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Biochemical prerequisites for resistance to biotic stress in apple (*Malus domestica* Borkh.)

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Abstract

The current study aimed to investigate the effect of an exclusion net and aphid infestation on some biochemical defense parameters of several apple varieties. The experiment was set up in 2021-2022 in the Agricultural University – Plovdiv, Bulgaria. During the first experimental year, the influence of exclusion net on phenolic content, DPPH radical scavenging, and salicylic acid in Super Chief, Fujion, Pinova, and Enterprise varieties were investigated. During the second experimental year, the activity of the enzymes guaiacol peroxidase, syringaldazine peroxidase, phenylalanine ammonia-lyase, and the content of salicylic acid in infested and uninfested leaves of Gemini and Modi varieties outside the exclusion net was compared. The results of the screening of Super Chief, Fujion, Pinova, and Enterprise varieties for the content of phenolic compounds and salicylic acid showed that all the varieties grown in the open field demonstrated higher content of gallic acid and salicylic acid, which clearly shows the response of plants to stressful conditions. Significantly increased values of phenylalanine ammonia-lyase, guaiacol peroxidase, and syringaldazine peroxidase in pest-infested leaves of Modi cultivar compared to non-infested leaves of the same cultivar were observed. In contrast, an increase in the activity only of guaiacol peroxidase, but not the other two enzymes, was observed in the Gemini variety. The increased activity of the peroxidase enzymes correlates with the lower content of gallic acid and quercetin (one of the substrates for the action of the enzymes) in the infested leaves.

Keywords: aphid, apple, phenols, peroxidase, phenylalanine ammonia-lyase, salicylic acid

INTRODUCTION

Apple (*Malus domestica* Borkh.) is Bulgaria's traditionally grown fruit tree. The total production for 2022 was about 46.4 million kg. It is used for fresh consumption and processing. To protect the fruit orchards from pests and diseases and ensure high commercial quality, farmers must provide multiple pesticide applications (Sauphanor et al., 2012). Pesticides have a significant effect on the environment. On the other hand, they are not always effective against exotic invasive species. These disadvantages suggest that alternative control methods are required.

Nowadays, different kinds of nets are widely used in crop production as mechanical barriers against abiotic and biotic factors that reduce crop productivity and fruit quality (Sivakumar and Jifon, 2018). Using nets also reduces the application of agrochemicals, making them an environmentally friendly alternative to chemical pesticides (Briassoulis et al., 2007). The application of exclusion nets is a non-chemical alternative to chemical protection. Netting has gained popularity in pome fruit orchards worldwide, with several designs to protect from adverse weather conditions, sunburn, and insect pests. According to many authors, exclusion nets are more effective than chemicals on some pests. Several studies declare that covering orchards with net is very effective against key pests on apple like codling moth (*Cydia pomonella* (L.); Lepidoptera: Tortricidae) (Alaphilippe et al., 2016; Candian et al., 2020; Marshall and Beers, 2021).

Enclosing orchards in netting is being evaluated as a pest control method, but these enclosures may also serve as an exclusion cage to natural enemies, thereby disrupting biological control (Marshall & Beers, 2022).

According to many researchers, nets reduce the intensity of solar radiation on the tree, decreasing the level of evapotranspiration (Manja & Aoun, 2019). This leads to lower plant water stress symptoms, higher photosynthesis, a more significant yield, and bigger fruit size (Amarante et al., 2010; Treder et al., 2016).

It is observed that some enzymes and secondary metabolites are involved in the plant's defense against pests (Cai et al., 2004; Xu et al., 2021). According to many authors, peroxidase (POD), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) act as significant biochemical markers in pest-resistant plants (Han et al. 2009; Sha et al. 2015). Phenylalanine ammonia-lyase activity is correlated with lignin, cellulose, and hemicellulose contents (Morrison and Buxton 1993), which could strengthen the mechanical barrier (Xu et al., 2021). Phenylalanine ammonia-lyases (PALs) are involved in resistance to plant pathogens, but the mechanism by which they contribute to plant immunity is unclear (Wang et al., 2019). PALs catalyze the deamination of L-phenylalanine to form trans-cinnamic acid, the first step in the phenylpropanoid pathway (Dixon et al., 2002). Transcinnamic acid is a precursor for the biosynthesis of various phenylpropanoid compounds, such as the cell wall component lignin and the defense-related phytohormone salicylic acid (SA), as well as the flavonoid phytoalexins naringenin and sakuranetin (Duan et al., 2014). Many phenylpropanoid compounds show broad-spectrum antimicrobial activity and are involved in plant defense against pests.

