ISSN 1330-9862
(FTB-2197)

original scientific paper

Biochemical Basis of Apple Leaf Resistance to *Erwinia amylovora* Infection

Marija Viljevac[1], *Krunoslav Dugalić*[1], *Ivna Štolfa*[2], *Edyta Đermić*[3], *Bogdan Cvjetković*[3], *Rezica Sudar*[1], *Josip Kovačević*[1], *Vera Cesar*[2], *Hrvoje Lepeduš*[1] *and Zorica Jurković*[1,4]*

[1]Agricultural Institute Osijek, Južno predgrađe 17, HR-31000 Osijek, Croatia

[2]Department of Biology, University of J. J. Strossmayer in Osijek, Trg Lj. Gaja 6, HR-31000 Osijek, Croatia

[3]Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, HR-10000 Zagreb, Croatia

[4]Croatian Food Agency, I. Gundulića 36b, HR-31000 Osijek, Croatia

Received: November 11, 2008
Accepted: February 13, 2009

Summary

Erwinia amylovora is the most frequently found necrogenic bacterium on apple (*Malus domestica* Borkh.) trees, which causes progressive necrosis and blight of host plants. Rapid spread of bacteria through the host tissue can lead to the loss of entire trees in one growing season. In this work, the aim is to investigate long-lasting biochemical responses in leaves of two apple cultivars (Enterprise and Golden delicious). Several histochemical (polyphenols, suberin and callose) and biochemical parameters (total polyphenols, superoxide dismutase – SOD, ascorbate peroxidase – APX and guaiacol peroxidase – GPOD) were screened 60 days after *Erwinia* inoculation in order to find their potential correlation with plant resistance mechanisms to the pathogen attack. Differential susceptibility to the pathogen attack observed between the investigated cultivars was in accordance with previous studies that characterized Enterprise as less susceptible and Golden delicious as more susceptible cultivar. Infected leaves of Golden delicious expressed symptoms seen as large brown areas at the abaxial side mostly placed at the leaf margin and necrosis also found peripherally, while damage in Enterprise leaves was observed as small brown spots and sporadic leaf edge necrosis. Increased SOD and GPOD activities combined with decreased polyphenol content as well as wide cuticle suberization in cultivar Enterprise should be considered as reliable biochemical parameters characterizing its ability to develop certain resistance to the pathogen infection. Furthermore, the absence of callose deposition in leaves of Enterprise confirmed our findings that thick suberized cuticle is likely the main defense mechanism that enables long-term efficient protection of apple leaves against biotic stress caused by *Erwinia* attack.

Key words: antioxidative enzymes, apple, callose, *Erwinia amylovora*, pathogen resistance, polyphenols, suberin

Introduction

Erwinia amylovora is the first bacterium identified as a plant pathogen which causes fire blight in the species of *Rosaceae* family, primarily in apple and pear. It is a Gram-negative bacterium belonging to the family *Enterobacteriaceae*. This necrogenic bacterium provokes progressive necrosis in aerial parts of susceptible plants.

*Corresponding author; Phone: ++385 31 515 572; Fax: ++385 31 515 579; E-mail: zorica.jurkovic@poljinos.hr

This paper was presented at the Congress of Croatian Society of Biochemistry and Molecular Biology in Osijek, Croatia, September 17–20, 2008

Fire blight symptoms vary depending on the host plant and climate conditions. Symptoms are initiated by water soaking, followed by wilting and rapid necrosis seen as scorched blackened parts of infected tissue (*1*). A rapid spreading of bacteria in susceptible host under optimal climate conditions can cause the loss of entire trees in a single growing season. The main sites of *E. amylovora* infection are blossoms and wounds. Blossom infection starts in the stigma with insects or raindrops as vectors. Rainfall washes bacteria into the nectar, which is rich with nutrients for bacterial growth. Since nectaries are noncutinised, bacteria have direct access to the underlying tissue and vascular system. Accumulation of bacteria in xylem blocks the water flow to distal parts of the plant causing death of those parts (*2*). Bacteria are able to survive the winter in cankers produced by deposition of cork layers around the infected tissues, which represents the host's attempt to limit the spread of the infection (*1*).

The control of *E. amylovora* spreading is difficult because it can survive as an endophyte or epiphyte, not only in infected plants, but also in symptomless plants or plant parts. Often, the source of infection is the rootstock highly susceptible to *E. amylovora*, which has been used for grafting of healthy or symptomless material. The bacterium was found as epiphyte or endophyte on symptomless fruit but there was no evidence of dissemination of *E. amylovora* by fruit (*3*). Leaf surfaces can be colonized by bacteria arriving from earlier blossom infections or active cankers but without any damage if bacteria remain on the surface. In case of invading bacteria into the leaf tissue, infected leaves showed either necrotic patches starting from the margin of the leaf blade or blackening of the petiole and leaf midrib depending on the way the infection took place (*4*).

