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Original scientific paper

Strawberry resistance to the aphid *Chaetosiphon fragaefolii* Cockerell (Homoptera: Aphididae)

Slobodan Milenković[1], Marijana Pešaković[2]*, Dejan Marčić[3] and Drago Milošević[4]

[1]Megatrend University, Faculty of Biofarming, Bačka Topola, Maršala Tita 39, 24300 Bačka Topola, Serbia
[2]Fruit Research Institute, Čačak, Kralja Petra I 9, 32000 Čačak, Serbia
[3]Institute of Pesticides and Environmental Protection, Banatska 31B, P.O. Box 163, 11080 Belgrade, Serbia
[4]University of Kragujevac, Faculty of Agronomy, Cara Dušana 34, 32000 Čačak, Serbia
*(*marijanap@ftn.kg.ac.rs)*

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SUMMARY

Cross-sections of leaf blades and petioles of different strawberry genotypes exhibiting different levels of resistance to *Chaetosiphon fragaefolii* were studied using the paraffin method, and staining with safranin, crystal violet and light green. Besides thicker cell walls of the cortex collenchyma in the more resistant genotypes, and a proportionally wider collenchyma than parenchyma in the cortex, their midribs are also encircled by a ring of more intensely stained lignified cells forming a physical ring. This stain reaction of cells to safranin and crystal violet occurs also in lateral vascular bundles, as well as in leaf palisade tissue. The tissue cross-sections of the sensitive genotypes revealed a predominance of green on the cellulose cell walls and protoplasts due to the reaction to light green SF, while stain reactions to safranin and crystal violet were not evidenced.

Keywords: Plant resistance; Strawberry; *Chaetosiphon fragaefolii*

INTRODUCTION

Plant resistance to insects in the broadest sense results from hereditary traits of plants that enable them to sustain less injury than plants with no such traits, i.e. either preventing or inhibiting the development of a specific insect species. Resistance is relative in character, as it is based on a comparison of plants by their sensitivity (Panda & Khush, 1995).

Research that includes the development, study and introduction of insect-resistant plant cultivars into production is of vital importance to modern agricultural production, especially to the concept of integrated insect control programmes. Resistant cultivars developed in recent years have greatly contributed to the attempts of agriculturalists to stabilize and enhance yields (Milenković, 2011).

The biology of strawberry and its growing methods, characteristics of the development cycles of its insect

pests and causal agents of diseases, as well as fruit residue limits are to be considered when designing a strawberry protection programme.

Strawberry resistance to the leaf aphid *Ch. fragaefolii* is now known to be a hereditary trait after some earlier findings had denied such strawberry resistance to aphids in spite of the fact that they reproduce faster on some cultivars than on others (Shanks & Barritt, 1974).

A number of hypotheses have been proposed on the resistance mechanism of strawberry to the strawberry leaf aphid. One of them suggests that *Ch. fragaefolii* develops on artificial food containing sucrose, and fails to develop on food containing glucose and fructose (Shanks & Finnegan, 1969). It was a basis for a proposition that the species, the availability of carbohydrates, and their transport are significant parameters of resistance. Analysis of the composition of phloems has revealed that *Fragaria chiloensis* clone Del Norte contains a high rate of sucrose, although it is somewhat lower than it is in the sensitive clone 'Totem'. Hence, it is not certain that high sucrose content is the principle cause of high rates of aphid mortality on the clone Del Norte (Crock, 1981). The study hypothesized that the plant root was the place of synthesis of a phenol-substance which governs plant resistance to aphids.

The first data on the sensitivity of strawberry species, cultivars, selections and hybrids in Serbia had been collected by Milenković (Milenković, 2000, Milenković et al., 1998, Milenković, 1992a, 1992b).

The objective of this investigation was to identify differences in the structures of leaf and petiole cross-sections of several strawberry genotypes that are sensitive or resistant to the strawberry leaf aphid.

