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Apolygus lucorum-induced resistance in *Vitis vinifera* L. elicits changes at the phenotypic, physiological, and biochemical levels

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ABSTRACT

The infestation of the pest *Apolygus lucorum* (Meyer-Dür, 1843) induced changes in *Vitis vinifera* L. 'Cabernet Sauvignon' at the growth, physiological, and biochemical levels, which were recorded in this study. Continuous infestation by *A. lucorum* for $>$ 4 d significantly affected plant height and leaf width, although there were no effects when infestation was for $\leq$ 4 d. Over the long term, *A. lucorum* mortality increased and their average survival time decreased. *A. lucorum*-based injury induced plant defense responses, and a spike in defense enzymes was observed. Additionally, infestation induced significant changes in protease inhibitor and secondary metabolite levels in the grape leaves. After *A. lucorum* infestation, total sugar, protein, and chlorophyll in the leaf decreased significantly. Puncture injury generally induced a milder response compared with *A. lucorum* infestation. In summary, *A. lucorum* infestation induced resistance in Cabernet Sauvignon by activating various physiological and biochemical defense responses. In addition to providing new insights into the coevolution of plants and insects, the results presented in this study may lead to the development of an effective, innovative, and environmentally friendly pest control method.

1. Introduction

Plants are stressed during growth by drought, high temperature, pathogens, physical damage, and herbivorous insects (Mao et al., 2011). To resist pathogens and insect invasions, plants have developed a complex defense system that uses active metabolic resistance reactions (Barah et al., 2013; Gong et al., 2020; Jiang et al., 2019). Plant defense systems are closely correlated with morphological characteristics, growth rhythms, and production of physical and chemical substances, including secondary metabolites (Xing et al., 2014; Zhang et al., 2018). Using endogenous plant resistance to resist external attacks is one of the most economical, effective, and environmentally friendly green management strategies, and it has been gradually applied to a variety of crops and forest products (Arimura et al., 2009). Increasing or inducing plant resistance has become an important approach in smart agriculture and modern plant protection.

Vitis vinifera L. 'Cabernet Sauvignon' is one of the main grape varieties cultivated in wine regions in China. In recent years, damage caused by the piercing and sucking insect *Apolygus lucorum* (Meyer-Dür, 1843) has become increasingly severe in wine grape-growing areas in China (Lu and Wu, 2011). Adults and nymphs badly damage the young tissues of Cabernet Sauvignon, resulting in great losses in yield and quality of wine grapes and wine. Currently, the damage caused by *A. lucorum* infestation and the defense responses in Cabernet Sauvignon induced by *A. lucorum* infestation have not been systematically studied. In addition, there is little information on how the degree and duration of *A. lucorum* infestation activity affect host plant molecular defense mechanisms, or how the effects of herbivory differ from those of only physical injury.

Induced resistance in plants typically only reduces the degree of damage within a certain range or improves tolerance, and the magnitude of the induced resistance is related to the intensity of the stimulus. Within a specific range, the stronger the stimulus is, the greater the resistance. However, after reaching a maximum, the level of induced

Abbreviations: AOS, allene oxide synthase; CAT, catalase; CK, control treatment; LOX, lipoxygenase; MDA, malondialdehyde; PAL, phenylalanine ammonia-lyase; POD, peroxidase; PPO, polyphenol oxidase; SOD, superoxide dismutase.

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resistance tends to remain unchanged or decrease. In addition, plants do not permanently acquire induced resistance, and when the inducing factor is absent, induced resistance also ends (Poelman et al., 2011; Sugio et al., 2011). Plant resistance to insects is most commonly expressed as changes in physical structure or in contents of physiological and biochemical substances (Geng et al., 2021).

Infestation by herbivorous insects can induce a defense mechanism in host plants, which can in turn negatively affect survival, growth and development, and egg deposition in insects, elevating their risk of predation or parasitism (Hu et al., 2007). Insect infestation-induced resistance in plants is associated with various physiological and biochemical changes, including increased production of defense enzymes, volatile terpenoids, protease inhibitors, secondary metabolites, and nitrogen-containing compounds, as well as changes in nutrient and chlorophyll profiles (Hu et al., 2007). Changes in activities of important antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT), are strongly associated with stress-induced resistance in plants (Li et al., 1998; Yue et al., 2003). Activities of enzymes involved with secondary metabolite production, such as phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX), and hydroperoxide lyase, also reflect levels of induced resistance in plants (Bruinsma et al., 2010; Liu et al., 2005). Examining the changes in plant defense enzymes with different degrees of biological or physical damage can help reveal physiological mechanisms of induced resistance, allowing researchers to achieve a comprehensive understanding of coevolution of host plants and insects.

When injured or stressed, plants quickly adjust their metabolism, and the injured parts produce secondary metabolites to defend against invasion or adverse environmental factors (Bouwmeester et al., 2003; Furlan et al., 2019; Tuominen and Salminen, 2017). Adjustments to metabolism in response to biological stress caused by herbivorous insects cause reduction in available food resources that support the growth and development of the latter (Koricheva, 2002) and often lead to production of large amounts of protease inhibitors, which can impair the digestion and metabolism of the insect pests (Kim et al., 2020; Stam et al., 2014). Additionally, many plants release specific chemical volatile substances or secondary metabolites, such as tannins, phenols, terpenes, flavonoids, alkaloids, lignin, and hydroxyproline glycoprotein (Moctezuma et al., 2014; Shi et al., 2014), to attract natural enemies of the insect herbivores (Sugio et al., 2011).

In this study, the resistance induced in *Vitis vinifera* L. in response to continuous infestation by *A. lucorum* was examined by determining changes in plant growth, defense enzymes, secondary metabolites, and nutrients. In addition to providing new insights into the coevolution of plants and insects, the results presented in this study may help in the development of novel chemical agents for the control of *A. lucorum* populations, which may emerge as an effective, innovative, and environmentally friendly pest control method in the future.

2. Methods

2.1. Materials

Cabernet Sauvignon grape plantlets were obtained from the Langes Winery (Qinhuangdao) Co., Ltd. (Heibei, China). Branches 20.0 cm in length and 0.5 to 1.0 cm in diameter were cut from plants in winter and stored in sand. Cuttings were planted in 10 cm-diameter pots in a mix of peat soil, vermiculite, and perlite at a volume ratio of 5:3:2 and grown at 25 °C to 30 °C and 70% to 80% humidity under 16 h of light per day. Cuttings were watered daily, and fertilizers and pesticides were not used. After two months of cultivation, robust seedlings were selected for experiments. Only one main vine (new shoot) was retained in each pot. The Institute of Plant Protection, Hebei Academy of Agriculture and Forestry Sciences, Baoding, China, provided the *A. lucorum*. They were maintained at 26 °C and 60% to 70% humidity with a 16:8 h light:dark photoperiod and were fed fresh green beans (*Phaseolus vulgaris* L.).

Two experimental treatments were established. (1) In the *A. lucorum* sucking and infestation treatment, five fully unfolded young leaves from the top of each grape plantlet were infested with five healthy *A. lucorum* 3rd instar nymphs that were picked with a clean brush. Sticky shellac was smeared under the 5th leaf to prevent movement, and plantlets were covered with 80-mesh net to prevent escape. Insects fed freely for 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, or 15 d and then were removed from the plantlet. (2) In the puncture injury treatment, five fully unfolded young leaves from the top of each grape plantlet were pricked approximately 100 times every day at 08:00 with a No. 0 insect pin to simulate insect feeding. Plantlets were covered with 80-mesh net, which was removed after 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, or 15 d. One control treatment (CK) was also established, and five fully unfolded young leaves from the top of each grape plantlet were covered with 80-mesh net. The net was removed after 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, or 15 d. Each treatment in each group was replicated three times.

2.2. Plant growth

Grape plantlets treated above were monitored regularly for 15 d after the treatments started. Damage to marked leaves (the first unfolded young leaves at the top) was photographed. Before initiating the experiment, the height of each plantlet and the width of the first unfolded young leaf (marked) at the top were measured. After 15 d, the height of each plantlet and the width of the marked leaf were measured again to determine increases in height and width (Nafisa et al., 2020).

2.3. Apolygus lucorum mortality

Five healthy 3rd instar *A. lucorum* nymphs were placed on Cabernet Sauvignon plantlets as described in 2.1. The density was based on field survey results. Insects fed freely and were observed at 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, and 7 d. The insect net was removed after all *A. lucorum* died. Nymph mortality and average survival time were determined. When all five nymphs of the initial infestation were dead, another five 3rd instar nymphs were placed immediately at the same location. Times of death of *A. lucorum* were recorded for the two infestations.

In no-feeding controls, 50 3rd instar nymphs were divided into 10 groups, placed in empty boxes covered with insect nets, and times of death were recorded. In green bean-fed controls, 50 3rd instar nymphs were divided into 10 groups, placed in boxes containing green beans covered with insect nets, and times of death were recorded.