One of the first defense responses to the bacterial infection is rapid generation of excess reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical (*5*). ROS have direct antimicrobial activities which reduce pathogen viability. On the other hand, the enhanced ROS production can also lead to oxidative damage of pigments, proteins, nucleic acids and lipids (*6*). Plants have developed efficient protective mechanisms against oxidative stress which utilize enzymatic and non-enzymatic components in order to scavenge ROS excess. The main enzymatic components involved in the response to pathogen attack are peroxidases (ascorbate peroxidase – APX and guaiacol peroxidase – GPOD), superoxide dismutase (SOD) and catalase (CAT), while phenolic compound accumulation represents one of the main non-enzymatic components of response.

Fire blight disease severity varies from extensive damage on a highly susceptible host to limited symptoms, or lack of symptoms on a resistant host. Our hypothesis is that the severity of symptoms is a consequence of different plant resistance capacity that could be revealed as changes in histochemistry and/or some biochemical parameters such as different activities of antioxidative enzymes or total polyphenol content. In this work, the aim is to investigate long-lasting biochemical responses in leaves of two apple cultivars after *E. amylovora* inoculation in order to find reliable biochemical parameters that could be correlated with pathogen resistance mechanisms.

Materials and Methods

Plant material

Field experiments were performed on six-year-old trees of two apple cultivars (Golden delicious and Enterprise) grafted on rootstock MM-106. Plants were grown in the experimental orchard of the Agricultural Institute Osijek, Croatia. The experiment was designed as a random block in 4 repetitions with a total of 8 plants of each cultivar.

Bacteria and inoculation procedures

Artificial infection of apple cultivars was performed with two isolates of *E. amylovora* (OS 5 and OS 6). These isolates originated from the same orchard (Agricultural Institute Osijek, Croatia) where sampling of plant material was done. It was reported that the used isolates belong to the Mediterranean group Pt2 (*7*). Inoculum was prepared by overnight culture of the isolates in nutrient broth at 28 °C with constant shaking. Isolates were pooled together and diluted with sterile distilled water to the final concentration of $3.9 \cdot 10^5$ CFU/mL. Inoculation was performed by spraying one branch per tree with the prepared bacterial suspension during the flowering period (stage F-F2 according to Baggiolini *et al.* (*8*)). Leaves used for biochemical and histochemical analyses were collected from the sprayed branch 60 days after the inoculation was done.

Enzyme extractions

For enzyme extraction, five replicates were taken from the control and infected leaves. Each sample consisted of 6 leaves. After the removal of the main veins and the addition of polyvinylpyrrolidone (PVP), leaf tissue was macerated into fine powder with liquid nitrogen using pestle and mortar. About 0.2 g of leaf powder were extracted with 1 mL of 100 mM potassium phosphate buffer, pH=7.0. After centrifugation for 15 min at 18 000×*g* and 4 °C, the supernatant was taken for superoxide dismutase and guaiacol peroxidase assays. Approximately 0.2 g of leaf powder were extracted with 1 mL of 100 mM potassium phosphate buffer, pH=7.0, with 5 mM sodium ascorbate and 1 mM EDTA, and centrifuged for 15 min at 18 000×*g* and 4 °C. The supernatant was taken for ascorbate peroxidase assay.

Measurements of enzyme activities

Determination of guaiacol peroxidase (GPOD; EC 1.11.1.7) activity was done according to Siegel and Galston (*9*). Guaiacol peroxidase activity was determined by monitoring the increase in absorbance at 470 nm over 2 min. The enzymatic reaction was initiated by the addition of 100 μL of protein extract to 900 μL of reaction mixture. Reaction mixture consisted of 5 mM guaiacol and 5 mM hydrogen peroxide in 0.2 M phosphate buffer (pH=5.8). The enzyme activity was expressed as ΔA_{470}/(min·g fresh mass).

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (*10*) by monitoring the decrease in the absorbance at 290 nm over 2 min. The enzymatic reaction was started by adding 10 μL of 12 mM H_2O_2 in 990 μL of the reaction mixture. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7.0) with 0.1 mM EDTA, 50 mM ascorbic acid and 100 μL of protein extract. The enzyme activity was expressed as ΔA_{290}/(min·g fresh mass).

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (*11*). Each sample consisted of 910 μL of reaction mixture, 80 μL of diluted enzyme with 50 mM potassium phosphate buffer (pH=7.8) and 10 μL of 1 mM riboflavin. The reaction mixture contained 50 mM potassium phosphate buffer (pH=7.8), 13 mM methionin, 75 μM NBT and 0.1 mM EDTA. The absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the NBT reduction rate.

Determination of polyphenol content

Fine powder obtained by maceration of leaf tissue in liquid nitrogen without PVP was used for the extraction of polyphenols. The extraction of polyphenols was done with 95 % EtOH at –20 °C for 72 h and homogenates were centrifuged at 10 000×*g* and 4 °C for 10 min. The polyphenol content was determined according to Randhir and Shetty (*12*) by measuring absorbance at 540 nm.

