

## **THE EFFECT OF *Monilinia seaveri* (Rehm) Honey INFECTION ON THE CONDENSED TANNINS CONTENT IN THE LEAVES OF *Prunus serotina* Ehrh**

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**Abstract.** The incidence of natural enemies of plants triggers in them a number of defensive reactions. In spite of many studies, the role of phenolic compounds in tree-pathogen interactions, has been still ambiguous. The aim of the present study has been to analyse the tannin content in the leaves of the black cherry (*Prunus serotina*) infested, to a different degree, by *Monilinia seaveri*. The observations of the health status of the leaves were carried out on marked trees for two years. The pathogen was identified based on the material obtained from laboratory cultures. The tannin content, expressed as (+)-catechin, was determined using the vanillin method. The greatest extent of disease development was observed at the end of July. It has been found that with disease symptoms covering > 25% of the leaf area, the (+)-catechin content in the diseased leaves was significantly higher compared to the healthy ones. The obtained results thus indicate, that at the damage level exceeding approximately 25% of the leaf area, mechanisms of the induced resistance are triggered in plants, that may prevent the further development of disease.

**Key words:** black cherry, Monilia disease, induced resistance, (+)-catechin

### **INTRODUCTION**

The black cherry, *Prunus serotina* Ehrh. (syn. *Padus serotina* (Ehrh.) Borkh.), Rosaceae, had been imported from North America into Europe at the beginning of the 17<sup>th</sup> century, as a decorative tree [Starfinger 1997]. A greater interest in this species in Poland started only at the beginning of the 19<sup>th</sup> century, for its white flowers, its fruit changing their coloration and leaves blushing in autumn. Since that time the plant became a welcome guest in parks and gardens in Poland [Hereźniak 1992, Siciński 1992].

Black cherry is a decorative fairly insusceptible to pathogens and herbivore pests. The leaf damage of the species, as reported so far from city parks and city forest areas,

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had been ascribed mostly to the foraging of leaf beetle *Gonioctena quinquepunctata* Fabr. (Coleoptera) and bird-cherry ermine *Yponomeuta evonymella* L. (Lepidoptera) [Tomalak 2006]. In the recent years however, first disease symptoms had been found, taking the form of necrotic patches on the plant leaves. The pathogen responsible for these symptoms had been identified as *Monilinia seaveri* (Rehm) Honey (syn. *Sclerotinia seaveri* Rehm.) [Halarewicz and Płaskowska 2011].

At the present time, gradually more and more attention is being paid to the investigation of resistance mechanisms, naturally protecting plants from infections. These mechanisms may take a form of permanent attributes of a plant like morphological and anatomical barriers or physiological plant properties, which is usually termed constitutive resistance, or they may involve producing defensive factors only at times when they are needed, which is called induced resistance. The induced resistance is expressed, in the first place, as hypersensitive response (HR), characterized by the rapid cell necroses in the vicinity of an infection [Heath 2000]. Moreover, local symptoms of HR are often accompanied by the onset of systemic acquired resistance (SAR) in remote plant tissues [Kombrink and Schmelzer 2001]. Apart from the HR signals, SAR is induced by salicylic acid (SA) and its functional metabolites [Złotek and Wójcik 2007]. Triggering of SAR results in systemic gene activation that is shown in plant as subsequent biosynthesis of protective secondary metabolites and inhibitory proteins in order to contain the disease [Agrios 1997, Pospieszny 2000].

Tannins are merely one group of secondary plant metabolites playing role in plant defensive response. They are classified as polyphenolic compounds and fulfill a variety of biological functions. Hence, they are permanently present in uninfected tissues of many plant species [Harborne 1993], including the leaf tissue of *Prunus serotina* [Buchalter 1969, Halarewicz 2011]. Tannins are usually classified into two groups: hydrolyzable tannins and non hydrolyzable tannins (condensed tannins, proanthocyanidins). The latter are, among other things, derivatives of flavan-3-ols, such as catechin [Buchalter 1969, Harborne and Baxter 1999]. In terms of function, hydrolyzable tannins act primarily against chewing phytophagous insects and animals [Lewis and Yamamoto 1989, Porter 1989, Harborne 1993].

