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Morphological and Biochemical Evaluation of Resistance in Some Vine Rootstocks to *Cicadatra Ochreata* Melichar

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ABSTRACT

Cicadas (*Cicadatra ochreata* Melichar) are prevalent in regions such as Iran, Afghanistan, southern Russia, Turkey, and Iraq. These pests stunt grapevine growth and dry out temperate fruit trees, posing significant agricultural challenges. This study aimed to evaluate the morphological and biochemical resistance of four grapevine rootstocks to Cicada infestation. The rootstocks assessed were Spoota, Nazemiyeh, Kober 5BB, and CH1. The experiment was conducted using a randomized complete block design with three replications, each containing three grapevine rootstocks per experimental unit. For each grapevine stem, five Cicada egg-laying branches were placed to allow nymphs to settle in the root zone after hatching. From the third year onward, the root zones of the grapevines were inspected in autumn for 2-3-year-old Cicada nymphs. Root samples were also collected to measure biochemical traits, including total phenolic compounds, flavonoid concentration, proline levels, and soluble sugar content. Among the rootstocks, Spoota exhibited the highest nymph establishment rate (3.3%), while CH1 showed the lowest rate. Notably, no nymph establishment was observed on Kober 5BB. None of the rootstocks experienced delays in germination or growth impairments. Biochemical analyses revealed that Kober 5BB had the highest total carbohydrate content (4.03 mg g^{-1}), while Nazemiyeh had the lowest. CH1 had the highest total phenol content (4.86 mg g^{-1}), whereas Nazemiyeh had the lowest (3.1 mg g^{-1}). Similarly, CH1 showed the highest total flavonoid concentration (1.67 mg g^{-1}), and Nazemiyeh exhibited the lowest (0.54 mg g^{-1}). No significant differences in proline content were observed among the rootstocks. Overall, the findings indicated that Kober 5BB was resistant to Cicadas, CH1 was highly tolerant, and both Nazemiyeh and Spoota were relatively tolerant.

Introduction

Iran is one of the world's leading producers of grapes and raisins, ranking behind countries such as China, Italy, the United States, Spain, France, Turkey, India, Argentina, and Chile. According to global statistics, grape production has shown a steady increase, with total production worldwide reaching 25.62 million tons in the 2021–2022 agricultural year, up from 24.54 million tons the previous year. Iran ranks eleventh globally in grape production, with an annual output of

approximately 1,990,000 tons (Anonymous, 2022). The grape Cicada pest (*Cicadatra ochreato* Melichar or *Psalmocharia alhageos*), belonging to the order Hemiptera and family Cicadidae, is a significant agricultural threat in Iran, Afghanistan, southern Russia, Turkey, and Iraq. This pest not only impacts grapevines but also damages other temperate fruit trees, including pomegranate, pistachio, quince, and walnuts, as well as nonproductive trees (Babaei, 1967). The primary damage caused by this pest stems from the long-term feeding of nymphs on the root sap of grapevines (Esmaeilli, 1991). Adult female Cicadas emerge from the soil after mating and lay eggs on one-year-old branches. Once hatched, the nymphs drop to the ground and burrow into the root zone. Over 4–5 years of feeding, the pest weakens the grapevines, significantly reducing their economic productivity and often causing them to dry out completely (Shekaryan and Rezvani, 2000). This damage has intensified in recent years due to drought stress in vineyards, rising temperatures, and reduced or infrequent irrigation, particularly in cicada-prone regions (Rasoli et al., 2023). (Fig. 1)