The *A. lucorum* were not exposed to any pesticides before or after experiments.

2.4. Enzyme activity

Sterile scissors were used to collect the top three leaves (approximately 2 g, with petioles removed) in each treatment at different points in time. After washing leaves with ammonia-free distilled water to remove dirt and impurities, they were wrapped in foil, snap-frozen using liquid nitrogen, and stored at −80 °C until enzyme activities and biochemical substances could be determined.

To obtain enzyme extracts, 5 mL of grape-leaf extraction solution (50 mM phosphate buffered saline, pH 7.8, 1% insoluble polyvinylpyrrolidone) was added to 0.5 g of grape leaves, and the ground homogenate was centrifuged at 15,000 rpm for 10 min. An SOD detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure SOD (EC:1.15.1.1) activity, and the guaiacol method (Li and Gong, 2008) was used to measure POD (EC:1.11.1.7) activity. The catechol rate method (Pan et al., 2018) was used to measure PPO (EC:1.10.3.1) activity, and a CAT detection kit (Nanjing Jiancheng Bioengineering Institute) was used to measure CAT (EC:1.11.1.6) activity. Detection kits (Nanjing Jiancheng Bioengineering Institute) were used to measure allene oxide synthase (AOS, EC:4.2.1.92), LOX (EC:1.13.11.12), and PAL (EC:4.3.1.24) activities.

Chitinase detection kit and β−1,3-glucanase detection kit procured from Solarbio (Beijing, China) were used to measure chitinase (EC3.2.1.14) and β−1,3-glucanase (EC3.2.1.58) activities, respectively.

2.5. Physiological and biochemical assays

Both trypsin inhibitor- and chymotrypsin inhibitor-like activities were measured using ultraviolet spectrophotometry (Shi et al., 2017).

Flavonoids were extracted by adding 3 mL of 70% ethanol to 0.1 g of leaf sample, followed by incubation for 2 h in a 70 °C water bath and then centrifugation at 6000 rpm for 5 min. The pellet was extracted a second time using the same method, and supernatants from the two extractions were combined. Next, 0.5 mL flavonoid extract was added to 4.5 mL of 1% aluminum chloride solution and incubated for 10 min. Absorbance was measured at 420 nm. The standard curve for flavonoids was prepared by measuring the absorbance of 0, 1, 3, 5, 10, and 15 mL of standard solution of 0.1 mg/mL rutin diluted to 50 mL with water (final concentrations of 0, 0.002, 0.006, 0.01, 0.02, and 0.03 mg/mL, respectively).

To measure tannins, 0.1 g of grape leaf was added to 10 mL of 80% acetone for 24 h. The solution was then filtered and centrifuged at 5000 rpm for 10 min. Next, 0.5 mL supernatant was added to 3 mL of 4% vanillin in methanol and 1.5 mL of concentrated hydrochloric acid, shaken well in a test tube covered with aluminum foil, and incubated in a water bath at 20 °C for 20 min. Absorbance was measured at 510 nm. The standard curve was prepared as described above using 0.1 mg/mL catechin.

To measure total phenols, 0.1 g of grape leaf was added to a 30-mL conical flask with 3.3 mL of 40% methanol, shaken well, transferred to a 5-mL centrifuge tube, and centrifuged at 6000 rpm for 5 min. Supernatant, 0.5 mL, was added to a conical flask with 2.5 mL of Folin reagent, followed by the addition of 2 mL of sodium carbonate (7.5 g/L) 5 min later. The mixture was incubated in a 50 °C water bath for 5 min and then allowed to cool. Absorbance was measured at 760 nm. The standard curve was prepared as described above using 0.1 mg/mL tannic acid.

Total sugar was extracted using the ethanol precipitation method. Grape leaf (0.1 g) was ground in distilled water in an ice bath and then transferred to a graduated test tube in a boiling water bath for full extraction, followed by centrifugation at 4000 rpm for 15 min. Total sugar content was determined using the anthrone colorimetry method (Yang et al., 2013). Extract, 0.5 mL, was mixed with reaction reagent containing 100 μg/L glucose solution, distilled water, anthrone ethyl acetate reagent, concentrated sulfuric acid, and glucose, incubated in a boiling water bath for 60 s, and allowed to cool. Absorbance was measured at 630 nm.

Proteins were extracted by grinding 0.2 g of grape leaf sample in 5 mL water on ice and then centrifuging at 4000 rpm for 20 min. Protein concentration was measured using the Coomassie brilliant blue method. Protein extract, 1 mL, was mixed with 5 mL of Coomassie brilliant blue (100 mg/L), and absorbance was measured at 595 nm. Free amino acids were extracted by adding 5 mL of 10% acetic acid to 0.5 g of ground grape leaf sample. The mixture was incubated for 2 to 6 h and then filtered. Free amino acid concentration was measured using the ninhydrin color method. Extract was mixed with ninhydrin and ascorbic acid and incubated in boiling water for 15 min, and after the solution cooled, the color reaction was performed. When blue-violet color appeared, the reaction mixture was diluted with 60% ethanol, and the absorbance was measured at 570 nm. Chlorophyll concentration was measured using the 95% ethanol extraction method (Pan and Han, 2017).

2.6. Statistical analyses

Three replicate plants were assessed per treatment. The SPSS 24 software (IBM, Armonk, NY, USA) was used for data analyses, and Excel 2010 (Microsoft, Redmond, WA, USA) was used for graphing. The statistical significance or variance between experimental groups was determined using one-way analysis of variance (ANOVA) with the Duncan post hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Phenotypic response

Apolygus lucorum only feeds on the young and tender parts of Cabernet Sauvignon, and the parts above the third fully unfolded leaf from the top are those most seriously damaged. After *A. lucorum* fed on young leaves in this study, small, black-brown necrotic sucking spots appeared first (Fig. 1A). Sucking spots gradually developed into rusty yellow spots on the 3rd day, and spots were completely rusty yellow on the 5th day. Subsequently, spots continued to develop into transparent withered spots or holes (Fig. 1B). Sucking spots of *A. lucorum* were notably different from punctured spots (Fig. S1).

Different infestation durations had significantly different effects on leaf phenotype ($P < 0.01$, Fig. 1B). After 24 h of infestation, only rusty yellow spots were observed on grape leaves on the 15th day, and any transparent, withered spots formed without aging, curling, or shrinking. After 48 h of infestation, leaf phenotype on the 15th day was not significantly different from that after 24 h of infestation, except spots were deeper and there were occasional holes. After 72 h of infestation, leaves were slightly curled on the 15th day, the number of spots increased obviously, and the spots were deeper and often connected to form large holes. After 96 h of infestation, on the 15th day, leaves were shrunken and deformed, leaf surface color was slightly uneven, and leaves were torn centered on the spots. After continuous infestation for 120 h, on the 15th day, leaves were severely shrunken with many rusty yellow spots, and the leaf surface was aged, thickened, and unevenly colored. After continuous infestation for 144 h or 168 h, leaves were aged, thickened, cracked, and highly lignified on the 15th day. Thus, the longer *A. lucorum* continuously pierced and sucked grape leaves, the more severe the damage (Table S1).

3.2. Plant growth

After *A. lucorum* infestation or puncture injury, plant height was affected to various degrees depending on length of treatment (Fig. 2A). After *A. lucorum* infested for 5 d, plant height was significantly lower than that in the control ($P < 0.05$). When *A. lucorum* infested for 6 d or 7 d, the decrease in plant height was significant compared with the control ($P < 0.01$). In other infestation-length treatments, *A. lucorum* did not significantly affect plant height. Regardless of treatment length, puncture injury did not significantly affect plant height, compared with the control (Fig. 2A).

After *A. lucorum* infestation and puncture injury, grape leaf width varied depending on length of treatment (Fig. 2B). When *A. lucorum* fed for 5 d, leaf width was significantly lower than that in the control ($P < 0.05$). When *A. lucorum* fed for 6 d or 7 d, the decrease in leaf width was significant compared with the control ($P < 0.01$). Even at day 15 when the insect net was removed, the leaf width was significantly smaller than that of the control ($P < 0.05$). Regardless of treatment length, puncture injury did not significantly affect leaf width, compared with the control (Fig. 2B). Thus, the effects of physical injury on plant growth were minor compared with those of insect infestation.

