BIOCHEMICAL RESPONSES OF PEACH LEAVES INFECTED WITH TAPHRINA DEFORMANS BERK/TUL.

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Abstract


The phytopathogenic fungus Taphrina deformans causing the so called “leaf curl disease” in peach trees leads to severe yield losses due to the development of leaf hypertrophy and subsequent necrosis and scission. Because of its economic importance, the molecular mechanisms underlying the onset and progression of the disease are of considerable interest to the agricultural science. In this study various biochemical parameters, including the activities of the antioxidant enzymes guaiacol peroxidase, syringaldazine peroxidase and catalase, total polyphenols and anthocyanin content, concentration of free proline, antiradical activity and quantity of plastid pigments, were characterized. All these were measured in both leaves with clear symptoms and distally situated leaves from the same plant that show no signs of the infection. The results demonstrate that the pathogen induces considerable biochemical changes concerning enzymatic and non-enzymatic elements of the plant defense and antioxidant systems. Moreover, it seems that the fungus provokes a systemic response detectable even in the tissues without observable symptoms.

Keywords: antioxidants, enzyme activity, leaf curl, Prunus persica, Taphrina deformans

INTRODUCTION

The leaf curl disease in peach (Prunus persica L. Batsch), caused by the phytopathogenic fungus Taphrina deformans Berk/Tul, is an economically important condition, which results in significant yield losses in the regions with temperate climate. In years characterized with a humid and cool spring season the inadequate pest management often leads to epiphytotic outbursts of the disease. Its typical symptoms include premature senescence, necrosis and scission of the affected hypertrophic leaves. This, in turn, affects negatively the development and resistance of the entire plant towards adverse environmental conditions. The embryonic infection of peach leaves with Taphrina deformans causes dramatic cytohistological changes, hypertrophy and hyperplasia, that manifest as chlorosis, thickening and deformation of leaves, shoots and fruit, and ultimate necrosis of the infected organs (Caporali, 1964).

Taphrina deformans Berk/Tul. is a bitrophic, dimorphic, monocyclic phytopathogenic fungus (Mix, 1935). It is able to affect not only individual leaves, but also systemically infect young shoots, which most often necrotize and die as a result. In some cases the symptoms of the disease manifest at later stages, in the period of June-July, which suggests summer infections with the pathogen (Agrios, 2005). However, such late symptoms may be also the consequence of systemically developed parasitizing mycelia in the young shoots, which causes leaf deformation by temperature decrease and humidity increase during cool and damp summer seasons. The fungus hyphae are entirely intracellular and obtain nutrients from adjacent cells, which determine the ability for systemic
development of the pathogen (Mehrotra and Aggarwal, 2013).

In normal conditions, pathogen attacks trigger both active and passive defensive mechanisms in plants. The active protection includes de novo synthesis of proteins, mainly pathogenesis-related (PR) proteins, and is regulated by an intricate network of signaling pathways. A prominent class among them is class III plant peroxidases. They participate in numerous physiological processes, including the hypersensitive response (HR),which induces programmed cell death PCD in order to limit the systemic spread of the invader (Almagro et al., 2008). At the same time, passive defenses count mainly on creating and supporting physical and chemical barriers which hinder the access to susceptible tissues. However, the establishment of the contact between the host and the pathogen, provokes a complex system of interactions. With their elicitor, chemical and toxic effects, the pathogenic organisms in many cases inhibit the adequate plant cell responses towards the ensuing biotic stress. The latter results in physiological, biochemical, morphological and ultrastructural changes in the host plant (Park, 2006).

An important aspect of the considerable current interest on the topic related to the leaf curl disease is the determination of the physiological and biochemical status of the infected leaves, which provides information for the extent of the biotic stress. Therefore, the aim of the present work was to investigate some biochemical markers of the enzymatic and non-enzymatic defense in peach during biotic stress, provoked by Taphrina deformans.

MATERIALS AND METHODS

Plant material and growing conditions: The samples for analyses were taken from healthy and naturally infected with Taphrina deformans peach trees (Prunus persica L. Batsch) of the variety Fayette, in the end of April 2014 and 2015, in the region of Plovdiv, Bulgaria. The following variants were studied: 1. healthy leaves of control non-infected plants; 2. red curled leaves, rich in anthocyanins, of infected plants; 3. chlorotic curled leaves of infected plants; 4. distally situated leaves, without symptoms of the disease, of infected plants.

Analyses: The plant material was harvested in the period of peak development of the disease. Each variant was tested in mixed samples in 4 replications.