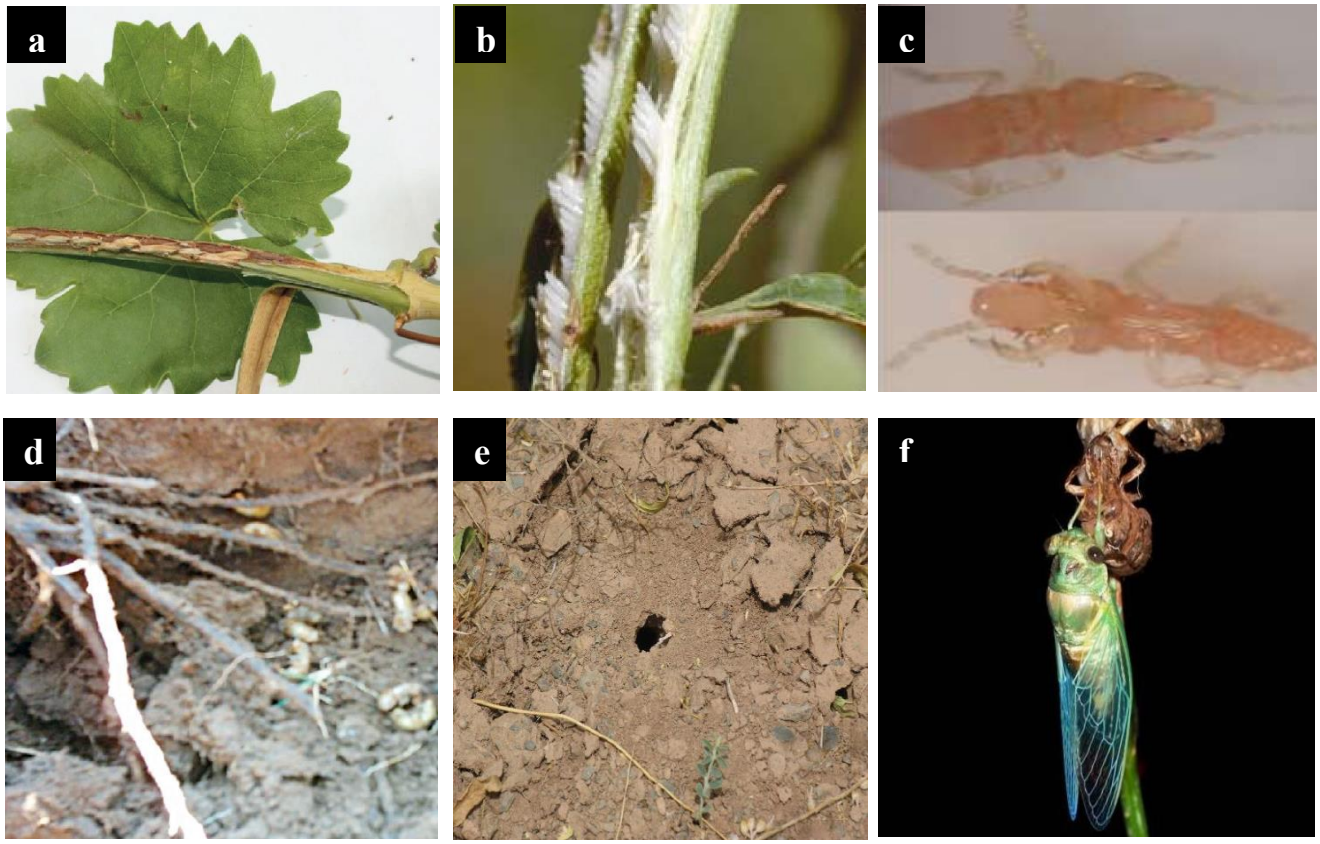

Fig. 1. Life cycle of and damage done by cicadas. a and b) egged-laying branch, c) freshly hatched nymphs, d) nymphs of different ages on the roots, e) place where the 5-year-old adult cicadas emerge from the soil, f) a view of the complete cicada pest insect after emerging from the pupal shell.

Currently, there is no practical and effective method to fully mitigate the damage caused by the grape Cicada pest. Chemical control strategies have achieved limited success (Valizadeh and Farahmand, 2009). However, certain cultural practices, such as summer pruning to remove egg-laying branches followed by their destruction, can partially reduce the pest population. One promising approach involves the use of kaolin clay, which forms a protective layer on plant surfaces, acting as a physical barrier against various pest insects. By disrupting visual and sensory stimuli, kaolin clay effectively deters pest attacks. In the United States, kaolin clay has been reported as a key inhibitor of pest oviposition in fruit orchards (Unruh et al., 2000; Knight et al., 2000; Glen et al., 1999). Similarly, its application has significantly reduced the oviposition of *Psalmocharis alhageos* in Iranian vineyards (Valizadeh et al., 2013). Although no studies have specifically examined the effectiveness of kaolin clay against periodic Cicadas, it is recommended as a low-risk

alternative for controlling Cicadas in nonproductive orchards (Pfeiffer et al., 2019).
The most reliable method for controlling grape Cicadas is the use of resistant rootstocks. Rootstocks resistant to other vineyard pests and diseases, such as grape crown gall (Mahmoodzadeh et al., 2004), phylloxera (Benheim et al., 2021), and nematodes (Ferris et al., 2012), have successfully demonstrated their effectiveness in pest management. Given that Iran is a center of diversity for grapevine varieties (Rasoli et al., 2015), it is plausible that cicada-resistant or tolerant rootstocks can be identified within this genetic pool. This research aimed to evaluate the morphological and biochemical resistance of selected grapevine rootstocks to the grape cicada pest.