3.3. Apolygus lucorum mortality

Some *A. lucorum* died after feeding on Cabernet Sauvignon leaves, and the mortality rate continued to increase with the increase in feeding time (Fig. 3A). From day 4 of feeding in the first infestation, *A. lucorum* mortality rate exceeded 50%. The mortality rate reached 99.14% after feeding for 7 d and then reached 100% after 8 d (Fig. 3A). When *A. lucorum* was fed green beans in the control, survival was 100%

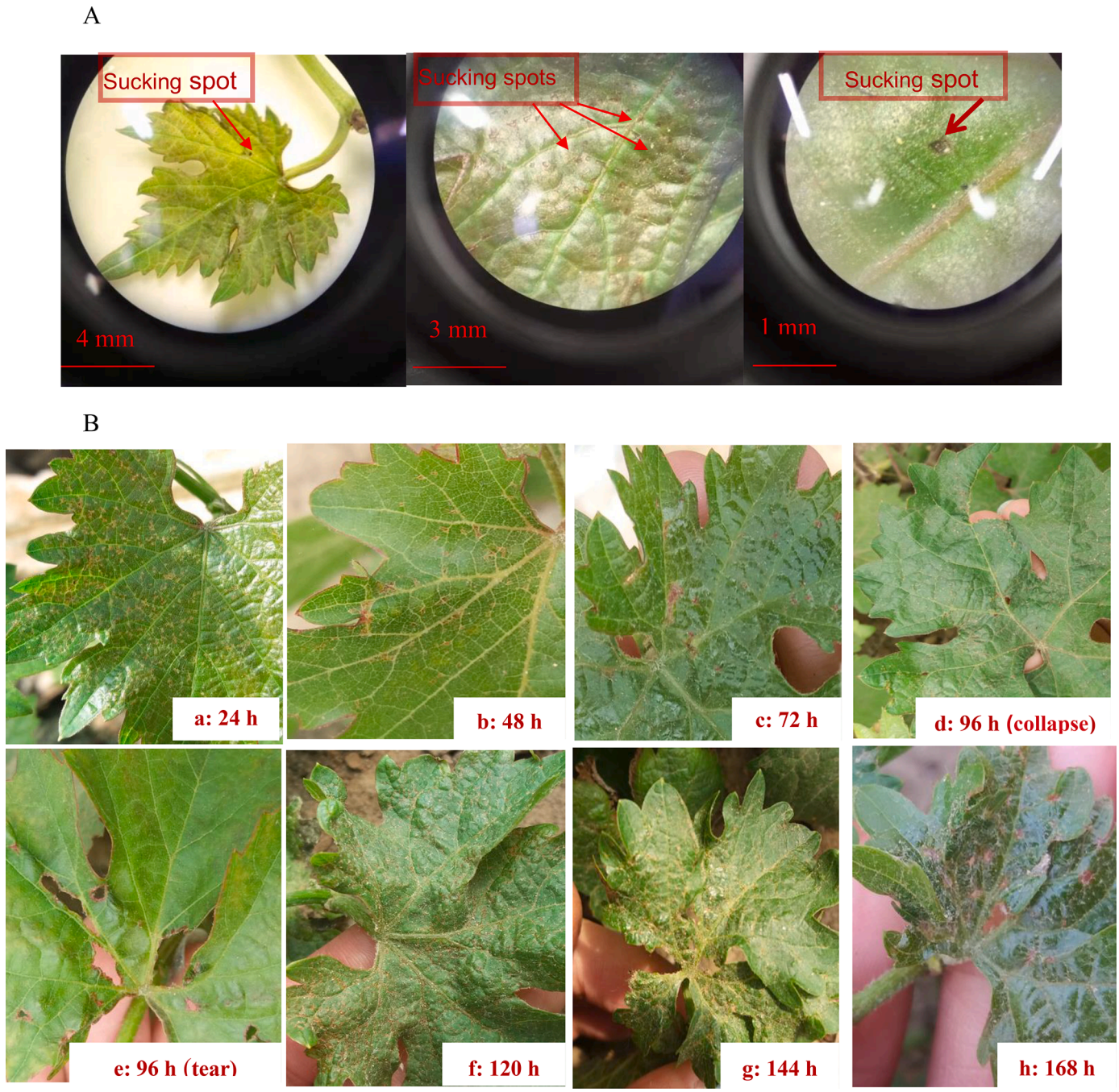


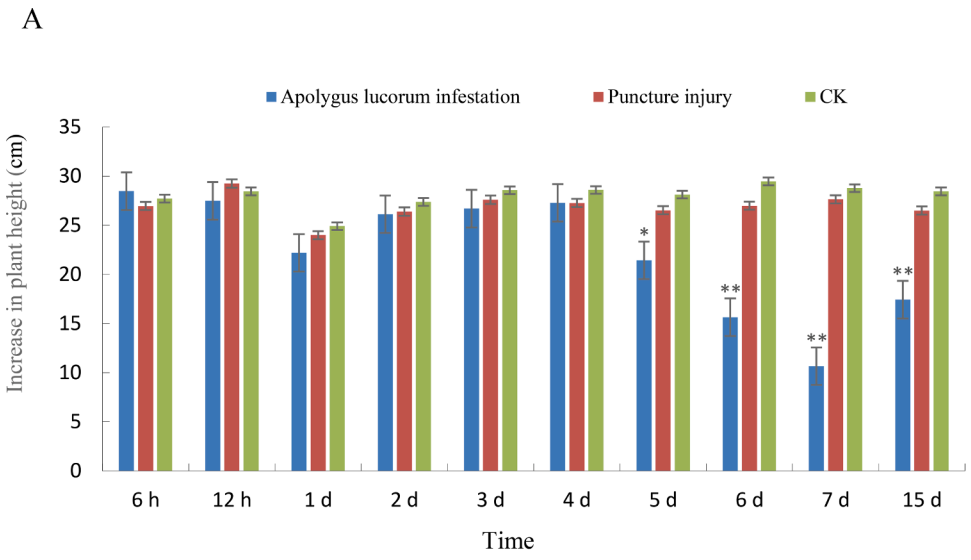
Fig. 1. Phenotypic changes in Cabernet Sauvignon leaves after *Apolygus lucorum* infestation. (A) Appearance of sucking spots caused by *A. lucorum* infestation. (B) Damage caused by continuous infestation of *A. lucorum* (A to H: 24 h to 168 h) after 15 days.

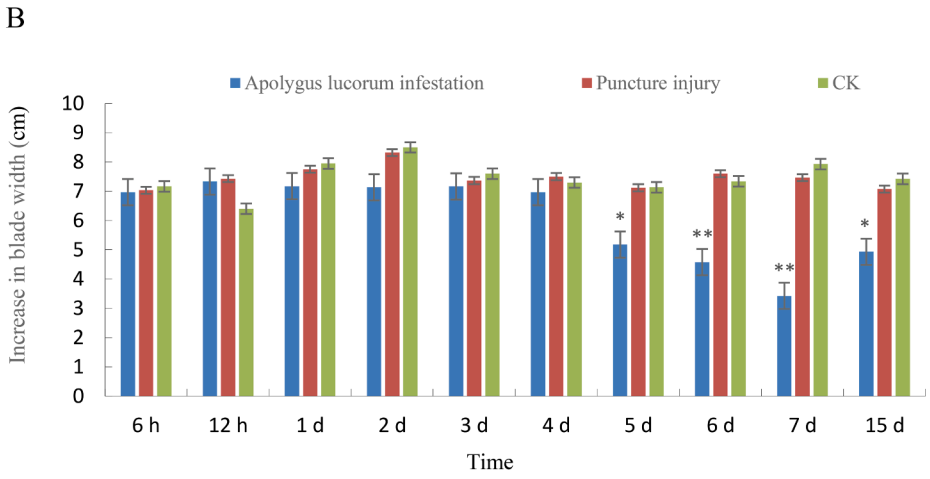
(Fig. 3A).

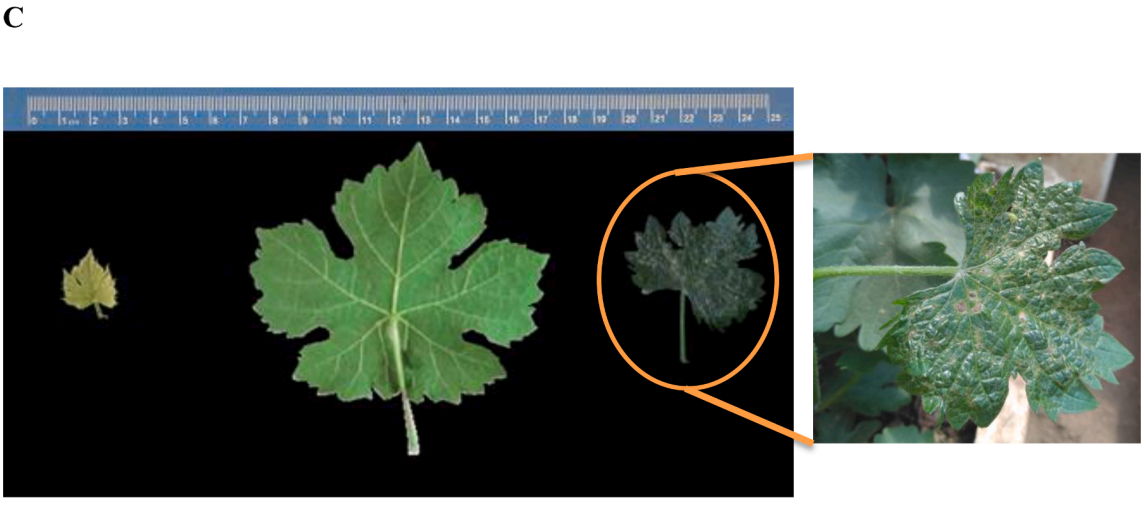
In the first infestation, 3rd instar nymphs survived 4.79 ± 0.41 d after feeding on Cabernet Sauvignon. In the second infestation, the nymphs survived 3.14 ± 0.89 d, which was significantly shorter than that in the first infestation ($P < 0.05$; Fig. 3B). These results suggested that insect-resistant substances were highly induced in grapes leaves with the first infestation, which were then rapidly accumulated by *A. lucorum* in the second infestation and accelerated their death.

