Enhanced sensitivity to higher ozone in a pathogen-resistant tobacco cultivar

Lefu Ye1,3,*, Xue Fu1,2,* and Feng Ge1,†

1 State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China
2 College of Agricultural Resource and Environment, HeiLongjiang University, Harbin 150086, PR China
3 State Key Laboratory of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Agricultural Academy of Sciences, Beijing 100101, PR China
* These authors contributed equally to the manuscript.
† To whom correspondence should be addressed. E-mail: gef@ioz.ac.cn

Received 8 April 2011; Revised 12 October 2011; Accepted 19 October 2011

Abstract

Investigations of the effects of elevated ozone (O₃) on the virus–plant system were conducted to inform virus pathogen management strategies better. One susceptible cultivar of tobacco (Nicotiana tabacum L. cv. Yongding) and a resistant cultivar (Nicotiana tabacum L. cv. Vam) to Potato virus Y petiole necrosis strain (PVYN) infection were grown in open-top chambers under ambient and elevated O₃ concentrations. Above-ground biomass, foliage chlorophyll, nitrogen and total non-structural carbohydrate (TNCs), soluble protein, total amino acid (TAA) and nicotine content, and peroxidase (POD) activity were measured to estimate the effects of elevated O₃ on the impact of PVYN in the two cultivars. Results showed that under ambient O₃, the resistant cultivar possessed greater biomass and a lower C/N ratio after infection than the susceptible cultivar; however, under elevated O₃, the resistant cultivar lost its biomass advantage but maintained a lower C/N ratio. Variation of foliar POD activity could be explained as a resistance cost which was significantly correlated with biomass and C/N ratio of the tobacco cultivar. Chlorophyll content remained steady in the resistant cultivar but decreased significantly in the susceptible cultivar when stressors were applied. Foliar soluble protein and free amino acid content, which were related to resistance cost changes, are also discussed. This study indicated that a virus-resistant tobacco cultivar showed increased sensitivity to elevated O₃ compared to a virus-sensitive cultivar.

Key words: Elevated ozone, open top chamber (OTC), potato virus Y petiole necrosis strain, resistance.

Introduction

Potato virus Y is one of the most common and destructive viruses that attack potatoes worldwide. Petiole necrosis strain (PVYN), vectored by the tobacco aphid, can devastate crops (Loebenstein et al., 2001), which led to selection of resistant cultivars designed to tolerate this effective plant virus.

The concentration of ozone (O₃), a major tropospheric photochemical oxidant, has risen by 0.5% to 2.5% per year in industrialized countries and is predicted to reach a global mean of >60 nl l⁻¹ by 2050 (Morgan et al., 2004). Elevated O₃ causes leaf damage in many plant species, inhibits photosynthesis, and reduces growth and yield accumulation (Horst et al., 1990; Schraudner et al., 1998; Morgan et al., 2003; Ashmore, 2005). O₃ reacts with the cell wall and cell membrane to produce reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (Kangasjärvi et al., 1994), and triggers a series of metabolic reactions (Kanofsky and Sima, 2000; Langebartels et al., 2000). Excess ROS can disrupt plant metabolism by causing irreversible damage to cell membranes, proteins, carbohydrates, and DNA (Apel and Hirt, 2004); furthermore, ROS availability influences the accumulation of infection-induced secondary metabolites (Clara et al., 2010). Even in the
absence of visible symptoms of O₃ damage, growth and development can be inhibited (Krupa, 2003; Ashmore et al., 2007); and this change can influence disease susceptibility and the effect is variable. For example, in wheat, leaf rust disease was strongly inhibited by O₃ (Tiedemann and Firsching, 2000), and the resistance of barley and fescue to B. sorokiniana was enhanced (Plazek et al., 2001). Conversely, for necrotrophic fungi, increased susceptibility was found after O₃ exposure (Manning and Tiedemann, 1995), and O₃ significantly increased disease incidence in pine seedlings (Bonello et al., 1993).