Material and methods

Plant rootstocks

The rootstocks used in this study included Spoota, Nazemiyeh, Kober 5BB, and CH1 (Table 1). Spoota, Nazemiyeh, and CH1 were developed through grapevine breeding programs by Iranian researchers and were obtained from the Takestan Grapevine Research Station (Rasoli and Golmohammadi, 2008; Rahmani et al., 2023; Mahmoudzadeh, 2015). Kober 5BB, originally introduced to Iran from France, was supplied by the Temperate Fruit Research Center (TFRC) of the Horticultural Sciences Research Institute (HSRI).

Table 1. Characteristics of the investigated vine rootstocks and their pedigree.

Rootstocks	Pedigree	Special trait	Reference
Spoota	*V. vinifera* × *V. riparia*	Crown gall resistance	(Mahmoudzadeh, 2015)
Nazemiyeh	*V. vinifera* × *V. riparia*	Crown gall resistance	(Mahmoudzadeh, 2015)
Kober 5BB	*V. vinifera* × *V. riparia*	Resistance to phylloxera, nematodes, and drought stress	(Minio et al., 2022; Dodson and Andrew, 2017)
CH1	*V. vinifera*	Drought resistant, Tolerant to lime soil	(Rasoli and Golmohammadi, 2008; Rahmani et al., 2023)

Characteristics of the experimental location

All investigations were conducted at the Grape Research Station in Takestan, Iran, located at 36°3'2'' N and 49°40'51'' E, at an altitude of 1,250 meters above sea level. The region receives an average annual precipitation of 220 mm, with most rainfall occurring during autumn and winter. It is classified as a temperate zone, characterized by hot summers and cold winters. The maximum and minimum recorded temperatures are 42°C and -30°C, respectively. The area has an average annual relative humidity of 52% and an evaporation rate of 1,800 mm a year. Additionally, the region experiences an average of 65 frost days annually.

Experimental design

This study was conducted using a completely randomized block design with three replications, each consisting of three vine rootstocks per experimental unit. Initially, Cicada egg-laying branches were collected, and their ends were placed in water-filled plastic capsules to prevent drying. Five branches were placed near the trunk of each grapevine rootstock to allow Cicada nymphs to settle in the root zone after hatching. These procedures were repeated over two years. The research design was based on the pest's biological characteristics (Esmaeilli, 1991; Shekaryan and Rezvani, 2000). If the pest established itself in the root zone, visible symptoms such as delayed spring germination, slow growth, and small leaves were used as initial indicators of Cicada establishment and rootstock sensitivity (Esmaeilli, 1991; Shekaryan and Rezvani, 2000). Beginning in the third year, the roots of the rootstocks were examined in autumn for the presence of 2–3-year-old Cicada nymphs, with the findings recorded as part of the morphological analysis. Additionally, root samples from each rootstock were collected for biochemical analysis to measure total phenolic compounds, total flavonoids, proline, and soluble sugar levels. For this purpose, 200 g of root samples were harvested from each Cicada-infected rootstock, wrapped in aluminum foil, transported to the laboratory under liquid nitrogen, and stored at -80°C until analysis.

Extraction and determination of the total phenolic and flavonoid compounds

The root samples were air-dried at room temperature under full shade to preserve their properties. Once dried, the material was ground into a fine powder using a grinder. A 50 g portion of the powdered root was placed into a Soxhlet extractor bag. The solvent chamber of the Soxhlet extractor was filled with 500 mL of methanol, ethanol, ethyl acetate, and chloroform solutions. After several hours of extraction, the obtained extract was filtered through filter paper and centrifuged at 12,000 rpm for 5 min. The supernatant was concentrated as much as possible using a vacuum rotary evaporator. The concentrated extracts were then transferred to sterile Petri dishes and placed in an incubator at 37°C for further drying. Once dried, the raw extracts were carefully scraped off using a scalpel under a sterile hood. The extracts were then transferred to separate sterile containers and stored at -22°C for subsequent analysis (Slinkard and Singleton, 1977).

