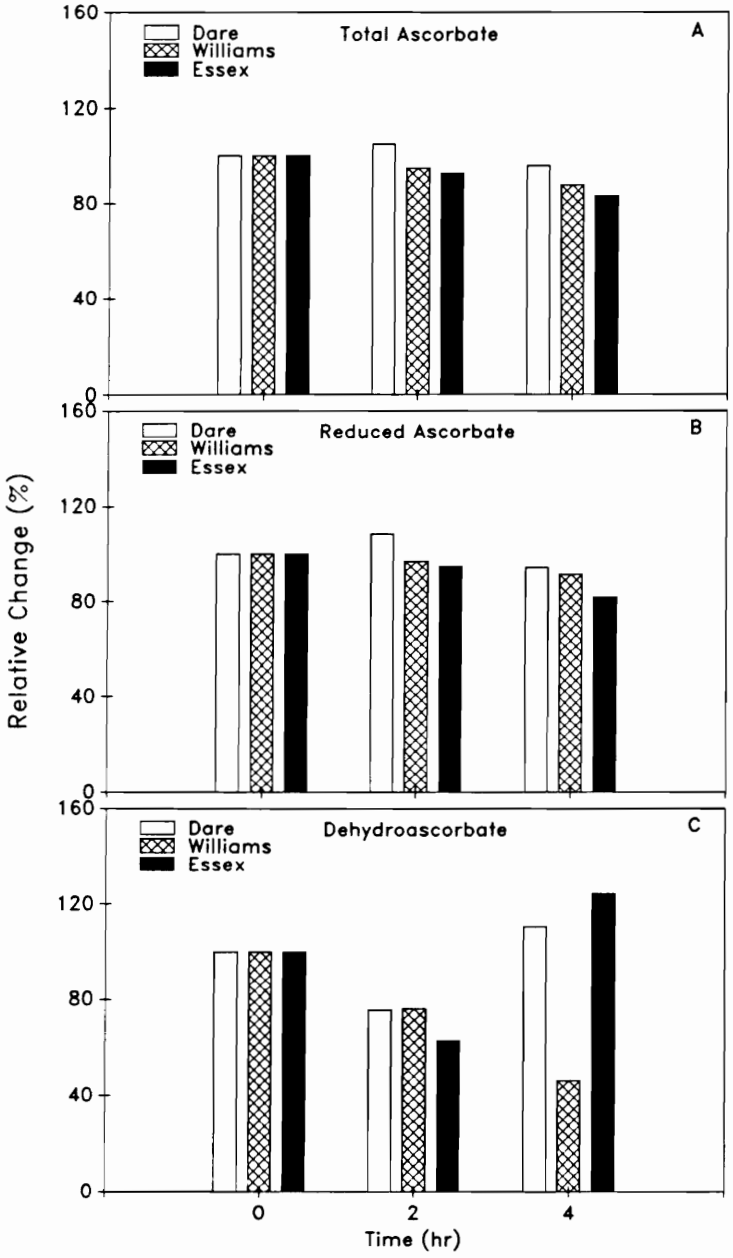


Fig. 19. Changes of A) total ascorbate, B) reduced ascorbate and C) dehydroascorbate concentrations of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 9-10 samples.

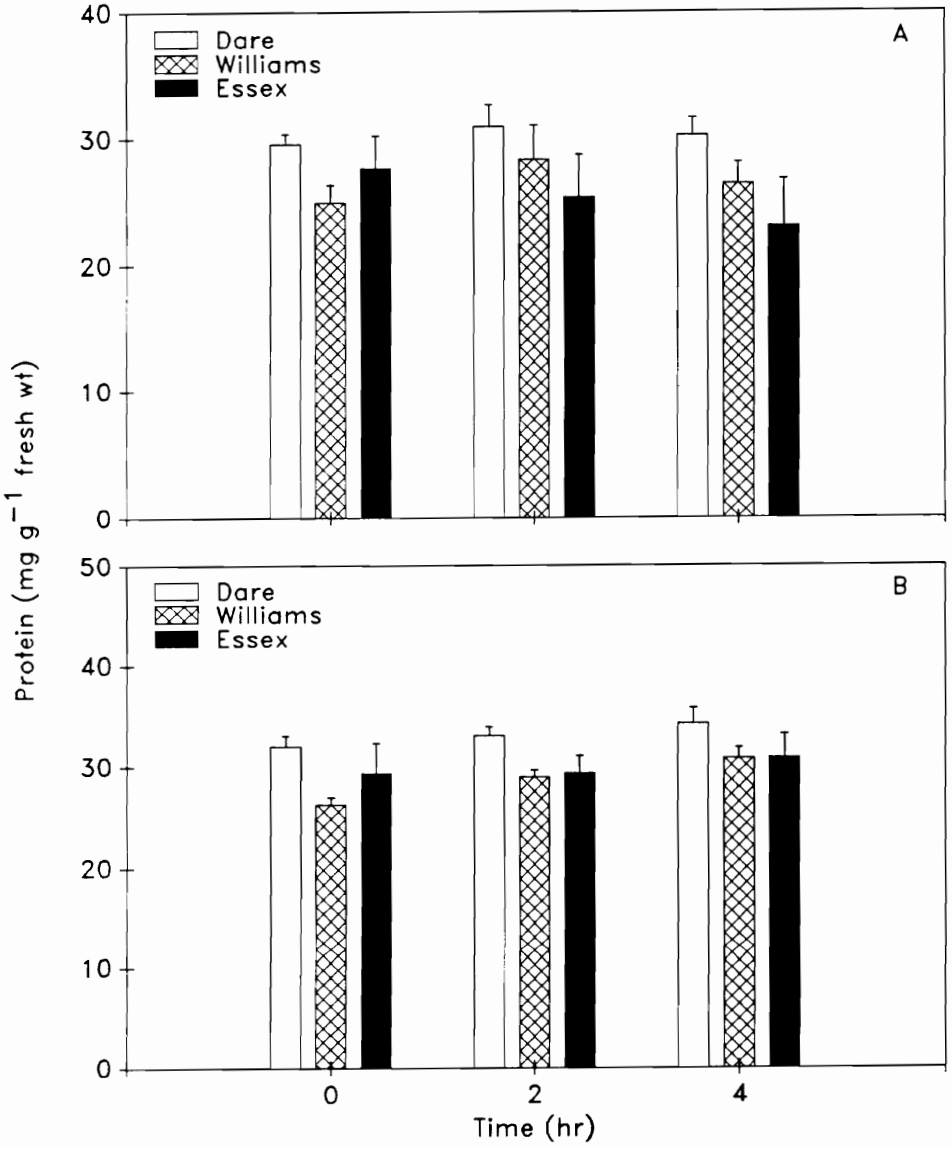


Fig. 20. Protein concentration of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 9-10 samples.