Unlike most fungal pathogens, whose infection periods are essentially non-coincident with periods of high ambient O₃ and thus present minimal interactive risks, viruses occur within high ozone periods and suppress host defences, which can exacerbate disease expression (Sandermann, 2000). Moreover, elevated O₃ altered the gene expression of plants and induced a host defence response (Bilgin et al., 2008). Thus, intensified research on interaction between higher O₃ and plant virus is needed to improve understanding and management of plant diseases in the face of current and future climate extremes (Coakley et al., 1999).

Reports on the impact of global change (not only elevated O₃) on plant diseases has been limited, with most work concentrating on the effects of a single atmospheric constituent or meteorological variable on the host and the pathogen, or the interaction of the two under controlled conditions (Coakley et al., 1999). Research to date has indicated that elevated O₃ damages plant tissues and increases risk to infection (Brennan and Leone, 1970; Reinert and Gooding, 1978; Heagle et al., 1992; Bilgin et al., 2008); however, little is known about the relative effects of elevated O₃ on resistant and susceptible cultivars of the same crop, which is needed to develop more holistic approaches to controlling plant disease (Oksanen and Saleem, 1999).

Investigating the variation in resistance of crops against virus pathogens under elevated O₃ is an important step to understand the effects of elevated O₃ on the efficacy of virus pathogen management strategies. In this study, the hypothesis was tested that elevated O₃ would reduce the relative resistance advantage of a resistant tobacco cultivar against PVYN infection. Two tobacco cultivars were used, one with resistance and one with susceptibility to PVYN infection, grown in open-top chambers (OTC) under ambient air and increased concentrations of O₃. Two questions were addressed: (i) what is the difference between resistant cultivar responses and susceptible cultivar responses to elevated O₃ and the interaction of O₃ with virus; (ii) how do assimilation rate, resistance costs, and other plant response variables related to virus infection change with exposure to elevated O₃?

Materials and methods

Sites and facilities

This experiment was conducted in eight hexagonal open-top chambers (OTCs), each 2 m in diameter, located at the Observation Station for Global Change Biology, Institute of Zoology, Chinese Academy of Sciences (CAS) in Xiaotangshan County, Beijing, China (40°11’ N, 116°24’ E). Two levels of atmospheric O₃ concentration, ambient (40 nl l⁻¹) and elevated (80 nl l⁻¹) were applied. Four open-top chambers were used for each O₃ treatment. O₃ came from an O₃ generator (3S-A15, Tonglin Technology Beijing, China) and then sent to the higher O₃ OTC entries using a fan (HB-429, 4.1 m³ min⁻¹, Ruyingh Mechanical and Electrical Equipment Company). Mixed air (O₂ and ambient air) was ventilated to each elevated O₃ OTC through columniform polyvinyl chloride pipes (inner diameter=11 cm) in the day time from 09.00 h to 17.00 h. In the control treatment, ambient air was ventilated to each OTC continuously. The air was changed twice per minute in each OTC through a hemispherical stainless steel sprayer (diameter=30 cm, at 1.5 m height) at a rate corresponding to approximately 15 m³ min⁻¹. The hemispherical sprayer was adjusted to make a homogeneous distribution of treated gas (monitored by the instrument mentioned below) throughout each OTC. O₃ concentrations were monitored within OTCs (AQL-200, Aeroqual).

The actual daily O₃ concentration (within 8 h) range was 40±10 nl l⁻¹ in the ambient chambers and 80±10 nl l⁻¹ in the elevated chambers (means ±SD; SD here referred to variation between hours and replicate chambers). O₃ concentration outside 8 h was not monitored continuously, but periodic examination showed a night-time value of ~0 nl l⁻¹ within all OTCs. The open tops of these chambers were covered with nylon net to prevent insects from entering. The plants were acclimated to the environment in the chamber for 48 h before initiating O₃ exposure. Air temperature was measured three times per day and did not differ significantly between the two sets of chambers (25.7±2.6 °C in the ambient O₃ chambers versus 27.5±3.2 °C in the elevated O₃ chambers) throughout the experiment.