MATERIAL AND METHODS

Plant material

The following strawberry genotypes were singled out for the study of leaf blade and petiole cross-sections: *F. chiloensis* clone Del Norte – a genotype that has been shown as resistant to *C. fragaefolii*, the hybrids zf/1/94/96 and 2/11/95/97 ('Senga Fructarina' x 'Del Norte') as moderately resistant genotypes, the hybrid da/15/94/96 ('Dana' x 'Del Norte') as moderately sensitive, and 'Čačanska Rana' as a genotype highly sensitive to *F. fragaefolii*. Samples were collected from leaves during their phase of intensive development. The samples were cut out from the upper third segment of the petiole, and from the leaf base bearing the main veins. The petiole cuttings were about 5 mm long, whereas the samples collected from leaves were rectangular in shape (10 x 5 mm).

Experimental procedure

Petiole cuttings were fixed for 48 hours in FPA (formalin : propionic acid : 70% ethyl alcohol, *5:5:90*). The samples were then placed in 70% ethyl alcohol and stored at 4°C prior to staining. The fixated material was further dehydrated in a series of concentration-rising ethyl alcohols. Upon irradiation in xylol, the material was stored in paraffin. Ten μm thick cross-sections of leaf blades and petioles were cut on a rotational microtom (Baird and Tatlock, England) and attached to glass plates after spreading in warm water. Upon deparaffinizing in xylol, the cross-sections were rinsed in a series of ethyl alcohols at decreasing concentrations, and then rehydrated and made ready for staining. The samples were stained by safranin, crystal violet and light green SF (Gerlach, 1969). The cross-sections were stained in 3% safranin for 1 h and subsequently in crystal violet for 1 min. The cross-sections were kept in a fresh 0.5% light green SF solution in 90% ethanol and were then left to remain in that stain for approximately 30 seconds, i.e. until the violet stain ceased to flow from cross-section.

Determination of leaf and petiole cross-sections

The thickness of collenchyma and parenchyma of the cortex, lignified ring encircling vascular bundles and cell walls of the collenchyma and parenchyma cells were measured by the automatic image analysis device QUANTIMET 500 MC (Leica, Austria). The thickness of layers and cell walls was determined by random linear method.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the MSTAT-C statistical computer package (Michigan State University, East Lansing, MI, USA). The Least Significance Difference (LSD) was used to compare treatment means, and treatments were declared different at $p \leq 0.05$ level of significance.

RESULTS

The study of cross-sections of leaf blades and petioles of several strawberry genotypes that had exhibited different sensitivity to the leaf aphid *Ch. fragaefolii* in previous investigations was performed on cross-section devices. 'Čačanska Rana' cultivar was singled out for the study from a group of sensitive strawberry genotypes. The hybrid da/15/94/96 was chosen to represent moderately sensitive genotypes, while resistant genotypes were represented by the moderately resistant hybrids zf/1/94/96 and 2/11/95/97, and Del Norte as a resistant clone.

The staining of leaf blade and petiole cross-sections by safranin, crystal violet and light green SF displayed differences in tissue structure among the five strawberry genotypes (hybrid 2/11/95/97, hybrid zf/1/94/96, 'Čačanska Rana', clone Del Norte, hybrid da/15/94/96) in terms of genotype resistance.

The results showed significant differences in leaf blade and petiole cross-sections of the clone Del Norte. Intensive stain reaction was also observed on vascular bundles of the leaf midrib of Del Norte clone. Intense stain reactions to safranin and crystal violet were particularly visible in the petiole cross-sections (Figure 1a) where vascular bundles were encircled in rings. In that resistant genotype, the leaf palisade tissue was stained along with the thick cuticle on the upper leaf surface.

The cross-sections of the moderately resistant hybrid zf/1/94/96 revealed a stain reaction covering the entire midrib, its outer edge in particular, lateral veins, and the palisade tissue. The petiole cross-sections (Figure 1b) displayed a stain reaction around the vascular bundle, which was not as unbroken as in clone Del Norte, but covering the entire vascular bundle nevertheless.

The midrib cross-sections of the moderately susceptible hybrid da/15/94/96 displayed thicker collenchyma cell walls, while some more intensely stained individual cells encircling vascular bundles were observed in petiole cross-sections (Figure 1c).

Cellulose cell walls stained with light green SF were predominantly green in the cross-sections of the midrib of 'Čačanska Rana'. It was only the large-sized tracheas that stained red. A similar stain reaction occured in the cross-sections of lateral veins and in leaf palisade tissue. The cross-section of the petiole (Figure 1d) revealed green tissues as well, whereas tracheas and a few cells around vascular bundles stained safranin and crystal violet.

