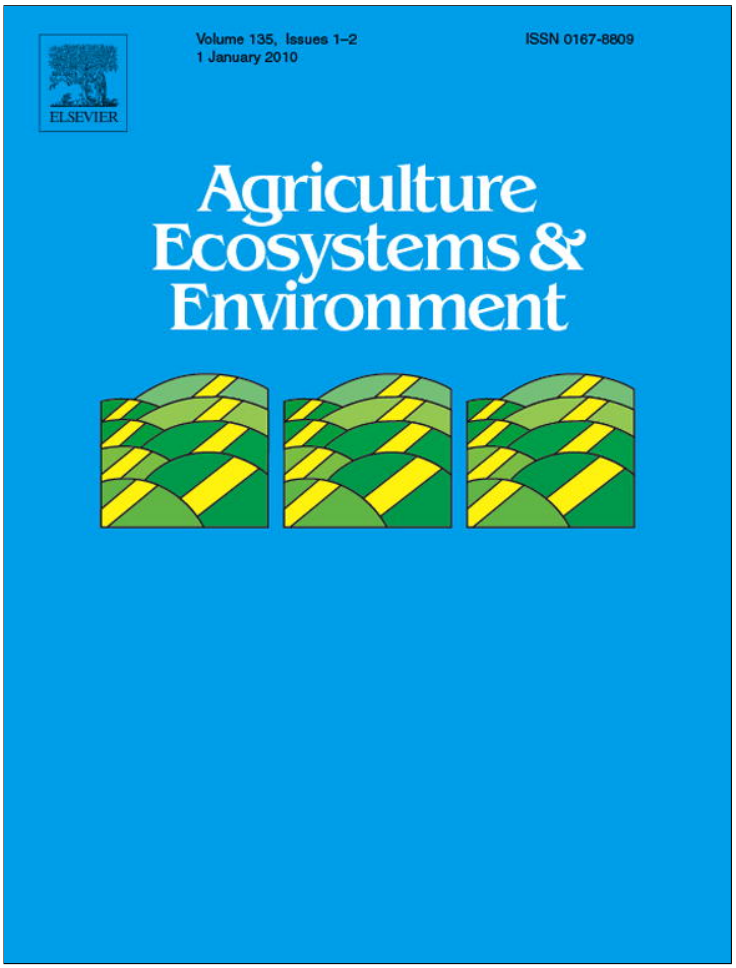

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Aphid and episodic O_3 injury in arugula plants (*Eruca sativa* Mill) grown in open-top field chambers

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ABSTRACT

Aphid attacks and tropospheric ozone (O_3) cause stress and considerable damage in both wild and cultivated plants. Induced defense responses to aphid attacks and O_3 exposure share signaling pathways with common points. We investigated the plant–aphid interaction under O_3 exposure using open-top O_3 chambers. Ozone leaf injury was lower in aphid-infested plants than in aphid-free plants, although herbivore damage was not evident. Aphid population growth was strongly affected by previous exposure to O_3 but no direct effect of O_3 was observed. The possibility that during O_3 episodes, herbivores may reduce O_3 damage on host plants and that the offspring of the exposed aphids have lower population growth rates opens new and intriguing questions about potential effects of future increased tropospheric O_3 levels on plant–insect interactions.

1. Introduction

Aphids damage crop plants due to photo-assimilate consumption and the toxic action of salivary enzymes. Their effect on growth is mainly due to the reduction of nutrient mass flow into the primary apical zone (Pollard, 1973). Harper and Kaldy (1982) found that the pea aphid (*Acyrthosiphon pisum*) infestation reduced growth, stem elongation and dry mass yield of alfalfa (*Medicago sativa*) under field conditions. Aphid-induced reduction in elongation rates results from a complex effect on nutrient fluxes, i.e. reversed nitrogen deposition rates of the growth zone, converted from a sink into a source tissue (Girousse et al., 2005). In response to herbivore damage, plants may express biochemical, physiological or morphological changes known as induced responses, some of which are activated by salivary enzymes (Karban and Myers, 1989). The signal is transmitted throughout the plant, establishing a communication among different plant tissues and conferring systemic acquired resistance (SAR) (Mudgett, 2005). In turn, plant responses may be in some cases beneficial to herbivores, resulting in induced susceptibility (Sauge et al., 2006). For example, avoidance of activation of defense responses by the greenbug aphid through induction of antioxidant proteins was observed in sorghum (Zhu-Salzman et al., 2004). Production of reactive oxygen species (ROS), particularly H_2O_2, has repeatedly been associated with defense activation in diverse plant–pathogen and plant–insect interactions (Bruxelles and Roberts, 2001; Scheel, 2002). Under normal circumstances, the production of ROS-detoxifying enzymes would appear to represent an attempt of the plant to cope with oxidative damage resulting from elevated H_2O_2 levels. However, their induction after an aphid attack results in an increased aphid population overwhelming the plant anti-stress system (Moran et al., 2002).