Tobacco cultivars and growth conditions

Two tobacco cultivars were obtained from the Institute of Tobacco, Chinese Academy of Agricultural Sciences in TsingDao city, Shandong province. Both tobacco (Nicotiana tabacum L.) cultivars (Yongding with susceptibility to PVYN and VAM with resistance to PVYN) were sown in trays on 25 May 2008. On 25 June, tobacco seedlings, one plant per pot, were transplanted into plastic pots (diameter:height of 10:12 cm) filled with 8:1 v/v of turfy soil: vermiculite. Sixteen pots of five-leaf-stage plants were randomly assigned to each chamber for O₃ treatment on 5 July 2008. Plants were irrigated sufficiently every other day using tap water and fertilized once a week with 100 ml of a 0.5% solution of NPK fertilizer (15-15-15). Pots were randomly exchanged every other day. Tobacco samples were harvested in another month of treatment.

PVYN infection

Potato virus Y petiole necrosis strain from potato plants was identified with RT-PCR and reproduced in tobacco plants in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Infected tobacco leaves stored in a −20 °C freezer were homogenized in 100 mM K-phosphate buffer, pH 7.0 (1 g of leaf material in 20 ml of buffer), to obtain a viral extract. When the plants had 6–7 leaves (on 19 July 2008), plants were mechanically infected with viral extracts by rubbing the virus liquid with carborundum powder on the dorsal face of the fifth leaf of the tobacco plant. After 5 min, the treated leaves were washed with distilled water. Eight tobacco plants from one OTC were infected with PVYN. Another eight plants without infection from the same OTC, as a control, were simultaneously inoculated with normal saline. Three days later, all plants were replaced into the OTC for another month of treatment.

Downloaded from https://academic.oup.com/jxb/article-abstract/63/3/1341/471540 on 27 July 2018
Elevated ozone reduces virus damage on two tobacco cultivars

Table 1. ANOVA results for the effects of ozone level, tobacco cultivars, and tobacco virus (PVYN) on the above-ground biomass and foliar nutrient constituents of tobacco

| Main effects and interactions | Dependent variable | Mass | TNCs | Nitrogen | C/N | Nicotine | POD | TAA | Protein |
|------------------------------|--------------------|------|------|----------|-----|----------|-----|-----|---------|
| Ozone (O)<sup>a</sup>        | n.s.               | *    | n.s. | *        | n.s.| n.s.     | n.s.| *  | *       |
| Cultivar (C)<sup>b</sup>     | ***                | ***  | ***  | ***      | *** | ***      | *** | n.s.| ***     |
| PVYN (P)<sup>c</sup>         | ***                | n.s. | n.s. | n.s.     | n.s.| n.s.     | n.s.| n.s.| n.s.   |
| O×C                         | **                 | n.s. | n.s. | n.s.     | n.s.| n.s.     | n.s.| n.s.| n.s.   |
| O×P                         | n.s.               | n.s. | n.s. | n.s.     | n.s.| n.s.     | **  | n.s.| n.s.   |
| C×P                         | **                 | n.s. | n.s. | n.s.     | **  | n.s.     | n.s.| n.s.| n.s.   |
| O×C×P                       | *                  | n.s. | n.s. | n.s.     | n.s.| **      | *** | n.s.| *      |

<sup>a</sup> Ozone levels (ambient and elevated O₃).

<sup>b</sup> Cultivars (susceptible and resistant).

<sup>c</sup> PVYN (with and without).

<sup>d</sup> TNCs:Nitrogen.

<sup>e</sup> Peroxidase.

<sup>f</sup> Total amino acids significance levels are indicated by *P <0.05, **P <0.01, ***P <0.001, and n.s. denotes non-significant.

Sampling

On 12 September 2008, after growing in the OTCs for 65 d, tobacco seedlings from each OTC were cut at ground level, weighed, and kept at −20 °C until laboratory examination. A 10 g wet foliage sample was dried at 80 °C to prepare for measuring water content, total non-structural carbohydrates content, and nitrogen content; a 0.2 g fresh foliage sample was prepared for soluble protein content examination; a 0.3 g foliage sample (the fifth upper expanded leaf) for chlorophyll content examination; a 0.5 g foliage sample for free amino acid content examination; and a 1.0 g foliage sample (the fifth upper fully expanded leaf) for peroxidase activity examination.

