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Elevated CO_2 alleviates damage from *Potato virus Y* infection in tobacco plants

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ABSTRACT

The assessment of plant resistance to viral infection under elevated carbon dioxide [ECO_2] conditions is a key step in the development of plant–pathogen management. We quantified the aboveground biomass, foliar TNCs (total non-structural carbohydrates): Nitrogen ratio, and nicotine content of a tobacco cultivar (NC_{89}) in two CO_2 treatments after *Potato virus* Y^N (PVY^N) infection. Foliar peroxidase (POD) activity, total amino acid content, soluble protein content, and chlorophyll content were also tested to explore the potential interaction of ECO_2 and PVY^N infection on tobacco secondary indices. ECO_2 increased plant aboveground biomass, did not significantly influence TNCs or nitrogen content; while PVY^N infection had adverse effects on biomass, markedly affected the later two indices; but no interactive effects between ECO_2 and PVY^N infection on these three indices were detected. As single factor, ECO_2 or PVY^N infection reduced chlorophyll content, while ECO_2 increased the soluble protein content; interaction between two factors was observed on free amino acid and nicotine content. Variations in POD activity revealed that CO_2 influenced infected plant primary production by reducing virus resistance cost. Results suggested that plants grown under ECO_2 have alleviated damage of the virus infection or ECO_2 delay the viral spread to some extent.

1. Introduction

Since industrial times, the atmospheric CO_2 concentration has risen from 280 ppm to 379 ppm in 2005 due to anthropogenic activities. Furthermore, the atmospheric CO_2 is predicted to double by the end of this century [1].

Elevated CO_2 [ECO_2] will enhance the plant photosynthetic rate, resulting in greater production of plant biomass or yield [2,3]. Kimball et al. [4] reviewed that grass biomass increased by 12%, grain increased by 10–15%, and potato increased by 28% under double CO_2 conditions. Increased atmospheric CO_2 would result in higher carbohydrate content in plant tissue and a decrease in nitrogen, phosphorus and some trace elements [5–7]. Changes of foliar nitrogen and carbon content have some effect on the secondary metabolites produced by plants due to the carbon and nitrogen present in leaves. Bezemer and Jones [8] indicated that the hydroxybenzene substance content in 13 out of 15 plant species increased, on average, by 31% under ECO_2 conditions. Chen et al. [9] found that the cotton hydroxybenzene and tannin content in transgenic cotton and the corresponding common cotton increased significantly under double CO_2 concentrations in comparison with the ambient CO_2 [ACO_2] condition. Matros et al. [10] studied the variations of secondary compounds in tobacco under different CO_2 concentrations and two nitrogen supply conditions. They concluded that foliar phenylpropanoids increased significantly and secondary metabolites, based on nitrogen nicotine content, decreased markedly under ECO_2 conditions. However, little is known about the response of a crop after virus infection under ECO_2.

Plant virus diseases severely constrain agricultural production worldwide, especially in less developed countries [11]. *Potato virus Y* (PVY) is a major plant virus for tobacco [12]. Its interaction with the host plant and epiphyte pathogen under ECO_2 conditions may change host susceptibility and the pathogen's infection capacity. Moreover, host resistance would vary with morphological, physiological, nutritional balance, and water balance changes. For example, a decrease in stoma density could enhance plant resistance to a pathogen that penetrates through stoma under ECO_2 [13]. Nitrogen content of aboveground wheat tissue decreased by 14% under ECO_2 conditions, resulting in a decrease in mildew susceptibility [14]; however, the biomass variation of barley infected with *Barley yellow dwarf virus* (BYDV) exceeds that of healthy plants under ECO_2 conditions [15]. Although several studies have focused on the effects of plant viruses on crop cultivars [16,17], seldom has research illuminated the influence of plant viruses under elevated CO_2 conditions.