3.4. Enzyme activities

After *A. lucorum* infestation and puncture injury, the activities of four defensive enzymes in Cabernet Sauvignon young leaves changed to varying degrees over time (Fig. 4A–D). In the two treatments, SOD (Fig. 4A), PPO (Fig. 4C), and CAT (Fig. 4D) activity increased significantly on the 1st day, compared with the control ($P < 0.05$). With the increase in time in *A. lucorum* infestation and puncture injury treatments, SOD activity gradually decreased, and in the *A. lucorum* infestation treatment, activity was significantly lower than that in the control on the 7th day ($P < 0.05$). Activity of POD (Fig. 4B) increased significantly from the 4th day in both treatments ($P < 0.01$ and $P < 0.05$ on different days and in different treatments compared with the control), and the increase with *A. lucorum* infestation was higher on the 5th and 7th days than that with puncture injury. Activity of CAT was consistently higher in *A. lucorum* infestation and puncture injury treatments than that in CK and was significantly higher on the 1st, 3rd, and 4th days ($P <$







Width of new leaf just unfolded	Leaf width after 15 d in CK	Leaf width after 15 d (*A. lucorum* infestation for 7 d)

Fig. 2. Growth of Cabernet Sauvignon after *Apolygus lucorum* infestation and puncture injury. (A) Increase in plant height (in cm). (B) Increase in leaf width (in cm). The values represent the increase in the plant height or leaf width 15 days after the initiation of the interventions compared with the initial state. The time on the x-axis indicate the duration of the interventions (in days). (C) Photographs of the leaf profiles 15 days after the initiation of the interventions in control (CK) and *Apolygus lucorum*-infested grape leaves (infestation duration of 7 d). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control (CK) for each length of treatment time.

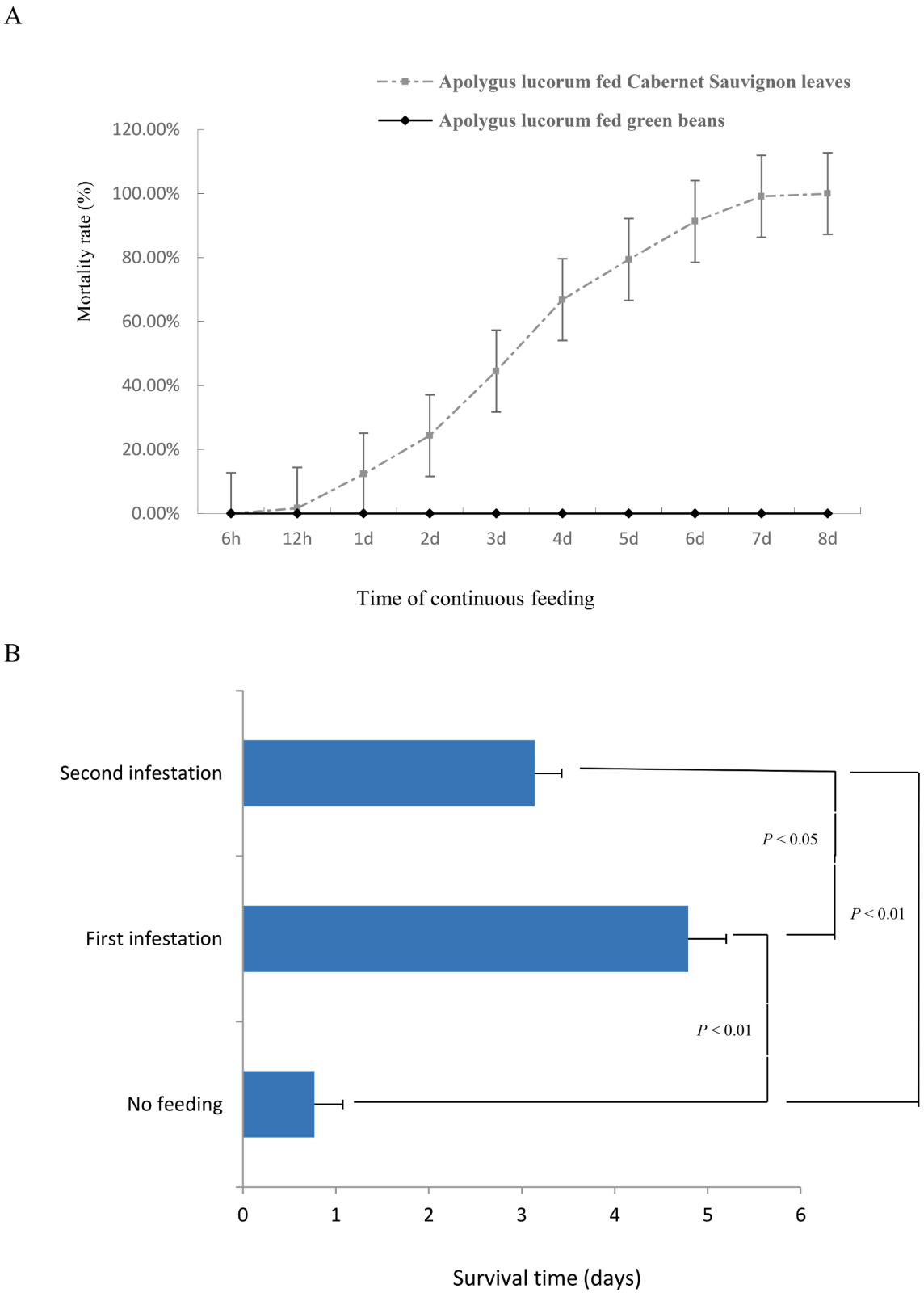
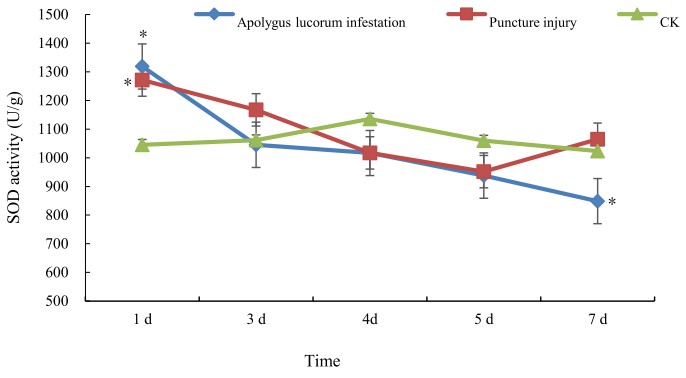


Fig. 3. Change in mortality of *Apolygus lucorum* after continuous feeding on Cabernet Sauvignon leaves. (A) Mortality rate continued to increase until day 8, when all *A. lucorum* died. The control was *A. lucorum* fed green beans. (B) Mean survival time of *A. lucorum* in the two infestations. The control was *A. lucorum* without feeding.