Flavonoids are widespread in the plant kingdom and form a group of plant secondary products. These compounds exhibit several physiological functions in response to environmental factors. As is often argued, flavonoids exhibit different biological activities to the plants that produce these compounds and animals that ingest flavonoids in their diet (Kazuki et al., 2013).

The antiradical activity index, expressed as % inhibition of the DPPH radical, gives an idea of the potential of plants to deal with radicals generated under stressful conditions. This potential is due to an extensive range of compounds such as phenols, flavonoids, vitamins, etc., which are present basally or are synthesized de novo in the affected plants.

MATERIALS AND METHODS

The study is performed in the period 2022-2023 in young apple orchard at the experimental field of the Agricultural University – Plovdiv. The varieties selected for analysis were Super Chief – a well-known old variety, and the modern Fujion, Pinova, Enterprise, Gemini, and Modi, recently introduced in Bulgaria. The trees were planted in four rows, according to a scheme of 4x2 m or at a density of 1250 pcs/ha with a supporting structure and drip irrigation. Of the rows, two were with exclusion net, and two adjacent to them, without exclusion net (in the open). Each apple variety was presented with four replications with six to eight plants each. Most of the plants (except Enterprise) are resistant to Scab (according to the producer). All the tested varieties have thick skin (except Modi).

Biochemical analyzes

The leaves for the biochemical analyzes were collected from shoots of chosen varieties based on the results of preliminary observation on the rate of infestation by aphids.

Enzyme extraction

The fresh plant sample (0.5 g) was ground with 5 ml ice-cold extraction buffer (pH 7.8), quartz sand, and 200 mg polyvinylpyrrolidone (PVP). Samples were centrifuged at 4°C for 10 min at 13500 rpm. The resulting clear supernatant was immediately used for analysis.

Guaiacol peroxidase

According to Bergmeyer et al. (1974), Guaiacol peroxidase activity was determined spectrophotometrically. Absorbance was measured at 436 nm against a blank. The results are expressed as IU mg/g fresh weight (FW).

Syringaldazine peroxidase

The activity of SPOD was determined spectrophotometrically by the method of Imberty et al. (1985). The absorbance was measured at 530 nm against a blank. The values obtained were expressed as IU mg/g FW.

Analyses of polyphenols, flavonoids, and DPPH

Plant material and extraction method

One gram of ground leaves and 10 ml of 70% acidic methanol were used to extract phenolic and flavonoid compounds. Each variant was placed in a capped tube for the duration of the extraction. Each variant was performed in 4 replicates. The extraction lasted for 24 hours at room temperature. After extraction, the samples were filtered, and the filtrates were used for analysis.

Polyphenols

Quantitative determination of total phenols was performed using the method of Singleton & Rossi (1965) with minor modifications (Koleva-Valkova et al., 2017). The absorbance was recorded spectrophotometrically at 760 nm. The quantity of total phenols was calculated from a standard gallic acid curve with the formula y = 1.421x + 0.0074 (R2 = 0.9997). It was expressed as mg of gallic acid equivalents per gram of fresh weight (Singleton & Rossi, 1965).

Total flavonoids

Total flavonoids were determined using the method described by Zhishen et al. (1999). The absorbance was measured spectrophotometrically at a wavelength of 510 nm. The total amount of flavonoids was calculated from a standard quercetin curve with the formula y = 0.0776x + 0.0244 (R2 = 0.9963) and was expressed as mg of quercetin equivalents per gram of fresh weight (Zhishen et al., 1999).

DPPH

DPPH reagent was used to determine the antiradical activity of plant extracts using the method of Brand-Williams et al. (1995), with minor changes (Koleva-Valkova et al., 2017). Absorption was recorded after 10 minutes of incubation at 517 nm against absolute alcohol. The DPPH radical scavenging activity of the sample was expressed as mg Trolox equivalent antioxidant capacity (TEAC) by the formula obtained from the standard curve (y = -0.1954x + 0.708, R2 = 0.9858).

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase activity is determined spectrophotometrically by following the formation of trans-cinnamic acid, which exhibits an increase in absorbance at 290 nm, according to Brueske (1980). The absorbance was recorded at 290 nm, and PAL activity was measured in terms of the amount of trans-cinnamic acid (t-CA) formed.

