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EFFECT OF SEQUENCES OF OZONE AND NITROGEN DIOXIDE ON PLANT DRY MATTER AND STOMATAL DIFFUSIVE RESISTANCE IN RADISH

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ABSTRACT

Ozone (O_3) is the most important gaseous air pollutant in the world because of its adverse effects on vegetation in general and crop plants in particular. Since nitrogen dioxide (NO_2) is a precursor of ozone, studying the implication of sequences of these two gases is very important. Hence, the effects of sequences of fluctuating levels of ozone (O_3) and/or nitrogen dioxide (NO_2) on growth and stomatal diffusive resistance were studied in Radish (*Raphanus sativus* L.). Nitrogen dioxide was applied either alone early in the day (06:00 to 10:00 hr), late (18:00 to 22:00 hr), both early and late (06:00 to 10:00 hr and 18:00 to 22:00 hr) or early with ozone (O_3 from 10:00 to 18:00 hr), late with ozone, and both early and late with ozone. Ozone was also applied alone (10:00 to 18:00 hr), while control plants (C) were not exposed to either gas. The exposure profiles for both gases approximated sine waves with peak concentrations of 120 parts per billion by volume; nl l^{-1}. In the case of O_3, this is close to the reported threshold for adverse effects, while with NO_2 it is below the reported threshold. Ozone alone had no effect on growth after 21 days; while NO_2 caused a significant increase in growth only when applied alone early. Exposures to NO_2 in sequence with O_3 had negative effects on growth. In the control treatment, stomata tended to be relatively closed in the morning and late afternoon. Nitrogen dioxide alone enhanced this closure, while O_3 alone or with NO_2 countered the closure.

Key Words: Plant growth, *Raphanus sativus*, stomatal resistance

RÉSUMÉ

L'Ozone (O_3) constitue un gaz le plus important dans la pollution de l'air au monde à cause de ses effets néfastes sur la végétation en général et les cultures en particulier. Etant donné que le dioxyde d'azote (NO_2) est un précurseur d'ozone, étudier les implications des séquences de ces deux gaz est très important. Ainsi, les effets des séquences de fluctuation des niveaux d'ozone (O_3) et/ou du NO_2 sur la croissance et la résistance diffusive de stomates étaient étudiés dans le Radis (*Raphanussativus* L.). Le dioxyde d'azote était appliqué soit seul tôt le matin (06 :00 à 10 :00hr), tard (18:00 à 22:00 hr), en même temps tôt le matin et tard (06:00 à 10:00 hr et 18:00 à 22:00 hr) ou tôt avec l'ozone (O3 à partir de 10:00 à 18:00 hr), tard avec l'ozone, et en même temps tôt et tard avec l'ozone. L'ozone était appliqué seul (10:00 à 18:00 hr), pendant que les plantes témoins (C) n'étaient exposées à aucun gaz. L'exposition des profiles pour les deux gaz a approximé une ondulation avec une concentration de 120 parties par millions par volume ; nl l^{-1}. Dans le cas de O_3, ceci est proche de la valeur limite pour des effets adverses reportés, pendant que pour NO_2 cette valeur est en deçà de la valeur limite. L'ozone à lui seul avait un effet sur la croissance après 21 jours, pendant que NO_2 une fois appliqué seul et tôt a induit une augmentation significative de la croissance. Les expositions au NO_2 en séquence avec O_3 avaient d'effets négatifs sur la croissance. Dans le traitement témoins, les stomates tendaient à être relativement fermées le matin et tard dans l'après midi. L'application du NO_2 seul avait induit cette fermeture alors que O_3 seul ou avec NO_2 a contré la fermeture.

Mots Clés: Croissance des plantes, *Raphanus sativus*, résistance de stomates

INTRODUCTION

Ozone is the most important of the phytotoxic gaseous air pollutants. NO_2 is the pollutant closely associated with ozone (Reinert and Gray, 1981; Cho *et al*., 2011) because of the role that NO_2 plays as a precursor of O_3 in polluted air. Since O_3 maxima typically occur during the early afternoons, while NO_2 maxima occur in the mornings and evening hours, the response of plants to O_3 exposures cannot be dissociated from daily sequences of exposure to NO_2 and O_3 under field conditions. Although there are very few studies of the effects of such sequences, it has become clear that the two gases have complicated interactive effects on vegetation. Runeckles and Palmer (1987) showed that pretreatment with NO_2 reduced the impact of O_3 on leaf growth and retention in wheat (*Triticum aestivum* L.) and radish (*Raphanus sativus* L.). Kress and Skelly (1982) reported less than additive interactions between O_3 and NO_2 in sweet gum (*Liquidambar Styraciflua* L.) and white ash (*Fraxinus americana* L.). Goodyear and Ormrod (1988) observed reduced biomass caused by the sequence NO_2-O_3 and no effect of the O_3-NO_2 sequence in tomato (*Lycopersicon esculentum* Hill.). Bender, Weigel and Jägen (1991) showed reduced N assimilation caused by the O_3-NO_2 sequence in bush bean (*Phaseolus vulgaris* L.).

Much of the early work on the effects of O_3 and NO_2 utilised "sudden" exposures to relatively high steady-state concentrations (>200 ppb) of either pollutant. Later work emphasized the importance of using exposures involving concentrations that increase to and decrease from a peak level (Bicak, 1978; Dann and Pell, 1989; Bahl and Kahl, 1995; Wellburn and Wellburn, 1996), since such exposure profiles more closely resemble those occurring in ambient air. Most studies involving open-top field exposure chambers have used exposures to fluctuating O_3 profiles (Heagle*et al*., 1988).