Chemical determination

Plant tissues were dried at 80 °C for 72 h and weighed. Leaves from each treatment were ground with a mortar and pestle for later use. Foliar nitrogen content was analysed using a CNH analyser (Coviella et al., 2002), and total non-structural carbohydrates were tested using a DNS (3,5-dinitrosalicylic acid) method (Suh et al., 2002). Foliar nicotine content was quantified by HPLC (Agilent 1100 Series LC System). The mobile phase consisted of 40% (v/v) methanol containing 0.2% (v/v) phosphoric acid buffered at pH 7.25 with triethylamine (Saunders and Blume, 1981). Fresh leaves were homogenized in 1:10 (fresh weight/buffer volume ratio) 100 mM phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA for 1.5 min at 4 °C. The homogenate was centrifuged at 10 000 g for 15 min and the supernatants were used to analyse soluble protein content by the Bradford assay (Bradford, 1976). The foliar content of total amino acid was examined using the absorbance spectrophotometry method (A570) by combining ninhydrin (Moore and Stein, 1954; Yang and Miller, 1963). Foliar chlorophyll content was quantified using the absorbance spectrophotometry method (Porra et al., 1989). POD activity in tobacco leaves was also examined using a commercial kit and following the methods directly (Nanjing Jiancheng Company, Nanjing, Jiangsu Province, China). One POD unit represents the amount of enzyme needed to catalyse 1 μg H₂O₂ min⁻¹ mg⁻¹ of total proteins present in the homogenate. Chemical variables described above were measured on two randomly selected samples from each treatment per OTC (=8 samples per OTC and 64 samples in total).

Data analysis

Response variables including the indirect yield index (periodic above-ground biomass accumulation), plant quality index (total non-structural carbohydrates, nitrogen content, TNCs:Nitrogen, free amino acid content, soluble protein content), plant virus resistance index (nicotine content), and oxidative stress response (POD activity) were analysed using ANOVA SPSS13.0.1 (SPSS Inc. Chicago, IL, USA), with O₃ concentrations as the main factor and PVY infection and tobacco cultivar as sub-factors in a split-split-plot design. The differences between means were determined using the least significant difference (LSD) test (SAS 6.12, SAS Institute Inc. USA, 1996). The data for C/N were transformed by the ASIN function.

Results

Typical symptoms that appeared on the leaves of virus-infected plants included mottling, leaf curling, and prominent veins. Symptoms were visible after 5–7 d of infection with PVYN

Interactions between higher O₃ and PVY were found for five growth and biochemical parameters (biomass, nicotine, POD, TAA, and protein) (Table 1).

Interactions of O₃ and virus on biomass of two cultivars

Healthy tobacco plants of the resistant cultivar possessed more biomass than the sensitive cultivar by 39.9% (P <0.0001) in ambient O₃. Virus infection negatively influenced the biomass of the sensitive cultivar by 17.8% (P=0.0008) and the resistant cultivar by 19.5% (P <0.0001) (Fig. 1) in ambient O₃ conditions. The advantage of biomass (37%, P <0.0001) was still found in the resistant cultivar rather than in the sensitive cultivar after virus infection in ambient air (Fig. 1). For biomass, there was a significant O×C×P interaction and the effect of the virus infection was less in elevated O₃ than that in ambient O₃ (Table 1; Fig 1).

No significant difference of biomass was found after infection in both cultivars in elevated O₃.
Interactions of O₃ and virus on nicotine content and POD activity of two cultivars

Foliar nicotine content of the resistant cultivar was significantly higher than that of the susceptible cultivar in all treatments. PVYN infection decreased nicotine by 31.7% \((P=0.0022)\) in the susceptible cultivar and by 46.8% in the resistant cultivar \((P <0.0001)\) under ambient O₃ (Fig. 2A). Elevated O₃ reduced the effect of PVYN in the resistant cultivar but not in the sensitive cultivar (Fig. 2A).

A three-way interaction \(O×C×P\) for POD was highly significant (Table 1). PVYN infection increased POD levels (+70.9%, \(P <0.0001\)) in the susceptible, but not for the resistant cultivar in ambient air and this difference was lost in elevated O₃, in which virus increased POD in both cultivars and a greater increase in POD was found in the resistant cultivar (+29.2%) than the susceptible one (+10.4%) after infection (Fig. 2B).