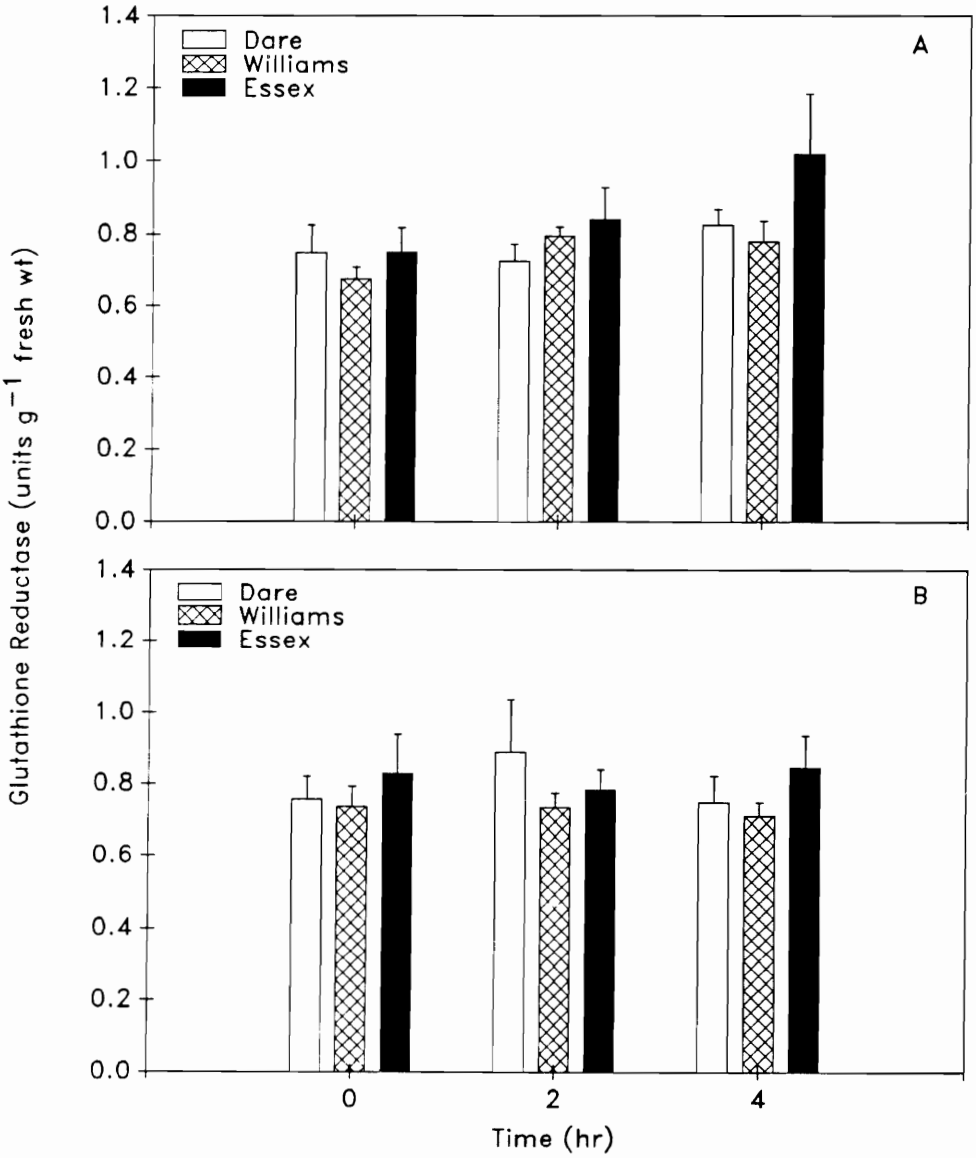


Fig. 21. Changes in glutathione reductase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