Histochemical analysis

Fresh hand-made leaf sections were used for histochemical detection of suberin and callose. Suberin was stained with the alcoholic Sudan III reagent, while the presence of callose was established with 0.05 % aniline blue in HEPES buffer, pH=9.25, under UV light (*13*). Leaf tissue was cut in small pieces and fixed for 24 h in 1 % glutaraldehyde in 0.05 M phosphate buffer, pH=6.8. Then, specimens were dehydrated in a series of alcohols (two changes in each) and embedded in methacrylate resin (Historesin, Leica, Germany). Semi-thin sections, 3 μm thick, were stained with 0.05 % toluidine blue 0 in benzoate buffer, pH=4.4, for polyphenol detection (*14*). The sections were analyzed using light and fluorescent microscope (Carl Zeiss Jena, Germany). Photographs were made using Panasonic DMC-LS80 camera, USA.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), N=5 for every leaf type. The mean values were compared using the least significant difference (LSD) test. Differences were considered significant at $p \leq 0.05$. All statistical analyses were done with Statistica 7.1. software (StatSoft, Inc. 2005, USA).

Results and Discussion

Investigated cultivars revealed different levels of sensitivity to artificial inoculation with *E. amylovora*. This was in accordance with previous reports (*15,16*) where cultivar Enterprise was reported to be less susceptible, while cultivar Golden delicious was described as more susceptible. Infected leaves expressed symptoms seen as large brown areas at the abaxial side mostly placed at the leaf margin and necrosis also found peripherally (Fig. 1). Described symptoms were totally absent from control leaves of cultivar Enterprise (Fig. 1a), while infected leaves of the same cultivar had only small brown spots and few necrotic areas (Fig. 1b). Symptoms were more pronounced in cultivar Golden delicious (Figs. 1c and d), where control leaves expressed a certain level of injury as well. Since Golden delicious is a second apple cultivar in Croatia concerning the production quantity and cultivation area, known to be fire blight susceptible, it was chosen as a control cultivar in our study and compared to the cultivar Enterprise, reported to be considerably less susceptible to *E. amylovora* infection. In accordance with previous reports (*15,16*), these two apple cultivars revealed different levels of sensitivity to artificial inoculation with *E. amylovora*. Disease symptoms were totally absent from control leaves of cultivar Enterprise (Fig. 1a), while infected leaves of the same cultivar had only small brown spots and few necrotic areas (Fig. 1b). In contrast, infected Golden delicious leaves displayed large brown areas on the abaxial side, mostly along the leaf margin, as well as necrosis found peripherally (Fig. 1d), and even control leaves expressed a certain level of injury (Fig. 1c). This is not surprising, because control leaves can be indirectly affected by the bacterial toxin amylovoran, which causes aggregation of cytoplasm and organelle disorganization of mesophyll cells (*17*).

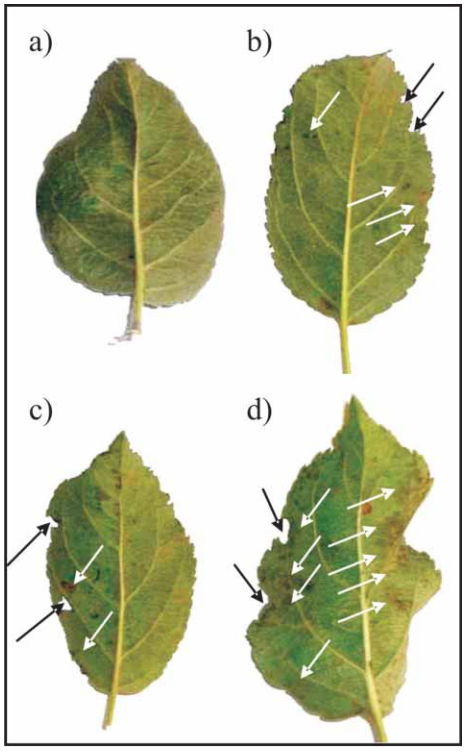

Fig. 1. Control (a, c) and infected (b, d) leaves of cvs. Enterprise (a, b) and Golden delicious (c, d). Infected leaves expressed symptoms seen as brown spots (white arrows) at the abaxial side of the leaf as well as necrosis on the leaf edge (black arrows)