The oxidative burst associated with insect attacks is similar to that observed in plants exposed to tropospheric ozone (O_3). This gas is the leading component of photochemical air pollution (Wohlgemuth et al., 2002; Baier et al., 2005), and its level is expected to triple within the next 40 years (Chameides et al., 1994). O_3-induced ROS accumulation is concentrated in the periveinal regions of the leaves (Schraudner et al., 1998; Wohlgemuth et al., 2002). It affects cell membranes and produces defined lesions on the adaxial side of the leaf. Symptoms produced by O_3 were originally described in tobacco (*Nicotiana tabacum* L.), and characterized as weather flecks (Wohlgemuth et al., 2002). Some plant species can reduce O_3 damage by the production of volatile isoprenoids, and it has recently been demonstrated that O_3 episodes are actually able to stimulate isoprenoid emission by leaves (Fares et al., 2008).

Despite the vast existing literature on the effects of O_3 on plants and the potential impact on food–web interactions, few studies have investigated plant–insect interactions under elevated O_3 conditions, and even fewer have focused on its impact on crop insect pest outbreaks. Previous studies have demonstrated points of convergence in the signaling circuitry of plants exposed to O_3 and aphid feeding. This "crosstalk" is manifested in common molecular or metabolic responses (Bostock, 2005). It seems clear that O_3-induced production of ROS could serve as a general alarm

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signal leading to cell death (apoptosis) and expression of defense genes (Rao et al., 2000) to many plant pathogens (Yalpani et al., 1994; Sharma et al., 1996; Langebartels et al., 2002) and insects (Moran et al., 2002). However, studies about aphid responses to elevated O_3 produced variable results (Awmack et al., 2004), with both decreases (Sharma et al., 1996; Von Tiedemann and Firsching, 2000; Holopainen, 2002) or increases (Percy et al., 2002; Kopper and Lindroth, 2003; Awmack et al., 2004) in insect growth or oviposition rate in comparison to those on control plants grown under filtered air, being recorded. It appears that subtle variations in the experimental design and in the particular combination of stressors might influence the outcome of the above results. For example, O_3 effect on aphid population development depends on shoot elongation during O_3 exposure (Holopainen and Kössi, 1998). The induction patterns associated with phloem feeding organisms on the *Brassicaceae* family are still under study, but have been shown to share many aspects of generalized stress responses, which may include a combination of plant defenses through abortion of infested organs, plant protection associated with tolerance, and induced susceptibility of the host plant by the aphids (Sauge et al., 2006). Areas of overlap in aphid induction of plant defense responses with other forms of biotic and abiotic stresses in this plant family are likely (Moran et al., 2002).

The parallels of expression between O_3-induced and aphid-induced responses in plants, including the increased oxidative stress common to both stimuli, prompted us to test the hypothesis that aphid-infection prior to O_3 exposure might reduce O_3 injury. We tested this hypothesis on arugula (*Eruca sativa* (P. Mill.)), a member of the *Brassicaceae* family. We also studied whether aphids (*Myzus persicae*) from populations that developed on arugula plants exposed to episodes of O_3 contamination were able to establish new infestations on unstressed plants.

2. Materials and methods

2.1. O_3 exposure in open-top chambers

Experiments were conducted at the IFEVA, Facultad de Agronomía, University of Buenos Aires experimental field (34°35′S, 58°29′W). Average maximum and minimum temperatures during the experimental period were 27.9 and 16.8 °C, respectively. Six 8 m^3 "open-top" chambers with crystal PVC walls mounted on a metal structure and tropospheric O_3 level regulation were used (Hogsett et al., 1985; Lefohn et al., 1986). Open-top chambers are a proven exposure technology in air pollution field research, developed to provide an environment that closely resembles ambient conditions, overcoming the limitations to the conduct of experiments under natural field conditions that was a feature of early O_3 studies (Heagle et al., 1973; Rogers et al., 1983).