When the daily cumulative exposures were equal, Musselman*et al*. (1986) found no differences in adverse effects of O_3 on the growth of *Phaseolus vulgaris* L. between square-wave (steady-state) and fluctuating exposures with the same peak concentration. In the present study, the daily fluctuating exposures lasted approximately twice as long as such steady-state exposure. However, if the duration of the daily exposure period and cumulative exposures were the same (achieved with peak levels in the fluctuating exposure double those in the square-wave) the fluctuating exposure led to greater adverse effects (Musselman *et al.*,1986). The present work therefore, points out the caution needed in making comparison among data obtained with different exposure profiles.

Since ozone dissolves in water to give superoxide, peroxyl and hydroxyl radicals (Hoigne and Baber, 1975; Staehelin and Hoigne, 1985). The mechanism of its toxicity has always been thought to be a result of the reaction of these highly reactive radicals with components of cell walls and cell membranes. An oxidative burst in plants' sensitive to ozone has been confirmed (Wohlgemuth *et al*., 2002; Pasqualini *et al*., 2003; Tiwariand Agrawal, 2011; Hasanuzzaman*et al*., 2012). In addition, ESR studies have detected free radicals (Runeckles and Vaartnou, 1997) at the early stages of ozone response. Further, ozone reacts directly with bio-molecules and constituents of plant surfaces. It causes lipid peroxidation (Tiwari and Agrawal, 2011), chlorophyll diminution (Saitanis *et al*., 2001), photochemical damage (Velikova1*et al*., 2005) and impairment of stomatal rhythms (Tiwari and Agrawal, 2011), among many other negative effects. At low levels (< 210 ppb 1-hr objective) and alone, nitrogen dioxide is rarely phytotoxic (Bennett *et al*., 1975). However, in combination with other pollutants like SO_2 (Bennett *et al*., 1970) or O_3, it enhances the phytotoxicity of such pollutants. At levels more than 30 times the ones used in the present study, Shimazaki (1988) found inhibition of photosynthetic electron transport in spinach leaves and Zeevaat (1976) found inhibition of pigment systems in several species. The objective of this study was to determine the effect of sequential exposure to NO_2 and/or O_3 on dry matter and stomatal diffusive resistance in radish.

METHODS AND MATERIALS

Plant materials. A greenhouse experiment was conducted with Radish (*Raphanus sativus* L. cv

Cherry Belle) plants grown from seed in 13 cm diameter pots containing about 982 cc standard potting soil (85 loam; 15% peat). A slow release fertiliser,14:14:14 NPK (Osmocote; Sierra Chemical Company), was applied to the standard potting soil at planting at 1g pot^{-1}. About 15 radish seeds, variety Cherry belle, were sown per pot and the seedlings were thinned to 4 plants per pot 10 days after sowing. Soon after thinning, they were transferred to a control growth chamber, where they acclimatised for 3 days before the commencement of exposure to various sequences of O_3 and NO_2 (4 pots per treatment). After 21 days of exposure to each respective treatment, whole plants were harvested, washed, separated (shoots and hypocotyls) and dried at 78°C in an oven for 3 days prior to weighing.

Growth chamber exposures. Growth chambers (Model EF7, Conviron Ltd.) were modified to allow the measurement of leaf diffusive resistance in *situ*. Plants were maintained in the growth chambers under a photoperiod of 16.5 hr (light of intensity 150 µmol m^{-2} s^{-1}) and 7.5 hr (dark) and day/night temperatures of 24 and18°C, respectively. The photoperiod started at 05:45 hr whereas the thermoperiod ran from 06:00 to 22:00 hr.

The chambers were supplied with air filtered through activated charcoal and Air Repair (alumina coated with $KMnO_4$) (Air Repair Products Inc.) to remove ambient O_3, NO_2 and NO. To simulate field conditions, exposures to NO_2 and O_3 both approximated sine waves, with <10 ppb minimum and 120ppb maximum concentrations. A computer programme written for a 21X Micrologger (Campbell Scientific Corp.) effected control of the exposure system. The programme provided O_3 exposures between 10:00 and 18:00 hr and NO_2 exposures between 06:00 and 10:00 hr (early NO_2) and between 18:00 and 22:00 hr (late NO_2). Only one chamber was used for NO_2 and O_3 exposures in the present study; the second chamber was available in the event of equipment failure. A third chamber with filtered air was used as a control. The complete range of seven treatments was obtained by moving plants from chamber to chamber as required.

Ozone and NO_2 levels in each chamber were monitored in sequence through a time share system operated by the data logger which controlled input to a Model 1003 AH O_3 monitor (Dasibi Environmental Corp.) and a Model 14D/E NOx monitor (ThermoElectron Corp.). The nitrogen oxides (NO_x) monitor provided outputs of both nitrogen dioxide (NO_2) and nitric oxide (NO) levels.

Ozone was generated by means of an ultraviolet source (Delzone Z0.300; Del Industries Ltd.) supplied with air from an oil free diaphragm pump. Nitrogen dioxide was supplied from a cylinder of compressed NO_2 in nitrogen (1%). Overall concentrations of NO_2 and O_3 in the chambers were adjusted using needle valve/flow meter combinations to produce the desired maximum concentrations (120 ppb). The selection of 120 ppb maxima for both pollutants was based in part on the 1 hour average concentrations that are defined in the Federal Air Quality Objectives for Canada (210 ppb for NO_2 and 82 ppb for O_3), and the Air Quality Standards for the United States (53 ppb, annual average for NO_2 and 120 ppb for O_3).