Total phenol content

The total phenolic content was measured using the Folin-Ciocalteu reagent, following the method described by Slinkard and Singleton (1977). To prepare the reagent mixture, 15 mL of Folin-Ciocalteu reagent was combined with 4 mL of a 1 M sodium carbonate (Na_2CO_3) solution. Subsequently, 0.5 mL of each plant extract (10 mg mL^{-1}) was added to the reagent mixture. The mixtures were allowed to react at room temperature for 15 min. Following this incubation period, the absorbance of the solutions was measured using a spectrophotometer at a wavelength of 765 nm. Standard curves were constructed using Gallic acid solutions with concentrations of 0, 50, 100, 150, 200, and 250 mg mL^{-1}. The total phenolic content in the samples was calculated and expressed as milligrams of Gallic acid equivalents (GAE) $gram^{-1}$ of dry weight (mg GAE g^{-1} DW).

Total flavonoid content

The total flavonoid content in the extracted plant samples was measured using a method described by Chang et al. (2002). Initially, 0.1 mL of a 10% aluminum chloride solution was mixed with 0.1 mL of a 1 M potassium acetate solution, followed by the addition of 2.8 mL of double-distilled water. Next, 0.5 mL of each plant extract solution was combined with 1.5 mL of ethanol and added to the aluminum chloride, potassium acetate, and water mixture. The final solution for each extract (5 mL) was incubated at room temperature for 30 min. After incubation, the absorbance was measured using a spectrophotometer at a wavelength of 415 nm. Total flavonoid content was expressed as mg of quercetin equivalent $gram^{-1}$ of dry weight (mg QE g^{-1} DW).

Measurement of proline

Proline content was determined using the method described by Bates et al. (1973). Fresh root samples (0.5 g) were homogenized in 4 mL of 3.0% sulfosalicylic acid. The homogenate was then centrifuged at 1000 rpm for 10 min. A 1 mL aliquot of the supernatant was mixed with 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid in a test tube. The mixture was incubated in a water bath at 100 °C for 60 min, followed by rapid cooling in an ice bath. After cooling, 4 mL of toluene was added to the mixture, and the solution was vortexed. The chromophore-containing toluene (upper layer) was carefully transferred to a new test tube. Finally, the absorbance was measured at 520 nm using a spectrophotometer, with toluene serving as the blank. The proline concentration was calculated using a standard curve and expressed as mg g^{-1}.

Measurement of soluble carbohydrates

Carbohydrates were extracted using the Anthrone method as described by Carrol et al. (1956). Fresh root samples (0.5 g) were crushed, and 5 mL of 95% ethanol was added. The supernatant was separated, and the residue was subjected to a second extraction with 5 mL of 70% ethanol. The combined extracts were centrifuged at 4500 rpm for 15 min and stored at 20 °C until carbohydrate analysis. For total carbohydrate determination, 100 µL of the extracted solution was mixed with 3 mL of freshly prepared Anthrone reagent (prepared by dissolving 150 mg of pure Anthrone in 100 mL of 72% sulfuric acid). The mixture was incubated in a boiling water bath for 10 min and then allowed to cool to room temperature. The absorbance of the samples was measured at 625 nm using a spectrophotometer.

Data analysis

Duncan's multiple range test was used to compare the averages of the varieties for the studied traits. Pearson's bivariate correlation coefficient was employed to assess the relationships between quantitative characteristics. The GGE biplot analysis method was applied to evaluate the associations between varieties and metabolites. Statistical analyses were performed using SPSS version 26 and GenStat version 12 software.

Results

The highest rate of nymph establishment in Cicadas was observed in the 'Spoota' rootstock, with an establishment rate of 3.3%, while the lowest rate occurred in the 'CH1' rootstock. Notably, no nymph establishment was recorded in the Kober *5BB* rootstock. Furthermore, none of the studied rootstocks exhibited any signs of Cicada pest activity in the leaves, such as delayed germination, growth stagnation, or weakened growth (Table 2).