0.05). Activity of PPO in *A. lucorum* infestation group was continuously significantly higher than that in CK and was significantly different on the 3rd, 4th, and 5th days ($P < 0.01$). On the 3rd day, PPO activity in the puncture injury group was significantly different from that in the CK group ($P < 0.01$). Activity of PPO was also significantly different between puncture treatment and *A. lucorum* infestation ($P < 0.01$). These results indicated that with external damage, stress protection mechanisms were activated in the young leaves of Cabernet Sauvignon. Changes in defensive enzyme activities were somewhat different between *A. lucorum* infestation and puncture injury, indicating that

A



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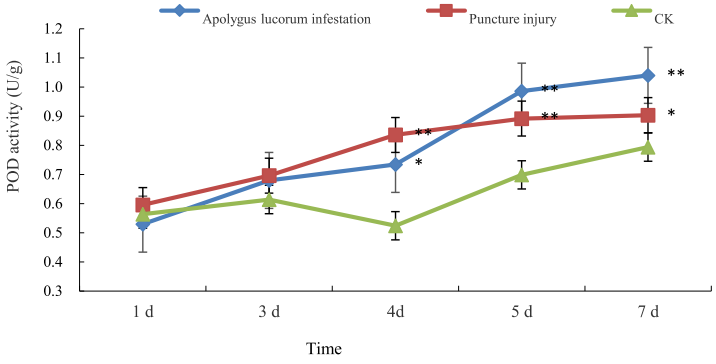

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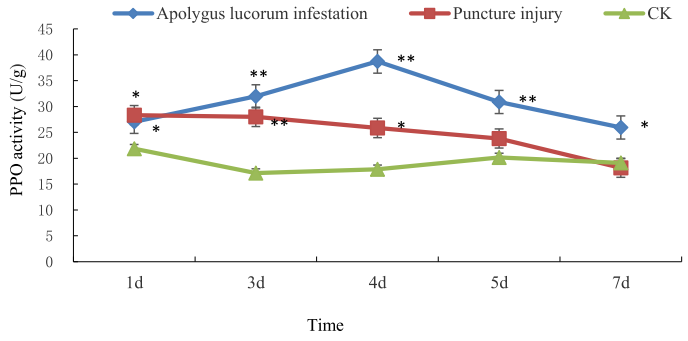

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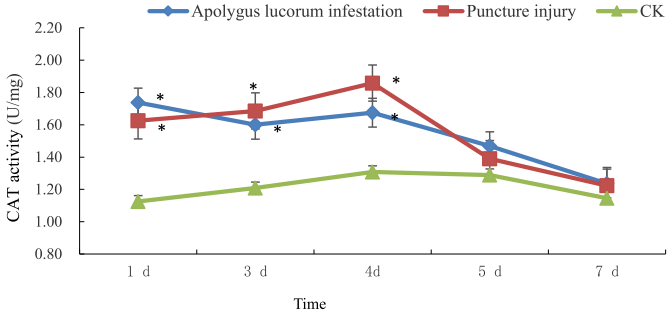

Fig. 4. Changes in enzyme activities in Cabernet Sauvignon leaves after *Apolygus lucorum* infestation and puncture injury. Activities of (A) superoxide dismutase (SOD), (B) peroxidase (POD), (C) polyphenol oxidase (PPO), and (D) catalase (CAT), (E) allene oxide synthase (AOS), (F) lipoxygenase (LOX), and (G) phenylalanine ammonia-lyase (PAL) were monitored following different lengths times of *A. lucorum* infestation and puncture injury, which generally increased after the two treatments to varying degrees. (H) β−1,3-glucanase and (I) Chitinase enzyme activity 4 d after initiation of *Apolygus lucorum* infestation or puncture injury. *Apolygus lucorum* infestation resulted in a significant increase in the activity of β−1,3-glucanase and chitinase compared with that induced by puncture injury. $*P < 0.05$ and $**P < 0.01$, compared with the control (CK) for each length of treatment time.

Fig. 4. (*continued*).

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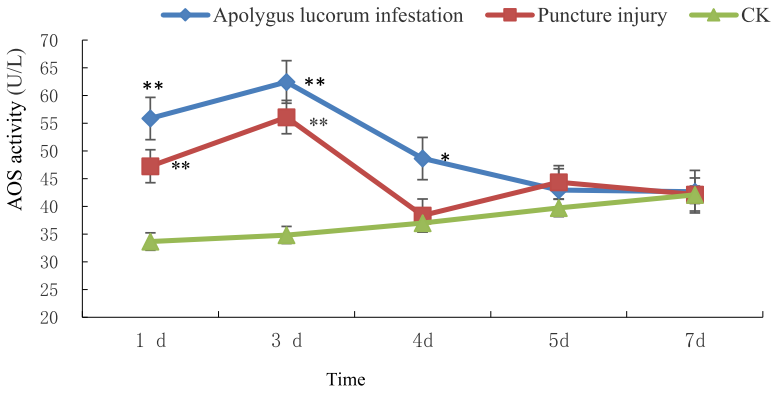

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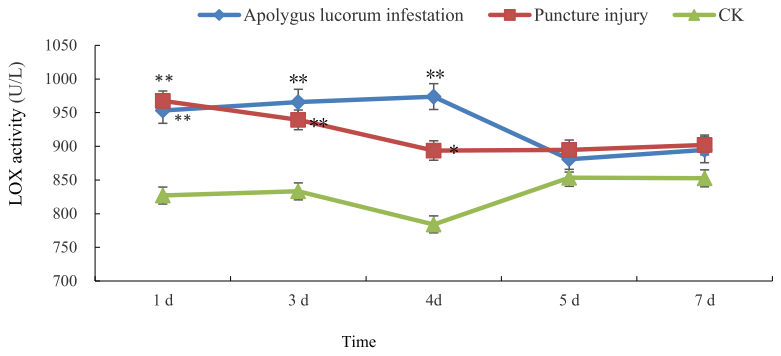

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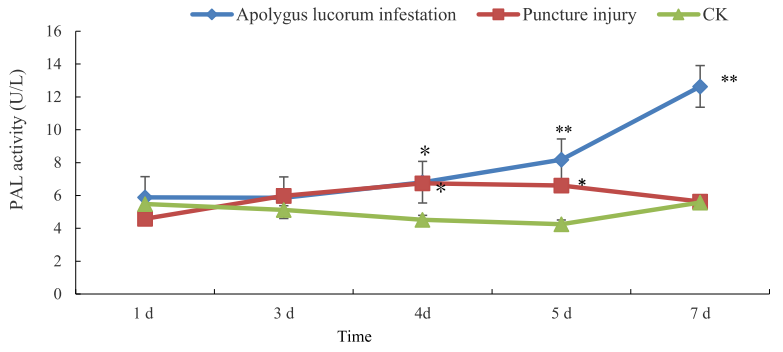

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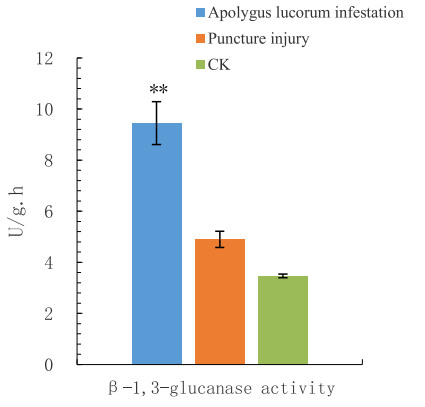

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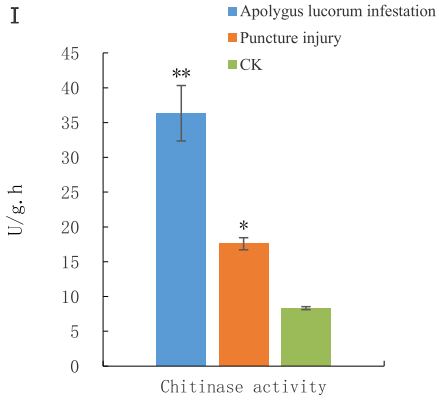

different protective mechanisms are activated in response to different stresses. Moreover, changes in enzyme activities were closely related to the duration of external stimuli.

After *A. lucorum* infestation or physical injury damaged Cabernet Sauvignon leaves, the activities of enzymes involved in the production of secondary metabolites, AOS, LOX, and PAL, in the top leaf tissues changed with treatment time (Fig. 4E–G). Compared with CK, leaf activity of AOS (Fig. 4E) and LOX (Fig. 4F) increased significantly ($P < 0.01$) during early stages of *A. lucorum* infestation and puncture injury. Leaf AOS activity began to decrease, and there was no significant difference between *A. lucorum* infestation and CK on the 5th day and between puncture injury and CK on the 4th day. Leaf LOX activity was not different between *A. lucorum* infestation and CK after 4 d. Leaf PAL activity (Fig. 4G) in the two treatments was significantly different from that in the control on the 4th day ($P < 0.05$). Subsequently, PAL activity increased significantly ($P < 0.01$) under the stimulation of *A. lucorum* infestation, whereas it decreased with puncture injury until there was no significant difference between treatment and control on the 7th day. These results suggested that stresses also activated defensive metabolic pathways and that changes in activities of those enzymes were closely related to duration of *A. lucorum* infestation and puncture injury.

Fig. 4H–I shows the β−1,3-glucanase and chitinase activities in the leaves of Cabernet Sauvignon, which were significantly increased after A. lucorum infestation. Puncture injury showed a significant increase in chitinase activity with no significant changes in β−1,3-glucanase activity as compared with that in the control.