Plants were exposed to eight air pollutant exposure treatments, namely control (C), O_3 alone (~O~), early NO_2 alone (N~~), late NO_2 alone (~~N), early NO_2 + late NO_2 (N~N), early NO_2 + O_3 (NO~), O_3 + late NO_2 (~ON) and early NO_2 + O_3 + late NO_2 (NON). These symbols are maintained in all the presented illustrations in this paper.

Typical exposure regimes for the seven treatments are shown in Figure 1. The curves were obtained with the distance weighted least squares (DWLS) smoothing function of SYGRAPH (SYSTAT Inc.) and show the typical daytime rise in ambient O_3, the tendency for NO_2 maxima to occur early and late in the day, and for NO to have a morning maximum. The data logger also recorded chamber temperatures and light intensities to provide assurance of the temperature and light regimes.

Stomatal resistance measurements and estimation of pollutant fluxes. A Model LI-1600 (LI-COR Corp.) steady state porometer was used to measure leaf diffusive resistance. Measurements were made on the seventh day after the beginning of exposure to NO_2/O_3 combinations in each growth experiment. In each treatment, readings were obtained on the first true

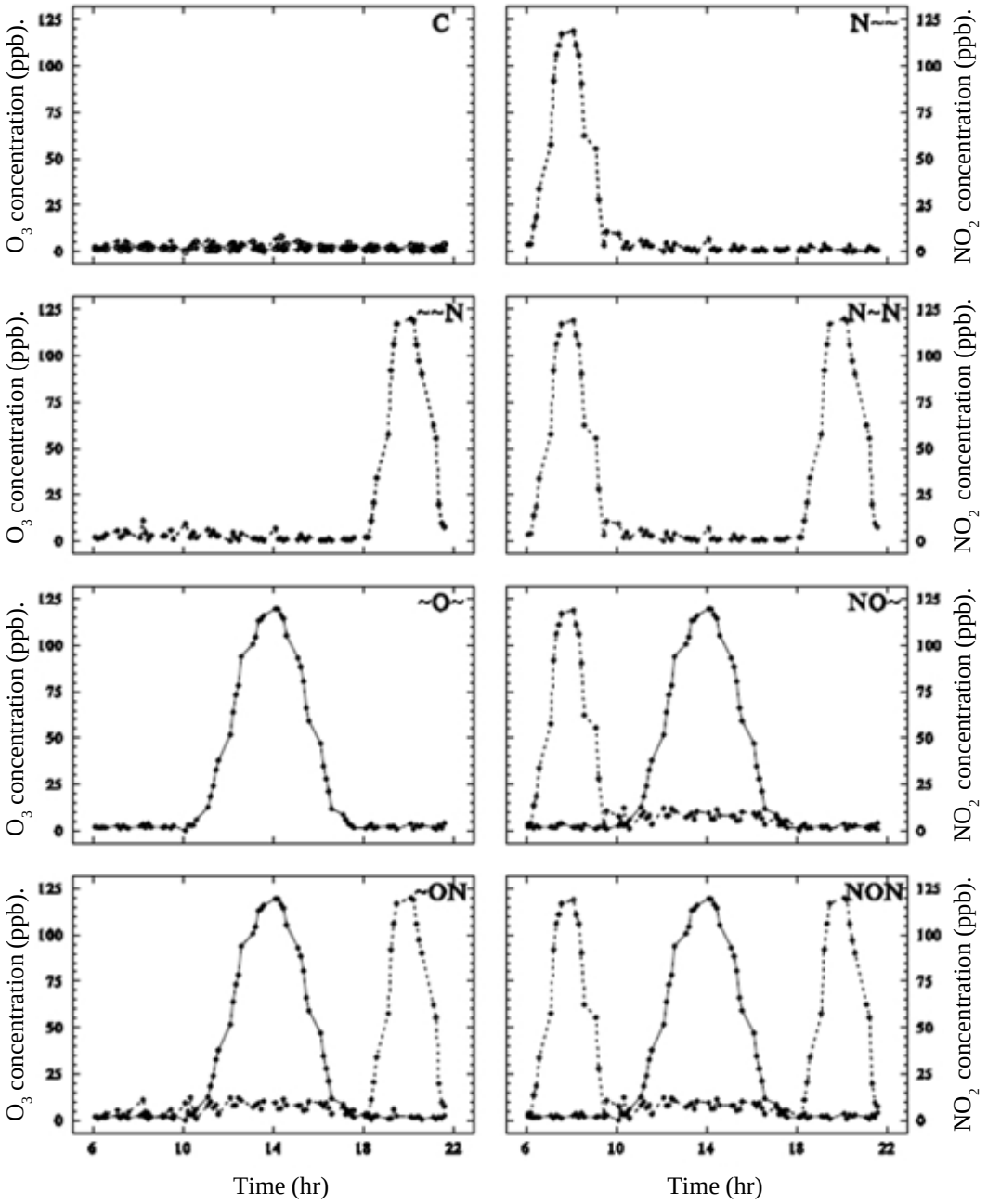

Figure 1. The exposure regime for the NO_2/O_3 sequences. The sequences were: charcoal filtered air C. exposed to O_3 alone (~O~), early NO_2 (N~~), late NO_2 (~~N), both late and early NO_2 (N~N) early NO_2 plus O_3 (NO~), O_3 plus late NO_2 (~ON), and both late and early NO_2 plus O_3 (NON). The early NO_2 (broken line) was applied from 06:00 to 10:00 hr late NO_2 was applied from 18:00 to 22:00 hr and O_3 (solid line) was applied from 10:00 to 18:00 hr.