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ECO_2 has the potential to accelerate plant–pathogen evolution, which may, in turn, affect virulence [13]. Each type of resistance puts a different form of selection pressure on the virus. Virologists and plant breeders can use these results to develop methods to characterize the combination of resistance mechanisms in the cultivars they breed and to determine whether the cultivar will put selective pressure on the virus to evolve more harmful types [18]. Responses of the "host plant-epiphyte" system to ECO_2 were always embodied as an infection variation of the pathogen or a susceptibility change of the host plants. Plant–pathogen interactions under increasing CO_2 concentrations have the potential to severely disrupt both agricultural and natural systems, and without experimental data we would be unable to predict future outcomes [13]. More research is needed to obtain base-line information on different disease systems [19], especially the responses of many crops to rising CO_2.

We present a study conducted in open-top chambers (OTC) to explore plant responses to PVY^N infection under ECO_2 in which the following questions were addressed: (1) how does tobacco respond to ECO_2, and (2) what are the different responses of tobacco cultivar to infection with PVY under ECO_2?

2. Materials and methods

2.1. Sites and facility

This experiment was conducted in eight octagonal open-top chambers (OTCs), each 4.2 m in diameter and located in the Observation Station of the 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 CO_2 concentrations, the ambient level (375 $\mu l\,l^{-1}$) and the double ambient level (750 $\mu l\,l^{-1}$), were applied continuously. Four OTCs were used for each CO_2 treatment. CO_2 concentrations were monitored continuously and were adjusted using an infrared CO_2 analyzer (Ventostat 8102, Telaire Company, USA) to maintain the assigned CO_2 concentrations. Details of the automatic control system for CO_2 and the OTCs are provided in reports by Chen et al. [9,20]. The actual mean CO_2 concentrations were 376 ± 22 $\mu l\,l^{-1}$ in the ambient chambers and 754 ± 33 $\mu l\,l^{-1}$ in the double ambient chambers. The open tops of these chambers were covered with nylon netting to prevent insect migration. Air temperature was measured three times a day and did not differ significantly between the two sets of chambers (24.8 ± 3.4 °C in the ambient CO_2 chambers versus 25.5 ± 4.6 °C in the elevated CO_2 chambers) throughout the field experiment.

2.2. Tobacco growth conditions

Plants of *Nicotiana tabacum* L. cv. NC_{89}, a common PVY-susceptible cultivar [21] was sown in trays on July 5, 2008. On August 5th, tobacco seedlings were transplanted into plastic pots (diameter: height = 10 cm: 12 cm) filled with an 8:1 ratio of turfy soil:vermiculite. Soil pH was 7.4, organic matter consisted of 13.7%, the available N was measured at 391.2 mg/kg (hydrolic N, 1 N NaOH hydrolysis), P was available at 279.8 mg/kg (0.5 M $NaHCO_3$ extraction), and there was 256.3 mg of K/kg of soil (1 N CH_3COONH_4 extraction). No chemical insecticides were used through the growing season. Individual plants were grown in their own pots. On August 15, 2008, 96 pots of tobacco plant were randomly assigned to chambers with CO_2 treatment after virus treatment described later in the next subsection. Plants were sufficiently irrigated every other day using tap water and fertilized once a week. Pots were randomly exchanged within chamber daily to minimize position effect within chamber and rotated weekly among similar chambers used to minimize chamber effects. In order to prevent contamination by other pathogens and cross-contamination between infected and uninfected plants, pots was covered by columniform cages with transparent net (40 cm diameter, 50 cm height) and separated by at least 50 cm.

2.3. PVY^N infection

Viral (*Potato virus Y*, PVY) was supplied by Beijing Agriculture and Forestry Academy of Sciences and kept in a −20 °C freezer until used. Virus extracts were obtained as described by Herbers et al. [22]. When plants grew to 6 or 7 leaves (on August 12, 2008), 96 plants in the similar growth conditions were selected for later treatments. Half of the chosen plants were mechanically infected with viral extracts by rubbing the virus liquid with carborundum powder on the adaxial surface of the fifth tobacco leaf. The other halves were simultaneously inoculated with normal saline as a control. After five minutes, the treated leaves were washed with distilled water. Six tobacco plants infected with PVY^N were randomly selected to place in one OTC, while another uninfected six plants were assigned in the same OTC as control; all plants were placed in the OTC for one month of treatment. Over all OTCs, treatments with plants that were uninfected with virus or non-infected with virus were represented by 24 replicate pots for each CO_2 concentration (6 plants per viral treatment × 4 OTC per CO_2 treatment = replication of 24 plants).