3.5. Physiological and biochemical responses

Under the stress of *A. lucorum* infestation, trypsin inhibitor (Fig. 5A) and chymotrypsin inhibitor (Fig. 5B) activities in Cabernet Sauvignon leaves increased significantly compared with those activities in CK ($P < 0.05$). After 7 d of continuous infestation, the increase in trypsin inhibitor activity was significant compared with CK ($P < 0.01$). After 4 d of continuous infestation, the increase in chymotrypsin inhibitor activity was significant compared with CK ($P < 0.01$). After puncture injury, trypsin inhibitor activity was significantly higher than that in CK only on the 3rd day ($P < 0.05$), whereas chymotrypsin inhibitor activity was

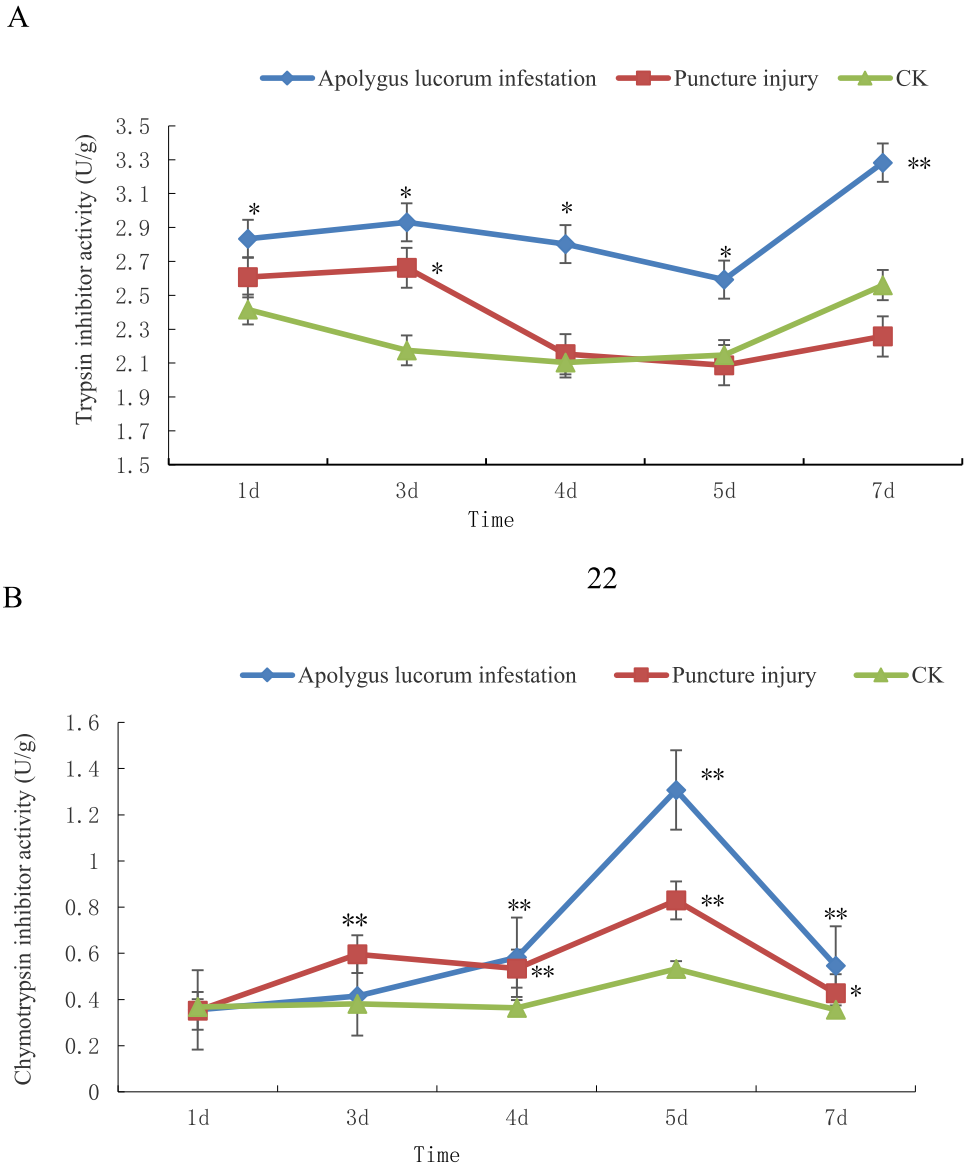

Fig. 5. Changes in activity of (A) trypsin inhibitor and (B) chymotrypsin inhibitor after *Apolygus lucorum* infestation and puncture injury. *Apolygus lucorum* infestation resulted in a significant increase in the activity of protease inhibitors compared with puncture injury. *$P < 0.05$ and **$P < 0.01$, compared with the control (CK) for each length of treatment time.

significantly higher than that in CK from the 3rd day ($P < 0.01$ (3 d, 4 d, 5 d) and $P < 0.05$ (7 d)). The different responses indicated that plants could distinguish between different external stresses (Fig. 5).

Infestation by *A. lucorum* led to significant increases in contents of flavonoids, tannins, and total phenols in Cabernet Sauvignon leaves (Fig. 6A-C). Flavonoids (Fig. 6A) and tannins (Fig. 6B) increased significantly after 1 d of infestation ($P < 0.05$), and on the 5th and 7th days, the increases in both were significant compared with CK ($P < 0.01$). There was a significant increase in total phenols (Fig. 6C) on the 4th day after *A. lucorum* infestation ($P < 0.05$). The increase was significant on the 5th and 7th days ($P < 0.01$). The content of malondialdehyde (MDA) (Fig. 6D) was significantly higher than that in CK after 4 d of *A. lucorum* infestation ($P < 0.01$), but it decreased thereafter until there was no significant difference on day 7. Compared with *A. lucorum* infestation, puncture injury caused similar but also different effects on the secondary metabolites in Cabernet Sauvignon leaves. The contents of flavonoids and tannins were also significantly higher than those in CK on day 1 ($P < 0.05$, Fig. 6A–B). However, with increases in infestation duration, flavonoid content decreased, whereas tannin content increased and was significantly higher than that in CK on the 3rd, 4th, and 7th days ($P < 0.01$). Total phenol content decreased significantly after 1 d of puncture injury ($P < 0.05$), whereas the content on the 4th and 5th days was significantly higher than that in CK ($P < 0.05$). Total phenols then decreased to the level in CK thereafter (Fig. 6C), which was significantly different from that in the *A. lucorum* infestation treatment. Contents of flavonoids, tannins, and total phenols were also significantly different between the two treatments at different treatment times. With *A. lucorum* infestation, flavonoids, tannins, and total phenols in grape leaves continuously accumulated, and the accumulation accelerated significantly after the 4th day. Puncture injury showed a similar pattern of change in the early stage, whereas stabilized accumulation occurred in the later stage. Thus, with extended treatment time, *A. lucorum* infestation led to continuous accumulation of secondary metabolites in Cabernet Sauvignon young leaves, indicating that changes in their contents were closely related to the duration of stimuli, i.e., the severity of the damage. In the later stage, contents of secondary metabolites induced by puncture injury decreased compared with those induced by *A. lucorum* infestation. These results indicated that plants could distinguish biological stress from physical stress and thus biosynthesized toxic secondary metabolites that could harm the insects causing damage.

Infestation by *A. lucorum* resulted in an overall decrease in leaf total sugar content (Fig. 7A), proteins (Fig. 7B), and chlorophyll (Fig. 7C). Compared with CK, infestation by *A. lucorum* on the 1st day caused a slight increase in total sugar content, but then, total sugar content decreased significantly on the 3rd day ($P < 0.05$), followed significant decreases on the 4th, 5th, and 7th days ($P < 0.01$). Protein content was also significantly different from that in CK after the 4th day ($P < 0.05$). Chlorophyll content was significantly lower ($P < 0.05$) than that in CK after the 3rd day and remained at a low level. Compared with CK, puncture injury caused a significant decrease in total sugars on the 4th day only ($P < 0.05$). There was no difference in protein content at each time point compared with that in CK. With puncture injury, chlorophyll content was only significantly different from that in CK and the *A. lucorum* infestation treatment on the 7th day ($P < 0.05$ and $P < 0.01$, respectively). These results suggested that compared with *A. lucorum* infestation, physical injury did not cause plants to aggressively change their nutrient levels. The content of free amino acids in leaves increased significantly after *A. lucorum* infestation (Fig. 7D). This result indicated that *A. lucorum* infestation significantly stimulated leaf protease activity and accelerated protein decomposition, thereby increasing free amino acids. The findings of Zhu et al. (2014) are similar.

4. Discussion

Apolygus lucorum prefers to feed on young leaves, shoots, flowers, and young fruits of fruit trees and crops. Because those parts are the most vulnerable, damage leads to reduced yields and quality of crops (Zhao, 2014). We previously found that damage to Cabernet Sauvignon caused by *A. lucorum* was closely related to the duration of continuous infestation, and not to insect age (unpublished observations). The nymph stage of *A. lucorum* is short and varies from 1 to 2 d to 3 to 4 d in different generations, whereas adult survival time is relatively long and can exceed 40 d. Therefore, to avoid the uncontrollable effects of egg deposition by adults during the experiment, 3rd instar nymphs of *A. lucorum* were used in this study.