leaf of each of 4 plants per pot, at approximately hourly intervals from 07:00 to 22:00 hr. In order to analyse the data obtained for each treatment, the readings were segregated by hour using the range: h ± 0.5, and the mean time and mean diffusive resistance calculated using the data for each leaf and experiment (N=16). The diffusive resistance data were normalised to the value at 14:00hr in order to remove leaf to leaf variation. Fluxes of the gases were computed from the relationship:

$$F = C/r_d$$

where F =flux (g $m^{-2}s^{-1}$ units), C =gas concentration (g m^{-3} units), and r_d=diffusive resistance of the gas (s m^{-1} units), obtained by inverse proportionality of the molecular diffusivity of the gas to that of water vapour (Runeckles, 1992).

Statistical analyses. SYSTAT/SYGRAPH (Systat Inc.) was used for all statistical analyses. Orthogonal contrasts were used to compare treatment means.

RESULTS

Effects of NO_2/O_3 sequences on growth. Nitrogen dioxide applied early during the day significantly ($P<0.05$) stimulated total dry matter accumulation per plant relative to control plants in filtered air (cf. C vs. N~~; Fig. 2A). The stimulation by early + late NO_2 was not significant (cf. C vs. N~N; $P<0.1$). The slight increase over the control due to late NO_2 was not significant, and late NO_2 had no effect on the response to early NO_2 (cf. N~N vs. N~~). Ozone alone had no significant effect on total dry matter accumulation (cf. C vs. ~O~), but significantly suppressed the stimulatory effects of early NO_2 (cf. N~~ vs. NO~) ($P<0.05$), late NO_2 (cf. ~~N vs. ~ON) ($P<0.1$) and early + late NO_2 (cf. NON vs. N~N) ($P<0.01$).

The effects of treatment on shoot dry matter production (Fig. 2B) were similar to those on total dry matter. Early NO_2, late NO_2, and early + late NO_2 tended to stimulate shoot dry matter production. Late NO_2 had no effect on early NO_2 (~~N vs. N~N). Ozone alone had no effect on shoot dry matter accumulation, but significantly ($P<0.05$) suppressed the stimulatory effect of early + late NO_2 (cf. NON vs. N~N). It also suppressed ($P<0.1$) the slight stimulation of early NO_2 (cf. NO~ vs. N~~) or late NO_2 (cf. ~ON vs. ~~N).

Hypocotyl dry matter accumulation also showed similar effects of treatment to total dry matter. As shown in Figure 2C, early NO_2 and early + late NO_2 resulted in stimulation relative to the control (cf. C vs. N~~; $P<0.01$; C vs. N~N; $P<0.05$). Ozone alone had no effect, but it significantly reduced the stimulation caused by NO_2 (cf. NO~ vs. N~~; $P<0.01$; ~ON vs. ~~N; $P<0.1$; NON vs. N~N; $P<0.01$). As a result of the effects of treatment on component dry weights, shoot to hypocotyl ratios relative to the control were marginally reduced by O_3 ($P<0.1$) while all the other treatments and treatment combinations were not significant (Fig.2D).

Stomatal conductance and gas fluxes. Figure 3 presents consolidated data obtained during the seventh day of treatment in each experiment, i.e. for plants having received six daily cycles of treatment before the measurements were made. Repeated exposure to early NO_2 tended to enhance early morning stomatal closure (cf. N~~ vs. C; Fig. 3), late NO_2 accelerated closure late in the day (cf. ~~N vs. C) and the combination of two daily exposures to NO_2 tended to increase the late afternoon resistance, which persisted through the following morning (cf. N~N vs. C). Thus, the combination of early + late NO_2 enhanced the early and late stomatal closures due to either early or late NO_2 alone (cf. N~N vs. N~~, N~N vs. ~~N).

In addition to suppressing the normal morning and evening closure of stomata in the control, exposure to O_3 tended to overcome both early and late closure of stomata induced by late NO_2 (cf. ~ON vs. ~~N) and to a lesser extent, the early closure caused by early NO_2 (cf. N~~ vs. NO~) or early + late NO_2 (cf. NON vs. N~N). Conversely, late NO_2 had no effect on the course of diffusive resistance caused by early NO_2 and O_3 (cf. NON vs. NO~), while early NO_2 tended to overcome the suppression of early stomatal closure caused by O_3 (cf. NO~ vs. ~O~).

The estimated fluxes of both gases are shown in Figure 4. The NO_2 flux reached a maximum of