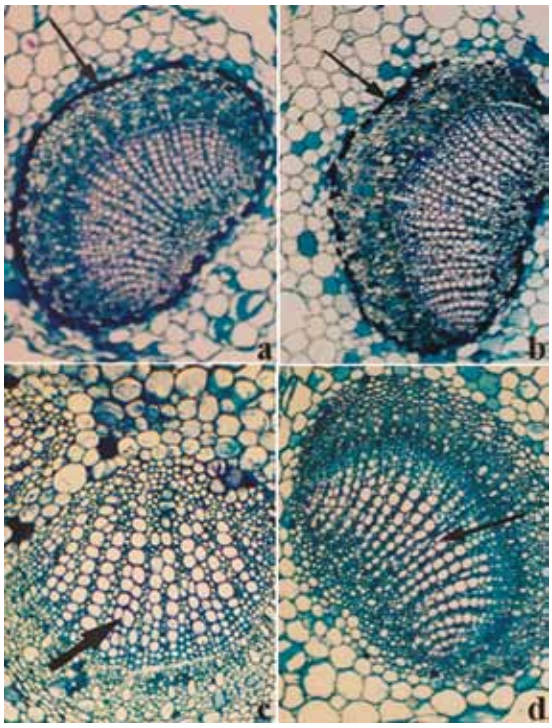

Figure 1. Cross-sections of the petiole: (a) *F. chiloensis* clone Del Norte; (b) hybrid zf/1/94/96; (c) hybrid da/15/94/96; (d) 'Čačanska Rana'

The midrib cross-sections of the moderately resistant hybrid 2/11/95/97 resembled those of hybrid zf/1/94/96, although the cell wall of cortex collenchyma was thicker in the former. Stain reaction was also visible in the palisade leaf tissue of this hybrid, and the cuticle on the upper leaf surface was similar to Del Norte clone. The intensity of staining of the cells encircling vascular bundles was somewhat lower in the phloem zone than in the xylem zone. Parenchyma tissue cells exhibited stain reaction of indistinct origin.

The intensity and type of staining enabled a qualitative description of tissue characteristics. The data shown in Table 1 and Figures 2-4 provide a basis for a more precise qualitative analysis of some specific traits of the tissue structure of different strawberry genotypes possibly affecting the mechanism of strawberry resistance to penetration by aphid stylets. Therefore, we measured the thickness of cell wall, as well as the lignified layer encircling vascular bundles, in cells of the cortex parenchyma and collenchyma. The thickness of layers of cortex parenchyma and collenchyma was also compared.

The analysis of variance showed a significant effect of the genotype on cell wall thickness in leaves and petioles. A significant effect of the genotype on the parenchyma/collenchyma ratio was also observed.