The condensed tannins, in turn, mainly defend plants against the action of microbes [Lewis and Yamamoto 1989, Cushnie and Lamb 2005, Min et al. 2008] and pathogenic fungi in particular [Hammerschmidt 2005]. This group of tannins may confine the infections by complexation with extracellular enzymes that pathogens produce or by interfering with the metabolism of the pathogen itself [Davis 1998].

The present study aimed to identify the dynamics of disease symptoms caused by *Monilinia seaveri* on black cherry leaves and establishing what threshold level of the leaf damage is critical in triggering the mechanisms of the plant's induced resistance for the pathogen, in the form of increased synthesis of condensed tannins, namely (+)-catechin.

## MATERIAL AND METHODS

**Study area.** The observations of the health status of the five trees of *Prunus serotina* infested by *Monilinia seaveri* were carried out in two vegetation seasons of 2010–

–2011 years. The trees were located in a house garden of a private estate within the boundaries of Wołów, a small town in SW Poland (51.33°N, 16.64°E). The trees formed a compact group of plants with the tallest two of them reaching the height of approximately 7 m and the remaining three, of 2–2.5 m height, being most likely root suckers of the mother plant.

**Meteorological data.** The course of the weather during the study period was variable (fig. 1). In 2010, the beginning of June was colder but all of July – much more torrid, compared to 2011. The temperature dynamics in August was similar in both the years. Also, the extensive rains were recorded at the turn of July and August in 2010 as well as in 2011, and torrential rains occurred after the 20<sup>th</sup> of August in 2010 and by the end of June in 2011. The meteorological data originate from the weather station of the Wrocław University of Environmental and Life Sciences at Swojec, located 11 km to the East from the center of Wrocław.

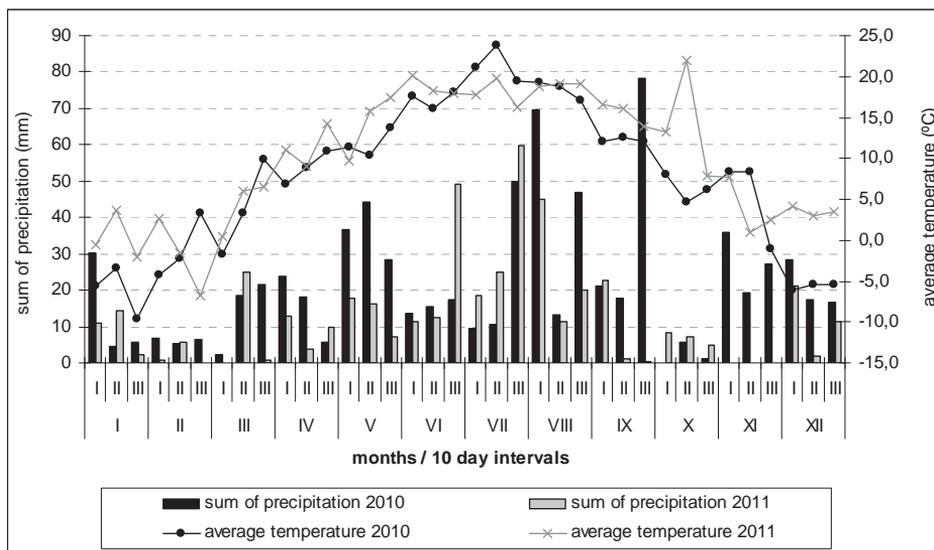


Fig. 1. The course of the weather during the study period of 2010–2011 (Wrocław, Swojec)

**Field observations.** The observations had been performed in 7–10 day intervals since mid-April until mid-September. Altogether 11 records had been made; their dates are given in Figure 2. On every tree 10 lateral shoots of 40 cm length were chosen randomly and inspected on each observation date. The health status of leaves on the inspected shoots was assessed based on a 4-grade infestation scale in which the grade 1 designates healthy, undamaged leaf and the grades 2, 3 and 4 are used for the leaves in which the diseased patches cover 1–24%, 25–50% and 51–75% of the leaf area (LA), respectively. Based on the recorded data, the infestation index ( $I_i$ ) was calculated individually for each infestation date, according to the formula:

$$I_i = \frac{\sum nc}{N},$$

where  $n$  – is the number of the leaves on a shoot, that are infested to the extent contained within a range of particular class,  $c$  stands for the central value of the respective class, whereas  $N$  is the total number of the leaves on the inspected shoot.