Interactions of O₃ and virus on foliar chemical contents of two cultivars

There were no significant \(O×P\) or \(O×C×P\) interactions on TNCs, nitrogen content, C/N ratio or chlorophyll content, so it can be concluded that there was no evidence of O₃ involved in these indices affecting the impact of virus infection.

However, there was significant \(O×C×P\) interaction on foliage soluble protein content (Table 1). PVYN had a greater effect on the sensitive cultivar (~34.9%) than on the resistant cultivar (~7.1%) in ambient air, but the effect of virus was similar (~21.3% versus ~18.8%) in both cultivars in elevated O₃ (Fig. 3A).

PVYN reduced the TAA content in the susceptible cultivar by 20.8% and in the resistant cultivar by 45.0% in ambient air; while in elevated O₃, PVYN increased TAA by 37.2% \((P <0.0001)\) in the resistant cultivar, but had no effect on the sensitive cultivar (Fig. 3B).

Discussion

Plant viruses decrease the output of plants and, therefore, breeding resistant cultivars is a strategy to control agricultural losses (Kang et al., 2005). A study was made to determine whether elevated O₃ could alter the responses of the two tobacco cultivars to PVYN. Although biomass decreased in the resistant cultivar (~20%) which was similar to the sensitive cultivar (~18%) after virus-infection in ambient air, the resistant cultivar had relatively greater biomass accumulation after infection (~37%) which was defined as the resistance advantage of this selected resistant cultivar. Elevated O₃ is well known to inhibit plant photosynthesis and growth processes resulting in significant negative effects on crop yields (Mckee et al., 2000; Sander-mann, 2005; Ashmore, 2005; Biswas et al., 2008; Reid and Fiscus, 2008). Some studies have estimated that current O₃ levels in East Asia will be high enough to cause substantial yield loss by 2020 (Aunan et al., 2000; Wang and Mauzerall, 2004; Ashmore, 2005, Sitch et al., 2007). In this study, elevated O₃ had negative effects on biomass of the resistant cultivar, however, O₃ was also found to remove the negative effect of PVYN in both cultivars which suggested some beneficial effects of this climate change might exist and the resistant cultivar was more sensitive to O₃. This finding also

---

Fig. 1. Differences of fresh above-ground biomass between susceptible and resistant tobacco cultivars with and without infection under ambient O₃ and elevated O₃. Ambient, ambient O₃; PVY, ambient O₃ and PVY infection; Ozone, elevated O₃; PV+Ozone, elevated O₃ and PVY infection. Yongding indicates the susceptible tobacco cultivar and Vam indicates the resistant tobacco cultivar. Values are the means (+1 SE) of eight replicates. Different lower-case letters indicate different levels of biomass among all treatments.

Fig. 2. Differences of (A) foliar nicotine content and (B) peroxidase activity between susceptible and resistant tobacco cultivars with and without infection under ambient O₃ and elevated O₃. Ambient, ambient O₃; PVY, ambient O₃ and PVYN infection; Ozone, elevated O₃; PV+Ozone, elevated O₃ and PVYN infection. Yongding indicates the susceptible tobacco cultivar and Vam indicates the resistant tobacco cultivar. Values are the means (+1 SE) of eight replicates. Different lower-case letters indicate different levels of nicotine content and POD activity among all treatments.
suggested that higher O₃ should be an additional consideration for the development of future cultivars.

Concentrations of carbohydrates and nutrients, as indices of quality, have been reported either to increase, decrease or to remain the same in response to elevated O₃ in previous studies (Saleem et al., 2001; Wustman et al., 2001; Oksanen, 2003; Oksanen et al., 2005; Valkama et al., 2007). In this study, rising O₃, virus, and both stressors had little effect on the C/N ratio of individual cultivars. The relative quality advantage (relatively lower C/N ratio) of the resistant cultivar to the sensitive one was found when virus infection, higher O₃ fumigation or double stressors were applied (~38%, ~25%, and ~51%) (Fig. 4A). This could be considered as further evidence for enhanced sensitivity of the resistant cultivar to higher O₃ concentrations.