The first line of defense against pathogen attack in vascular plants is cuticle on the surface of the plant body. It consists of a polymer matrix called cutin surrounded and overlaid with wax. Frequently, the cuticle is also embedded with suberin, which further contributes to reducing water loss and uncontrolled transport of dissolved ions and gases, provides an outer barrier protecting the organ from pathogens (*18*). Usually, the thickness of the cuticle was considered the most important structural feature that distinguishes pathogen resistant plant species, cultivars or lines from the susceptible ones (*19*). Histological and histochemical characterization of cuticles in the leaves of two apple cultivars under investigation is shown in Fig. 2. Cultivar Enterprise (A, B, E and F) was characterized by a thick suberized cuticle in both control (A and E) and infected (B and F) leaves, while the cuticle of cultivar Golden delicious (C, D, G and H) was much thinner and less suberized in control (C and G) and infected (D and H) leaves. The thick suberized cuticle that was observed in leaves of cultivar Enterprise (Fig. 2) may be an important resistance mechanism against *Erwinia* attack. Staining tissue sections with phloroglucinol-HCl in order to check the lignin presence in the epidermal cell walls appeared negative in all cultivars (photos not shown), indicating that lignin was absent.

As reported by Franke and Schreiber (*20*), the final step in biosynthesis of suberin involves linking aliphatic polyester with polyaromatic domain (hydroxycinnamic acids and derivates) synthesized in phenylpropanoid pathway by apoplastic peroxidase enzymes. The presence of hydrogen peroxide is needed for oxidation of such polyphenolic substrates by peroxidases (*21*). In order to investigate the biochemical background of the observed differences in leaf structure (Fig. 2) between the studied cultivars, the concentration of total polyphenols and GPOD activities were determined. The mean values of total polyphenols in infected and control leaves of the two investigated cultivars are shown in Fig. 3a. Control leaves of cultivar Enterprise had 9.87 mg per g FM of polyphenols, while the mean value in the infected leaves of the same cultivar was significantly lower (7.59 mg per g FM). In contrast, there were no significant differences observed in the polyphenol content between the control and infected leaves of cultivar Golden delicious (9.87 and 9.02 mg per g FM in control and infected leaves, respectively). The values of GPOD activities were shown to be increased in infected leaves compared to control ones in both investigated cultivars (Fig. 3b). The mean values of GPOD activities were as follows: 8.18 and 14.23 ΔA_{470}/(min·g FM) in control and infected leaves of Enterprise, respectively, and 9.66 and 12.54 ΔA_{470}/(min·g FM) in control and infected leaves of Golden delicious

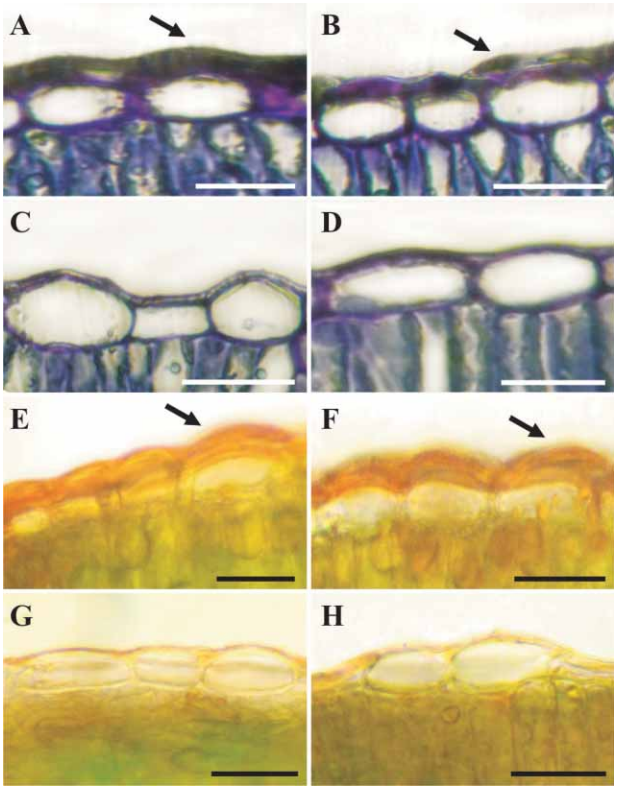

Fig. 2. *In situ* detection of polyphenols (A-D) and suberin (E-H) in transversal cross-sections of control (A, C, E and G) and infected (B, D, F and H) apple leaves. Cv. Enterprise (A, B, E and F) was characterised by thick suberized cuticle (arrows), while cuticle of cv. Golden delicious (C, D, G and H) was thinner and less suberized. Scale bar=20 μm