**Pathogen identification.** In order to identify the pathogen taxonomically, a sample of 20 infested leaves was taken on each observation date from each one of the trees. The sampled material was superficially disinfected in 0.5% sodium hypochlorite, diluted from concentrated reagent (Chempur, Piekary Śląskie), for the time of 1 minute. Next, one diseased fragment of every leaf was divided into 6 inoculum fragments and these were placed on solidified PDA medium. The growing pathogen colonies were then grafted onto PDA medium slants and diagnosed. After 14 days the fungus was identified based on conidial spore morphology, according to the available literature [Batra 1991].

**Material collection for analyses.** All samples of leaves for biochemical analyses were collected in 2011. Branches in shaded portions of each tree, northerly facing and situated ca 120 cm above ground level were marked. It is known that the tannin content in a plant is dependent on light access to it [Żmuda et al. 2008], therefore particular attention was paid to ensure that the sampled leaves would come from branches exposed to similar lighting conditions. The material was sampled on three occasions, at the beginning of June, July and August. The leaf blades of *P. serotina* sampled from the same branches were assigned to classes, depending on the extent of the cover of pathogenic patches on them. About thirty leaves were assigned to each one of such infestation classes per every sampled tree. Three leaf blades from each category were used to prepare the anatomical slides. These were necessary to produce visual images of the condensed tannins incidence and distribution in the plant cells. The slides were stained according to Jensen's method [1962] and the ultra thin slices were cut using microtome. All the other leaves were immediately frozen and stored at  $-20^{\circ}\text{C}$  until the time of chemical analysis.

**Sample handling.** Freeze-dried powdered plant material (0.5 g) was used for extraction. Samples in 80% methanol ( $10\text{ cm}^3$ ) were sonicated for 30 min at room temperature, then centrifuged. To  $2\text{ cm}^3$  of distilled water and  $3\text{ cm}^3$  of chloroform,  $2\text{ cm}^3$  of the extract were added, in order to remove the chlorophyll. After liquid-liquid extraction,  $0.5\text{ cm}^3$  of a methanol-water layer was used for determination of condensed tannin content by vanillin method [Broadhurst and Jones 1978]. In short,  $2.5\text{ cm}^3$  of vanillin-hydrochloride reagent (*i.e.* equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol, mixed just before analysis) was added to  $0.5\text{ cm}^3$  of the sample and after 20 minutes absorbance at 500 nm was measured against a blank sample. A standard curve was plotted with (+)-catechin hydrate (Aldrich). The measurements were carried out in 3 replicates for each sample of the plant material.

**Statistical analysis.** Data was analyzed using Kruskal-Wallis ANOVA and Mann-Whitney U-test, in Statistica 9.0 package (StatSoft), at  $p < 0.05$ .

## RESULTS

The mycological analysis had confirmed unambiguously that the observed disease symptoms, in the form of regular brown patches on the black cherry leaves (fig. 2), were caused by *Monilinia seaveri* – as from all the sampled, infested leaves only the one fungus had been isolated. The infestation of the leaves of *Prunus serotina* by the pathogen remained at a relatively similar level during two consecutive vegetational seasons of



Fig. 2. Symptoms of infection by *Monilinia seaveri* in the form of abundant necrotic spots on the leaf of *Prunus serotina*, July 2011

2010–2011 (fig. 3). First disease symptoms in particular season had been always noticed in mid – June, with gradual increase towards the full summer. The greatest extent of damage had been recorded by the end of July both years; the proportion of the infested leaves in samples being 33% and 36% in 2010 and 2011 respectively. The necrotic patches never covered > 50% of the leaf (LA). In the first year of the study the infestation index ( $I_i$ ) had reached its highest value by the end of July (5.40). In 2011,  $I_i$  reached the level of 5.17 at the beginning of August, and since the middle of that month an intensified leaf dropping was observed, resulting in the declining proportion of the infested leaves in the sampled material, that can be seen in Figure 3. Considered the course of the weather during the study years (fig. 1) and the dynamics of disease symptoms observed on the leaves (fig. 3), one may conclude that warm and wet summers are conducive to the development of *M. seaveri*.