Three chambers were used for the control treatment (ozone-free ambient air) and three for the O_3 treatment (charcoal filtered air with added O_3). O_3 chambers received a daily fumigation of 90 min that increased O_3 concentration to 100–120 ppb. O_3 was generated by a spark discharge-type O_3 generator (Hogsett et al., 1985) and ambient air was pumped through an activated charcoal filter. Ozone was continuously monitored using a Model 450 Ozone Monitor API-Teledyne Instrument (Teledyne Advanced Pollution Instrumentation San Diego, CA).

2.2. Plants and aphids

Two arugula seeds were planted in each pot filled with a 50:25:25 soil:peat moss:sand mixture and kept in a glasshouse during 4 weeks. Seedlings were thinned to one per pot after 5 days and were well watered throughout the experiment. Aphids (*M. persicae*) were cultured on 2-week-old arugula plants in a glasshouse under controlled temperature conditions (24–26 °C). New plants were added at weekly intervals as older, damaged, plants were removed.

2.3. Experimental procedures

Two experiments, each repeated twice, were carried out to investigate the effects of aphid/host plant interactions in response to O_3 exposure. In the first experiment, 4 weeks after planting four arugula plants were infested with six aphids per plant, and placed in each open-top chamber (both ozone and control), together with four uninfected plants. All plants from every treatment were covered with individual voile bags to prevent aphid migration between plants. The number of aphids per plant was recorded every other day. Plant lesions and chlorotic leaf symptoms were visually estimated as percent reduction of green tissue. Plants were harvested after 10 days; roots were carefully washed and separated from the shoots, and both were dried at 70 °C and weighed.

In the second experiment a group of naturally infested arugula plants with 20–30 aphids was exposed to O_3, 4 h a day during 3 consecutive days, while another group of infested plants remained in the control chambers. Immediately after O_3 exposure nymphs were transferred to 3-week-old plants that had never been either infested or exposed to O_3 before. Six aphids were placed on each plant and counted 5, 10 and 15 days after the beginning of the experiment.

2.4. Production of antioxidants

An experiment was set up to determine the production of antioxidants by aphid-infested arugula plants. Aphid induction of antioxidants in leaves was estimated through two parameters of oxidative stress: thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation (Yagi, 1976), and total radical-trapping antioxidant potential (TRAP) (Lissi et al., 1992). Each plant was originally infested with 20 aphids, and the aphid population was later allowed to increase without restriction. Leaf samples were harvested and frozen immediately after aphid infestation, and 3 and 6 days later. The chain reactions that occur during lipid oxidation lead to peroxide formation, and secondary products like aldehydes. A colorimetric technique was used by which the thiobarbituric acid joined to malondialdehyde (MDA) forms a Schiff base with an absorption peak at 532 nm. The TRAP index reflects the free radicals that react with a solution of antioxidants and so it is a measure of the quantity of antioxidants in a sample.

2.5. Statistics

Percent of green tissue and shoot–root rate of plants growing under the different treatments were analyzed by means of split-plot design analysis of variance (ANOVA). Main-plot effects were the atmospheric treatments (O_3-free vs. elevated). The subplot factor was herbivory (presence vs. absence of aphids). All interactions between ozone and herbivory were included as subplot interactions. As experimental replication of the fumigation treatment involves an entire open-top chamber, individual plants within each chamber are not true replicates. As a result, the plant response variables for each treatment pot were calculated. Then we obtained the mean value of plant biomass and percentage of green tissue per chamber on each sampling date. The effect of O_3 exposure on aphid population growth was analyzed by repeated measures ANOVA, and one way ANOVA was used to compare antioxidant production of aphid-infested and uninfested plants. InfoStat Professional, Argentina (InfoStat, 2008) was used for all statistical analyses.

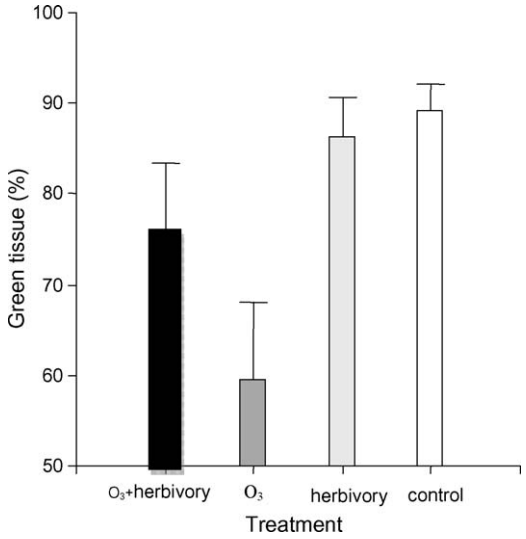

Fig. 1. Leaf damage caused by aphid infestation and O_3 exposure, estimated as remaining percentage of green tissue (mean + SE).