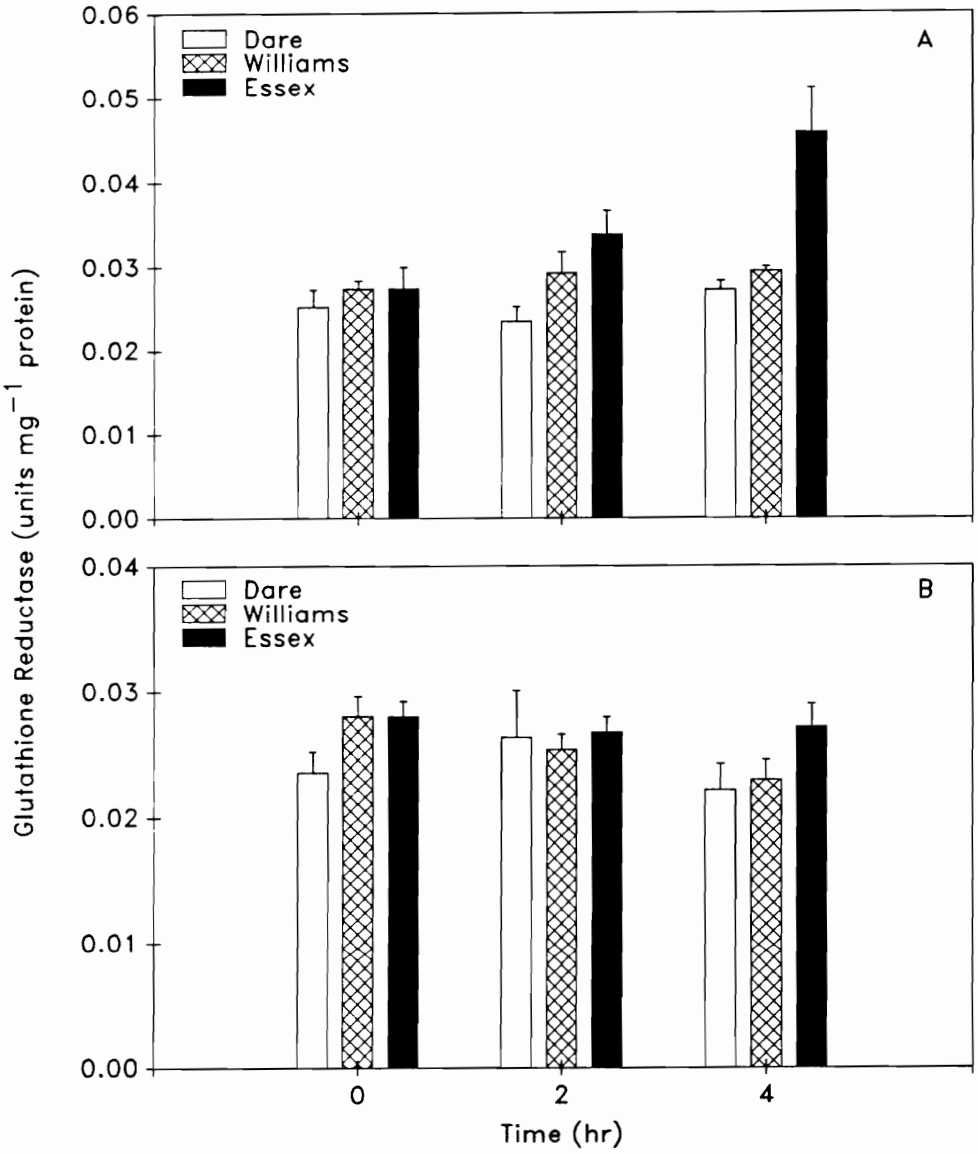


Fig. 22. Changes in glutathione reductase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

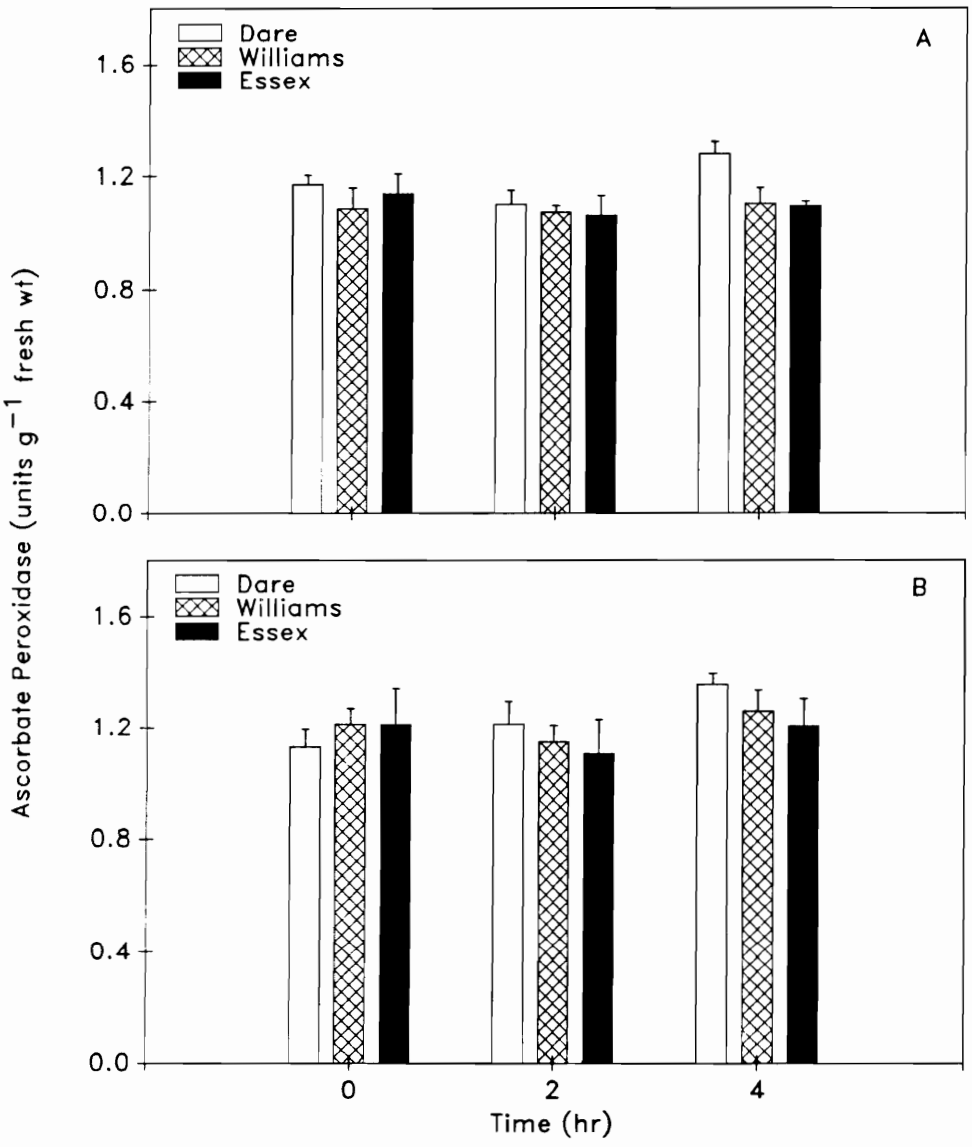


Fig. 23. Changes in ascorbate peroxidase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