Seasonal variation in the (+)-catechin content of the healthy and infected leaves of *P. serotina* is shown in table 1. The differences in the metabolite content in the healthy leaf blades of the black cherry were demonstrated for the first time in the work of Halarewicz [2011]. The significant increase in the synthesis of the metabolite was seen between the beginning of June and July, after which its concentration insignificantly

decreased in August. In the present study, the (+)-catechin content in all the infected leaves was higher in July, but only in the most extensively diseased leaf blades (25–50%) was it significantly different between the sampling dates (tab. 1). Tissue

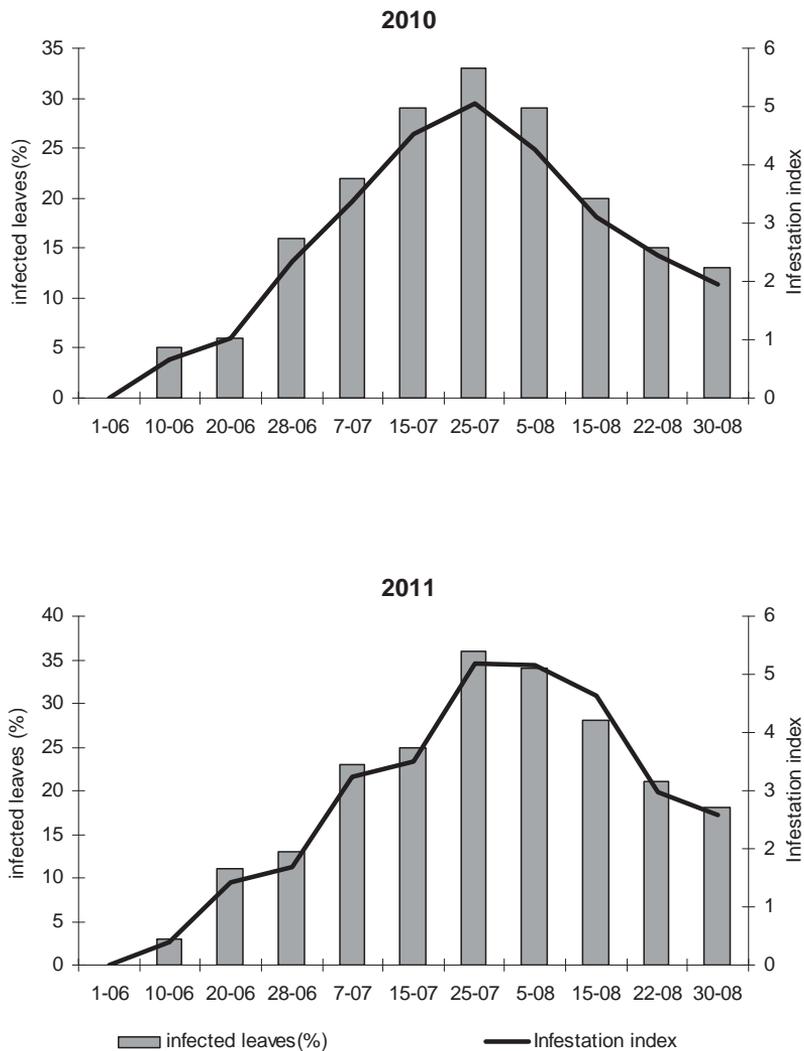


Fig. 3. The dynamics of disease symptoms caused by *Monilinia seaveri* on the leaves of the black cherry in 2010–2011