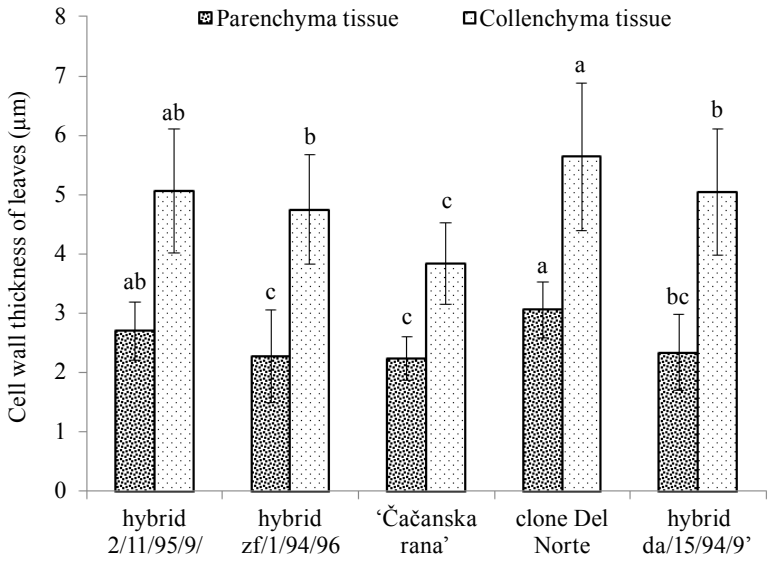

Figure 2. Cell wall thickness of leaves

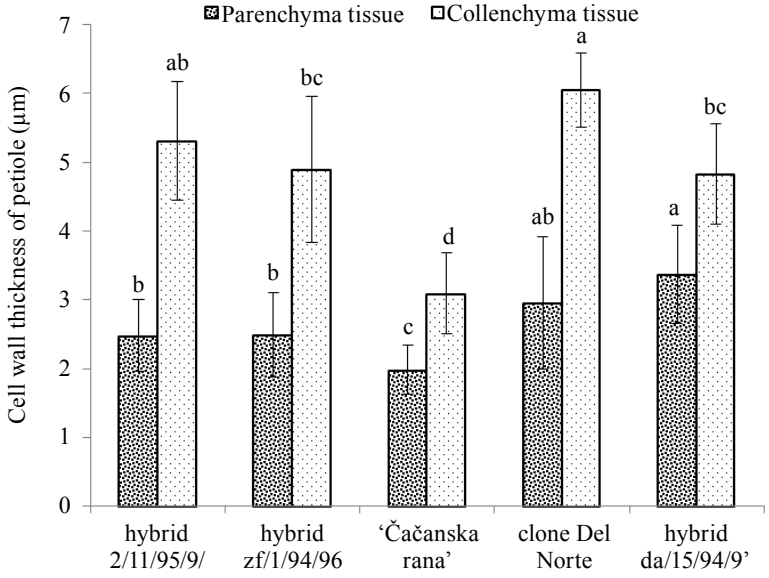

Figure 3. Cell wall thickness of petiole

The data on cell wall thickness in leaves are presented in Figure 2. The thickness of leaf cell walls in the parenchyma tissue encircling vascular bundles of the midrib ranges from 2.24 µm in 'Čačanska Rana' to 3.06 µm in Del Norte clone. The thickness of cell walls of the leaf cortex collenchyma was also the lowest in 'Čačanska Rana' (3.84 µm), and highest in Del Norte clone (5.64 µm).

Data analysis referring to measurements of the petiole tissue was somewhat different (Figure 3). The thinnest cell walls were found in 'Čačanska Rana' (1.98 µm in parenchyma, 3.09 µm in cortex collenchyma). Nevertheless, the parenchyma cell wall of the clone Del Norte was 2.96 µm thick, while the cortex collenchyme cell wall was 6.05 µm.

The relative ratio of cortex parenchyma and collenchyma thickness is presented in Figure 4. The highest parenchyma/collenchyma ratio of leaves was observed in 'Čačanska rana' (5.07) and the lowest in the hybrid zf/1/94/96 (1.53). Among the genotypes tested, 'Čačanska rana' exhibited the highest values of parenchyma/collenchyma ratio for the petiole (11.41), whereas the clone Del Norte had the lowest (5.11).

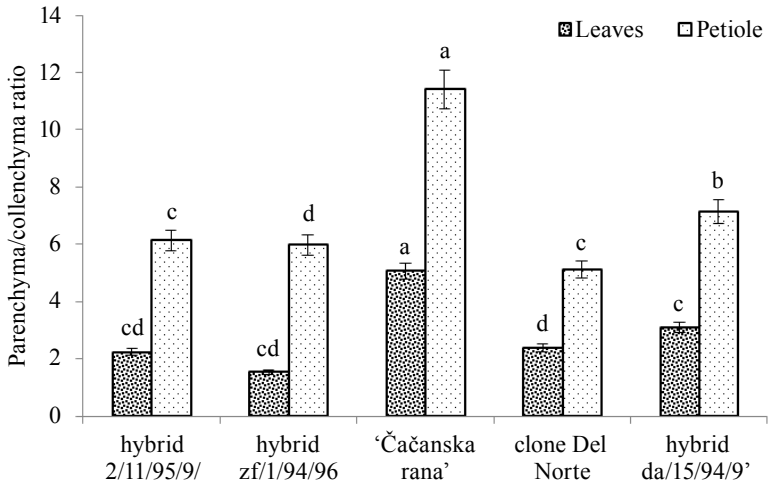

Figure 4. Parenchyma/collenchyma ratio in leaves and petioles

The thickness of lignified layers in leaves and petioles was also measured (Table 1). As shown in Table 1, no layer of lignified cells encircling vascular bundles was found in the cross-sections of the susceptible genotypes (i.e. 'Čačanska rana' and hybrid da/15/94/96).