2.4. Sampling

On September 15th, after being grown in the OTC for one month, tobacco seedlings from each OTC were cut at ground level, weighed, and immediately refrigerated at −20 °C until laboratory examination, except that the 5th upper expanded fresh leaf of each plant was removed and used to measure the following variables. Approximately 0.2 g was used to for soluble protein content examination, 0.5 g for free amino acid content examination, 0.3 g for chlorophyll content examination and 1.0 g for peroxidase (POD) activity. About 10 g of foliage sample was dried at 80 °C to determine total non-structural carbohydrate content, nitrogen content and nicotine content examination.

2.5. Chemical determination

Partial plant tissues were dried at 80 °C for 72 h, and weighed. Leaves from each treatment were ground in a mill for later use. Foliar nitrogen content was analyzed using a CNH analyzer [23], and total non-structural carbohydrates were tested using the DNS (3,5-dinitrosalicylic acid) method [24]. TNC:N ratio was then obtained from these above two values (foliar nitrogen content and total non-structural carbohydrates content) for the same tobacco plant. Foliar nicotine content was quantified by HPLC analysis (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 [25]. On the other hand, 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 $10{,}000 \times g$ at 4 °C for 15 min, and the supernatants were subjected to soluble protein content and POD content analysis. Soluble protein content was determined by the Bradford [26] assay. POD activity in tobacco leaves was also examined using corresponding recommended methods of the reagent kit (Nanjing Jiancheng Company, Nanjing, Jiangsu Province, China). One POD unit represents the amount of enzyme needed to catalyse 1 μg H_2O_2 per minute and per milligram of total proteins present in the homogenate. Total amino acids were analyzed using the absorbance spectrophotometry method by combining ninhydrin [27]. Chlorophyll content

Table 1
P values from ANOVAs for the effect of CO_2 level and tobacco virus (PVY^N) infection on the aboveground biomass and foliar nutrient constituents of tobacco.

Response	Mass	Nitrogen	TNCs	POD	TAA	Protein	Nicotine	Chlorophyll
[a]CO_2	<0.001***	0.405	0.259	0.001**	0.082	0.006**	0.896	0.025*
[b]Virus	0.020*	0.001**	0.005**	0.100	0.145	0.178	0.313	0.003**
$C \times V$	0.076	0.069	0.376	0.150	0.014*	0.402	0.009**	0.385

Significance levels are indicated by $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Where Mass is aboveground biomass; TNCs is total foliar non-structural carbohydrates content; Protein is the soluble protein in leaf; TAA is total free amino acid and POD is peroxidase enzyme activity in foliage.
[a] CO_2 levels (ambient and elevated CO_2).
[b] Virus (uninfected and infected).

Table 2
CO_2 and virus effect (ratio change) on the treatments and corresponding *P*-value.

	[a]CO_2 effects		[b]Virus effects	
	Uninfected plant	Infected plant	Ambient CO_2	Elevated CO_2
Biomass	+13.8 (0.294)	+44.4** (0.001)	−23.6* (0.026)	−3.1 (0.966)
Nitrogen	−7.6 (0.853)	+16.2 (0.233)	+17.9 (0.280)	+48.3** (0.002)
TNCs	+21.4 (0.474)	+3.6 (0.997)	−25.6 (0.329)	−36.5* (0.041)
C/N	+54.3 (0.374)	−5.8 (0.999)	−37.8 (0.655)	−62.1 (0.050)
Protein	+34.3 (0.050)	+16.9 (0.347)	+18.8 (0.401)	+3.4 (0.978)
TAA	+65.3 *(0.025)	−11.3 (0.900)	+18.0 (0.789)	−36.6* (0.038)
POD	−25.1 (0.172)	−50.7 ** (0.004)	−2.0 (0.998)	−35.5 (0.141)
Nicotine	−15.6 (0.152)	+17.9 (0.203)	−20.0 (0.051)	+11.8 (0.487)
Chlorophyll	−15.3 (0.120)	−9.2 (0.653)	−20.5* (0.029)	−14.8 (0.236)