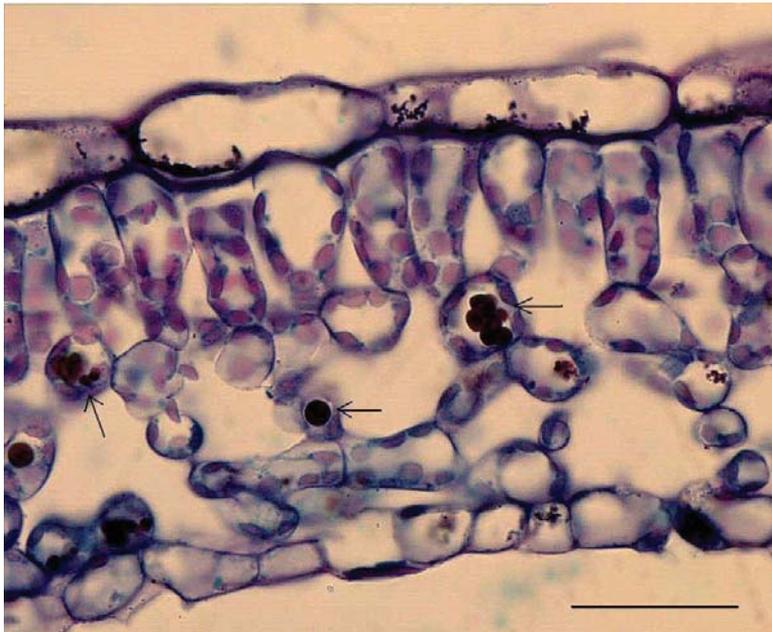
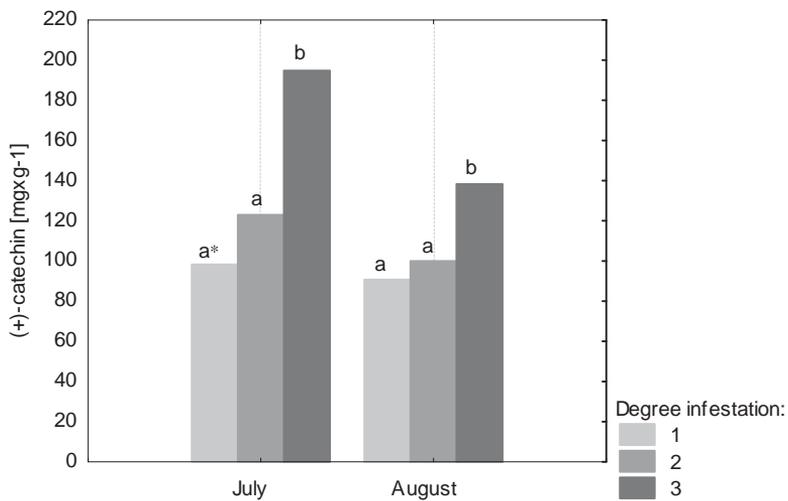


Fig. 4. Cross-section through the leaf of *Prunus serotina* infested by *Monilinia seaveri*, July 2010. The arrows indicate cells containing granulated tannin particles. Scale bar 30  $\mu\text{m}$



\* means in month for different degree infestation accompanied by the same letter do not differ significantly (Kruskall-Wallis ANOVA,  $p < 0.05$ ;  $n = 15$ )

Fig. 5. Concentration of condensed tannins in *Prunus serotina* leaves as dependent on the degree infestation (see tab. 1) for two summer months separately, expressed as (+)-catechin

Table 1. Concentration of condensed tannins in *Prunus serotina* leaves in consecutive summer months, expressed as (+)-catechin

Scale of leaf infestation		June	July	August	H / U	p
Degree	leaf area infested	mean $\pm$ SD	mean $\pm$ SD	mean $\pm$ SD		
1	0%	33.57 $\pm$ 14.80a	98.28 $\pm$ 23.86b	90.75 $\pm$ 29.38b	17.6754*	0.0001*
2	1–24 %	no data	123.03 $\pm$ 22.51a	99.97 $\pm$ 17.83a	18.0000**	0.0521**
3	25–50 %	no data	194.86 $\pm$ 23.03b	138.30 $\pm$ 17.96a	4.0000**	0.0015**
4	51–75%	no data	no data	no data	-	-

\* means of months (in row) accompanied by the same letter do not differ significantly (Kruskall-Wallis ANOVA,  $p < 0.05$ ;  $n = 15$ )

\*\* means of months (in rows) accompanied by the same letter do not differ significantly (Mann-Whitney U-test,  $p < 0.05$ ;  $n = 15$ )

localization of the tannin compounds in *P. serotina* leaf in July, with disease symptoms covering 10% of the leaf blade is shown in Figure 4.