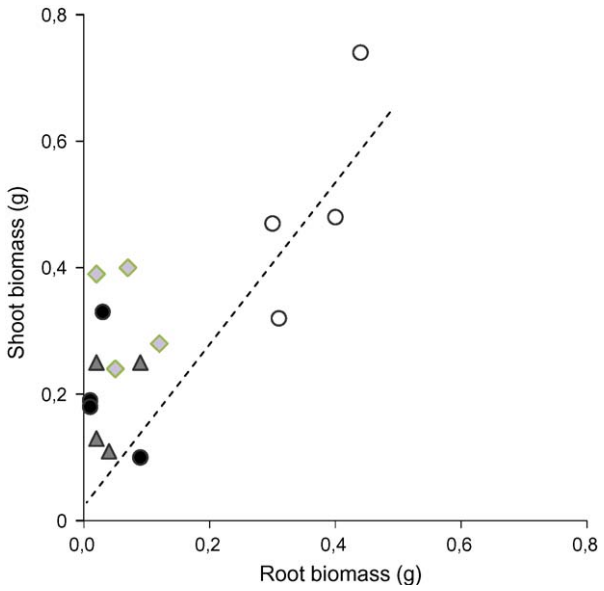

Fig. 2. Aphid infestation (◇), O_3 exposure (▲), and aphids + O_3 (●) modulate shoot/root biomass relationship of arugula plants. The dashed line represents the fitted linear regression model for the control plants (○) ($R^2 = 0.66$).

3. Results

All plants exposed to O_3 developed typical O_3 injury symptoms (necrotic patches and mottling on the leaf surfaces). The reduction in green leaf tissue due to O_3 damage was lower in aphid-infested plants (interaction $P = 0.03$) (Fig. 1, Table 1). However, the proportion of green tissue in leaves of infested plants did not differ from the control ($P = 0.10$) (Fig. 1, Table 1).

Plant size and allometry were affected by both biotic and abiotic stress factors. O_3 exposure and aphid feeding produced a 4–5-fold reduction in plant biomass, mainly due to a decrease in root growth (Table 1). As a result, shoot–root ratios of aphid-infested plants were twice that of the control (Fig. 2, Table 1).

The growth of the aphid population was similar in O_3 and O_3-free environments ($P > 0.05$) (Fig. 3). However, aphid population growth in ozone-free environments was strongly influenced when aphids came from plants that had been exposed to ozone ($P = 0.0059$).

At the end of the experimental period the level of aphid infestation of the O_3-challenged arugula plants was one half of that observed for the aphid population growing on control plants (Fig. 4).

After 3 days of aphid infestation, the level of TBARS in infested leaves was higher than that of the control plant tissues ($P < 0.05$) but returned to control levels 6 days after the start of infestation ($P > 0.05$) (Table 2). TRAP from aphid-infested plant tissues remained always significantly lower ($P = 0.01$) than those from control plants; three and 2-fold lower for the shortest and the longest aphid-infection period, respectively (Table 1). No damage was observed on the foliage of aphid-infested plants.

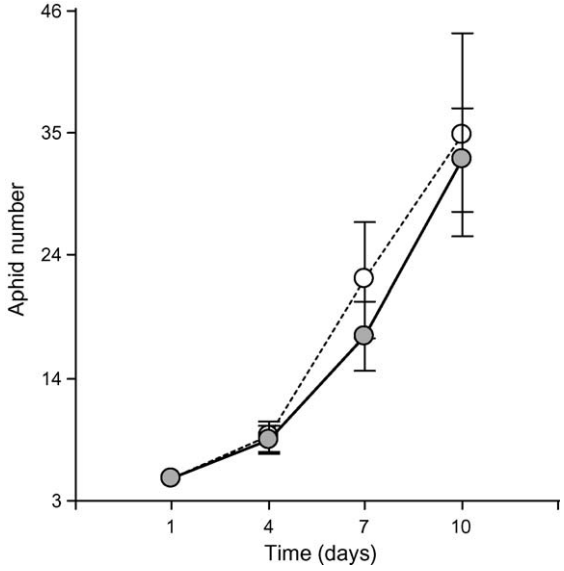

Fig. 3. Growth of the aphid population in O_3 (●) and O_3-free (○) environments.