Salicylic acid

The content of salicylic acid in the leaves of infested plants was determined according to Warrier et al. (2013) using 1 g of fresh plant material. The sample extraction was made using distilled water followed by centrifuge at 10 000 rpm for 10 min, and 100 µl of the supernatant was mixed with 0.1% freshly prepared ferric chloride. The volume of the reaction mixture was made up to 3.0 ml, and the complex formed between Fe^{3+} ion and SA, which is violet in color, was determined by spectrophotometry, measuring the absorbance of the complex in the visible region (at 540 nm). The results were calculated using a standard curve.

Statistical analysis

The results were statistically processed with the SPSS program using a one-way ANOVA dispersion analysis and Duncan's comparative method, with the validity of the differences determined at a 95% significance level. After the mean value, the different letters (a, b, c, d) show statistically significant differences between the variants.

RESULTS AND DISCUSSION

During the first year of the experiment, the content of phenols, flavonoids, salicylic acid, and radical scavenging potential of three modern apple varieties and a traditional one grown in the open field and under an exclusion net, were analyzed. The content of polyphenols in leaves is presented in Figure 1. The results show that under the exclusion net, the content of polyphenols in the leaves of all the tested variants decreases compared to the uncovered plants. In the trees grown without a net, the highest phenol content is observed in the leaves of the Pinova variety, followed by Enterprise. Super Chief and Fujion varieties demonstrate lower values. Under the exclusion net, the value decreases by 8%, 12%, 19%, and 7%, respectively, compared to the uncovered ones.

The salicylic acid (SA) content is presented in Figure 2. The results show that in all the tested varieties, the content of SA is lower under the net than that of the uncovered trees. In Super Chief, the content reduction under the net is the greatest. In the leaves of the uncovered trees, salicylic acid is 4906 µg/g, and under the net – 1887 µg/g (62% lower). In Fujion, without a net, the value of SA is 5568 µg/g and under the net – 4500 µg/g (19% lower compared to the uncovered trees). In Pinova, in the open field, the value is 4394 µg/g and 2312 µg/g under the net (47% lower). Enterprise shows the highest values of SA without and with exclusion net (6012 µg/g and 5269 µg/g, respectively). The covered trees have a 12% lower content of SA in the leaves compared to those grown in open fields.

Results from the analysis of the DPPH radical scavenging potential are presented in Figure 3. The data show different responses in the tested varieties. In Super Chief, the value of the parameter increases by 12% under the net, compared to the uncovered tress.

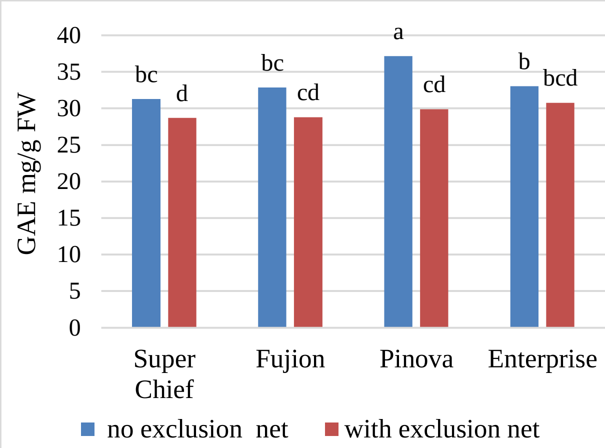

Figure 1. Content of polyphenols (GAE mg/g FW) in leaves of Super Chief, Fujion, Pinova, and Enterprise apple varieties

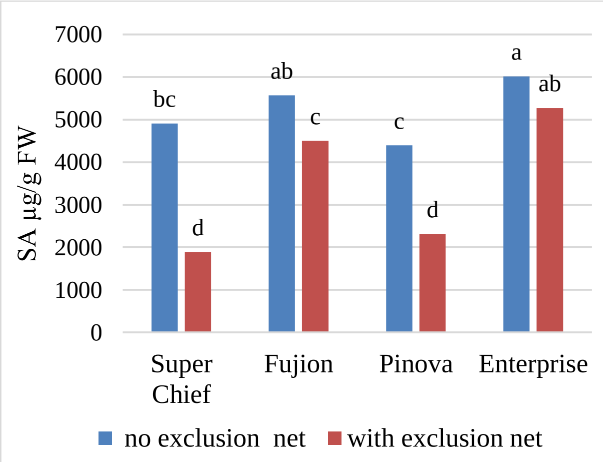

Figure 2. Content of salicylic acid in leaves of Super Chief, Fujion, Pinova, and Enterprise apple varieties

The scavenging activity decreased under the net in the leaves of the other tested varieties compared to open field conditions. In Fujion, the radical scavenging activity decreases by less than 1%. In Pinova, the decrease under the net is by 6%, and in Enterprise – by about 5%.