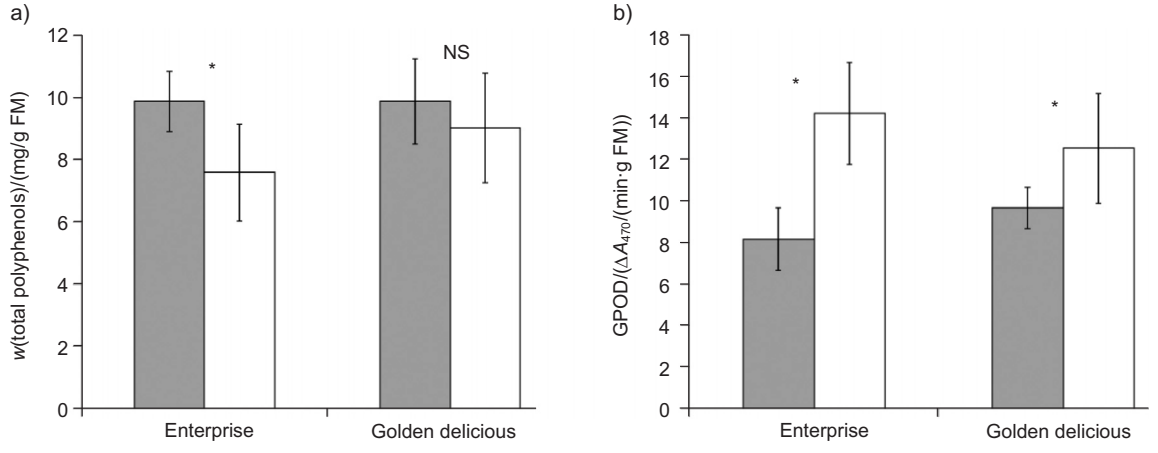

Fig. 3. The mean values of (a) total polyphenols and (b) GPOD activity in control (grey) and infected (white) leaves of cvs. Enterprise and Golden delicious; *p<0.05, NS not significant, FM fresh mass. Bar indicates standard deviation

cultivar. It was shown that changes in GPOD activity reported in response to environmental stresses (*e.g.* drought stress) reflect modified cell wall biochemistry (*22*). Since there was no significant difference between GPOD activities in infected leaves of Golden delicious and Enterprise cultivars ($p>0.05$), a decisive role of GPOD in cuticle thickening is questionable. However, it should be noted that cultivar Enterprise revealed much higher induction of GPOD activity upon *Erwinia* infection (74 %) than Golden delicious (30 %). This might be in accordance with the assumption that the observed increase in GPOD activity in cultivar Enterprise (Fig. 3b) is related to suberin biosynthesis and cuticle thickening, which provides its lower sensitivity to *Erwinia* attack compared to the leaves of Golden delicious. This, however, needs to be further investigated by measuring GPOD activities in intracellular washing fluid and/or in cell wall preparations.

Besides emphasized role in the secondary metabolic modifications of the cell wall (*e.g.* suberization and lignification), GPOD enzymes play a role in the plethora of plant physiological responses such as auxin catabolism (*23,24*), heavy metal loads (*25*), wound healing (*26*), UVB and ozone protection (*27*), pathogen defences (*28*) and aging (*29*). Together with other antioxidative enzymes (SOD, APX, CAT, *etc.*), GPOD enzymes are involved in scavenging of the reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) or singlet oxygen ($^1O_2$) produced during pathogen attack (*30*). Therefore, in this study the activities of SOD and APX, the main antioxidative enzymes in green tissues, were investigated. The results of APX assay are shown in Fig. 4a. Significantly decreased APX activities were found in infected leaves of both investigated cultivars: values in Enterprise were 8.07 ΔA_{290}/ /(min·g FM) in control and 4.95 ΔA_{290}/(min·g FM) in infected leaves, and 9.93 and 6.35 ΔA_{290}/(min·g FM) in control and infected leaves of Golden delicious, respectively. Mean values of SOD activities in infected and control leaves of the investigated cultivars are represented in Fig. 4b. The mean value of SOD activity in control leaves of Enterprise was 48.72 U/g FM, while in infected leaves this value increased (64.19 U/g FM). On the contrary, the mean values of SOD activities significantly decreased in infected leaves of Golden delicious (34.53 U/g FM) in comparison with control leaves (58.63 U/g FM). Venisse *et al*. (*31*) provided evidence of the involvement of an oxidative stress upon infection of pear (*Pyrus communis* L.) with *E. amylovora*. They showed that activities of APX and GPOD in leaves increased, which indicates their involvement in defense response. Investigation of the effects of *Fusarium* on tomato (*Solanum lycopersicum* L.) root done by Mandal *et al*. (*6*) revealed similar results including increased SOD activity as a key enzyme involved in the elimination of superoxide anions and production of hydrogen peroxide. Increased activities of GPOD and SOD in *Erwinia*-infected leaves of cultivar Enterprise that we report here (Figs. 3b and 4b) are consistent with previously mentioned findings. However, the activities of APX decreased in infected leaves of both investigated cultivars (Enterprise and Golden delicious) (Fig. 4a). Since APX is known as the main H_2O_2 scavenging enzyme in chloroplasts (*32*), the decrease in its activity might be connected with oxidative damage that would lead to chloroplast disorganization and degradation of photosynthetic pigments, resulting in the appearance of brown spots on the *Erwinia*-infected leaves that were revealed in the present investigation (Figs. 1b and d). Also, the observed data on the changes in the antioxidant enzyme activities corroborate the theory of the hypersensitive response (HR), with which plants cope with pathogen attack (*33*). HR is accompanied by the production of ROS, namely H_2O_2 and also involves salicylic acid (SA) and NO, which activate many plant defense responses, including programmed cell death (PCD). This increase in ROS abundance during HR is achieved by SA and NO, which suppress the biosynthesis and activity of APX (*34*) and CAT (*35*), the key H_2O_2 scavenging enzymes. Furthermore, the increase in H_2O_2 production in infected tissue of cultivar Enterprise was achieved due to the increase in SOD activity (Fig. 4b). These observations are in agreement with the general idea of the role of antioxidant enzymes in the plant response to pathogen attack.