Table 1. Thickness of the lignified layer of leaves and petioles of strawberry genotypes (μm)

Genotypes	Thickness of lignified layer	
	leaves	petioles
Hybrid 2/11/95/97	16.57	8.20
Hybrid zf/1/94/96	21.83	11.82
'Čačanska rana'	-	-
Clone Del Norte	25.94	18.97
Hybrid da/15/94/96	-	-

The thickness of the layer forming physical rings around vascular bundles in the leaf blade cross-sections ranged from 25.94 μm (clone Del Norte) to 16.57 μm (hybrid 2/11/95/97), and from 18.97 μm (clone Del Norte) to 8.20 μm (2/11/95/97) in the petiole cross-sections.

DISCUSSION

Staining fresh cross-sections of alfalfa organs by fluroglucinol, Brewer et al. (1986) provided data showing considerable differences in three levels of lignification of particular tissues. The levels were as follows: a) xylem b) xylem and phloem c) xylem, phloem, and interfascicular space. The authors reported unbroken rings of lignified cells formed at the highest lignification level around vascular bundles. The paper is of particular importance to our research as it investigated the feeding of cicadas which, at least in some aspects, are similar to leaf aphids. Phloem was the primary feeding site for *Empoasca flavescens* (F.) in susceptible selections of *Ricinus spp*., while it was the spongy tissue in resistant selections (Jayaraj, 1967). Morphologically, resistant and susceptible selections differ in the thickness of their secondary cell rings, formed lateral to the leaf veins. In susceptible rice cultivars, *Nephotettix virescens* Ishihara feeding was centred in the phloem, and in resistant ones in the xylem (Auclair et al., 1982). Besides, the fact that the aphid stylet always entered the phloem of the susceptible 'Totem', and only 57% of the totally analyzed time of feeding in 'Del Norte' (McKay, 1985), may be in some aspects associated with our results.

Studying cell lignification in alfalfa, Brewer et al. (1986) suggested that differences were not based on more or less intensive lignification, but on an earlier beginning of lignification in resistant clones. Apart from these factors, the display of resistance was also influenced by leaf hairs and the smaller diameter of tissue cross-section.

In order to reach plant phloem, aphids must overcome plant defences, either physically or chemically, or both (Guerrieri & Digilio, 2008). However, plants respond to aphid attack by activating defence genes that lead to the production of physical barriers and/or chemical toxic compounds (direct resistance). According to Guerrieri and Digilio (2008) the constitutive presence of trichomes, thorns and thick cell walls all add up to a specific type of direct resistance that prevents any feeding activity. These mechanical barriers can be produced in response

to the feeding activity of an invading aphid, and in such a case they are referred to as direct induced defences.

Besides the presence of a lignified layer around vascular bundles in the leaf and petiole of both the susceptible and resistant genotypes examined in our experiment, certain differences were recorded in the thickness of cell walls of cortex parenchyma and collenhyma, as well as in the thickness of layers and relative ratio of thickness of collenchyma and parenchyma. The relative ratio of cortex parenchyma and collenchyma thickness provided data for an analysis of correlation between strawberry resistance and anatomical characteristics of the cross-sections of its leaf blades and petioles. Leaf cross-sections of the susceptible 'Čačanska Rana' revealed that parenchyma tissue was 5.07 times thicker than the tissue of cortex collenchyma. In addition, petiole cross-sections displayed an advantage of parenchyma thickness, having the value of 11.41. In the clone Del Norte, parenchyma tissue was 2.39 times thicker than cortex collenchyma in leaf cross-sections, and 5.11 in petiole cross-sections, a difference that was only half as high as it was in 'Čačanska Rana'. These results infer that the thickness of cortex collenchyma, compared to parenchyma, was much greater (about twice) both in the leaf blades and petioles of the resistant clone Del Norte, as related to the susceptible selection 'Čačanska Rana'. Lower relative thickness ratios of parenchyma and cortex collenchyma tissue were found in the other resistant genotypes (hybrid 2/11/95/97 and hybrid zf/1/94/96) as compared to the susceptible genotype (hybrid da/15/94/96). Being a firmer and more resistant tissue than the parenchyma, collenchyma probably adds to the resistance quality that the clone Del Norte has against *Ch. fragaefolii*.