Significance levels are indicated by $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.
[a] Comparison of elevated O_2 to ambient CO_2 in the non-infected and infected plants.
[b] Comparison of infected plants to non-infected ones under ambient CO_2 and elevated O_2.

was tested using the absorbance spectrophotometry method [28]. Chemical variables described above were measured of two randomly selected samples from each treatment per OTC (=4 samples per OTC in the same CO_2 treatment and 16 samples total).

2.6. Data analysis

The effect of CO_2 and virus on indirect yield index (periodical aboveground biomass accumulation), plant quality indices (total non-structural carbohydrates, nitrogen content, free amino acid content, soluble protein content, and nicotine content), and the plant virus resistance index (POD activity) were analyzed by ANOVA using SPSS13.0.1 (SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Tukey's HSD (Honestly Significant Differences) test in the post hoc multiple comparisons at $P<0.05$ (SAS 6.12, SAS Institute Inc., USA, 1996).

3. Results

Typical symptoms appear on the leaves of infected plants including mottling, leaf curling, and prominent veins 5–7 days after infected with PVY.

The CO_2 concentration significantly ($P<0.001$) impacted the aboveground biomass, POD ($P=0.001$), soluble protein ($P=0.006$) and chlorophyll ($P<0.025$) content of the tobacco plants. PVY^N infection significantly influenced ($P=0.003$) tobacco foliar chloro-

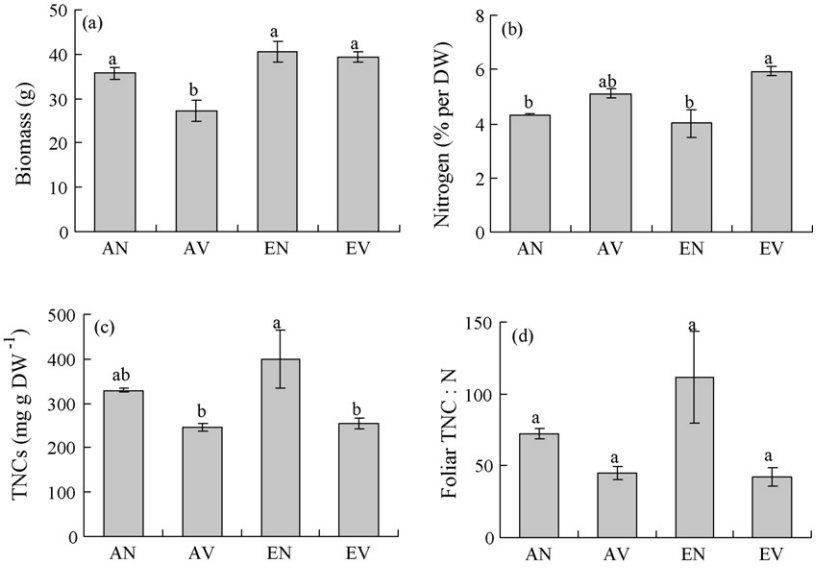

Fig. 1. (a) Aboveground biomass, (b) foliar nitrogen content, (c) total foliar non-structural carbohydrates (TNCs) content and (d) C:N ratio of 10-week-old tobacco (NC89) uninfected and infected with virus (PVY^N) grown under ambient (375 ppm) and elevated (750 ppm) CO_2. Values represent means ± SE of $n=8$ separate plants from one typical experiment. AN = uninfected tobacco in ambient CO_2, AV = infected tobacco in ambient CO_2, EN = uninfected tobacco in elevated CO_2, EV = infected tobacco in elevated CO_2, Different lowercase letters indicate significant differences among the four treatments (Tukey's HSD test, $P<0.05$).