4. Discussion

The response induced by aphid attack strongly decreased the level of tissue injury resulting from several episodes of O_3 exposure, even though the growth of the aphid population was negatively influenced by the pollutant. Our assessment of plant responses to the interaction of both stressors was done under field conditions including daily and seasonal weather fluctuations. Although an agroecosystem and its environment cannot be completely recreated within the open-top chamber, plants and aphids can be grown under realistic field conditions (Strain and Thomas, 1992). The arugula plants used in our experiments were

Table 1
The effect of ozone exposure (O_3) and aphid feeding (herbivory) on the growth of Arugula plants, as determined by split-plot ANOVAS.

Treatment	Total biomass (g)		Root biomass (g)		Shoot/root ratio		Green leaf tissue (%)	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
O_3	33.07	0.001	63.50	0.0002	1.72	0.23	5.68	0.07
Herbivory	11.45	0.01	36.30	0.0009	5.91	0.05	2.7	0.10
$O_3 \times$ Herbivory	12.20	0.01	32.82	0.0012	0.01	0.09	467	0.03

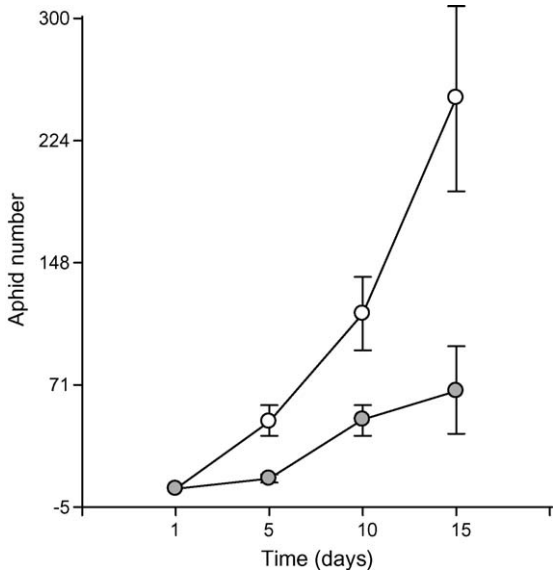

Fig. 4. O_3 effect on aphid population growth. Aphids were exposed to O_3 (●) or O_3-free (○) conditions and then transferred to the control plants.

Table 2
Parameters of oxidative stress measured in arugula plants after varying periods of aphid infestation. TBARS: thiobarbituric acid reactive substances and TRAP: total radical-trapping antioxidant potential.

Treatment	TBARS (μM/g leaf)	TRAP (μM)
Control	1,15 ± 0,2	111 ± 15
After 3 days infestation	4,65 ± 1,1**	38 ± 7**
After 6 days infestation	1,98 ± 0,1	53 ± 8*

* $P < 0.05$ (*F*-test).
** $P < 0.01$ (*F*-test).

highly sensitive to O_3. Exposed plants developed typical visible symptoms of injury consisting of necrotic patches and mottling on the leave surface. It could be argued however, that an important limitation of the OTCs is the elevation of air temperature that can occur within them, because reduced air movement in the chambers compared to open areas, can lead to greater solar heating (Olszyk et al., 1980). Thus the increased chamber temperatures can confound the responses to the primary variable of interest and compromise experimental objectives. Although the same temperature elevation should occur in all chambers (elevated ozone and control), the relative sensitivity of plants to ozone could be difficult to predict from their sensitivity determined in OTCs. Some experiments have addressed these critical questions, and no interactions were found at least for CO_2-temperature (Norby et al., 1999). Nevertheless temperature monitoring during the experimental period revealed that differences between temperature inside and outside the chambers never exceeded 1.5 °C.