When the differences in the (+)-catechin content between the healthy and infected leaves were analyzed on the same sampling date, a regular pattern was observed (fig. 5). In the leaves of *P. serotina* with low level of infestation by *M. seaveri*, both in July and August, only small alterations in (+)-catechin content were observed compared to the healthy leaves. At the same time the increase of the area of infected leaves was accompanied by the significantly enhanced (+)-catechin synthesis.

## DISCUSSION

The leaf infection of the black cherry by the pathogenic *Monilinia seaveri* had triggered, in the first place, the typical hypersensitive response, that has been also reported in other trophic systems [Kombrink and Schmelzer 2001]. The infestation, observed on the leaves of *Prunus serotina* in the form of necrotic spots, remained, for the two consecutive seasons, at a relatively constant average level of 35%, which is moderate compared to the infestation observed in forest areas, where the leaf litter remains at the forest floor for the winter time. In a 3-year research in such habitats, accomplished by Halarewicz and Płaskowska [2011], the disease symptoms had been found on approximately 54% of the sampled leaves and the necrotic spots were covering nearly all the LA. Presumably, the leaf raking and incinerating, performed in autumn each year in the garden observed in the present study, had played a role in reducing the volume of primary inoculum in spring time.

It appears that the seasonal accumulation of the (+)-catechin in the healthy leaves of *Prunus serotina* should not necessarily be considered as a uniform process. In the study of Riipi et al. [2002] the concentrations of most phenolic compounds in white birch leaves increased in the spring and then decreased throughout most of the summer, but concentrations of catechins remained low throughout the season. It therefore seems

likely that, for the multiple functions of the condensed tannins in plants, their accumulation is variable and that is why differences can be observed between plants. Among the external abiotic factors only the lighting conditions (full insolation) cause the increase in the tannin content in leaves, yet this is only true for some of the tree species, namely *Prunus* sp. and *Corylus avellana* [Žmuda et al. 2008]. So far, no direct relation has been found between the air temperature and humidity and the content of these compounds in plant tissues. Nevertheless, the weather conditions throughout the season induce the development of pathogenic fungi, that becomes transduced, indirectly, into plant defensive response.

Another, separate issue is the fluctuating content of the condensed tannins in the leaves with disease symptoms caused of *Monilinia seaveri*, as compared to the uninfected leaves. According to Bonello et al. [2006], the defensive biochemical mechanisms aimed at pathogenic colonization, that have evolved in trees, trigger the relevant changes in the metabolism of infected cells in a relatively short time following their infection. Therefore the low response level of *P. serotina* to the infection by *M. seaveri* observed in the present study might be more likely associated with the host tolerance reaction than with the direct mobilization of biochemical response. Witzell and Martin [2008] have also shown that in natural forest stands the tolerance to negligible levels of disease rather than activation of defensive mechanisms may be more ecologically viable, as it brings about lower energetic costs associated with the latter.

In some woody plants phenolics (especially condensed tannins) have been studied as markers for detection of plant resistance to pathogens. Based on the research accomplished in several clones of *Salix myrsinifolia* it has been found that infection by pathogenic rust (*Melampsora* spp.) increased the concentrations of the studied phenolic compounds irrespectively of the level of the infection [Hakulinen 1998, Hakulinen and Julkunen-Tiitto 2000]. Contrary to that, no significant increase in condensed tannin content was observed in response to inoculation of the pedunculate oak (*Quercus robur* L.) seedlings with oak powdery mildew (*Microsphaera alphitoides* Griff. et Maubl.) [Grzebyta et al. 2005]. The significant increase in (+)-catechin content of the leaves of *P. serotina* that show disease symptoms on > 25% of their leaf area, indicates the involvement of the condensed tannins in the plant response to the infection by *M. seaveri*. A similar biochemical response of a plant to a pathogen attack was observed in Norway spruce (*Picea abies* (L.) Karst.). Lieutier et al. [2003] and Brignolas et al. [1995] had recorded increases in phloem (+)-catechin synthesis of the observed trees caused by wound inoculations with blue-stain fungus *Ceratocystis polonica* (Siem.). Evensen et al. [2000] had demonstrated that the infection by *C. polonica* resulted in the increased (+)-catechin content in the bark extract from the infected trees compared to healthy plants. Presumably, the resistance of *P. abies*, to the bark beetle-associated fungus such as *C. polonica* is specifically dependent on the ability of this tree to activate the synthesis of phenolic compounds [Brignolas et al. 1995].