It was established that the number of aphid-infested shoots is higher under the exclusion net (data not presented) probably because there are almost no natural enemies (like birds and parasitoids) under the nets, and there is no chemical control in the experimental orchard. Regarding the preference of the aphids, the highest percentage of infested shoots was established on Gemini trees, and the lowest was on Modi (data not presented). Therefore, these varieties were selected to analyze some biochemical markers of plant resistance to biotic stress in the second experimental year.

In the open field, the phenol content is higher in the uninfested leaves of Gemini than in Modi (Figure 4). This result could be related to pest preference and the higher rate of damage and stress. There is no difference regarding the infested and uninfested leaves in Modi. This parameter is not affected by the aphid infestation.

Data about the contents of flavonoids and salicylic acids in leaves are presented in Figure 5 and Figure 6. It is seen that the contents of the parameters are higher in the uninfested leaves in both of the tested varieties compared to the infested ones. There are no statistically proven differences between the uninfested leaves of Gemini and Modi and between the infested leaves of both varieties.

In the study of Czerniewicz et al. (2017), *Sitobion avenae* infestation induced biosynthesis of different types of flavonoids within tissues of resistant triticale plants.

The compounds of the phenylpropanoid series play a significant biochemical role in the resistance of plants to various stresses. In plants, tolerance to biotic and abiotic stress is usually regulated by phenolic compounds, the main secondary metabolites synthesized by the phenylpropanoid pathway. Polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) are two important enzymes involved in the phenylpropanoid pathway and related to phenolic metabolism. These enzymes and antioxidant enzymes such as peroxidase (POX) are specifically regulated according to developmental stage and tissue (Amri et al., 2021).

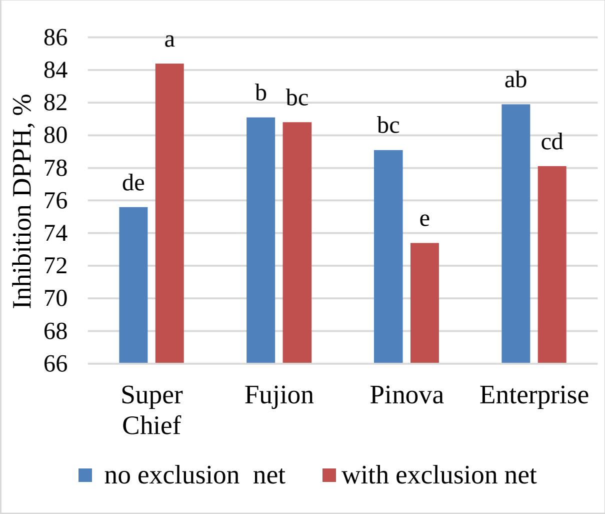

Figure 3. DPPH radical scavenging in leaves of Super Chief, Fujion, Pinova, and Enterprise apple varieties

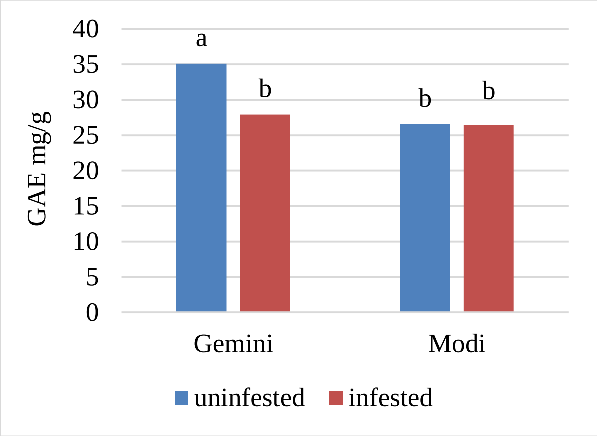

Figure 4. Content of polyphenols in infested and uninfested leaves of Gemini and Modi apple varieties in open field condition