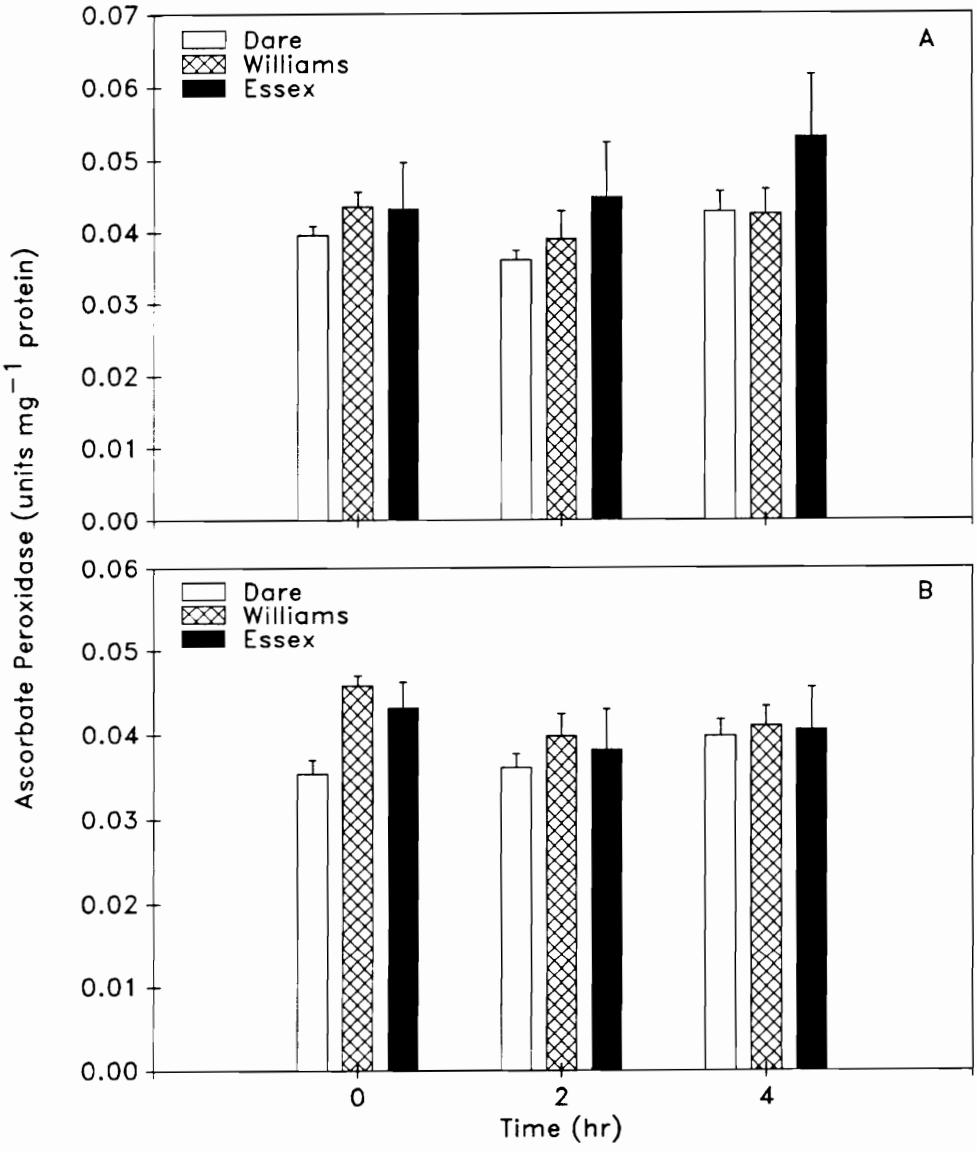


Fig. 24. Changes in ascorbate peroxidase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