Antioxidant enzymes: The activities of three antioxidant enzymes were measured. These were guaiacol peroxidase, syringaldazine peroxidase and catalase. To obtain the enzyme extract, 1 gram of fresh plant material was homogenized in 5 ml ice-cold 0.1 M Tris-HCl (pH 7.8) extraction buffer containing 1 mM DTT and 1mM EDTA. After that the samples were centrifuged at 13,500 g (4°C for 10 min). The supernatant was used to determine the enzymatic activities spectrophotometrically on a UV/VIS spectrophotometer Pharo 300, according to the methodology of Mocquot et al. (1996).

Catalase (CAT) (EC 1.11.1.6): The activity of this antioxidant enzyme was measured by the method of Aebi (1984) at 240 nm wavelength. The reaction mixture in the cuvette contained: 2.4 ml 0.1M KH$_2$PO$_4$ (pH 7.0), 500 μl 5 mM H$_2$O$_2$ and 100 μl enzyme extract.

Syringaldazine peroxidase (SPOD) (EC 1.11.35): This group of peroxidases is characteristic for the apoplast and is able to use syringaldazine as the electron donor. SPOD participates in the processes of lignification. SPOD activity was measured at 550 nm, following the method of Imberty et al. (1985) in a quartz cuvette containing the following reaction mixture: 2.55 ml 0.1 M Tris-HCl buffer (pH 7.5), 300 μl 10 mM H$_2$O$_2$, 50 μl 3.5 mM syringaldazine and 100 μl enzyme extract.

Guaiacol peroxidase (GPOD) (EC 1.11.1.7): The activity of this group of enzymes was measured at 436 nm according to Bergmeyer (1974). The reaction mixture in the cuvette contained: 2.3 ml 0.1 M KH$_2$PO$_4$ (pH 7.0), 300 μl 5 mM H$_2$O$_2$, 300 μl guaiacol and 100 μl enzyme extract.

Total phenolics content: The total amount of phenolic compounds in the plant extracts was determined with the reagent of Folin-Ciocalteu (Waterman and Mole, 1994) following the methodology of Singleton et al. (1999), with slight modifications. The samples (1 g of fresh leaf material) were ground with quartz sand and 10 ml 60% acidic methanol, and submerged in an ultrasound bath for 15 min. The homogenized material was then transferred to suitable tubes, which were carefully sealed and left for 15 hours in the dark at room temperature for extraction. During the incubation period, the tubes were periodically stirred. Afterwards, the tubes were centrifuged and the supernatant, which was used for the measurement of total phenolics, anthocyanins and antiradical activity, was carefully collected in new clean tubes. For the determination of total phenolics were mixed 40 μl of extract, 3,160 μl distilled water, 200 μl Folin-Ciocalteu reagent and after a minute were added 600 μl 20% NaCO$_3$. The test tubes were left for 2 hours at room temperature for the reaction to occur. After that the extinction at 765 nm wavelength. Total phenolics were calculated as gallic acid equivalents (GAE) using a standard curve and are presented as mg/g fresh weight. The standard curve was prepared with gallic acid (Sigma-Aldrich, St. Louis, MO) in the range 0–500 mg/l.

Total anthocyanin content: The measurement of the amount of monomeric anthocyanins was performed with the pH-differential method (Guisti and Wrolstad, 2001). The plant extracts with acidic methanol (as described in the procedure for phenolics measurement) were diluted with a buffer (0.025 mol/l potassium chloride), adjusted to pH = 1 with HCl, and another buffer (0.4 mol/l sodium acetate) with pH = 4.5. Each sample was diluted to a certain extent with the first buffer with pH = 1.
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(which gives the value DF) and the absorption was measured at 520 nm and 700 nm. A second aliquot of each sample was diluted to the same extent with the second buffer with pH = 4.5 and again the extinction at 520 nm an 700 nm was measured. To calculate total anthocyanin content, the absorption values were used in the following formula:

\[ A = (A_{520} - A_{700})_{pH \text{ 1.0}} - (A_{520} - A_{700})_{pH \text{ 4.5}} \]

\[ TA = (A^* MW^* DF^*1000)/\varepsilon^*1 \]

where: the molar extinction coefficient (\(\varepsilon\)) for cyanidin-3-glucoside = 26,900 (M\(^{-1}\) cm\(^{-1}\)), the molecular weight (MW) of cyanidin-3-glucoside = 449.2 g/mol and the dilution factor (DF) demonstrates the times dilution of the sample. The results (TA – total anthocyanins) are expressed as mg cyanidin-3-glucoside chloride/100 g fresh plant material used.