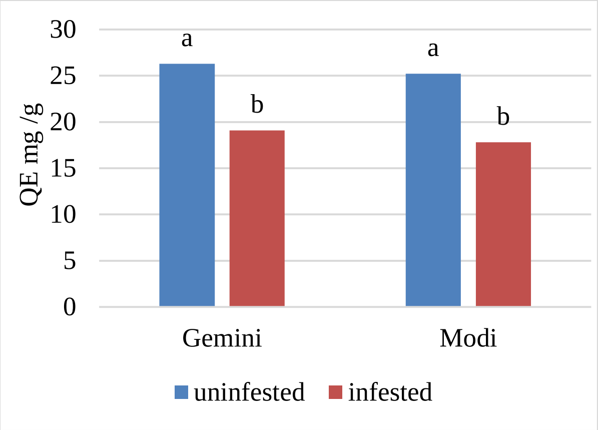

Figure 5. Content of flavonoids in leaves of Gemini and Modi apple varieties in open field condition

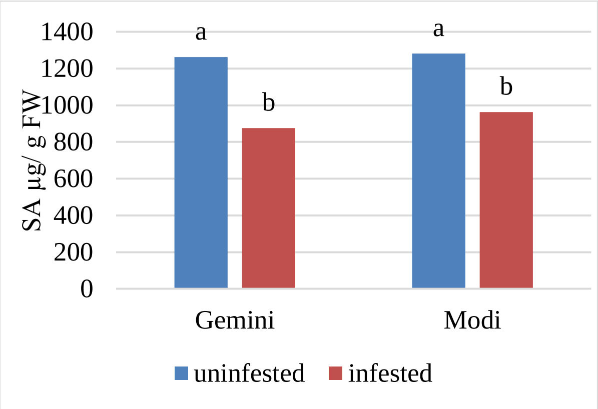

Figure 6. Salicylic acid content in leaves of Gemini and Modi apple varieties in open field condition

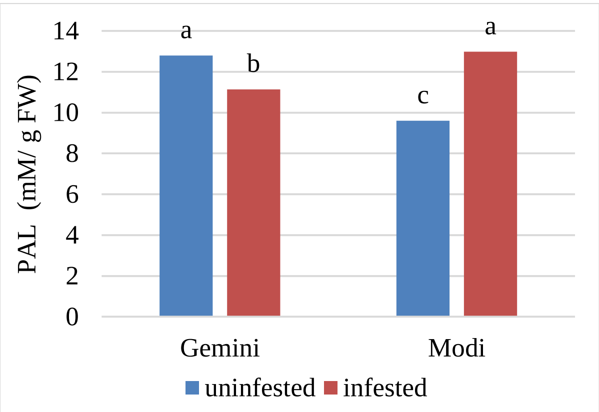

Figure 7. Activity of phenylalanine ammonia-lyase in leaves of Gemini and Modi apple varieties in open field condition

Figure 7. presents the activity of Phenylalanine ammonia-lyase in leaves of Gemini and Modi apple varieties. The enzyme activity in the uninfested leaves is higher in Gemini than in Modi. In contrast, the PAL activity in the infested leaves is higher in Modi than in Gemini. In Gemini, the enzyme activity decreases in the infested leaves compared to the uninfested, and in Modi, PAL activity increases when the leaves are infested with aphids. Activation of plant resistance mechanisms against the feeding of insect pests is an important phenomenon that protects host cells (Czerniewicz et al., 2017). Phenylalanine ammonia-lyase (PAL) is the key enzyme that catalyzes the deamination of L-phenylalanine to form trans-cinnamic acid, which is the first step in the phenylpropanoid pathway. This enzyme plays an essential role in the biosynthesis of phenolic compounds and phytoalexins. It is also responsible for inducing the synthesis of salicylic acid (SA), which causes systemic resistance in many plants. Many phenylpropanoid compounds show broad-spectrum antimicrobial activity and are involved in plant defense against pests (Wang et al., 2019). In a study with triticale, (Czerniewicz et al., 2017) observed an increased activity of PAL in a resistant cultivar compared to the susceptible one when the plant was attacked by the grain aphid (*Sitobion avenae* F.).

The activity of guaiacol peroxidase GPOD is presented in Figure 8. The enzyme activity is similar in the uninfested leaves in both of the tested cultivars. In the leaves with aphid colonies, in Modi, the GPOD activity is 23% higher than in Gemini. The increase of the activity in the aphid-infested leaves is stronger in Modi than in Gemini (187% and 221%, respectively).