Table 2. Number of initial and hatched eggs, established nymphs, and their symptoms in aerial organs in the four studied rootstocks.

Rootstocks	Initial population of pest eggs	Number of hatched eggs	Number of establishment nymphs	% Establishment	symptoms
Spoota	1320	1240	41	3.3	No
Nazemiyeh	1325	1280	32	2.5	No
Kober 5BB	1310	1280	0	0	No
CH1	1305	1185	8	0.67	No

The studied rootstocks displayed a wide diversity in biochemical trait profiles. Kurtosis and skewness values ranged from +2 to -2, indicating a normal distribution of the data points and allowing variance analysis without the need for data transformation (Table 3). Variance analysis of the biochemical characteristics revealed that the rootstock type had a significant influence on the levels of total carbohydrates, phenols, and flavonoids in the roots. However, the proline content in the rootstocks had no significant difference (Table 4).

Table 3. Mean values and variation parameters of total carbohydrates, phenols, and flavonoids in four grapevine rootstock root samples.

Rootstocks		Total carbohydrates (mg g^{-1})	Phenol (mg g^{-1})	Flavonoid (mg g^{-1})	Proline (µM g^{-1})
CH1	Mean ± Std.D*	1.92 ± 0.760	4.86 ± 0.993	1.67 ± 0.401	94.97 ± 30.282
	Minimum	1.351	3.782	1.236	72.022
	Maximum	2.789	5.735	2.018	129.293
	Kurtosis	0.23	0.27	0.44	0.12
	Skewness	1.453	-0.909	-1.088	1.451
Kober 5BB	Mean ± Std.D	4.02 ± 0.972	4.71 ± 0.819	0.81 ± 0.197	120.17 ± 24.54
	Minimum	2.588	3.951	0.664	105.748
	Maximum	5.862	5.582	1.039	148.511
	Kurtosis	0.01	0.41	0.22	0.63
	Skewness	1.000	0.527	1.398	1.731
Spoota	Mean ± Std.D	1.60 ± 0.509	3.52 ± 0.388	0.77 ± 0.098	113.80 ± 33.15
	Minimum	1.216	3.205	0.664	79.531
	Maximum	2.183	3.958	0.857	145.711
	Kurtosis	0.81	0.25	0.63	0.15
	Skewness	1.426	1.152	-1.008	-0.321
Nazemiyeh	Mean ± Std.D	1.12 ± 0.214	3.10 ± 1.22	0.54 ± 0.124	122.08 ± 26.116
	Minimum	0.898	1.966	0.406	94.040
	Maximum	1.325	4.397	0.643	145.711
	Kurtosis	0.53	0.29	0.44	0.12
	Skewness	-0.456	0.522	-1.390	-0.739

*: Standard deviation

A comparison of the mean total carbohydrate levels in the roots of the investigated rootstocks revealed significant differences among all rootstocks. The highest carbohydrate content (4.03 mg g^{-1}) was observed in the Kober 5BB rootstock, while the Nazemiyeh rootstock exhibited the lowest carbohydrate level (Fig. 2).

Table 4. Mean square values of the variance analysis of total carbohydrates, phenols, and flavonoids in four grapevine rootstocks root samples based on a randomized block design.

S.O.V.	D.F.	Total Carbohydrates	Phenol	Flavonoid	Proline
Rootstocks	3	4.928*	2.264*	0.744**	459.307
Replication	2	1.415	1.858	0.142	745.554
Error	6	0.755	0.482	0.028	851.675