**Antiradical activity:** This parameter was measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Beta et al. 2007) in extracts obtained in the way described above for total phenolics. The incubation mixture contained 100 µl extract and 3.9 ml 6×10\(^{-5}\) mol/l DPPH (0.06 µmol/l). The extract absorption was determined at 515 nm at min 0 and 30 from

1: Activity of antioxidant enzymes in peach leaves: variant 1 – control (healthy leaves from non-infected plants), variant 2 – infected leaves with anthocyanin coloration, variant 3 – chlorotic infected leaves, variant 4 – distally situated leaves of infected plants, with no evident symptoms. A) Guaiacol peroxidase, B) Syringladazine peroxidase, C) Catalase. The same letters above the bars indicate lack of statistical significance at P < 0.05.
RESULTS

The activity of the antioxidant enzymes catalase, guaiacol and syringaldazine peroxidases in healthy and infected with *Taphrina deformans* peach leaves is presented on Fig. 1. A considerable increase in the activity of guaiacol peroxidase was observed in variants 2 (3.89 U/g FW) and 3 (1.79 U/g FW) in comparison to the control (0.46 U/g FW), which may be due to a role of the enzyme in the defensive mechanisms against the pathogen (Fig. 1A). The analysis of syringaldazine peroxidase demonstrated a significant augmentation of the enzyme activity in all infected variants in comparison to the control (0.02 U/g FW) (Fig. 1B).

In the leaves with clearly observable symptoms (variants 2 and 3) the values of the enzyme activity reached 0.44 U/g FW, while in the distally situated leaves without symptoms they were 0.27 U/g FW, which was still 10 times higher than the controls. Together, the increase in the activity of both peroxidases suggests their involvement in the defensive system of the host plant against the fungal infection.

Interestingly, the measurement of catalase activity (shown on Fig. 1C) demonstrated an opposite trend: in this case the highest values were observed in the healthy leaves (variant 1 – 0.2 U/g FW), followed by the leaves of infected plants with no symptoms of the disease (variant 4 – 0.1 U/g FW). In the other 2 variants, in which the symptoms were manifested, the enzyme activity was significantly lower (variant 2 – 0.04 U/g FW and variant 3 – 0.08 U/g FW). This phenomenon can be explained with the existing competition between catalase and peroxidases for the same substrate – hydrogen peroxide.

Tab. I summarizes the results of the analyses of total polyphenols content, anthocyanin quantity and antioxidative activity. Leaves infected with *Taphrina deformans* had decreased amounts of polyphenol compounds, which was more prominent in the variants with manifested symptoms (Tab. I, variant 2 – 48.51 mg and variant 3 – 69.84 mg) in comparison to the control (variant 1 – 110.11 mg). These results are correlated with the ones obtained for the activity of syringaldazine peroxidase. In variants 2 and 3, characterized with the highest peroxidase activity, were detected the lowest concentrations of total polyphenols. This is not surprising since phenolic compounds

<table>
<thead>
<tr>
<th>Variants</th>
<th>Total polyphenols mgGAE/g FW</th>
<th>Anthocyanins as cyanidin-3-glucoside mg/100g FW</th>
<th>Antiradical activity %DPPH/g FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110.11a</td>
<td>0.175d</td>
<td>12.76 (100%)b</td>
</tr>
<tr>
<td>2</td>
<td>48.51c</td>
<td>9.018a</td>
<td>8.05 (61%)c</td>
</tr>
<tr>
<td>3</td>
<td>69.84b</td>
<td>0.335c</td>
<td>12.94 (101%)b</td>
</tr>
<tr>
<td>4</td>
<td>105.98a</td>
<td>1.334b</td>
<td>15.67 (123%)a</td>
</tr>
</tbody>
</table>

All the values are presented as mean. The data in the columns followed by the same letter (a, b, c) are not significant for *P* < 0.05

<table>
<thead>
<tr>
<th>Variant</th>
<th>Chl. a</th>
<th>Chl. b</th>
<th>Carotenoids</th>
<th>Chlorophyll a/b</th>
<th>Chlorophylls (a+b)/carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.65a</td>
<td>0.81b</td>
<td>1.33a</td>
<td>3.28a</td>
<td>2.6b</td>
</tr>
<tr>
<td>2</td>
<td>2.04b</td>
<td>0.88a</td>
<td>1.03b</td>
<td>2.32b</td>
<td>2.83a</td>
</tr>
<tr>
<td>3</td>
<td>1.76c</td>
<td>0.84b</td>
<td>0.89c</td>
<td>2.1b</td>
<td>2.91a</td>
</tr>
<tr>
<td>4</td>
<td>2.16b</td>
<td>0.70c</td>
<td>1.05b</td>
<td>3.1a</td>
<td>2.82a</td>
</tr>
</tbody>
</table>