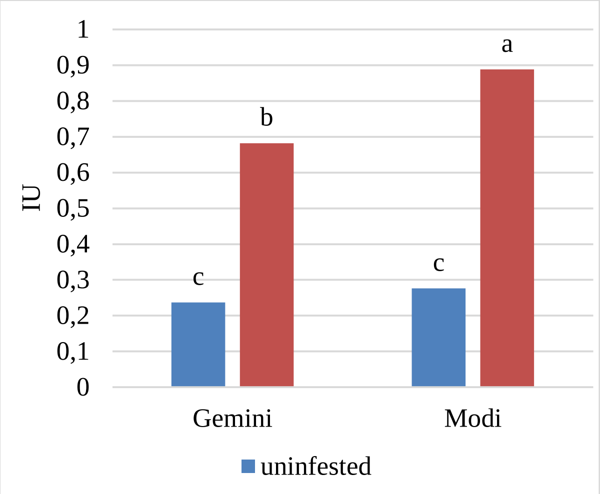

Figure 8. Activity of guaiacol peroxidase GPOD in leaves of Gemini and Modi apple variety in open field conditions

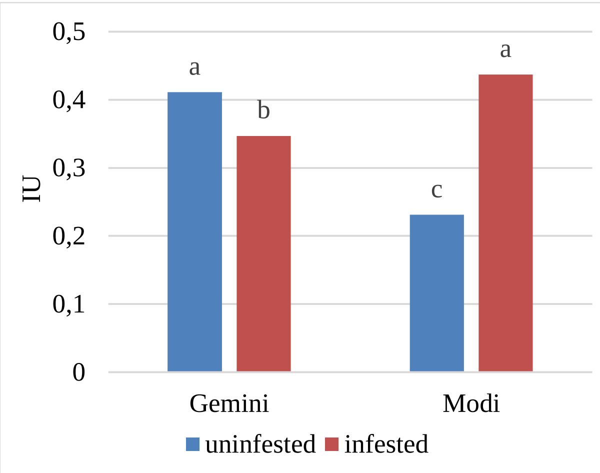

Figure 9. Activity of syringaldazine peroxidase (SPOD) in leaves of Gemini and Modi apple variety in open field conditions

The SPOD activity is presented in Figure 9. It is seen that Gemini and Modi respond differently to the aphid infestation. In Gemini, the enzyme's activity decreases in the infested leaves compared to the uninfested. In Modi, it is the opposite – the SPOD activity is enhanced by 47% in the aphid-infested plants compared to the uninfested. Compared to the infested leaves of Gemini, in Modi, the activity is 21% higher.

The increased PAL values in the infested plants of the Modi variety compared to the uninfested ones correlated with the increased activity of both investigated peroxidases, representing a defense reaction. At the same time, a decreased content of flavonoids and salicylic acid and no change in total phenolics was observed. It can be assumed that under the conditions of biotic stress, in the less affected variety Modi, the synthesized phenylpropanoid compounds are used in direct protection, as well as like substrates for the peroxidases (guaiacol and syringaldazine), which have a crucial role also in quenching the resulting oxidative stress.

On the other hand, the more heavily attacked cultivar Gemini showed lower activity of PAL and SPOD in infested compared to uninfested plants and lower levels of phenolics, flavonoids, and salicylic acid. An increase was observed only in GPOD, which indicates highly developed oxidative stress. These results are correlated with the degree of attack and plant damage. It is likely that the weaker enzyme activity, leading to lower levels of phenylpropanoid compounds, is the reason for the weaker defense and is, therefore, a predisposing factor for the increased attack.

CONCLUSIONS

The current experiment investigated the antioxidant defense system of different apple varieties in an experimental orchard. It was established that the value of antioxidative compounds decreases when the plants are grown under an exclusion net. This could be associated with lower stress levels in the case of covered trees. It was also observed that there is a difference between the varieties regarding the antioxidant defense system as a response to aphid infestation. Modi variety demonstrates activation of the antioxidant defense system after aphid infestation. In the attacked leaves, higher GPOD, SPOD, and PAL activities were measured. On the other hand, in Gemini, the peroxidases and PAL decreased their activity in the infested leaves compared to the uninfested. These responses could be used as markers of pest resistance in apple varieties.

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