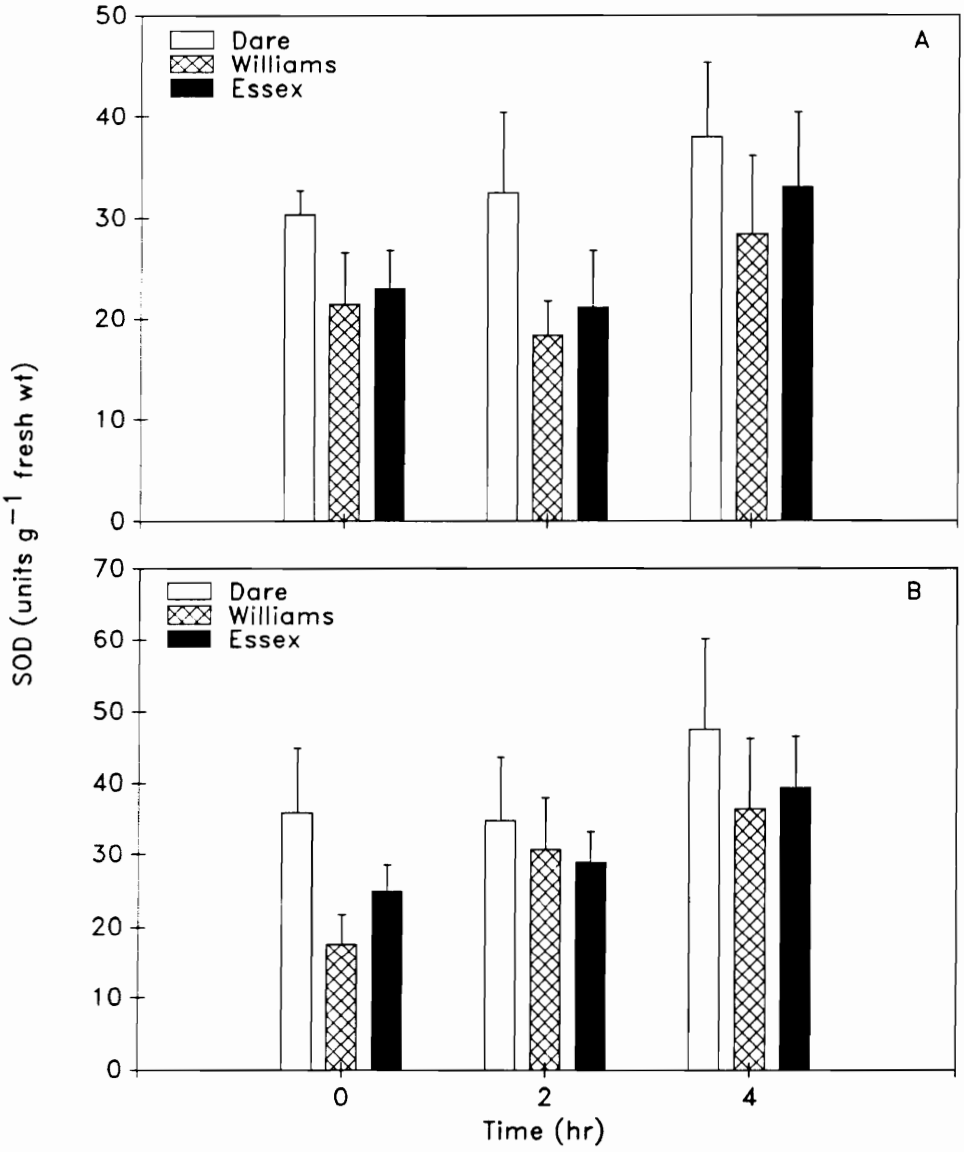


Fig. 25. Changes in superoxide dismutase total activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

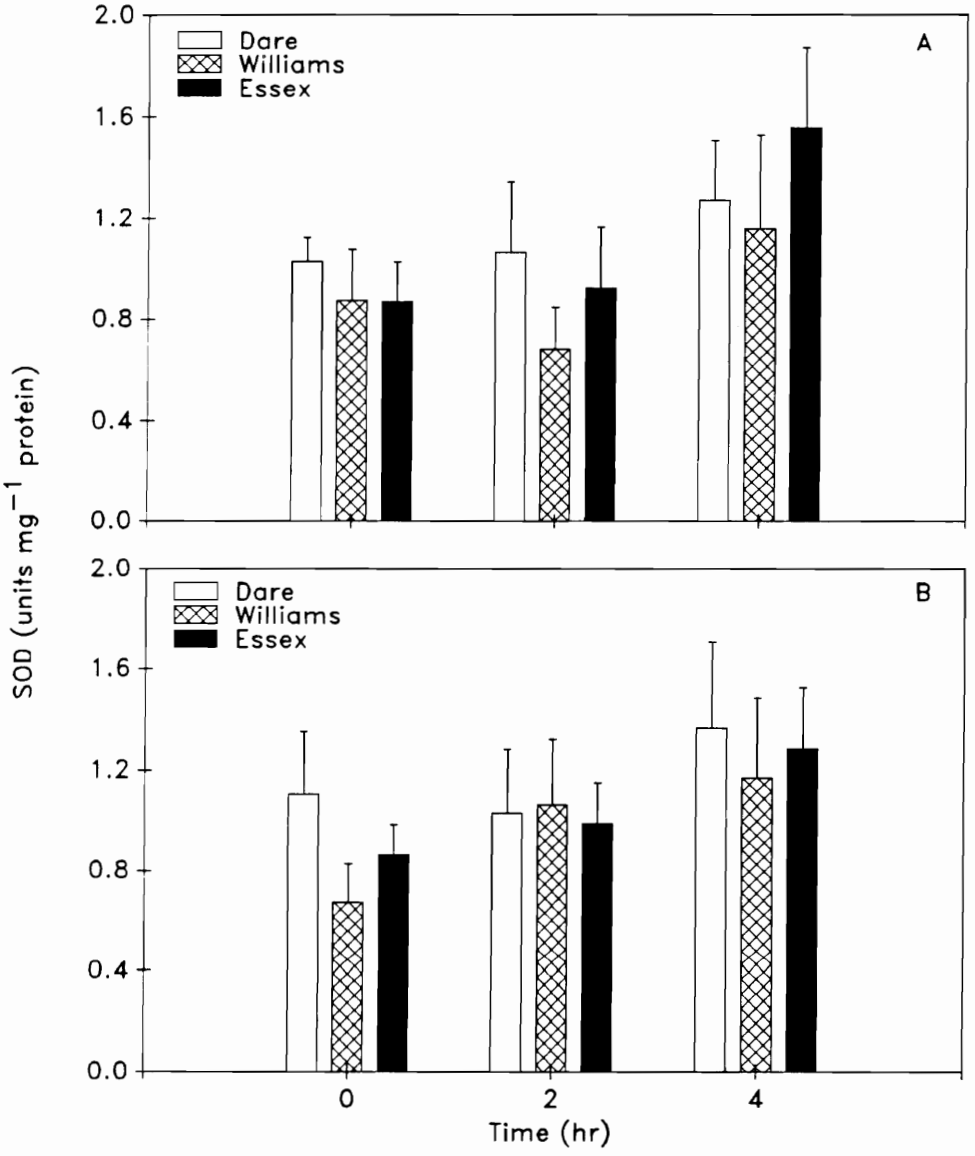


Fig. 26. Changes in superoxide dismutase specific activity of soybean cultivars exposed to A) 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 or B) filtered air for 4 hr. Bars represent the mean ± SE of 5 samples.