*: $P < 0.05$, **: $P < 0.01$

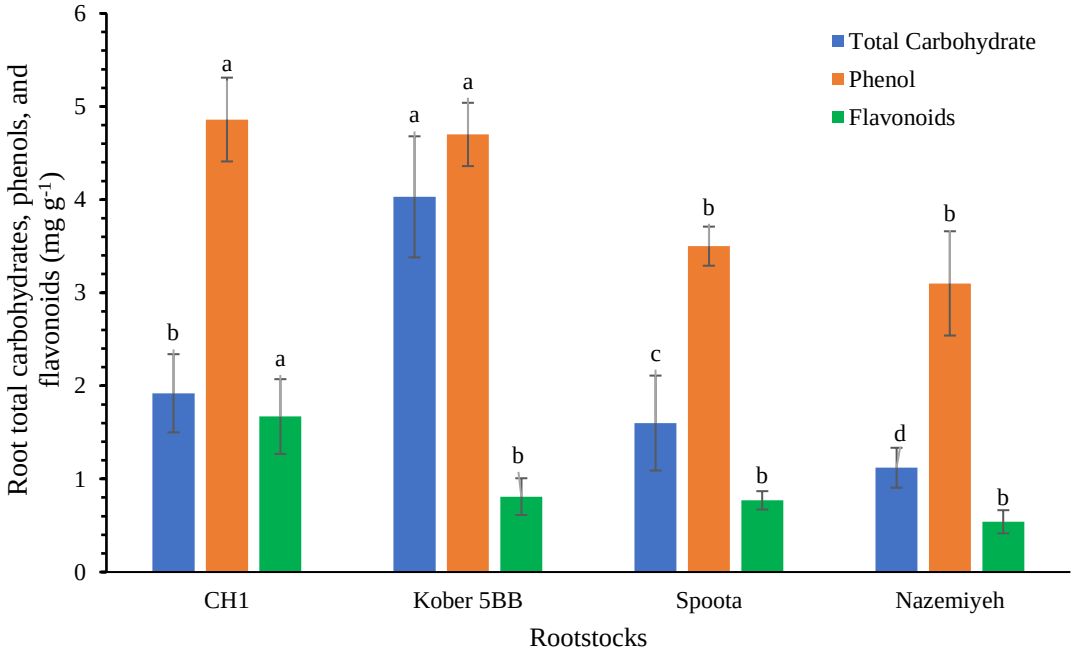

Fig. 2. Mean comparisons of the root samples, total carbohydrates, phenols, and flavonoids in four grapevine rootstocks at $\alpha = 0.05$ by Tukey's test.

In the comparison of mean phenol content in the roots of the investigated rootstocks, the highest levels were recorded in the CH1 (4.86 mg g^{-1}) and Kober 5BB (4.74 mg g^{-1}) rootstocks, with no significant difference between the two. The lowest phenol content (1.3 mg g^{-1}) was observed in the Nazemiyeh rootstock (Fig. 2). For total flavonoid content, the highest level (1.67 mg g^{-1}) was found in the CH1 rootstock, which significantly differed from the levels in the other rootstocks. No significant differences were observed in total flavonoid levels among the Kober 5BB, Spoota, and Nazemiyeh rootstocks. The lowest total flavonoid content (0.54 mg g^{-1}) was recorded in the Nazemiyeh rootstock (Fig. 2). To summarize the biochemical traits and identify the rootstocks associated with their production, a principal component analysis (PCA) was performed. The first and second components accounted for 98.64% and 1.35% of the total variation, respectively. The CH1 rootstock exhibited the highest production of both total flavonoids and phenols, while the Kober 5BB rootstock had the highest carbohydrate content. Proline was located outside the PCA polygon and was produced at similar levels across all rootstocks (Fig. 3).

The bilateral correlation between the studied characteristics and the establishment percentage of Cicada nymphs is presented in Table 5. The establishment percentage of vine Cicada nymphs exhibited a significant negative correlation with total carbohydrates, phenols, and flavonoids in the roots. Among these, the strongest negative relationship was observed between the establishment percentage and phenol content.