The severity of *A. lucorum* damage was closely related to the duration of continuous piercing and sucking on young grape leaves. Growth of Cabernet Sauvignon plantlets was not significantly affected by continuous infestation of *A. lucorum* for 4 d or less. However, after continuous infestation for more than 4 d, leaves gradually became severely damaged, and significant differences in plant height and leaf width were observed. Moreover, with longer infestation durations, *A. lucorum* mortality gradually increased, and the average survival time of individuals greatly decreased. The largest increase in mortality rate occurred when *A. lucorum* fed on leaves continuously for 4 d, exceeding 50%. The average survival time of *A. lucorum* feeding on Cabernet Sauvignon was 4.79 d, and all pests died before day 8.

Plants do not continuously express defensive substances when experiencing environmental stress but constantly adjust and change their defensive strategies according to the degree of injury and type of stress (Zhang et al., 2017). Our study observations reveal that early pest infestation in Cabernet Sauvignon did not impede the growth and development of the plant. Later, under the persistent stress of *A. lucorum* infestation, plants began to produce toxic secondary metabolites, which eventually accumulated in *A. lucorum* accelerating their death.

When plants are subjected to pests, diseases, or other stresses, they often adapt to the environment by inducing or inhibiting the expression of enzymes, including SOD, CAT, POD, and PPO (Bruinsma et al., 2010; Li et al., 1998; Liu et al., 2005; Yue et al., 2003). External environmental stresses usually cause oxidative damages in plants, and these enzymes function in a series of antioxidant reactions to remove active oxygen species. In this study, under the stress of *A. lucorum* infestation or puncture injury, the activities of the four defense enzymes in young leaves of Cabernet Sauvignon all increased to varying degrees. Compared with *A. lucorum* infestation, the resistance induced by puncture injury was slower, and the magnitude of induction was lower. In addition to antioxidant enzymes, plants also have enzymes, such as AOS, LOX, and PAL, which are involved in the formation of secondary metabolites (Bruinsma et al., 2010; Liu et al., 2005) and thus are key enzymes that control substance metabolism. The activities of AOS and LOX increased rapidly in the early stage. LOX is actively involved in the synthesis of jasmonic acid and serves as an enzymatic marker of the jasmonic acid-mediated defense signaling pathway (Westwood and Stevens, 2010). In addition, LOX can induce the production of volatile substances, which might have a defensive role against *A. lucorum*.

The glucan pathway is thought to be closely related to the induction of resistance in plants (Xie et al., 2015). The peritrophic membrane of insects contains chitin, and degradation of chitin by chitinase can adversely affect the digestion of insects. Production of chintinase in plants induced by insect infestation suggests that chitinase also plays an important role in the anti-insect response (Gong et al., 2019). Our data reveal that *A. lucorum* infestation caused a significant increase in the β−1,3-glucanase and chitinase activities in the leaves of Cabernet Sauvignon. Puncture injury, however, only elevated chitinase activity, suggesting that insect infestation and physical injury elicit different responses through different resistance mechanisms.

Secondary metabolites are important substances in plant resistance to insects, and their contents are continuously adjusted according to changes in the environment to help plants grow and develop (Bouwmeester et al., 2003; Tuominen and Salminen, 2017). Plant secondary metabolites can affect insect growth and reproduction, and many studies show that small molecules such as flavonoids, tannins, and phenols are

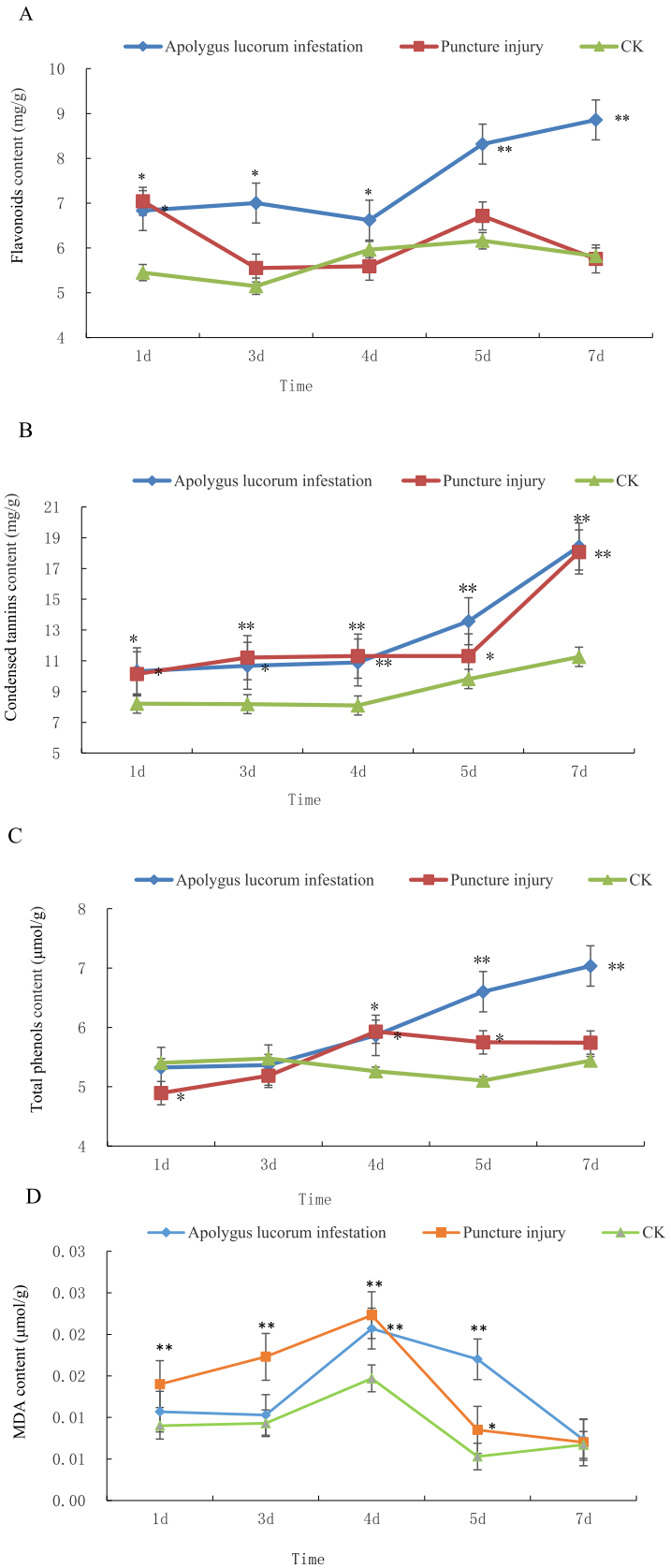

Fig. 6. Changes in secondary metabolite contents after *Apolygus lucorum* infestation and puncture injury. Mass fractions of (A) flavonoids, (B) condensed tannins, (C) total phenols, and (D) malondialdehyde (MDA). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control (CK) for each length of treatment time.