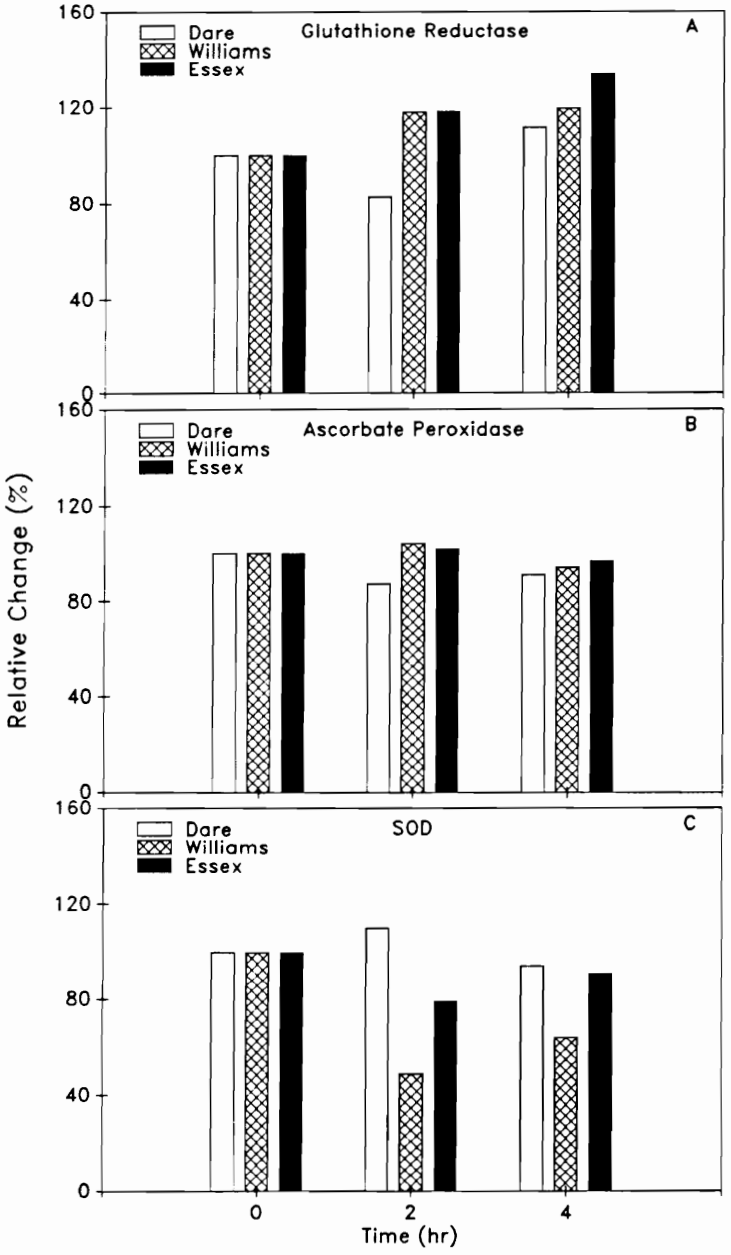


Fig. 27. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD total activities of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 5 samples.

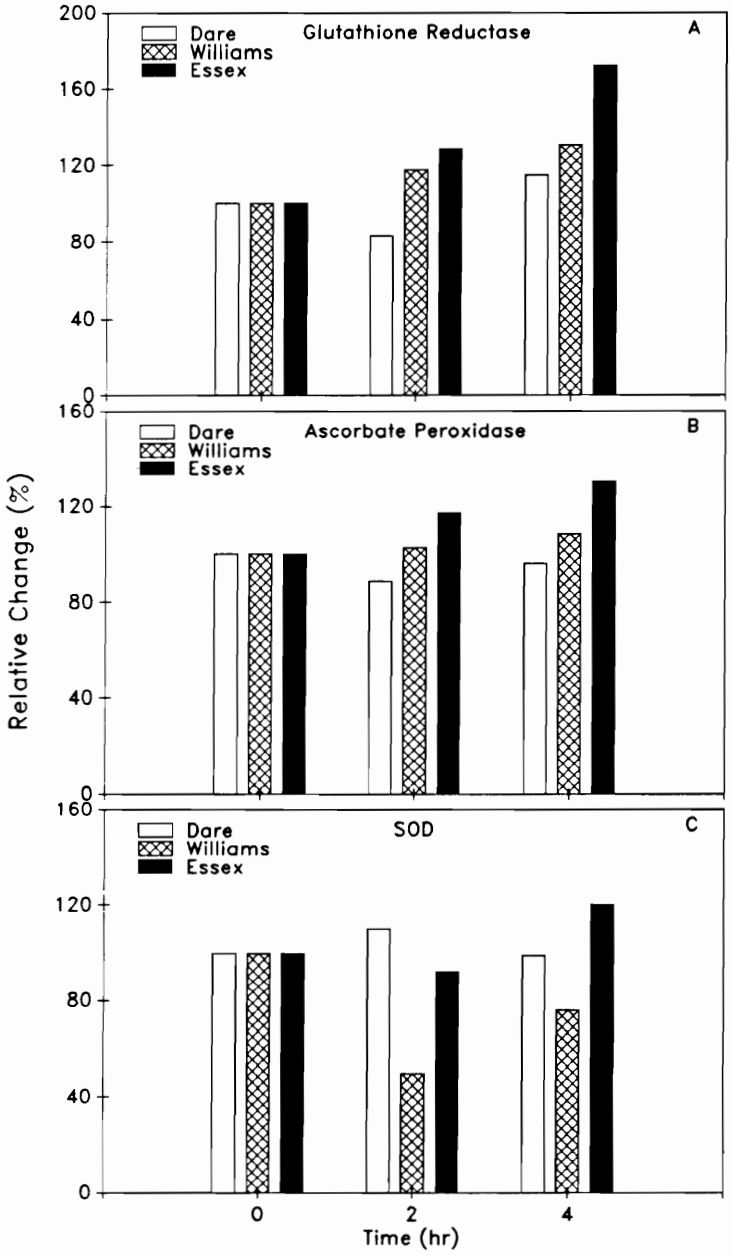


Fig. 28. Changes of A) glutathione reductase, B) ascorbate peroxidase and C) SOD specific activities of soybean cultivars exposed to 0.7 μl l^{-1} SO_2 and 0.2 μl l^{-1} O_3 for 4 hr relative to control plants. Relative changes represent mean differences of 5 samples.