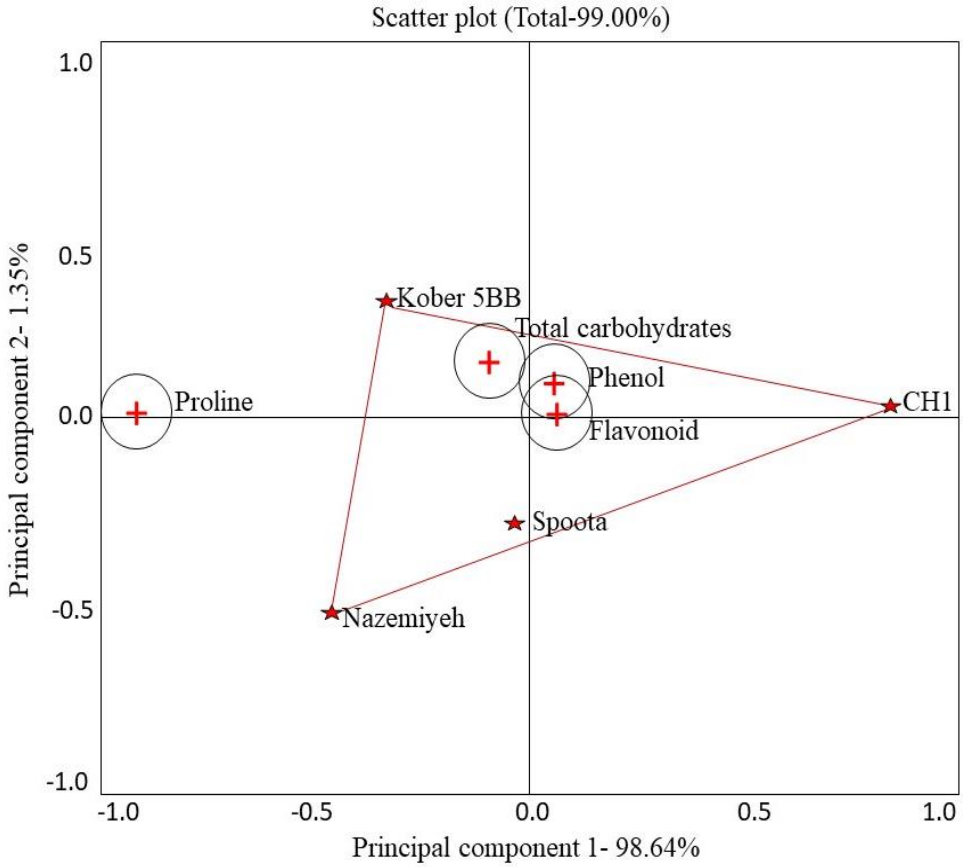

Fig. 3. A graphical analysis of which product- which rootstock according to the first and second principal components biplot by genotype product biplot interaction method.

Table 5. Bilateral Pearson's correlation of the total carbohydrate, phenol, flavonoids, proline, and Cicada nymph establishment. (Degree of Freedom = 2).

	Total Carbohydrate	Phenol	Flavonoids	Proline	Establishment on root
Total Carbohydrate	1	0.696*	0.054	0.163	-0.781**
Phenol		1	0.753*	-0.582*	-0.887**
Flavonoids			1	-.967*	-0.497*
Proline				1	0.262
Establishment on root					1

*: $P \leq 0.05$, **: $P \leq 0.01$.

Given that no vine Cicadas were observed in the Kober 5BB rootstock, and it exhibited high levels of total proline, phenols, and carbohydrates, the Kober 5BB rootstock was identified as resistant to vine Cicada pests (Table 6).

Table 6. Final summary of the resistance mechanism of the four grapevine rootstocks to the *cicada* pest based on biochemical characters, pest establishment on root, and symptoms in aerial organs.

Rootstocks	Establishment on root %	symptoms	Total Carbohydrate	Phenol	Flavonoids	Proline	Final Evaluation Result
Spoota	3.3	No	Low	Low	Low	Medium	Tolerate
Nazemiyeh	2.5	No	Very Low	Low	Low	High	Tolerate
Kober 5BB	0	No	High	High	Low	High	Resistant
CH1	0.67	No	Medium	High	High	Low	Very Tolerate

Discussion

The CH1 rootstock exhibited a very low percentage of Cicada pest infection and showed no visible symptoms of infestation. It demonstrated high levels of total phenol and flavonoid production in the roots, making it highly tolerant to Cicada pests. Similarly, the Spoota and Nazemiyeh rootstocks had relatively low infection levels and low production of total phenols and flavonoids in the roots. These rootstocks also displayed no visible symptoms of infestation, suggesting they were moderately tolerant to Cicada pests. In this study, the primary criterion for evaluating rootstock resistance to Cicadas was the pest population in the roots and the presence of symptoms affecting grapevine growth. Among the studied rootstocks, pest establishment was entirely absent only in the Kober 5BB rootstock. Moreover, no symptoms of pest contamination were observed in any of the rootstocks, a finding consistent with the results of Lawo et al. (2011). Their research used pest establishment in the roots and leaves, along with feeding activity, as the main indicators of resistance to the phylloxera pest. A similar observation was reported in Alexandrov's study (2016).