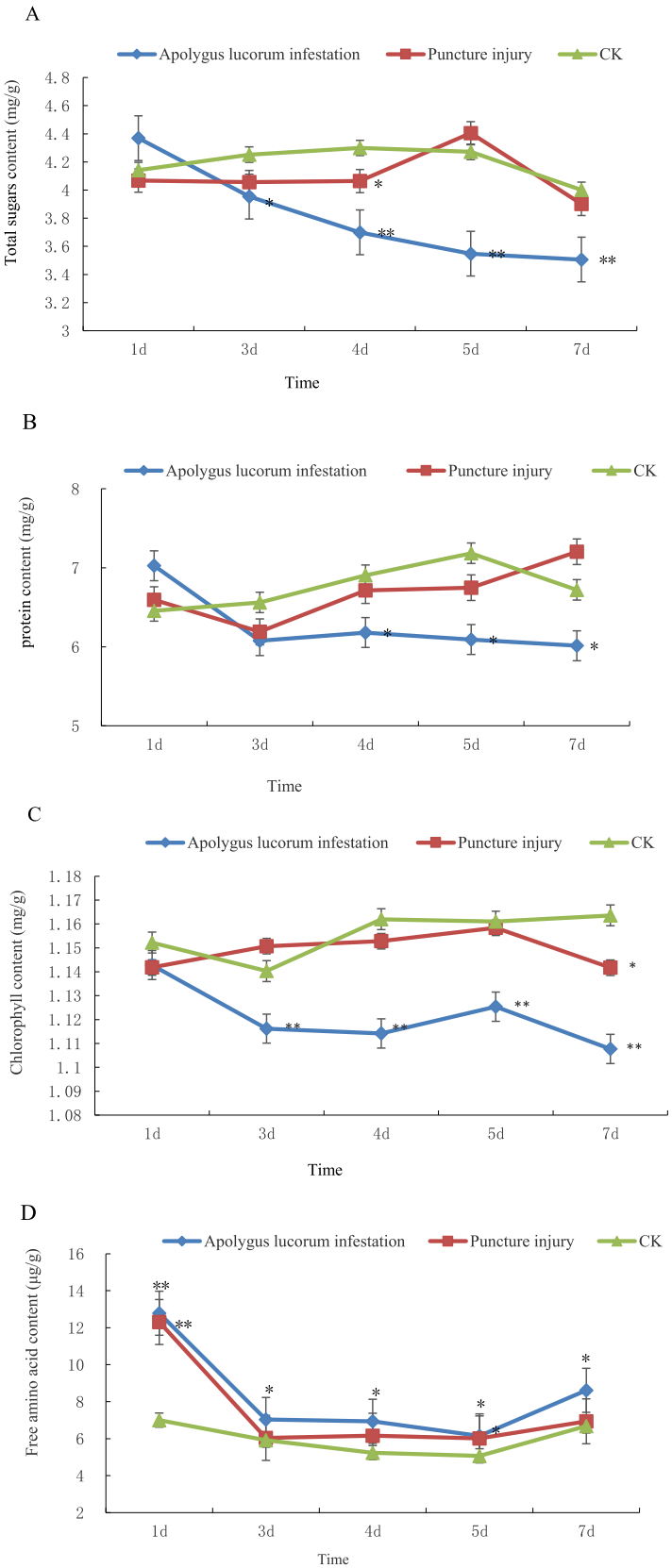

Fig. 7. Changes in nutrient and chlorophyll contents after *Apolygus lucorum* infestation and puncture injury. (A) Total sugars and (B) protein contents slightly decreased, whereas (C) chlorophyll content decreased significantly after *A. lucorum* infestation. (D) Free amino acid content increased after *A. lucorum* infestation. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control (CK) for each length of treatment time.

key players in plant resistance to insects (Koricheva, 2002; Moctezuma et al., 2014; Shi et al., 2014). The secondary metabolites are produced by the plant primarily through the phenylpropanoid pathway. The key regulatory enzymes involved in the phenylpropanoid pathway include phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) (Ganapathy et al., 2016; Koes et al., 2005; Vogt, 2010; Winkel-Shirley, 2001). The enzymes PAL and TAL catalyze analogous reactions, whereby PAL acts on the substrate L-phenylalanine to convert it into trans-cinnamic acid (Koukol and Conn, 1961), while TAL catalyzes the conversion of L-tyrosine to p-coumaric acid (Neish, 1961). The activation of the phenylpropanoid pathway is both PAL- and TAL-dependent, which eventually leads to the generation of numerous downstream phenolic metabolites of the phenylpropanoid pathway, such as phenolics or salicylates, tannins, flavonoids, coumarins, lignins, and anthocyanins (Dixon et al., 2002; Dixon and Paiva, 1995; Jones, 1984; Morrison and Buxton, 1993). Changes in PAL and TAL enzymatic activities in plant tissues in response to various physical and chemical-based stimuli have been documented earlier (Jones, 1984; Ju et al., 1995; Schmidt et al., 2004).

Our data revealed statistically significant changes in PAL starting at day 4 after the introduction of physical stimuli (Fig. 4G), while changes in flavonoid content (Fig. 6A) were evident as early as day 1. This leads us to conclude that this early onset of changes in flavonoid content as observed in Fig. 6A may be elicited by TAL instead of PAL. Even though TAL enzyme dynamics has not been a part of this study, our group plans to explore this interesting enzyme candidate in future studies.

Our data showed that *A. lucorum* infestation led to activation of the plant defense system that elicited the accumulation of flavonoids, tannins, and total phenols. With the increase in secondary metabolites in grape leaves, *A. lucorum* mortality increased significantly after 4 d (Fig. 3). Enzymatic reactions induced by *A. lucorum* were similar to yet significantly different from those induced by puncture injury. Therefore, saliva of *A. lucorum* might have been an extra stimulus in biochemical reactions in Cabernet Sauvignon leaves. Thus, as *A. lucorum* infestation duration increased, plant exposure to salivary secretions also increased, which may have mediated an increase in the activity of enzymes supporting plant resistance.

Substances with trypsin inhibitor and chymotrypsin inhibitor activities are important anti-nutritional factors in plants that can also inhibit digestive enzyme functions in herbivores and indirectly alleviate their damage by limiting growth and development (Kuwar et al., 2019; Zong et al., 2003). Both *A. lucorum* infestation and puncture injury increased such activities, although the pattern of increase was different. Infestation by *A. lucorum* caused a rapid and continuous increase in trypsin inhibitor- and chymotrypsin inhibitor-like activities, whereas puncture injury mainly caused an increase in chymotrypsin inhibitor-like activity. These results indicated again that plants could distinguish between types of damage and respond accordingly. The intensity of changes was also closely related to duration of the stress, consistent with the results reported previously by Shi et al. (2017).

Nutritional relations are the most important relations between herbivorous insects and plants. Plants respond to defense signal molecules and transduce the signals to increase the expression of resistance genes while also downregulating the expression of genes that control nutrient synthesis to affect the selection and feeding behaviors of insects (Gao et al., 2012; George et al., 2011). However, reductions in nutrient synthesis also affect plant growth and may explain why varieties with higher resistance usually have lower yield (Lauder et al., 2019; Zhu et al., 2005). In this study, *A. lucorum* infestation caused a decrease in total sugar and protein contents in Cabernet Sauvignon leaves. There are two possible explanations for these results. First, injury negatively affected carbohydrate and nitrogen metabolism, preventing the normal synthesis of sugar and protein. Second, Cabernet Sauvignon actively reduced its fitness to prevent the continuous infestation of *A. lucorum*. Puncture injury did not significantly affect nutrient contents in Cabernet Sauvignon leaves. This difference between *A. lucorum* infestation and physical injury further strengthened the conclusion that plants could distinguish between stresses and respond accordingly and economically. In this study, *A. lucorum* infestation significantly decreased chlorophyll content in Cabernet Sauvignon leaves, which might lead to a decline in photosynthesis and decrease in productivity. In this study, the effects of increasing lengths of *A. lucorum* infestation duration on *Vitis vinifera* L. 'Cabernet Sauvignon' were evaluated by assessing the physical appearance and growth, defense enzyme activities, production of secondary metabolites, and nutritional contents of the plant. In addition, the effect of induced resistance in Cabernet Sauvignon on *A. lucorum* survival and mortality was examined. Our study observations reveal that early pest infestation in Cabernet Sauvignon did not impede the growth and development of the plant; however, the expression of antioxidant enzymes and volatile repellent substances were observed. In the later stage, under the stress of long-term *A. lucorum* infestation, Cabernet Sauvignon began to produce toxic secondary metabolites, which accelerated the death of *A. lucorum* but also caused declines in growth and even serious damage to the plant itself. The results also showed that plants could accurately distinguish between different types of stress and respond accordingly.

The underlying molecular mechanisms supporting the plant resistance have not been examined in this study. However, our research group plans to screen for key genes that are related to plant resistance and determine their biological functions and regulatory mechanisms in the near future. Among the above-mentioned metabolic pathways of defense enzymes and secondary metabolites, the functional genes that are excellent candidates for evaluation of the development of plant resistance include those encoding the following proteins: mitogen-activated protein kinase kinase kinases (MEKK), WRKY transcription factor, ethylene response factor 1 (ERF1), jasmonate zim-domain (JAZ), MYC2 transcription factor, calmodulin-binding proteins (CaMBP), calcium-dependent protein kinase (CPK), protein kinase AVRPPHB susceptible1 (PBS1), respiratory burst oxidase homolog (RBOH), calmodulin-like proteins (CML), enhanced disease susceptibility 1 (EDS1), protein phosphatase type 2C (PP2C), SNF1-related protein kinases type 2 (SNRK2), pyrabactin resistance 1-like (PYL), xyloglucan endotransglucosylase/hydrolase (XTH), and 1-aminocyclopropane-1-carboxylic acid synthases (ACS).

5. Conclusions

The long-term *A. lucorum* pest infestation in *Vitis vinifera* L. elicited a defense response that involved reductions in its total sugar, protein, and chlorophyll contents, with a simultaneous increase in trypsin and chymotrypsin inhibitors, tannins, total phenols, and other secondary metabolites. Our data provide evidence of the ability of the plant's defense system to differentiate between pest-based infestation and general puncture wounds. Increased plant resistance against *A. lucorum* eventually led to an increase in mortality and decrease in survival time of the herbivore pest. The results presented in this study provide the knowledge base for the potential development of a novel, effective, innovative, and environmentally friendly pest control method against *A. lucorum*.

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Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.110985.

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