All the values are presented as mean. The data in the columns followed by the same letter (a, b, c) are not significant for *P* < 0.05
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are the substrate of syringaldazine peroxidase. In the experiment with anthocyanin quantification, as expected the most abundant levels were detected in variant 2 (9.01 mg/100 g FW).

In the leaves infected with Taphrina deformans the antiradical activity was also altered. It was considerably reduced, mostly in variant 2 (61% of the control), in which were detected the lowest amounts of polyphenols. It must be noted, that the latter are known to neutralize free radicals. In the other variants the antiradical activity was enhanced.

In Tab. II the quantities of plastid pigments, as well as their ratio, are presented. In the infected leaves it was observed a significant reduction of chlorophyll a (with 23, 34 and 19% for variants 2, 3 and 4, respectively) and carotenoids (with 23, 33 and 23% for variants 2, 3 and 4, respectively). The chlorophyll b content was affected to a lesser extent.

Finally, the abundance of another important antistress primary metabolite – free proline, was also tested and the results are presented on Fig. 2. Proline is an α-amino acid with a crucial role for the maintenance of the water potential of plant cells. In peach leaves infected with Taphrina deformans an increase in proline concentration was detected, but only in the variants with clear disease symptoms (variants 2 and 3 – 102% and 119% of the control, respectively).

DISCUSSION

We expand on the previous studies on the topic by analyzing various biochemical parameters not only in samples from leaves with clearly observable symptoms (variants 2 and 3), but also from tissues which do not show any defects (variant 4).

The data of the experiments indicate that in peach tree leaves the activity of some antioxidant enzymes and the concentration of some of the non-enzymatic components of the plant defense system are influenced by the infection with Taphrina deformans. Similar conclusions were drawn also by other authors studying the leaf curl disease in peach (Ciobanu, 2012). The most potent guaiacol peroxidase was measured in infected leaves with accumulated anthocyanins (Fig. 1A, variant 2), followed by the chlorotic infected leaves (Fig. 1A, variant 3). A similar result was observed with syringaldazine peroxidase as well. This may be an indication of synergistic effects of both enzymes in the response to the pathogen.

Moreover, the much higher activity of syringaldazine peroxidase in the infected organs can be linked to the role of the enzyme in the oxidation of phenolic compounds to quinones, which are more toxic for the pathogens (Mayer, 2006). Thus, by the upregulation of syringaldazine peroxidase activity, the host plant can limit or even halt the spread of the invader. This enzyme was more active even in the leaves which did not manifest any symptoms (Fig. 1B, variant 4).

The reported reduced antiradical activity in variant 2 (Tab. I) is in accordance with the data, obtained for the total polyphenols content, which is also significantly lower in infected leaves. The decrease in total polyphenols in cell walls weakens them and makes them more susceptible to the influence of the pathogen. As a result, the affected cells are more prone to hypertrophy (Semerdjieva et al., 2014). The induced biosynthesis of anthocyanins, found in all infected leaves (even those which are distal and without symptoms) can be also considered as a part of the defensive systems of the host (Tab. I).

The reasons for anthocyanin accumulation only in some of the infected leaves are not yet fully elucidated. Apart from their antioxidant properties, anthocyanins are also a carbohydrate reserve, since they exist in a glycosylated form (most often glucose). It can be presumed that their biosynthesis is stimulated by regulatory molecules released from the pathogens, which use the carbohydrates as a nutrient source or structural elements (Petit and Schneider, 1983).

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\[ 2: \text{Free proline content in healthy and infected with Taphrina deformans leaves of peach trees. Variants 1 – control (healthy leaves from non-infected plants), variant 2 – infected leaves with anthocyanin coloration, variant 3 – chlorotic infected leaves, variant 4 – distally situated leaves of infected plants, with no evident symptoms. The same letters above the bars indicate lack of statistical significance at } P < 0.05. \]
The considerably lowered catalase activity, found in the infected leaves can be related to their more active peroxidases since the primary substrate of catalase is hydrogen peroxide but it is consumed by the competing peroxidases. Other authors (Buonaurio and Montalbini, 1993) have also reported a correlation between reduced catalase and increased peroxidase activities, which is in accordance with our results. In another study, it was demonstrated that catalase was induced in infected peach leaves, but only in the initial phase of the disease. With the advancement of the infection catalase activity gradually diminished (Ciobanu, 2012).