The present study also found that the CH1 rootstock had higher levels of secondary metabolites compared to other rootstocks, further supporting its superior tolerance to Cicada pests. Lawo et al. (2011) similarly noted that rootstocks resistant to phylloxera exhibited higher levels of phenolic and ethanol compounds than other rootstocks. A review of the scientific literature revealed that studies on the resistance of grapevine cultivars and rootstocks to Cicada pests are extremely limited. This scarcity is primarily due to the restricted geographical range and bioecological behavior of the pest. Additionally, research in this area is challenging because the Cicada pest remains active for a minimum of five years, from egg hatching to adulthood, within the root expansion zone.

While the mechanism of Cicada resistance in grapevine rootstocks remains poorly understood, resistance and susceptibility to different species are often expressed phenotypically through growth and development traits. Differences in the number of nymphs replaced on the roots and symptoms such as growth stagnation in shoots and leaves among cultivars and rootstocks suggest a form of horizontal resistance involving multiple genes. This phenomenon is consistent with findings by Ferris et al. (1982), who noted similar patterns in other plants. Conversely, Anwar and McKenry (2002) reported that vine rootstock resistance to nematodes is monogenic.

In the current study, no cicada nymphs were replaced on the Kober 5BB rootstock, and the pest did not feed on it. This indicates that the resistance in Kober 5BB could be due to an antibiosis mechanism. Plant phenols, including polyphenols, flavonoids, and phenolic glycosides, are vital components of plant defense against herbivorous insects. These secondary metabolites act as antifeedants, toxins, feeding inhibitors, or chemicals that reduce digestibility, either directly or indirectly (Nemera and Bekana, 2023; Singh et al., 2021; Kumar et al., 2014).

Natural plant compounds with insecticidal and repellent properties often contain polyphenols, which are abundant in fruit crops (Boeckler et al., 2011). Under biotic and abiotic stressors, plants produce phenolics—especially phenols and flavonoids—which inhibit fungal growth and limit pathogen adhesion and invasion (Lattanzio et al., 2006). These compounds also influence interactions between plants and herbivores, affecting insect physiology and mediating the exposure of various herbivore feeding guilds to plant defenses. Generally, plant phenolics serve as adaptive traits that have evolved to chemically deter herbivores. Additionally, phenols help plants defend not only against herbivores but also against competing plants and microorganisms. Following insect damage, phenols often undergo quantitative and qualitative changes, while oxidative enzyme activity increases (Var et al., 2011).

In the Nazemiyeh, Spoota, and CH1 rootstocks, the Cicada pest fed on the roots without causing visible side effects, indicating that resistance in these rootstocks is tolerance-based (Ahman, 2009). Since resistance to Cicadas varies across rootstocks, it can be inferred that resistance genes differ among *Vitis* species, depending on the geographical origin of the parental rootstock. Factors such as root damage, increased leakage of sugars and amino acids from damaged tissues (which enhance pest feeding potential), and physiological changes in host tissues all contribute to the extent of nutrition and damage caused by grapevine Cicadas (Khan and Husain, 1989; Mai and Abawi, 1987; Hewezi et al., 2010).

Conclusions

The results of this study showed that grapevine Cicadas did not establish themselves in the Kober 5BB rootstock. This rootstock exhibited relatively high levels of repellent chemical compounds in the root zone, including proline, phenols, and total carbohydrates, making it resistant to Cicada

pests. The CH1 rootstock displayed a very low percentage of Cicada infestation and no visible symptoms such as growth stagnation, delayed germination, or spring awakening. Additionally, CH1 had elevated levels of phenolic and flavonoid compounds in its roots, classifying it as highly tolerant to grapevine Cicadas. The Spoota and Nazemiyeh rootstocks experienced relatively low levels of Cicada infestation and produced lower amounts of total phenols and flavonoids in their roots. Nevertheless, these rootstocks showed no signs of delayed growth or germination in early spring, indicating tolerance to Cicada pests.

A significant positive correlation was observed between the percentage of pest establishment in the roots and the levels of root metabolites, underscoring the role of these compounds in enhancing resistance to Cicadas. These rootstocks hold potential for use in the biological control of Cicada pests as grafting rootstocks for commercial grapevine cultivars. However, before large-scale implementation, it is crucial to evaluate the compatibility of these rootstocks with grafted commercial varieties. Such assessments should include the reproductive performance and the quantitative and qualitative yield of the commercial varieties to ensure successful vineyard establishment.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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