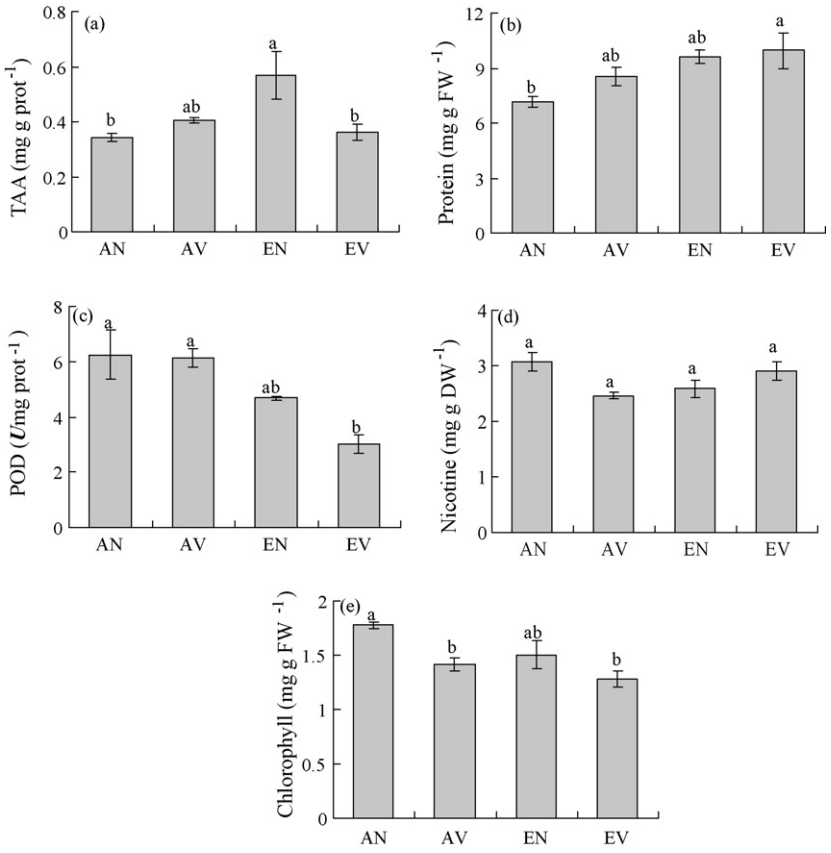

Fig. 2. Foliar chemical contents of 10-week-old tobacco (NC89) uninfected and infected with virus (PVY^N) grown under ambient (375 ppm) and elevated (750 ppm) CO_2. (a) Free amino acid content, (b) soluble protein content, (c) foliar peroxidase (POD) activity, (d) nicotine content, (e) chlorophyll content. Values represent means ± SE of $n = 8$ separate plants from one typical experiment. AN = uninfected tobacco in ambient CO_2, AV = infected tobacco in ambient CO_2, EN = uninfected tobacco in elevated CO_2, EV = infected tobacco in elevated CO_2, different lowercase letters indicate significant differences among the four treatments (Tukey's HSD test, $P < 0.05$).

phyll content and significantly affected biomass ($P = 0.020$), nitrogen content ($P = 0.001$) TNCs ($P = 0.005$) and C/N ratio ($P = 0.013$). The interaction between CO_2 concentrations and PVY^N infection significantly impacted the tobacco foliar free amino acid content ($P = 0.014$) and nicotine content ($P = 0.009$) (Table 1).