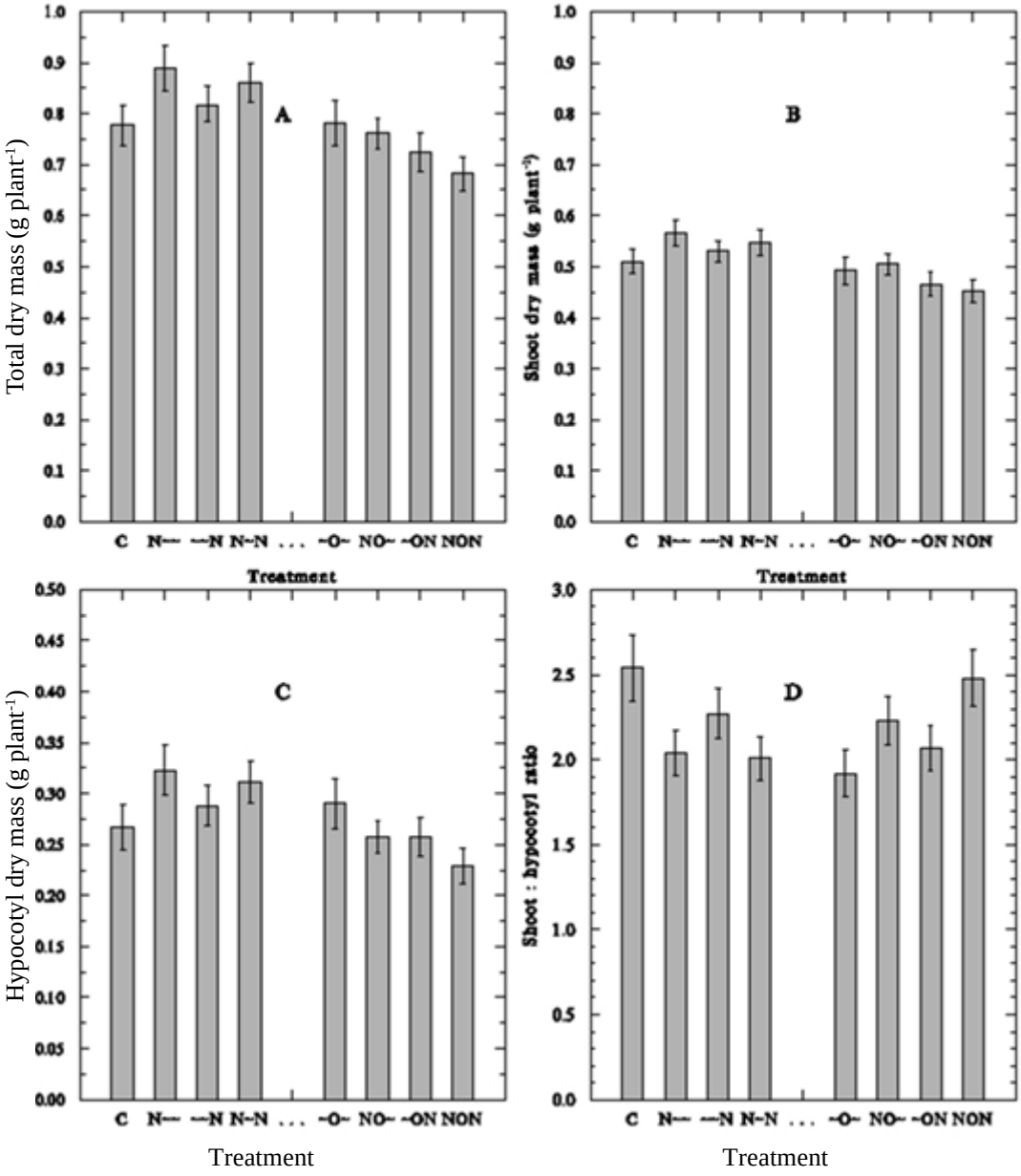

Figure 2. Effects of treatment on total (A), shoot (B), hypocotyl (C) dry shoot to hypocotyl ratio (D). Error bars are standard errors (n=64).

0.5 µg m^2s^1 in the absence of O_3. However, in the presence of O_3 the NO_2 fluxes approximately doubled (cf. NO~, ~ON, and NON). The O_3 flux was not affected by exposure to NO_2 and reached maxima of about 1.5 µg m^2s^{-1}.

DISCUSSION

The present study employed fluctuating exposures, to both NO_2 and O_3, in which the maximum concentration (120±5 ppb) was

