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RESEARCH PAPER

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

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Abstract

Investigations of the effects of elevated ozone (O_3) 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 (PVY^N) infection were grown in open-top chambers under ambient and elevated O_3 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 O_3 on the impact of PVY^N in the two cultivars. Results showed that under ambient O_3, the resistant cultivar possessed greater biomass and a lower C/N ratio after infection than the susceptible cultivar; however, under elevated O_3, 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 O_3 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 (PVY^N), 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 (O_3), 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 O_3 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). O_3 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 O_3 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 O_3 (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 O_3 exposure (Manning and Tiedemann, 1995), and O_3 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 O_3 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 O_3 altered the gene expression of plants and induced a host defence response (Bilgin *et al.*, 2008). Thus, intensified research on interaction between higher O_3 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 O_3) 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 O_3 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 O_3 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 O_3 is an important step to understand the effects of elevated O_3 on the efficacy of virus pathogen management strategies. In this study, the hypothesis was tested that elevated O_3 would reduce the relative resistance advantage of a resistant tobacco cultivar against PVY^N infection. Two tobacco cultivars were used, one with resistance and one with susceptibility to PVY^N infection, grown in open-top chambers (OTC) under ambient air and increased concentrations of O_3. Two questions were addressed: (i) what is the difference between resistant cultivar responses and susceptible cultivar responses to elevated O_3 and the interaction of O_3×virus; (ii) how do assimilation rate, resistance costs, and other plant response variables related to virus infection change with exposure to elevated O_3?

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 O_3 concentration, ambient (40 nl l^{-1}) and elevated (80 nl l^{-1}) were applied. Four open-top chambers were used for each O_3 treatment. O_3 came from an O_3 generator (3S-A15, Tonglin Technology Beijing, China) and then sent to the higher O_3 OTC entries using a fan (HB-429, 4.1 m^3 min^{-1}, Ruiyong Mechanical and Electrical Equipment Company). Mixed air (O_3 and ambient air) was ventilated to each elevated O_3 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 m^3 min^{-1}. The hemispherical sprayer was adjusted to make a homogeneous distribution of treated gas (monitored by the instrument mentioned below) throughout each OTC. O_3 concentrations were monitored within OTCs (AQL-200, Aeroqual).

The actual daily O_3 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). O_3 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 O_3 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 O_3 chambers versus 27.5±3.2 °C in the elevated O_3 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 PVY^N and VAM with resistance to PVY^N) 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 O_3 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 an NPK fertilizer (15-15-15). Pots were randomly exchanged within the chamber and rotated among the chambers carefully to prevent the infected and non-infected plants from cross-infection with the same O_3 treatments to minimize position effects within chamber and chamber effects.

PVY^N 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 PVY^N. 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.

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

Main effects and interactions	Dependent variable							
	Mass	TNCs	Nitrogen	C/N[d]	Nicotine	POD[e]	TAA[f]	Protein
Ozone (O)[a]	n.s.	*	n.s.	*	n.s.	n.s.	*	*
Cultivar (C)[b]	***	***	***	***	***	***	n.s.	***
PVY^N (P)[c]	***	n.s.	n.s.	n.s.	***	***	n.s.	***
O×C	**	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.
C×P	**	n.s.	n.s.	n.s.	*	**	n.s.	n.s.
O×C×P	*	n.s.	n.s.	n.s.	n.s.	***	n.s.	*

[a] Ozone levels (ambient and elevated O_3).
[b] Cultivars (susceptible and resistant).
[c] PVY^N (with and without).
[d] TNCs:Nitrogen.
[e] Peroxidase.
[f] 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 to 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* at 4 °C 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 H_2O_2 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 O_3 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 PVY^N.

Interactions between higher O_3 and PVY were found for five growth and biochemical parameters (biomass, nicotine, POD, TAA, and protein) (Table 1).

Interactions of O_3 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 O_3. 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 O_3 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 O_3 than that in ambient O_3 (Table 1; Fig 1). No significant difference of biomass was found after infection in both cultivars in elevated O_3.

Interactions of O_3 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. PVY^N 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 O_3 (Fig. 2A). Elevated O_3 reduced the effect of PVY^N 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). PVY^N 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 O_3, 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 O_3 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 O_3 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). PVY^N 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 O_3 (Fig. 3A).

PVY^N 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 O_3, PVY^N increased TAA by 37.2% (P <0.0001) in the resistant cultivar, but had no effect on the sensitive cultivar (Fig. 3B).

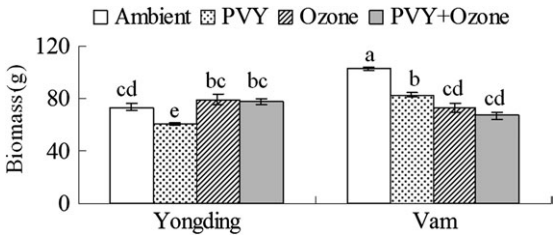

Fig. 1. Differences of fresh above-ground biomass between susceptible and resistant tobacco cultivars with and without infection under ambient O_3 and elevated O_3. Ambient, ambient O_3; PVY, ambient O_3 and PVY^N infection; Ozone, elevated O_3; PV+Ozone, elevated O_3 and PVY^N infection. Yongding indicates 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.