The depth and the manner of stylet penetration vary with aphid species. Most aphids populating herbaceous plants penetrate 100-200 μm into plant tissue. Pollard (1958) reported 106-118 μm for *Aphis gossypii*. *Adelges piceae* Ratzeburg penetrates *Abies pectinata* down to as much as 1.6 mm (Kloft, 1955). Gabrys et al. (1997) reported that the spacing between epydermis and phloems of *Sinapis alba* ranged from 250-400 μm, and that *Brevicoryne brassicae* (L.) penetrated beyond that layer. In our investigation, depending on genotype, the spacing between the epydermis and vascular bundles in leaf blade cross-sections ranged from 150-300 μm. Bearing in mind especially the data reported by McKay (1985), showing that the stylet of *Ch. fragafolii* rested in the phloem for 58% of total feeding time on 'Del Norte', whereas the stylet always ended in the phloem on 'Totem', we may assume that *Ch. fragaefolii* also 'chooses' mesophyll rather than phloem for feeding in resistant cultivars.

The inhibition of stylet penetration is one of the best forms of 'defence' of crop species against attacks by leaf aphids. Birch (1984) reported that young larvae of *Aphis fabae* Scopoli, *Acyrthosiphon pisum* (Harris) and *Megoura viciae* Buckton were incapable of penetrating the cuticle of some wild species of *Vicia* and so starved to death, which was not observed on grown *Vicia* species.

One of the properties of vascular bundles of both resistant and moderately resistant strawberry genotypes which may govern their display of resistance to *Ch. fragaefolii* is an intense stain reaction of a layer of cells to safranin and crystal violet due to cell lignification. Lignified cells form a physical ring which prevents penetration of aphid stylets. Such cells formed unbroken rings in the cross-sections of the resistant clone Del Norte.

The thickness of the layer encircling vascular bundles, displaying more intense stain reaction due to lignification, was highest in the resistant clone Del Norte, followed by the moderately resistant hybrid zf/1/94/96 and the hybrid 2/11/95/97. The leaf blade and petiole cross-sections of the susceptible 'Čačanska Rana' showed almost no evidence of lignified cells around vascular bundles (sporadically 1-2 cells in several cross-sections). In the moderately susceptible genotype (hybrid da/15/94/96), the cross-sections showed individual cells with slightly stained lignified walls, but failing to form unbroken rings in either segment of the cross-section. The beginning of the process of lignification is obviously earlier, and the rate and intensity of the process higher in resistant strawberry genotypes. Brewer et al. (1986) reported a similar observation that lignification started earlier in resistant alfalfa plants than in susceptible ones.

The results presented in this study reveal significant differences in degrees of lignification around vascular bundles of the resistant and moderately resistant strawberry genotypes compared to susceptible. Furthermore, these aspects of investigation are considered highly useful in strawberry breeding programmes relating to strawberry resistance to aphids, as well as for further investigation of the resistance of cultivated plants to insect pests.

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Otpornost jagode prema biljnoj vaši *Chaetosiphon fragaefolii* Cockerell (Homoptera: Aphididae)

REZIME

Parafinskom metodom i bojenjem preseka safraninom, kristal ljubičastim i svetlo zelenim SF proučavana je građa preseka liske i lisne drške genotipova jagode različite otpornosti prema *Ch. fragaefolii*. Kod otpornijih genotipova pored debljih zidova ćelija kolenhima primarne kore i većeg udela kolenhima u odnosu na parenhim u primarnoj kori, oko glavnih provodnih snopova postoji prsten jače obojenih lignifikovanih ćelija koje obrazuju mehaničku saru. Ova bojena reakcija ćelija na safranin i kristal ljubičasto postoji i na sitnijim provodnim snopovima i u palisadnom tkivu lista. Na presecima tkiva osetljivih genotipova dominira zelena boja celuloznih zidova ćelija i protoplasta zbog bojene reakcije na svetlo zeleno SF, dok su odsutne bojene reakcije na safranin i kristal ljubičasto.

Ključne reči: Otpornost biljaka; jagoda; *Chaetosiphon fragaefolii*