The impact of *Taphrina deformans* on infected leaves causes a significant decrease of the quantity of plastid pigments. This effect was observed in a number of other studies with the same pathogen. Raggy (1966, 1967), for example, reported that chlorophyll concentrations in the curled leaves were lowered by 30% to 50% in comparison to controls, while according to Nikolae (2009) the reduction of this parameter amounts to 53,62%. Similarly, in the variety Redhaven Montalbini and Buonaurio (1986) documented a difference of 30% between infected and healthy leaves. They found that the decreased chlorophyll content was not caused by elevated chlorophyllase activity, as is the case with some virus infections, and Nicolae and Mitrea (2009) suggested that the reason is the inhibited biosynthesis of the pigments. Moreover, other authors like Marte and Gagiulo (1972), Syrop (1975), Bassi et al. (1984) and Huang et al. (1993) presumed that in curled leaves the number of chloroplasts could be severely reduced and the remaining chloroplasts – characterized with damaged structure of both the thylakoid network and the granae. In our study, the analysis of the ultrastructure of mesophyll cells of leaves infected with *Taphrina deformans* confirm the hypothesis that chloroplasts degenerate with the progression of the disease (unpublished results).

In the genome of *Taphrina deformans* are found genes, responsible for the biosynthesis of compounds, related to the pathogenesis – proteases, which allow the digestion of plant tissues; secondary metabolites, which facilitate the interactions of the fungal pathogen with the environment, including the host plant; and hormones which are responsible for the typical hypertrophy and hyperplasia symptoms (Cisse et al., 2013). Therefore, the structural changes in the affected leaves are related to this hormonal disbalance provoked by the pathogen. In particular, the enhanced cellular growth and water content are in correlation with an increased concentration of auxins, while the stimulated cellular division and other growth anomalies are influenced by elevated cytokinin activity. Indeed, it has been shown that the cytokinin activity and the levels of indol-3-acetic acid and tryptophan are higher, respectively with 81% and 65%, in leaves infected by *Taphrina deformans* (Sziraki et al., 1975, Ymada et al., 1990). The significant accumulation of auxins and cytokinins in these leaves probably induces their ability to attract photoassimilates. According to Goodman et al. (1986), the photoassimilates are redirected from healthy leaves, which show a higher CO\textsubscript{2} fixation, to infected ones in order to partially compensate for their carbohydrate shortage.

Finally, the measurement of the concentration of free proline indicated that it was increased in the infected leaves with observable symptoms (Fig. 2). This positively correlates with the strong hydration of the damaged cells (Piperkova and Vasilev, 2000). Similar results were obtained also by Raggy (1967), who worked with peach trees infected with the same pathogen. He observed elevation of the levels of the free amino acids proline, ornithine and glycine during the incubation period of the infection. This phenomenon could be explained by the presence of chemicals (elicitors) released by the fungus, which stimulate the biosynthesis of such compounds or by increased protein degradation in the cells of the host plant. The second hypothesis was supported by the fact that the total protein content in infected leaves was also reduced (Raggy, 1967). In another study of the same author (Raggy, 1987) it was shown that the water content of infected leaves is from 2,4 up to 9,6 times higher than in healthy leaves. It is well known that free proline serves as an osmolite and is accumulated in response to water stress: water deficit, salt stress, low-temperature stress, waterlogging conditions (Olgun et al., 2008, Hayat et al., 2012). In plants attacked by *Taphrina deformans*, the higher proline content may be caused by chemical stimuli secreted by the pathogen and/or the hyperhydrated status of the affected cells.

**CONCLUSION**

The analyzed biochemical markers confirm that the curled leaf disease caused by *Taphrina deformans* induces serious changes in the biochemical status of the infected plants, which are detectable not only in the tissues with observable symptoms, but also in distally situated ones. These changes include the elevation of the activity of antioxidant enzymes (peroxidases); reduced polyphenols content and plastid pigments; alterations of antiradical activity, anthocyanin and free proline concentrations. On one hand these results suggest a systemic character of the pathogen infection and development and on the other hand demonstrate that the entire plant goes in a state of alert by inducing its defensive systems.
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Authors' contribution
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REFERENCES


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