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 O_3 could alter the responses of the two tobacco cultivars to PVY^N. 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 O_3 is well known to inhibit plant photosynthesis and growth processes resulting in significant negative effects on crop yields (Mckee *et al.*, 2000; Sandermann, 2000; Ashmore, 2005; Biswas *et al.*, 2008; Reid and Fiscus, 2008). Some studies have estimated that current O_3 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 O_3 had negative effects on biomass of the resistant cultivar, however, O_3 was also found to remove the negative effect of PVY^N in both cultivars which suggested some beneficial effects of this climate change might exist and the resistant cultivar was more sensitive to O_3. This finding also

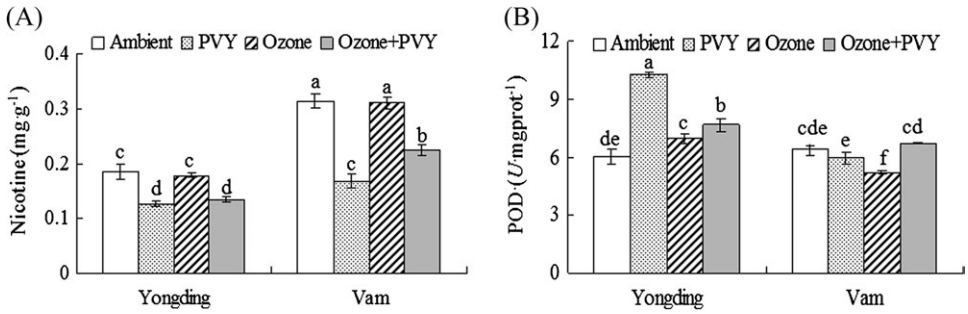

Fig. 2. Differences of (A) foliar nicotine content and (B) peroxidase activity between susceptible and resistant tobacco cultivars with and without infection under ambient O_3 and elevated O_3. Ambient, ambient O_3; PVY, ambient O_3 and PVY^N infection; Ozone, elevated O_3; PVY+Ozone, elevated O_3 and PVY^N 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 O_3 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 O_3 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 O_3, 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 O_3 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 O_3 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 O_3, 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 O_3 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 O_3, which suggests that a simple relationship between high chlorophyll content and O_3 sensitivity does not exist.

Elevated O_3 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 O_3 directly stimulated POD activity in the susceptible cultivar and reduced POD activity in the resistant cultivar and PVY^N infection also increased POD activity in the sensitive cultivar, but not in the resistant cultivar in ambient O_3, which indicated that the resistant cultivar may have produced less ROS than the susceptible cultivar. However, this advantage was lost in elevated O_3. Nicotine (insecticidal metabolite) content was negatively affected by infection in both cultivars, not only in ambient air but also in elevated O_3; moreover, a smaller extent of nicotine reduction after infection was found in higher O_3 for the resistant cultivar, which suggested that virus resistance after infection decreased less in higher O_3 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

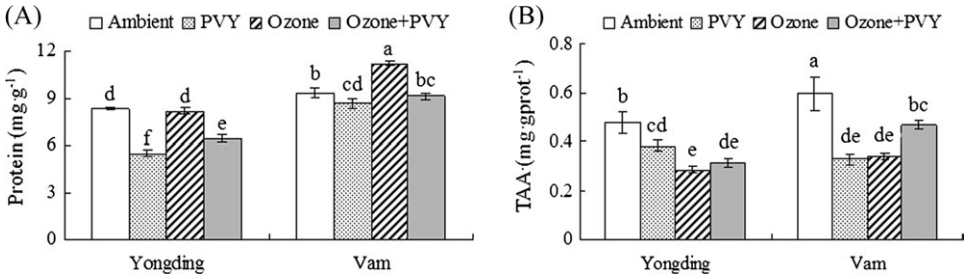

Fig. 3. Differences of (A) foliar soluble protein content and (B) total amino acid (TAA) content between susceptible and resistant tobacco cultivars with and without infection under ambient O_3 and elevated O_3. Ambient, ambient O_3; PVY, ambient O_3 and PVY^N infection; Ozone, elevated O_3; PVY+Ozone, elevated O_3 and PVY^N infection. Yongding indicates the susceptible tobacco cultivar and Vam indicates the resistant tobacco cultivar. Values are the means (±1 SE) of eight replicates. Different lowercase letters indicate different levels of pProtein and TAA content among all treatments.

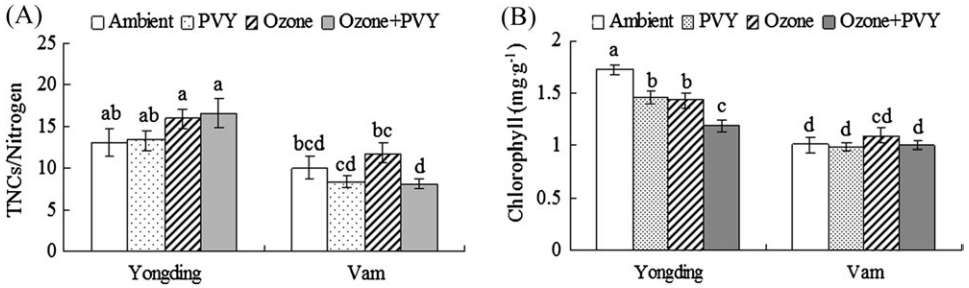

Fig. 4. Differences of (A) TNCs:Nitrogen (the ratio of non-structural carbohydrates content to nitrogen content) and (B) foliar chlorophyll content between resistant and susceptible tobacco cultivars with and without infection under ambient O_3 and elevated O_3. Ambient, ambient O_3; PVY, ambient O_3 and PVY^N infection; Ozone, elevated O_3; PVY+Ozone, elevated O_3 and PVY^N 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 TNCs:Nitrogen and chlorophyll content among all treatments.

this study, PVY infection reduced the foliar amino acid content of both tobacco cultivars in ambient O_3, 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 O_3 condition after infection; whereas, this merit against pests would be lost for the infected-resistant tobacco cultivar in elevated O_3.

This study indicated that a virus-resistant tobacco cultivar showed increased sensitivity to elevated O_3 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.

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