Another quick response of plants to different biotic and abiotic stresses that involve increase in plasma membrane permeability influenced by ROS is callose (β-1→3 glucan) biosynthesis (*17*). *In situ* detection of callose in

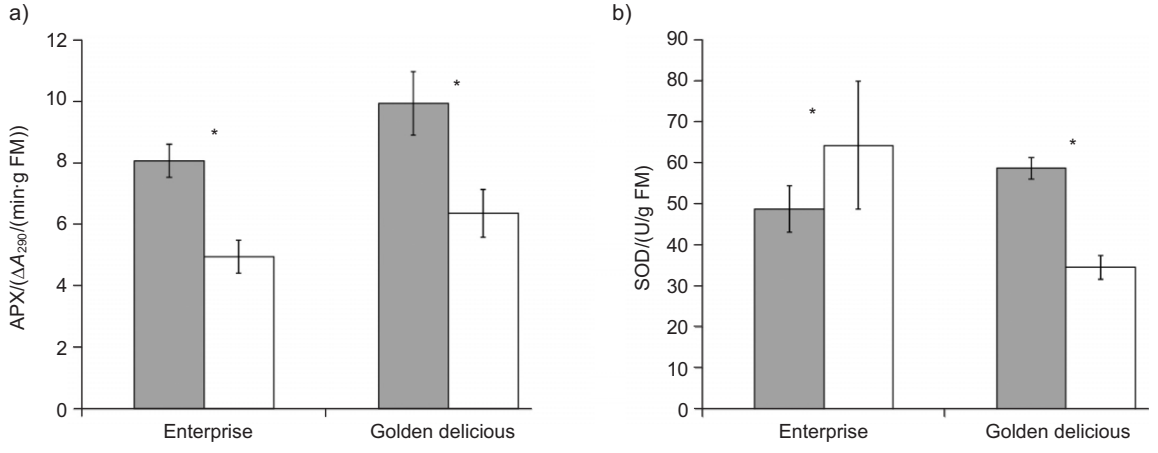

Fig. 4. The mean values of (a) APX and (b) SOD activities in control (grey) and infected (white) leaves of cvs. Enterprise and Golden delicious; *$p<0.05$, NS not significant, FM fresh mass. Bar indicates standard deviation

upper and lower leaf epidermis of the investigated apple cultivars is shown in Fig. 5. Since thick suberized cuticle was sufficient to prevent penetration of bacteria in the leaf of cultivar Enterprise, callose deposits were absent from the epidermal cell layer of both control and infected leaves of cultivar Enterprise (Figs. 5a-d). In contrast, callose clusters were detected in the leaves of cultivar Golden delicious (Figs. 5e-h) and were more abundant in the infected leaves (G and H) compared to the control (E and F).

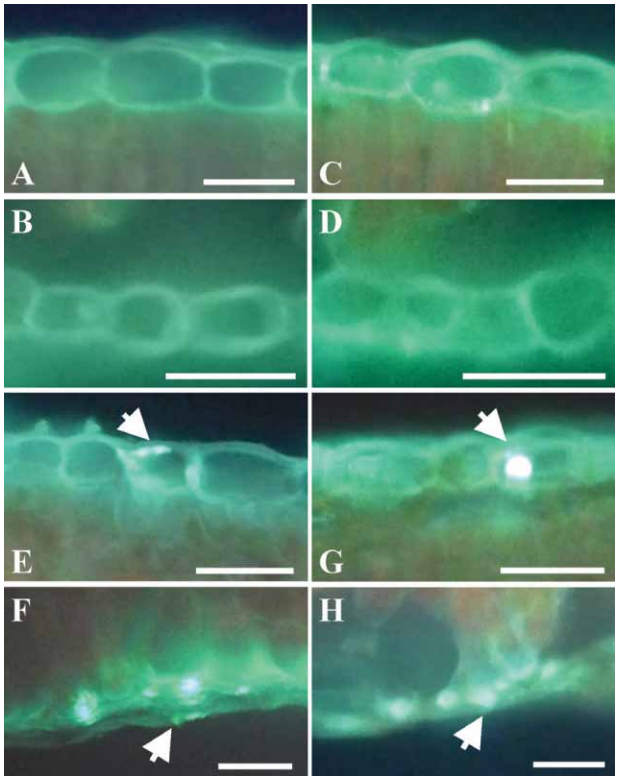

Fig. 5. *In situ* callose detection in cells of upper (A, C, E and G) and lower (B, D, F and H) apple leaf epidermis. The callose was absent from epidermal cell layer of control (A, B) and infected (C, D) leaves of cv. Enterprise. In cv. Golden delicious callose was shaped as small clusters (arrows) in control leaves (E, F), while in infected ones clusters were more abundant (G, H). Scale bar=20 μm