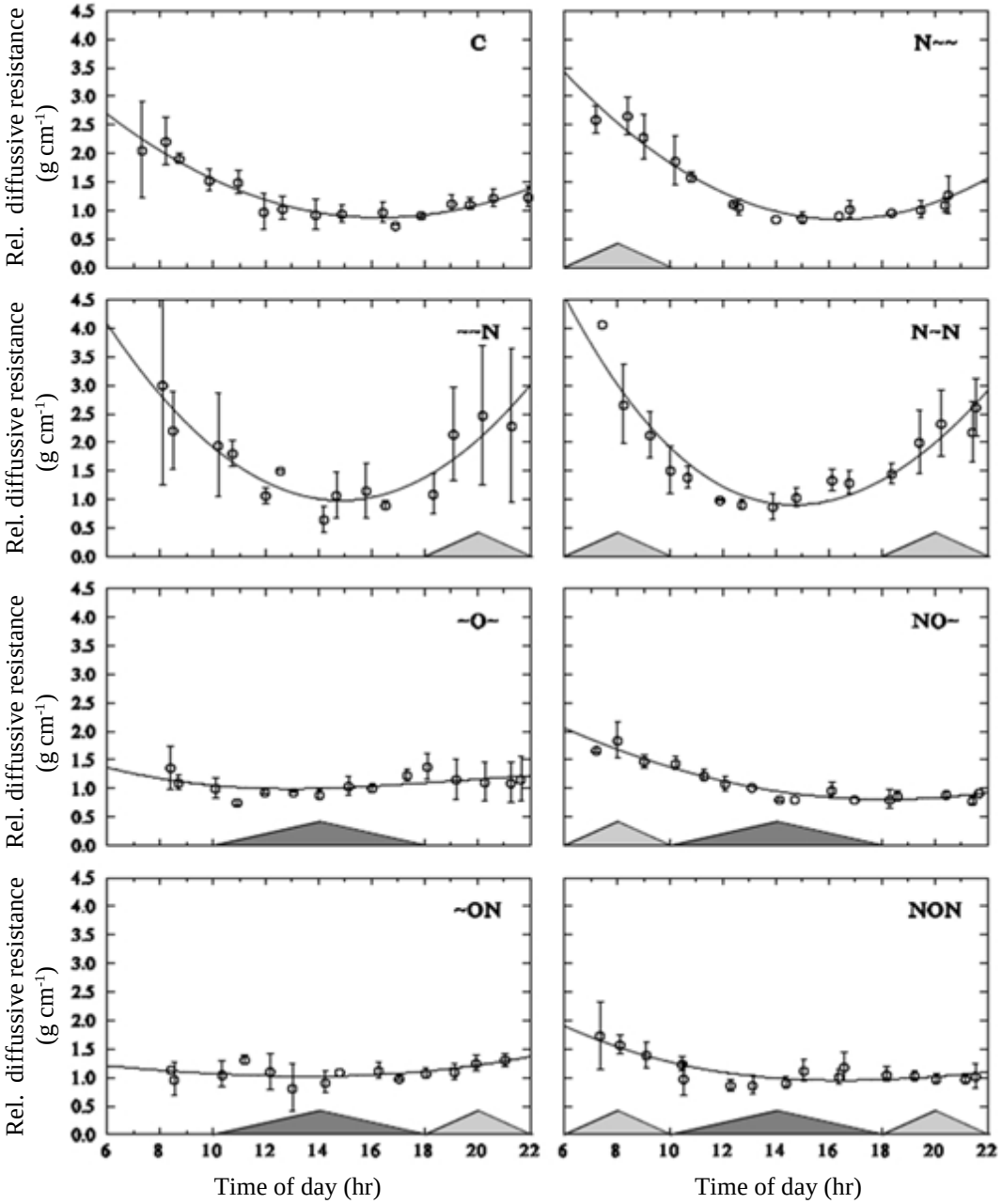

Figure 3. Time course of relative diffussive resistance by treatment. Error bars are standard errors (n=6). The shaded triangles indicate the periods of exposure to NO_2 (light shading) and O_3 (dark shading).

maintained for approximately one hour at the middle of the exposure period. The sine-wave time course used resulted in total exposures to either pollutant that amounted to one half of those that would have occurred with "square-wave" exposures in which 120 ppb was maintained throughout the exposure periods. 120 ppb NO_2 is below the Canadian Maximum Acceptable 1-hour objective, while 120 ppb O_3 is about 50% greater than the Canadian 1-hour objective, and equal to the U.S. standard.