Based on the present analyses it may be concluded that the catechin content in the leaves of *P. serotina* decreases by the end of the plant vegetation irrespectively of the pathogenic infection, and that it is lower in August, compared to July. Perhaps this trend should be seen as a background for the less apparent differences in (+)-catechin concentration between healthy and diseased plants observed in August. Nevertheless, this

statement may not necessarily apply to all individuals of *P. serotina* infected by *M. seaveri*. Even in a particular tree species in trophic relation with a particular pathogen, highly specific interactions are observed between the plant's secondary metabolites and its natural enemy. Hakulinen et al. [1999] have demonstrated that in the leaves of willow (*Salix myrsinifolia* Salisb.), the (+)-catechin levels induced by *Melampsora* rust may both depend on the individual plant's genotype and on the stage of rust development.

Regardless of the abundant and diverse research on the subject, the role of phenolics in tree-pathogen interactions remains still unclear. It also requires further study in order to better understand the regulation of phenolic metabolism in attacked and diseased trees, before the potential of these compounds can be used practically [Witzell and Martin 2008]. As Martin-Tanguy [1997] suggests, it is also possible that the activity of phenolics provides indirect plant protection against the fungal damage, by their working as antioxidants or as activity modulators of other metabolites (polyamines). Such function of phenolics may be then more closely integrated with tolerance than with direct defense mechanisms [Kuć 1982, 2001, Haukioja and Koricheva 2000].

Therefore, in spite of some degree of ambiguity found in the published literature on the role of condensed tannins in plant-pathogen interactions, the data reported in the present paper support, in our view, the hypothesis linking (+)-catechin fluctuations in *P. serotina* to the infection by *M. seaveri*.

## CONCLUSIONS

The mechanisms by which the induced resistance of plants affects the process of disease development in them are not completely understood yet. Hence, any work that provides a case study on the subject may be valuable in shedding some extra light on it. The presented data had helped to establish that there is indeed a relation between the extent of leaf infestation of the black cherry by *Monilinia seaveri* and the concentration level in them of (+)-catechin, the compound belonging to the condensed tannins group. The obtained results point at the fact that it must be no less than the approximate 25% LA damage level, that is capable of inducing the systemic resistance response in plant. This resistance is then evinced as the increased concentration of (+)-catechin in plant tissue, which constitutes an effective defensive measure, likely protecting the plant leaves against the further, excessive infestation by *M. seaveri*.

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## WPLYW INFEKCJI *Monilinia seaveri* (Rehm) Honey NA ZAWARTOŚĆ TANIN SKONDENSOWANYCH W LIŚCIACH *Prunus serotina* Ehrh

**Streszczenie.** Obecność wrogów naturalnych wywołuje w roślinach różne reakcje obronne. Pomimo wielu prac badawczych, rola związków fenolowych w oddziaływaniach drzewo-patogen jest wciąż niejednoznaczna. Celem badań była analiza zawartości tanin w liściach czeremchy amerykańskiej (*Prunus serotina*) porażonych w różnym stopniu przez *Monilinia seaveri*. Obserwacje zdrowotności liści prowadzono na znakowanych drzewach przez 2 lata. Identyfikacji patogenu dokonano na podstawie materiału z hodowli laboratoryjnej. Zawartość tanin, wyrażonych jako (+)-katechina, określono metodą waniolinową. Największe nasilenie choroby zaobserwowano pod koniec lipca. Stwierdzono, że przy zmianach chorobowych obejmujących więcej niż 25% powierzchni liści zawartość (+)-katechiny była istotnie większa w porównaniu z liśćmi zdrowymi. Uzyskane wyniki wskazują na to, że przy opisanym poziomie uszkodzenia liści w roślinach zostają uaktywnione mechanizmy indukowanej odporności, co chroni je przed dalszym rozwojem choroby.

**Słowa kluczowe:** czeremcha amerykańska, monilioza, odporność indukowana, (+)-katechina