CHAPTER 5. SUMMARY AND CONCLUSIONS

These experiments demonstrated that soybean cultivars, characterized as differentially sensitive to ozone (O_3) based on foliar symptoms, responded differently, physiologically and biochemically, to the pollutants O_3 and sulfur dioxide (SO_2). It was recognized that individual plants within a cultivar also could respond differently, to some extent, to these pollutants. In order to obtain responses of physiological and biochemical characteristics of soybean cultivars to the pollutants within the 4 hr exposure period, the concentrations of O_3 or SO_2 used in this study were well above the ambient levels found commonly: $\approx$ 2x normal episodic O_3 concentrations and $\approx$ 5 to 15x normal SO_2 concentrations.

Under O_3 and SO_2 stress, singly or in combination, all cultivars exhibited foliar symptoms except cv Essex which showed no injury when exposed to SO_2 alone. Typical O_3 injury appeared as dark brown to black stippling on the upper leaf surface 24 hr after fumigation. Sulfur dioxide induced bifacial, intercostal bleaching which appeared 24 hr after fumigation. Occasionally, water soaking symptoms were seen during SO_2 exposure in cvs Dare and Williams. The combination of O_3 and SO_2 fumigation resulted in injury, similar, but more severe, to that induced by O_3 alone, on both sides of leaf surface with some chlorophyll bleaching when foliage was examined under a dissecting microscope with transmitted light.

Net photosynthesis (Pn), stomatal conductance (Cs), intercellular CO_2 concentration (Ci) and transpiration (Ts) were inhibited in all cultivars by O_3 and SO_2, singly or in combination. It appeared that O_3 affected Cs ($\approx$ 70%) much

greater than Pn (≈ 30%) while SO_2 affected Cs (45~65%) slightly more than Pn (30~45%). Thus, Cs was inhibited to a greater extent by O_3 than by SO_2, while Pn was suppressed more by SO_2 than by O_3, particularly in cv Williams. The combination of O_3 and SO_2 fumigation resulted in 80% and 55% reductions in Cs and Pn, respectively, which was greater than that caused by O_3 or SO_2 alone, but less than an additive effect of both pollutants. Reduced pollutant fluxes and integrated doses in combined exposures, which were 50% and 25-30%, respectively, of those when fumigated alone, could contribute to the lack of synergistic or additive effects.

The attainment of an equilibrium Pn during exposures suggested a balance between pollutant uptake and detoxification of the pollutant or its byproducts in the leaves. The increase in WUE in all cultivars, particularly in cv Williams, and the lack of an increase in Ci at the beginning of O_3 exposure suggested that O_3 imposed effects on Cs prior to Pn. However, SO_2 apparently affected Pn first since Cs responded slightly, σ_{ns} contributed more than 90% to a change in limitation of Pn and WUE declined in the beginning of SO_2 fumigation. As O_3/SO_2 exposure inhibited Pn and Cs immediately and substantially, changes in WUE could not be used to distinguish between pollutant effects on Pn and Cs. However, the decline in Ci in the first 15 min of O_3/SO_2 fumigation suggested that Cs was affected first by the pollutants.

Under O_3 stress, stomata were affected substantially by O_3, but stomatal closure did not fully account for the suppressed Pn since Cr declined and σ_{ns} remained high during O_3 fumigation. A higher Cs, leading to a higher O_3 flux and integrated dose, in cv Dare than in cvs Williams and Essex may have contributed to

the continuous decline of Pn during the recovery period in the former cultivar. The higher Ci in cv Dare during O_3 exposure suggested that mesophyll processes associated with CO_2 fixation were inhibited to a greater extent, as reflected in a higher σ_{ns}, than in cvs Williams and Essex. Both cvs Williams and Essex exhibited similar O_3 fluxes and integrated doses, yet cv Essex maintained a higher Pn than cv Williams from the last hr of O_3 fumigation into the recovery period. Although the degree of O_3-induced foliar injury between cvs Dare and Essex was similar, the higher Pn in cv Essex was indicative of a greater potential to accommodate O_3 stress. This experiment indicated that high Cs during O_3 exposure was detrimental to carbon fixation in the plant and the effectiveness of metabolic processes to dispose of O_3 byproducts is more important than visible injury in determining the physiological sensitivity of soybean cultivars to O_3. Therefore, the use of gas exchange responses to quantify pollutant effects on stomatal and internal (metabolic) components of photosynthesis provides a more sensitive and reliable estimation of cultivars sensitivity than does foliar symptomology.

Under SO_2 stress, cv Essex had a significantly higher Pn than cvs Dare and Williams. Inhibition of Cs by SO_2 in cv Williams was the greatest, while it was similar in cvs Dare and Essex. The lower SO_2 flux and integrated dose in cv Williams did not have any positive impact on its Pn which was the most inhibited among cultivars. This experiment demonstrated that cv Essex has the most efficient photosynthetic system, genetic capacity for SO_2 resistance and/or best detoxifying system for SO_2, and cv Williams the least effective, among the cultivars. It also indicated that there was no correlation between the estimated total absorbed SO_2 dose and Pn reduction. Under O_3/SO_2 stress, Pn and Cs was higher in cv Essex than cvs Dare and Williams for the first 1 hr of fumigation. Thus, cv

Essex had the highest pollutant flux when O_3/SO_2 reached target concentrations and the highest pollutant integrated dose. This high pollutant uptake rate could contribute to cv Essex being unable to maintain a higher Pn to the end of fumigation, since gas exchange of all cultivars was inhibited to similar extent at this time. Apparently, the combination of O_3 and SO_2 exposure was too toxic for cv Essex to express its tolerance to SO_2 or O_3.

The endogenous antioxidant components varied among soybean cultivars and experiments. In general, total glutathione, reduced glutathione (GSH), total ascorbate and reduced ascorbate (AA) were significantly higher in cv Essex than other cultivars. The GRase activity also was higher (statistically insignificant) in cv Essex. The endogenous SOD activity was variable between experiments, possibly resulting from different analytic methods, although cv Williams always had the lowest SOD activity in all experiments. These endogenous characteristics suggested that cv Essex was able to scavenge pollutants or their byproducts more efficiently than the other cultivars.