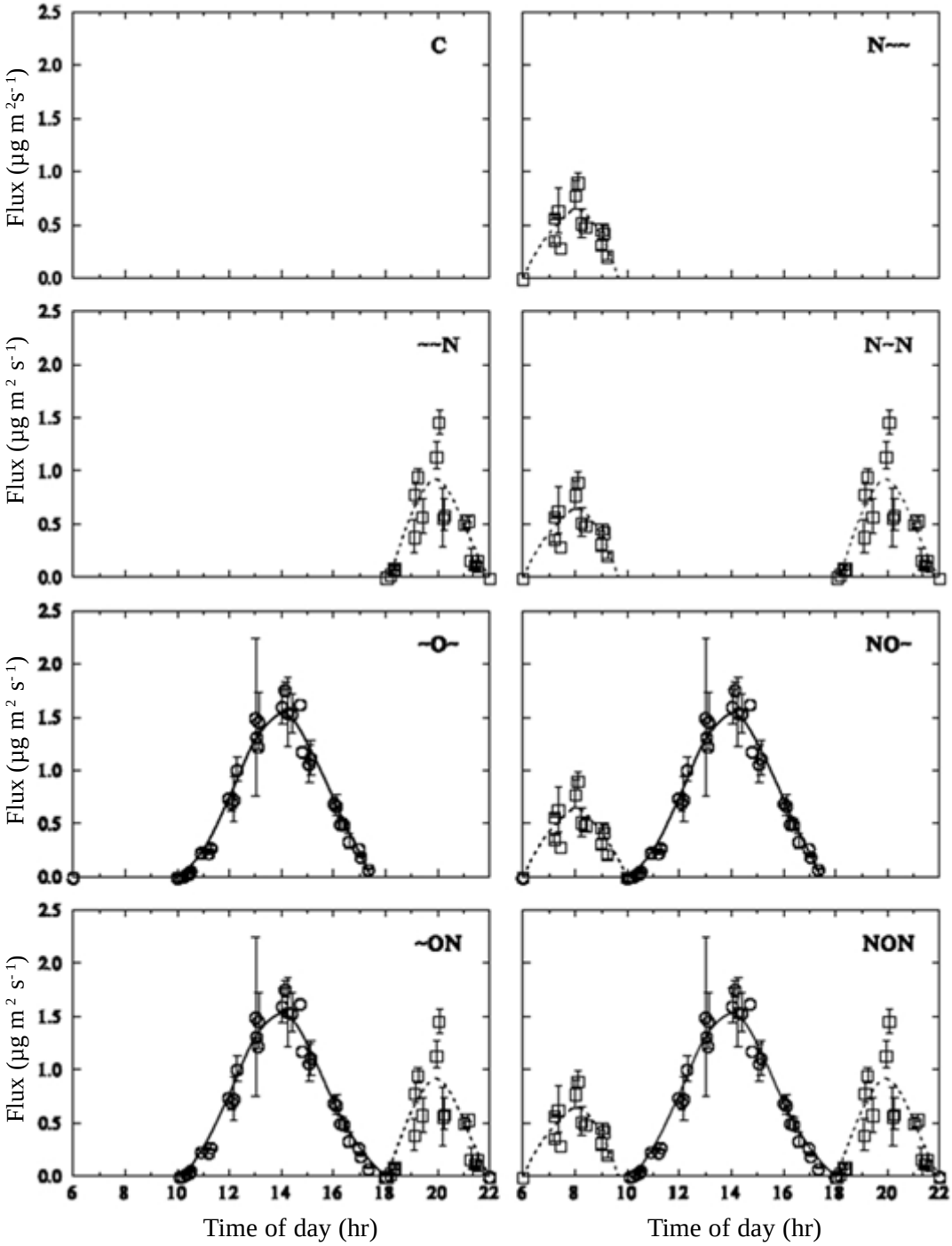

Figure 4. Time course of estimated fluxes of NO_2 (broken line) and O_3 (solid line) by treatment. Error bars are standard errors (n=16); for data without error bars, N=1.

Effects of NO_2/O_3 on growth. The observation that in spite of the low exposures used, repeated exposures to NO_2 regardless of the time of day, were found to increase significantly (or tend to increase) total shoot and hypocotyl dry matter accumulation suggested that the NO_2 must have been use as an N source by the plants. No visible symptoms of foliar injury were observed, which is in keeping with the reports that such NO_2-induced injury is uncommon in radish (see review by Kostka-Rick and Manning, 1992) even when "sudden" and relatively high concentrations are applied as square waves (200-400 ppb, Reinert and Gray, 1981; 400 ppb, Reinert and Heck, 1982; 3000, Sanders and Reinert ,1982; 200 ppb: Godzik *et al.*, 1985). In all these studies the tendency was for NO_2 to cause increased biomass. Using relatively lower early exposure concentrations (80-100 ppb for 3 hr) daily for 40 days, Runeckles and Palmer (1987) also found that NO_2 increased the biomass of radish and bean (*Phaseolus vulgaris*). The observations in the present study thus confirm previous work and suggest that NO_2 was probably used as an additional source of N as was suggested in spinach (*Spinacia oleracea*) (Yoneyama and Sasakawa, 1979).

The lack of growth inhibition by O_3 observed was unexpected since radish is ranked as 'sensitive' relative to other crops (Tingey*et al*., 1973; Ormrod *et al.*, 1984) and the cultivar used, Cherry Belle, is considered a sensitive cultivar (Wellburn and Wellburn, 1996). Ozone-induced reduced growth in radish has been widely documented (Tingey*et al*.,1973; Reinert *et al*., 1982; Reinert and Heck, 1982; Reinert *et al*., 1972Reiner*tet al.*, 1982; Reinert and Sanders, 1982; Kostka-Rick and Manning, 1992). These studies, however, also tended to involve higher concentrations of O_3 (>200 ppb) than those used in the present investigation. In some cases where concentrations in the range 150-370 ppb were applied, no biomass changes were observed despite 13-26% leaf injury (Tingey and Reinert, 1975; Reinert and Gray, 1981; Ormrod *et al*., 1984). The present results, therefore, suggest that, because of the type of exposure profile used and the maximum concentration achieved, the actual uptake of O_3 was insufficient to exceed the threshold for adverse effects on radish dry matter production, in spite of the effects on stomatal conductance discussed below.

The combinations of O_3 and NO_2, irrespective of sequence, caused no visible injury but either significantly suppressed or tended to suppress the stimulatory effect of NO_2 and confirmed the previous reports for radish exposed to the NO_2 O_3 sequence (Runeckles and Palmer, 1987) or to NO_2+O_3 mixtures (Reinert and Sanders, 1982; Sanders and Reinert, 1982; Reiner*tet al.*, 1982). Suppression of NO_2-induced stimulation of growth by O_3 was also reported in wheat and bean (Runeckles and Palmer, 1987).

Stomatal conductance and gas fluxes. The increase in diffusive resistance after 6 days of exposure to NO_2 regardless of sequence can be explained by adverse effects on guard cell abscisic acid signal transduction or calcium based signalling or both (Martin *et al*., 2002). NO_2-induced stomatal closure has been reported for other species (soybean, *Glycine max*, Carlson, 1983; *Euonyous japonica*, Natori and Totsuka, 1984), although Carlson (1983) used higher exposure levels (up to 600ppb) than those used in the present study. However, Sandhu and Gupta (1989) reported decreased stomatal resistance in bean (*Phaseolus vulgaris*) exposed daily to 100 ppb for 7 hrs per day for 3 weeks.

The Ozone suppression of stomatal closure and disruption of the apparent diurnal cycle of stomatal activity of radish to O_3 was contrary to the generally held view that O_3 induces stomatal closure even at relatively low concentrations (e.g. Beckerson and Hofstra, 1979; 150 ppb; Barnes and Pfirrman, 1992; 75 ppb). However, the review by Darrall (1989) lists O_3-induced stomatal closure in four species (one of which was radish), opening in two, and no effect in one, at concentrations <200 ppb. In the present study, the repeated daily exposure to daily maxima of 120 ppb O_3 apparently caused the stomata to lose their ability to respond to the daily photoperiod and thereby failed to close at night. This confirms the findings by Hassan *et al*. (1994) who reported stomatal opening in radish caused by 80 ppbO_3, and those of Runeckles and Rosen (1977) who found earlier that repeated exposure to 50 ppb caused stomatal response in bean (*Phaseolus*

vulgaris) to be sluggish with reduced diurnal opening-closing cycles. Evidence suggest that ozone exposure has a marked effect on the calcium based signalling in guard cells (Martin *et al.*, 2002).