For the susceptible cultivar, single stressor or double stressor applications resulted in decreased chlorophyll content (Fig. 4B); meanwhile, chlorophyll content in the resistant cultivar remained steady after either treatment with O₃, virus or both stressors. That meant there was no correlation in chlorophyll content changes with biomass variation in either cultivar. Pleijel et al. (2006) demonstrated that the increased sensitivity of the modern cultivar to O₃ was associated with a higher photosynthetic rate and leaf chlorophyll content. In our study, relatively lower chlorophyll content in the resistant cultivar showed more sensitivity to higher O₃, which suggests that a simple relationship between high chlorophyll content and O₃ sensitivity does not exist.

Elevated O₃ typically increases peroxidase activity which increases the oxidation of cellular proteins, and hence decreases the soluble protein content (Pell et al., 1997; Loreto and Velicova, 2001; Calatayud et al., 2003; Biswas et al., 2008). Our studies showed that O₃ directly stimulated POD activity in the susceptible cultivar and reduced POD activity in the resistant cultivar and PYV⁻N infection also increased POD activity in the sensitive cultivar, but not in the resistant cultivar in ambient O₃, which indicated that the resistant cultivar may have produced less ROS than the susceptible cultivar. However, this advantage was lost in elevated O₃. Nicotine (insecticidal metabolite) content was negatively affected by infection in both cultivars, not only in ambient air but also in elevated O₃; moreover, a smaller extent of nicotine reduction after infection was found in higher O₃ for the resistant cultivar, which suggested that virus resistance after infection decreased less in higher O₃ for the resistant cultivar.

Currently available disease management options include the use of host cultivars that support lower vector and virus populations (Van Den Bosch et al., 2006). In one previous case, tobacco foliar free amino acid content was found to be significantly and positively correlated with aphid abundance on individual plants (Fu et al., 2010). By the same token, in
in this study, PVY infection reduced the foliar amino acid content of both tobacco cultivars in ambient O₃, which suggested a smaller aphid density could be supported by the plant. Furthermore, the resistant cultivar could resist aphid infestation better than the susceptible one under ambient O₃ condition after infection; whereas, this merit against pests would be lost for the infected-resistant tobacco cultivar in elevated O₃.

This study indicated that a virus-resistant tobacco cultivar showed increased sensitivity to elevated O₃ compared with a virus-sensitive cultivar. One explanation might be the different response of photosynthetic rate (chlorophyll content) changes to stressor effects and another reason might be the different responses of resistance costs (POD activity and nicotine content) between cultivars.

Acknowledgements

We thank the editor and the two anonymous reviewers for their valuable comments and suggestions. We also appreciate Professor Marvin Harris of Texas A&M University for the prior reading of the manuscript. We are grateful to Professor Wang Xifeng from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences for providing potato virus Y and Li Xianfeng for his help in the experiment. This study was funded by the ‘National Basic Research Program of China’ (973 Program) (No. 2012CB114103), the Innovation Program of the Chinese Academy of Science (KSCX2-EW-N-005), the National Nature Science Fund of China (Nos 31000854, 30921063), and the Open Project Program Foundation of National Nature Science Fund of China (Nos 31000854, 30921063), and the Open Project Program Foundation of Engineering Research Center of Biological Control, Ministry of Education (20070101).

References

Aunan K, Bernsten TK, Seip HM. 2000. Surface ozone in China and its possible impact on agricultural crop yields. Ambio 29, 294–301.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55, 373–399.

Ashmore MR. 2005. Assessing the future global impacts of ozone on vegetation. Plant, Cell and Environment 28, 949–964.

Ashmore MR, Buker P, Emberson LD. 2007. Modeling stomatal ozone flux and deposition to grassland communities across Europe. Environmental Pollution 146, 659–670.

Bilgin DD, Aldea M, O’Neill BF, Benitez M, Li M, Clough SJ, Delucia EH. 2008. Elevated ozone alters soybean–virus interaction. Molecular Plant–Microbe Interactions 21, 1297–1308.