ECO_2 led to an increase of aboveground biomass, TNCs, C/N, amino acid content, and soluble protein content. However, decreases in nitrogen content by 7.6% ($P = 0.853$) and nicotine content by 15.6% ($P = 0.152$) POD by 25.1% ($P = 0.172$) and chlorophyll content by 15.3% ($P = 0.120$) were seen for tobacco plants without PVY^N infection when compared with those under ambient CO_2 conditions (Table 2, Figs. 1 and 2).

Under ACO_2, PVY^N infection led to decrease of aboveground biomass by 23.6% ($P = 0.026$), TNCs by 25.6% ($P = 0.329$), C/N by 37.8% ($P = 0.655$), nicotine content by 20.0% ($P = 0.051$), POD by 2.0% ($P = 0.998$), and chlorophyll content by 20.5% ($P = 0.029$). Meanwhile, increase of nitrogen content by 17.9% ($P = 0.280$), TAA by 18.0% ($P = 0.789$) and protein by 18.8% ($P = 0.401$) were observed when compared with uninfected plants (Figs. 1 and 2).

Under ECO_2 conditions, PVY^N infection increased nitrogen content by 48.3% ($P = 0.002$), protein by 3.4% ($P = 0.978$) and nicotine by 11.8% ($P = 0.487$). Meanwhile, decrease of biomass by 3.1% ($P = 0.966$), TNCs by 36.5% ($P = 0.041$), C/N by 62.1% ($P = 0.050$), TAA by 36.6% ($P = 0.038$), POD by 35.5% ($P = 0.141$) and chlorophyll by 14.8% ($P = 0.236$) were observed when compared with uninfected plants (Figs. 1 and 2).

For infected plants, ECO_2 led to increase of biomass by 44.4% ($P = 0.001$), TNCs by 3.6% ($P = 0.997$), Nitrogen by 16.2% ($P = 0.233$), soluble protein by 16.9% ($P = 0.347$) and nicotine by 17.9% ($P = 0.203$). Meanwhile, decrease of C/N by 5.8% ($P = 0.999$), TAA by 11.3% ($P = 0.900$), POD by 50.7% ($P = 0.004$) and chlorophyll by 9.2% ($P = 0.653$) were seen when compared infected plants under ECO_2 condition with infected plants under ACO_2 condition (Figs. 1 and 2).

4. Discussion

Plant virus diseases severely constrain agricultural production worldwide [18], but the roles played by pathogens in determining an ecosystem response to ECO_2 have rarely been examined [15,13]. Chakraborty et al. [19] suggested that ECO_2 might have many positive effects on agricultural production, including better pest and disease resistance of the C_3 crop. However, that study mainly referred to resistance against fungal disease and herbivore feeding. Therefore, studies on the variation of plant resistance to virus infection under ECO_2 are needed.

ECO_2 levels directly impact plant physiology and result in an increase in the photosynthetic rate, which alters the growth and aboveground biomass [29,12,30,31]. On the other hand, Malmstrom and Field [15] found that the biomass of BYDV-infected barley decreases by 50–60% compared with healthy barley under ACO_2, while the biomass of infected barley decreases by 39–40% compared with healthy plants under ECO_2. Generally speaking, few studies on pot-grown plants have discovered an insignificant growth response to ECO_2 [32,33]. In the present study, significant increase in the aboveground biomass of tobacco plants in response to ECO_2 was observed. Under ACO_2 and ECO_2 conditions, the biomass of infected tobacco plants decreased by 23.6% and 3.1%, respectively, in comparison with uninfected plants. The positive effect of increasing CO_2 should not be overestimated, especially

considering real ecosystems contain both insects and pathogens. On the other hand, the negative effect of virus infection on yield or biomass could be alleviated by ECO_2.