Recently, DebRoy *et al.* (*36*) have shown that molecular mechanisms of *Erwinia* pathogenesis on apple leaves involve the suppression of callose deposition in the cell wall, which greatly reduces the ability of leaves to resist bacterial infection. Therefore, even the presence of callose as early defense mechanism in leaves of Golden delicious cultivar characterized by thin cuticle is not sufficient to provide adequate protection against *Erwinia* attack.

Conclusion

In conclusion, results of this study demonstrate a difference in sensitivity of the investigated apple cultivars to *Erwinia* infection. Cultivar Enterprise exhibited lesser damage than cultivar Golden delicious. Such different susceptibility to the pathogen attack could be attributed to distinct structural and biochemical features of the leaves. Increased SOD as well as considerably thicker suberized cuticle that were observed in cultivar Enterprise may be important parameters characterizing the resistance of this cultivar to the pathogen infection. Furthermore, the absence of callose deposition from leaves of cultivar Enterprise supports our conclusion that structural changes of the epidermal cell walls, namely increased cuticle thickness and suberization, are the main defense mechanisms that enable efficient protection of apple leaves against *E. amylovora* attack.

Acknowledgements

This work was supported by the scientific research grants to Zorica Jurković (073-1781844-1930), Hrvoje Lepeduš (073-0731674-1673), Vera Cesar (073-0731674-0841), Josip Kovačević (073-0731673-0552) and Bogdan Cvjetković (178-1781844-2692).

References

1. J.A. Eastgate, *Erwinia amylovora*: The molecular basis of fireblight disease, *Mol. Plant Pathol. 1* (2000) 325–329.
2. C.S. Oh, S.V. Beer, Molecular genetics of *Erwinia amylovora* involved in the development of fire blight, *FEMS Microbiol. Lett. 253* (2005) 185–192.
3. T. Tsukamoto, K. Azegami, T. Matsuura, T. Ohara, Y. Inoue, A. Mizuno, K. Yoshida, H. Bessho, S. Kimura, M. Goto, Infection frequency of mature apple fruit with *Erwinia amylovora* deposited on pedicels and its survival in the fruit stored at low temperature, *J. Gen. Plant Pathol. 71* (2005) 296–301.
4. EPPO Data Sheets on Quarantine Organisms No. 52: *Erwinia amylovora*, *EPPO Bulletin, 13* (1983).
5. R. Mittler, Oxidative stress, antioxidants and stress tolerance, *Trends Plant Sci. 7* (2002) 405–410.
6. S. Mandal, A. Mitra, N. Mallick, Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *lycopersici, Physiol. Mol. Plant Pathol. 72* (2008) 56–61.
7. E. Halupecki, C. Bazzi, S. Jock, K. Geider, D. Đermić, B. Cvjetković, Characterization of *Erwinia amylovora* strains from Croatia, *Eur. J. Plant Pathol. 114* (2006) 435–440.
8. M. Baggiolini, E. Keller, H.G. Milaire, H. Steiner: *Visual Control in the Cultivation of Apple*, OILB/SROP Brochure, OILB, Stuttgart, Germany (1980) p. 96 (in German).
9. B.Z. Siegel, W. Galston, The isoperoxidases of *Pisum sativum*, *Plant Physiol. 42* (1967) 221–226.
10. Y. Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts, *Plant Cell Physiol. 22* (1981) 867–880.
11. C.N. Giannopolitis, S.K. Ries, Superoxide dismutases. I. Occurence in higher plants, *Plant Physiol. 59* (1977) 309–314.
12. R. Randhir, K. Shetty, Developmental stimulation of total phenolics and related antioxidant activity in light- and dark--germinated corn by natural elicitors, *Process Biochem. 40* (2005) 1721–1732.
13. V. Cesar, C.H. Bornman, Anatomy of vegetative buds of Norway spruce (*Picea abies*) with special reference to thir exchange from winter to spring, *Nat. Croat. 5* (1996) 99–118.
14. T.P. O'Brien, M.E. McCully: *The Study of Plant Structure: Principles and Selected Methods*, Termarcarphi Pty. Ltd., Melbourne, Australia (1981).