In the sequences of NO_2 and O_3, repeated exposures to O_3 countered the NO_2-induced stomatal closure, leading to diffusive resistances during the morning and evening that were intermediate between those in the control and those exposed to O_3 alone. As a result these effects on diffusive resistance, the estimated flux of NO_2 was increased when followed by O_3 (during the previous daily cycles) or preceded by O_3 Hence, although O_3 alone had no significant effect on growth, it suppressed the growth stimulation induced by NO_2, in spite of an approximate doubling of the amounts of NO_2 taken up by the leaves. Conversely, since the effects of NO_2 were to increase diffusive resistance early and late in the day, NO_2 exposures had no apparent effect on O_3 flux.

SUMMARY

The observed effects on growth and diffusive resistance lead to several conclusions. First, despite the fact that NO_2 alone caused increased stomatal closure early and late in the day relative to the control, the increases in dry matter production must have resulted from increased rates of CO_2 assimilation. Stimulations of apparent photosynthesis by low levels (100 ppb) of NO_2, based on CO_2 uptake, have been reported by Sabaratnam*et al*. (1988) and Sandhu and Gupta (1989), although Carlson (1983) reported no effects with exposure as high as 600 ppb. Inhibition of net photosynthesis by high exposure levels has been reported by several workers (Wellburn, 1990), and occasionally for low levels (50 ppb; Bull and Mansfield, 1974).

Second, the O_3-induced suppression of the normal pattern of stomatal closure suggests that CO_2 assimilation would not be limited by any stomatal-dependent reduced CO_2 flux throughout the photoperiod with constant illumination and, hence, might be greater than in the controls in filtered air if O_3 did not inhibit the photosynthetic process. However, O_3-induced inhibition of net CO_2 assimilation has been widely reported, as has O_3-induced stimulation of dark respiration although responses of dark respiration are varied, with several reports of O_3-induced inhibitions (Runeckles and Chevone, 1992).

Amthor (1988) and Amthor and Cumming (1988) partitioned respiration into its growth and maintenance components and reported significant O_3-induced increases in maintenance respiration in bean (*Phaseolus vulgaris*). Hence, under the exposure conditions used in the present study, any potential for increased CO_2 assimilation resulting from the constant illumination photoperiod and reduced stomatal closure was negated either by direct inhibition of photosynthetic CO_2-fixation or by increased diversion of assimilation to maintenance, or both.

Third, if no NO_2-induced stimulation of CO_2 assimilation persisted in the presence of O_3, the tendency for the stomata to remain open in the NO_2-O_3 sequences in contrast to exposures to NO_2 alone suggests that dry matter accumulation should have been further stimulated. Conversely, the stomatal closure induced by NO_2 relative to O_3 suggests that, if the reduction in CO_2 assimilation or increase in assimilate utilization for repair resulting from O_3 alone were maintained then growth should have been further reduced. The observations that growth in NO_2-O_3 sequences was consistently less than in NO_2 alone therefore suggests that the inhibition of CO_2 assimilation or stimulation of assimilate utilisation for repair induced by O_3 overcame the NO_2-induced stimulation of CO_2 assimilation.

In view of the lack of the effect of O_3 on growth, the only conclusion possible, therefore, appears to be that increased CO_2-assimilation was nullified by increased maintenance/repair respiration.

With the NO_2 exposures, the increases in growth are compatible with the suggestion of increased CO_2-assimilation, resulting from the uptake of NO_2 early in the photoperiod, in spite of reduced stomatal conductance early and late in the day. However, it is not possible from these data alone to elucidate the mechanisms involved. Although Holmes *et al*. (1989) showed that in N-limited cultures of the alga, *Selenastrum minutum*, provision of NO_3^- effectively caused

cessation of CO_2-fixation until the supply of NO_3^- was exhausted; such a mechanism appears unlikely in the present studies in which the plants were well supplied with N. The effects of NO_2 alone on growth show that late NO_2 exposures caused no significant effects relative to the control, although the estimated flux of NO_2 was comparable to those observed in other NO_2 treatments. The NO_2 was taken up during the exposure in the light, and would result in the formation of nitrate and nitrite on dissolution in water. Since the plants had been maintained in light up to and during this exposure, the levels of nitrate and nitrite reductases should have been close to maximal, up to the end of the photoperiod

Whatever the mechanism by which NO_2 alone may have stimulated dry matter accumulation, this was suppressed by the mechanism involved in the O_3-induced effect. If the effects of O_3 alone on growth were the result of both increases CO_2-fixation and respiration then the tendency for additional exposures to NO_2 to reduce growth relative to O_3 alone suggest that the effects were on CO_2-assimilation rather than on maintenance/repair respiration.

CONCLUSION

The exposures to NO_2 and O_3 sequences led to effects on growth and diffusive resistance that suggest increased CO_2-assimilation and maintenance/repair respiration resulting from O_3 exposures. Although increased CO_2-assimilation can account for increased growth resulting from NO_2 exposures, O_3 reduces the NO_2-induced stimulation of CO_2-assimilation. Although no effects of O_3 alone could be demonstrated on radish growth, the effects of the different sequences of exposures to NO_2 and O_3 show clearly that observations made on individual pollutants may not reflect effects occurring in exposure situations that more closely resemble those likely to occur in polluted ambient environments. Lastly, the observed effects on stomatal diffusive resistance probably imply an adverse effect on the guard cell signal transduction or calcium signalling or both.

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