An ECO_2 level alters the carbon-to-nitrogen ratio (C/N) and the production of plant nutrients and secondary metabolites, especially in C_3 plants [5,30,34]. Therefore, carbon-based secondary compounds would increase and nitrogen-based secondary materials would decrease, especially while nitrogen fertilization was not enough provided [5,10]. Matros et al. [10] found that foliar phenylpropanoids increased significantly and secondary metabolites, based on nitrogen nicotine content, decreased markedly under the ECO_2 condition. Our results suggest that, both ECO_2 and virus infection negatively influence chlorophyll content; under ECO_2 treatment, both the chlorophyll content and the environmental CO_2 concentration influence the photosynthetic rate, which determines the biomass accumulation of tobacco. Double CO_2 concentrations and slightly decreased chlorophyll content without much variation of other environmental factors lead to a slightly increased photosynthetic rate, which gave rise to rare nitrogen nutrition as a limiting factor in field, resulting in slightly decreased nitrogen content and slightly increased sugar content. Upon virus infection, ECO_2 could alleviate the damage caused by viral infection to chlorophyll; combined with double CO_2, biomass accumulation did not vary significantly. It seemed like the higher CO_2 could reverse the negative effect of PVY infection on chlorophyll content, which should correlate with the steady biomass production under ECO_2. Moreover, in this study, an increase of C/N under ECO_2, a significant decrease in C/N during PVY infection, and a marginally significant decrease of C/N in infected plants compared to healthy plants under ECO_2 were observed. However, we did not find a correlation between C/N ratio (or nitrogen content) and nicotine content (a nitrogen-based secondary metabolite), especially in infected tobacco plants under ACO_2 conditions. This finding is inconsistent with a previous hypothesis, which claimed that carbon-based secondary compounds would increase and nitrogen-based secondary materials would decrease when the C/N rate was elevated during increased CO_2, especially while nitrogen fertilization was scarce [5,10]. Moreover, defensive chemicals have long been thought to be costly for plants because of the resources consumed in their biosynthesis, their toxicity to the plant itself or the ecological consequences of their accumulation [35]. Similarly, in our study, taking increase of soluble protein content as a response of the plant to PVY infection, ECO_2 had a similar effect on soluble protein and could strengthen this costly response. NC_{89} is common susceptible cultivar, in which the amino acid content was higher while infected by PVY or while treated with ECO_2. However, infected plants under ECO_2 totally reversed this response and led to a decrease in amino acids; on the other hand, as one studies documented that POD may arise from the oxidative burst triggered upon pathogen attack [36], in our study, no POD activity response to PVY infection was detected while ECO_2 led to a significant decrease of POD in infected tobacco plants compared to healthy plants.

In general, ECO_2 impacts plant physiology by increasing the photosynthesis rate, growth, aboveground biomass, yield, and carbon: nitrogen (C:N) ratio and reducing nitrogen concentrations, impacting the production of plant nutrients and secondary compounds [29,5,30,31]. However, in present study, the overall capacity of ECO_2 to increase biomass accumulation may have been counteracted by virus infection in the ECO_2 + PVY treatment. Alternatively, infected tobacco under ECO_2 treatment may have invested more in long-term chemical defence (especially in secondary metabolites synthesis) than healthy plants. ECO_2 increased plant biomass and foliar soluble protein content. PVY infection basically had an opposite effect on these major factors in comparison with the effects of ECO_2, excluding the impact on nicotine content. Furthermore, nicotine synthesis did not reach significant levels throughout any of these variations. Moreover, ECO_2 and PVY infection had similar negative effects on tobacco chlorophyll content without accumulative characteristic, which partially elucidated the cause of the biomass variation under ECO_2 after PVY infection in addition to higher CO_2 (photosynthetic substance). Our results also suggest that, first, if two factors have opposing effects on the plant-specific index, their effects might not necessarily counteract one another, or two factors have similar effects on specific plant index, their effects might not necessarily have accumulative characteristics. Here, we refer to the effect of ECO_2 and PVY infection on tobacco TNCs content, nitrogen content, and nicotine content. Second, all findings reconfirmed that it is necessary to study the effects of multiple factors on the plant index, instead of deducing potential results from the influence of a single factor. Therefore, it seemed reasonable to obtain these results, which suggested in the present study that CO_2 could protect a plant from virus damage in seedling period, or at least delay the viral damage.

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