15. J.A. Crosby, J. Janick, P.C. Pecknold, J.C. Goffreda, S.S. Korban, 'Enterprise' apple, *HortSci. 29* (1994) 825–826.

16. J. Tsiantos, P. Psallidas, Fire blight resistance in various loquat, apple and pear cultivars and selections in Greece, *J. Plant Pathol. 86* (2004) 227–232.

17. S. Fink: *Pathological and Regenerative Plant Anatomy*, Gebrüder Borntraeger, Berlin, Germany (1999) pp. 561–571.

18. M. Pollard, F. Beisson, Y. Li, J.B. Ohlrogge, Building lipid barriers: Biosynthesis of cutin and suberin, *Trends Plant Sci. 13* (2008) 236–246.

19. F.M. Davis, G.T. Baker, W.P. Williams, Anatomical characteristics of maize resistant to leaf feeding by southwestern corn borer (Lepidoptera, Pyralidae) and fall armyworm (Lepidoptera, Noctuidae), *J. Agric. Entomol. 12* (1995) 55–65.

20. R. Franke, L. Schreiber, Suberin – A biopolyester forming apoplastic plant interfaces, *Curr. Opin. Plant Biol. 10* (2007) 252–259.

21. T. Gaspar, C. Penel, D. Hagege, H. Greppin: Peroxidases in Plant Growth, Differentiation, and Development Processes. In: *Biochemical, Molecular and Physiological Aspects of Plant Peroxidases*, J. Lebaryewski, H. Greppin, C. Penel, T. Gaspar (Eds.), University M. Curie-Sklodowska, Lublin, Poland (1991) pp. 249–280.

22. B.R. Lee, K.Y. Kim, W.J. Jung, J.C. Avice, A. Ourry, T.H. Kim, Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (*Trifolium repens* L.), *J. Exp. Bot. 58* (2007) 1271–1279.

23. S. Hiraga, K. Sasaki, H. Ito, Y. Ohashi, H. Matsui, A large family of class III plant peroxidases, *Plant Cell Physiol. 42* (2001) 462–468.

24. J. Tyburski, P. Jasionowicz, A. Tretyn, The effects of ascorbate on root regeneration in seedling cuttings of tomato, *Plant Growth Regul. 48* (2006) 157–173.

25. W. Geebelen, J. Vangronsveld, D.C. Adriano, L.C. van Poucke, H. Clijsters, Effects of Pb-EDTA and EDTA on oxidative stress reactions and mineral uptake in *Phaseolus vulgaris*, *Physiol. Plant. 115* (2002) 377–384.

26. M.A. Bernards, W.D. Fleming, D.B. Llewellyn, R. Priefer, X. Yang, A. Sabatino, G.L. Plourde, Biochemical characterization of the suberization-associated anionic peroxidase of potato, *Plant Physiol. 121* (1999) 135–145.

27. V.M. Rao, G. Palyath, P.D. Ormrod, Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*, *Plant Physiol. 110* (1996) 125–136.

28. M. Sedlářová, A. Lebeda, Histochemical detection and role of phenolic compounds in the defense response of *Lactuca* spp. to lettuce downy mildew (*Bremia lactucae*), *J. Phytopathol. 149* (2001) 693–697.

29. H. Lepeduš, V. Gaća, V. Cesar, Guaiacol peroxidases and photosynthetic pigments during maturation of spruce needles, *Croat. Chem. Acta, 78* (2005) 355–360.

30. E. Vranová, D. Inzé, F. Van Breusegem, Signal transduction during oxidative stress, *J. Exp. Bot. 53* (2002) 1227–1236.

31. J.S. Venisse, G. Gullner, M.N. Brisset, Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*, *Plant Physiol. 125* (2001) 2164–2172.

32. K. Asada, Ascorbate peroxidase – A hydrogen peroxide-scavenging enzyme in plants, *Physiol. Plant. 85* (1992) 235–241.

33. D.F. Klessig, J. Durner, R. Noad, D.A. Navarre, D. Wendehenne, D. Kumar, J.M. Zhou, J. Shah, S. Zhang, P. Kachroo, Y. Trifa, D. Pontier, E. Lam, H. Silva, Nitric oxide and salicylic acid signaling in plant defense, *Proc. Natl. Acad. Sci. USA, 9* (2000) 8849–8855.

34. R. Mittler, X. Feng, M. Cohen, Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco, *Plant Cell, 10* (1998) 461–473.

35. S. Dorey, F. Baillieul, P. Saindrenan, B. Fritig, S. Kauffmann, Tobacco class I and II catalases are differentially expressed during elictor-induced hypersensitive cell death and localized acquired resistance, *Mol. Plant-Microbe Interact. 11* (1998) 1102–1109.

36. S. DebRoy, R. Thilmony, Y.B. Kwack, K. Nomura, S.Y. He, A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants, *Proc. Natl. Acad. Sci. USA, 101* (2004) 9927–9932.