Biswas DK, Xu H, Li YG, Sun JZ, Wang XZ, Han XG, Jiang GM. 2008. Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. Global Change Biology 14, 46–59.

Bonello P, Heller W, Sandermann H. 1993. Ozone effects on root-disease, susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedling of Scots pine (Pinus sylvestris L.). New Phytologist 124, 653–663.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Brennan E, Leone IA. 1970. Not on the interaction of tobacco mosaic virus and ozone in Nicotiana sylvestris. Journal of Air Pollution Control Association 470.

Calatayud A, Iglesias DJ, Talón M, Barreno E. 2003. Effects of 2-month ozone exposure in spinach leaves on photosynthesis, antioxidant systems and lipid peroxidation. Plant Physiology and Biochemistry 41, 839–845.

Clara S, Mathilde LM, Floriant B, et al. 2010. The differential spatial distribution of secondary metabolites in Arabidopsis leaves reacting hypersensitively to Pseudomonas syringae pv. tomato is dependent on the oxidative burst. Journal of Experimental Botany 61, 3355–3370.

Coakley SM, Kimball BA, Brooks TJ, et al. 1999. Climate change and plant disease management. Annual Review of Phytopathology 37, 399–426.

Coviella CE, Stipanovic RD, Trumble JT. 2002. Plant allocation to defensive compounds: interactions between elevated CO₂ and nitrogen in transgenic cotton plants. Journal of Experimental Botany 53, 323–331.

Fu X, Ye LF, Ge F. 2010. Elevated CO₂ shifts the focus of tobacco plant defences from cucumber mosaic virus to the green peach aphid. Plant, Cell and Environment 33, 2056–2064.

Heagle AS, McLaughlin MR, Miller JE, Joyner RL. 1992. Response of two white clover clones to peanut stunt virus and ozone. Phytopathology 82, 254–258.

Horst M, Jacqueline MO, Alan RW. 1990. Atmospheric ozone interacts with stress ethylene formation by plants to cause visible plant injury. Journal of Experimental Botany 42, 17–24.

Kangasjärvi J, Talvinen J, Utriainen M, Karjalainen R. 1994. Plant defense systems induced by ozone. Plant, Cell and Environment 17, 783–794.

Kang BC, Yeam I, Jahn MM. 2005. Genetics of plant virus resistance. Annual Review of Phytopathology 43, 581–621.

Kanofsky JR, Sima PD. 2000. Assay for singlet oxygen generation by Arabidopsis leaves reacting hypersensitively to Pseudomonas syringae pv. tomato. Journal of Air Pollution Control Association 50, 373–381.

Krupa SV. 2000. Joint effects of elevated levels of ultraviolet-B radiation, carbon dioxide and ozone on plants. Methods in Enzymology 319, 512–520.

Loebenstein G, Berger PH, Brunt AA, Lawson RH, Stipanovic RD, Trumble JT. 2001. Isoprene produced by leaves protects underground parts of tomato plants. New Phytologist 150, 299–307.

Loreto F, Velicova V. 2003. Effects of elevated CO₂ and ozone on plants. Environmental Pollution 124, 653–663.
Elevated ozone reduces virus damage on two tobacco cultivars

and ultraviolet-B (UV-B) radiation on plant diseases. *Environmental Pollution* **88**, 219–245.

Mckee IF, Mulholland BJ, Craigon J, Black CR, Long SP. 2000. Elevated concentrations of atmospheric CO₂ protect against and compensate for O₃ damage to photosynthetic tissues of field-grown wheat. *New Phytologist* **146**, 427–435.

Moore S, Stein WH. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *Journal of Biological Chemistry* **211**, 907–913.

Morgan PB, Ainsworth EA, Long SP. 2003. How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. *Plant, Cell and Environment* **26**, 1317–1328.

Morgan PB, Bernacchi CJ, Ort DR, Long SP. 2004. An in vivo analysis of the effect of season-long open-air elevation of ozone to anticipated 2050 levels on photosynthesis in soybean. *Plant Physiology* **135**, 2348–2357.