Increases of total glutathione, GSH and GSSG were consistent in all cultivars exposed to SO_2 or O_3/SO_2, although the magnitude of change was variable. The increase in GSH accounted for the increase in total glutathione, since GSSG, which increased substantially, only constituted a small portion of total glutathione ($\approx 5\%$). This result indicated a *de novo* synthesis of glutathione, as an excess sulfur storage pool, which could be either important for protecting the photosynthetic apparatus against SO_2 or partly responsible for the detoxifying of SO_2. Relative to the controls, during SO2 fumigation, GSSG increased moderately at 2 hr ($\approx$ 60%) and dramatically at 4 hr ($\approx 173\sim260\%$). The increase was highest

in cvs Williams and Essex (≈ 260%) and lowest in cv Dare (≈ 173%). However, GSSG increased moderately at 2 hr (≈ 70%) and maintained at the same level at 4 hr in all cultivars during O_3/SO_2 fumigation. The considerable increase of GSSG could result from an accumulation of H_2O_2 which could directly oxidize GSH to GSSG. Total glutathione and GSH increased similarly (≈ 30%) among cultivars exposed to SO_2, and increased more in cv Dare (≈ 30%) than in cvs Williams and Essex (≈ 15%) exposed to O_3/SO_2. The GRase activity did not change under SO_2 stress even though GSSG increased substantially, indicating that maximum enzyme activity or GRase affinity for GSSG (Km for GSSG) might be affected by SO_2 exposure. The GRase activity increased in all cultivars exposed to O_3/SO_2 with cv Essex showing a higher increase than cvs Dare and Williams. This response could result from either enzyme activation or *de novo* synthesis.

Ozone fumigation did not induce consistent responses of antioxidant components among cultivars. No change in glutathione or GRase activity was found except a small decline of GSSG in cv Williams (25%) and slight increases of GSSG in cv Essex (15%) and of GRase activity in cv Dare (20%) relative to the controls. Decrease of total ascorbate and AA and increases of dHAA and APase activity in cv Essex exposed to O_3 could result from H_2O_2 scavenging. The primary reaction product of APase is the monodehydroascorbate radical (MDA) which could disproportionate to AA and dHAA in the absence of MDA reductase. Dehydroascorbate, which constitutes approximately 10% of the total ascorbate, declined in cv Dare and increased in cv Williams exposed to O_3 which could be due to changes in MDA (and/or dHAA reductase) reductase activity since total ascorbate and AA remained unchanged. Stimulation of SOD activity in cv Dare could stimulate the dismutation of $O_2^{\cdot -}$ to H_2O_2, while the decline of SOD activity

in cvs Williams and Essex exposed to O_3 could result from H_2O_2 inhibition. Although cv Essex has the greatest O_3 resistant potential considering its gas exchange responses and endogenous glutathione and ascorbate levels, it did not appear to be substantially more resistant than the other cultivars in our experiments. It is possible that the O_3 concentration used might have overtaxed the antioxidant system in cv Essex.

Sulfur dioxide fumigation resulted in a slight increase of AA in cvs Dare and Williams ($\approx$ 17%). Inhibition of APase activity in cv Williams by SO_2 exposure could contribute to its continuous decline of Pn (no equilibrium attainment) during SO_2 fumigation as H_2O_2 removal could be inhibited. Increase of SOD activity in cv Dare exposed to SO_2 suggested the formation of $O_2^{\cdot -}$ and H_2O_2, although AA and APase specific activity did not increase until the end of SO_2 fumigation. The decline of SOD activity at the end of SO_2 fumigation in cv Essex could result from H_2O_2 inhibition. If H_2O_2 was responsible for the suppression of Pn, due to its inhibition of sulfhydryl enzymes in Calvin cycle, then the photosynthetic apparatus in cv Essex was less sensitive to H_2O_2, and SOD activity was more sensitive to H_2O_2, than in cvs Dare and Williams exposed to SO_2. This suggested that SOD did not play a role in SO_2 sensitivity among cultivars in our study. This experiment indicated that cv Williams was most sensitive, while cv Essex was least sensitive, to SO_2.

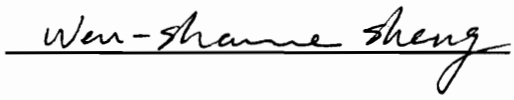
Relative to the controls, the decline of SOD activity in cv Williams after O_3/SO_2 exposure suggested a H_2O_2 inhibition. A reduction in dHAA level of cv Williams could be related to a stimulation of enzymes associated with dHAA reduction. The decrease of AA and increases of dHAA, APase and SOD specific

activity in cv Essex should dispose of $O_2^{\cdot -}$ and H_2O_2 formed, but these changes apparently were insufficient to avoid cellular damages. This experiment demonstrated that O_3/SO_2 exerted considerable effects on the physiological and biochemical processes of soybean cultivars. Although responses of antioxidant components were considerable among cultivars, the toxic effects from O_3/SO_2 fumigation were too adverse to be overcome.

Overall, these experiments characterized responses of gas exchange and the antioxidant system of three soybean cultivars to O_3 and/or SO_2 under specific environmental conditions. Importance of the antioxidant components appears to be different among the cultivars which could relate to their sensitivity to pollutants. However, potential constituents for pollutant resistance are difficult to determine under conditions when antioxidant systems are maximally, or over, stressed. Similar experiments at somewhat lower pollutant concentrations and longer exposure durations would add to an understanding of the role of antioxidants in modifying oxidative, air pollutant stress.

Vita

Wen-Shame Sheng was born October 27, 1957 in Taiwan. She received a B.S. degree in Plant Pathology in June of 1979 from National Chung-Hsing University in Taiwan and a M.S. degree in Plant Pathology in June of 1981 from the same university. After working as a research assistant in the Department of Plant Pathology at Asian Vegetable Research and Development Center in Taiwan for one year, she entered the Department of Plant Pathology at Rutgers University, NJ in September 1982 to study the honeybee spiroplasma and the production of monoclonal antibody against honeybee spiroplasma. In September 1985, she transferred to the Department of Plant Pathology, Physiology and Weed Science at Virginia Polytechnic Institute and State University to study the effects of air pollutants on soybean plants.



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