Aphid feeding did not cause any visible leaf damage. This is consistent with results of experiments performed with another species of the same plant family (*Arabidopsis thaliana*), which showed lack of necrosis or other symptoms after 72–96 h of *M. persicae* infestation (Moran et al., 2002). This could reflect the maintenance of a favorable oxidative-reduction condition in the plant by the aphid salivary sheaths (Miles and Oertli, 1993). Size and allometry of arugula plants were impacted by both biotic and abiotic stress factors. Stress reduced individual plant growth. In the aphid-infested plants in particular, the magnitude of the reduction was greater for roots than shoots, suggesting that compensation growth and repair metabolism preventing leaf damage may compromise root growth. However, aphid-produced hormonal changes could be also responsible for higher shoot–root ratios in aphid-infested plants (Ilarduya et al., 2003). This could explain in part the reduction of O_3 damage on plants pre-infested with aphids, which increased the antioxidant production in those plants. Exposure to biotic or abiotic stress factors generally induces ROS production in cells that in turn activates an antioxidant system (Perl-Treves and Perl, 2002). Ilarduya et al. (2003) reported that aphids induced biochemical and physiological changes in the host plants that included ROS production and Gomez et al. (2004) argued that, depending on the aphid–plant combination, these changes included an increase in the foliar antioxidant response. In our study, ROS production in the aphid-infested plants is supported by the high TBARS levels. In turn the low TRAP levels detected on aphid-infested plants might be an indicator of antioxidant consumption and neutralization of the oxidative burst, which correlates with the absence of visible foliar damage in infested plants and the lower leaf injury in O_3-exposed plants. Moreover, herbivores might increase the emission of volatile compounds with an O_3-scavenging activity, contributing to the protection against O_3 damage. Some Mediterranean *Quercus* species emit isoprenoids such as monoterpenes, with antioxidant activity which allows these plants to scavenge O_3 at leaf level (Loreto et al., 2004; Fares et al., 2008). Production of those isoprenoids is stimulated by O_3 episodes (Fares et al., 2008). Herbivores, i.e. mites, can induce the emission of volatile organic compounds, similar to those induced by O_3 (Vuorinen et al., 2004). Arugula plants produce several aromatic compounds (Mastelic et al., 2008; Jirovetz et al., 2002); however, in our experiments arugula plants exposed to O_3 expressed unmistakable foliar injuries. This suggests that the volatiles produced by O_3 exposure did not have scavenging activity, or they were not produced in sufficient amount.

Aphid nymphs born from adults exposed to and feeding on O_3-exposed plants were less capable of infesting new plants than those nymphs from the control plants. This could be a consequence of the direct effect of O_3 on aphids, an indirect effect through the plant response to O_3 or a combination of both. Because aphids were able to maintain population growth rates on O_3-exposed plants similar to those growing on untreated control plants, it seems that host facilitation induced by aphids would hide the negative effect on the aphids caused by O_3, which became apparent when the insects were transferred to unstressed plants. Previous studies have suggested that O_3 by itself produces no effect on insect ability to reproduce or feed (Agrell et al., 2005). This information and our results are consistent with the idea that the reduced ability of the insect population to colonize and infest new plants was the result of aphid–plant interaction rather than the direct effect of the O_3 on the individuals.

The integration of studies on plant performance, responses and insect behavior, coupled with the biochemical approach used here seems useful to better define plant–aphid interactions under O_3 stress. Our experiments bring new evidence that aphids may not only reduce O_3 damage on plants, but also that aphid attack intensity could be modified in areas with periods of high O_3 concentration. On the other hand, this model takes no account of the potential adaptation of aphids to atmospheric changes. It is possible that more O_3-tolerant clones will prosper as the less-tolerant currently present decline. This information has implications for pest outbreaks and merits further investigation. It could be expected that the convergence of responses to different stressors in agricultural systems, inducing oxidative stress in plant tissues, might render a plant phenotype functionally adaptive or maladaptive to a particular stress. A better understanding of these interactions will provide insight into the role of

aphids in ecological communities, and will lead to the development of novel and durable ways to manage biotic and abiotic resistance traits for crops.

5. Conclusions

Our results provide evidence that plant responses to insect herbivore will be strongly influenced by future levels of tropospheric ozone. The outcome of plant–aphid–O_3 interactions will depend, at the molecular level, on the balance between ROS and antioxidant production, resulting from the shared responses to both stressors. This balance will depend not only on the relative magnitude, but also on the timing and order of the stress factor. Stressor interactions were evident also at the individual plant level and in aphid population growth. However, the stochasticity in the occurrence of high levels of ozone and aphid plant challenge makes it difficult to make inferences of the consequences of these interactions at individual and population levels.

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