Oksanen E. 2003. Physiological responses of birch (Betula pendula) to ozone: a comparison between open-soil-grown trees exposed for six growing season and potted seedlings exposed for one season. *Tree Physiology* **23**, 603–614.

Oksanen E, Saleem A. 1999. Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch. *Plant, Cell and Environment* **22**, 1404–1411.

Oksanen E, Riikonen J, Kaakinen S, Holopainen T, Vapaavuori E. 2005. Structural characteristics and chemical composition of birch (Betula pendula) leaves are modified by increasing CO₂ and ozone. *Global Change Biology* **11**, 732–748.

Pell EJ, Schlaghaufer CD, Arteca RN. 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. *Physiologia Plantarum* **100**, 264–273.

Plazek A, Hura K, Rapacz H. 2001. The influence of ozone fumigation on metabolic efficiency and plant resistance to fungal pathogens. *Journal of Applied Botany* **75**, 8–13.

Pleijel H, Eriksson AB, Danielsson H, Bondesson N, Selldén G. 2006. Differential ozone sensitivity in an old and a modern Swedish wheat cultivar-grain yield and quality, leaf chlorophyll and stomatal conductance. *Environmental and Experimental Botany* **56**, 63–71.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975**, 384–394.

Reid CD, Fiscus EL. 2008. Ozone and density affect the response of biomass and seed yield to elevated CO₂ in rice. *Global Change Biology* **14**, 60–76.

Reintir RA, Gooding Jr GV. 1978. Effect of ozone and tobacco streak virus alone and in combination on *Nicotiana tabacum*. *Phytopathology* **68**, 15–17.

Saleem A, Loponen J, Pihlaja K, Oksanen E. 2001. Effects of long-term open-field ozone exposure on leaf phenolics of European silver birch (Betula pendula Roth). *Journal of Chemical Ecology* **27**, 1049–1062.

Sandermann JH. 2000. Ozone/biotic disease interactions: molecular biomarkers as a new experimental tool. *Environmental Pollution* **108**, 327–332.

SAS Institute. 1996. SAS/STAT user’s guide. Cary, NC: SAS Institute.

Saunders JA, Blume DE. 1981. Quantitation of major tobacco alkaloids by high-performance liquid chromatography. *Journal of Chromatography* **205**, 147–154.

Schraudner M, Moeder W, Wiese C, Van Camp W, Inzé D Langebartels C, Sandermann Jr H. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco BelW3. *The Plant Journal* **16**, 235–245.

Sitch S, Cox PM, Collins WJ, Huntonford C. 2007. Indirect radiative forcing of climate change through ozone effects on the land-carbon sink. *Nature* **448**, 791–795.

Suh HJ, Noh DO, Choi YM. 2002. Solubilization of onion with polysaccharide-degrading enzymes. *International Journal of Food Science Technology* **37**, 65–71.

Tiedemann AV, Firsching KH. 2000. Interactive effects of elevated ozone and carbon dioxide on growth and yield of leaf rust-infected wheat. *Environmental Pollution* **108**, 357–363.

Valkama E, Koricheva J, Oksanen E. 2006. Effects of elevated O₃, alone and in combination with elevated CO₂, on tree leaf chemistry and insect herbivore performance: a meta-analysis. *Global Change Biology* **13**, 184–201.

Van Den Bosch F, Akudibilah G, Seal S, Jeger M. 2006. Host resistance and the evolutionary response of plant viruses. *Journal of Applied Ecology* **43**, 506–516.

Wang XP, Mauzerall DL. 2004. Characterizing distributions of surface ozone and its impact on grain production in China, Japan and South Korea: 1990 and 2020. *Atmospheric Environment* **38**, 4383–4402.

Wustman BA, Oksanen E, Karnosky DF, Noormets A, Isebrands JG, Pregitzer KS, Hendrey GR, Sober J, Podila GK. 2001. Effects of elevated CO₂ and O₃ on aspen clones varying in O₃ sensitivity: can CO₂ ameliorate the harmful effects of O₃? *Environmental Pollution* **115**, 473–481.

Yang SF, Miller GW. 1983. Biochemical studies on the effect of fluoride on higher plants. *Biochemical Journal* **88**, 505–509.