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Cultivar-specific and ulvan-induced resistance of apple plants to Glomerella leaf spot are associated with enhanced activity of peroxidases

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ABSTRACT. Glomerella leaf spot (GLS) is an important disease of apple plants, and the use of the algal polysaccharide ulvan represents a new technology for its control. This study aimed to verify whether the defense mechanisms involved in cultivar-specific and ulvan-induced plant resistance to GLS are associated with changes in the activities of peroxidase and β-1,3-glucanase. Seedlings were first sprayed with ulvan or water and then inoculated with *Colletotrichum gloeosporioides* 6 days later. The disease severity was recorded daily on both young and old leaves up to 10 days after inoculation, and the enzyme activities were monitored from 24 to 72h after inoculation (HAI). Although the young leaves were more susceptible to GLS, ulvan reduced approximately 66% of the disease severity in both of the leaf age groups. Additionally, the cultivar-specific and ulvan-induced resistance was associated with enhanced peroxidase activity at 24 and 72 HAI, respectively. Both the resistant and susceptible seedlings exhibited similar glucanase activities.

Keywords: *Colletotrichum gloeosporioides*, *Malus domestica*, *Ulva* sp., induction resistance, peroxidase, glucanase.

Resistências cultivar-específica e induzida por ulvana à mancha foliar de Glomerella em macieira são associadas com aumento da atividade de peroxidases

RESUMO. Mancha foliar de Glomerella (MFG) é uma doença importante da macieira e o uso do polissacarídeo algal ulvana representa uma nova tecnologia para o seu controle. Este estudo teve como objetivo verificar se os mecanismos de defesa envolvidos na resistência de planta cultivar-específica e induzida por ulvana a MFG estão associados com mudanças na atividade de peroxidase e *β*-1,3-glucanase. Plântulas foram primeiro pulverizadas com ulvana ou água e então inoculadas com *Colletotrichum gloeosporioides* 6 dias mais tarde. A severidade da doença foi registrada diariamente em ambas folhas jovens e velhas até 10 dias após a inoculação. Atividade enzimática foi monitorada de 24 a 72h após a inoculação (HAI). Embora, folhas jovens foram mais suscetíveis a MFG, a ulvana reduziu aproximadamente 66% da severidade da doença em ambos grupos etários de folhas. Adicionalmente a resistência cultivar-específica ou induzida por ulvana foi associada com um aumento da atividade de peroxidase às 24 e 72 HAI, respectivamente. Ambas plântulas resistentes e suscetíveis exibiram atividade similar de glucanase.

Palavras chaves: *Colletotrichum gloeosporioides*, *Malus domestica*, *Ulva* sp., indução de resistência, peroxidase, glucanase.

Introduction

Glomerella leaf spot (GLS) is an emerging disease of apple plants (*Malus domestica* Borkh.) growing in regions with a humid subtropical climate, such as the southeastern USA (GONZALEZ et al., 2006) and South Brazil (BECKER et al., 2000). GLS often occurs during a rainy summer and has become a major concern, particularly to Brazilian apple producers, because the most widely grown cultivar (cv. Gala) is highly susceptible, making GLS the main summer crop disease in the region. The disease can potentially lead to severe defoliation (above 75%), reducing the yield and weakening the trees. In addition, the production costs can increase by approximately 20% due to the application of fungicides (BECKER et al., 2000; GONZALEZ et al., 2006; KATSURAYAMA; BONETI, 2009).

GLS is a disease caused by the fungi *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Colletotrichum acutatum* JH Simmonds, with the former species being the more prevalent and aggressive (CRUSIUS et al., 2002; GONZALEZ et al., 2006; KATSURAYAMA; BONETI, 2009). The disease incubation period is usually short, and the first symptoms appear as reddish-purple spots as early as two days after inoculation. Between eight and ten days after infection, the spots develop into

necrotic lesions of irregular shape, and the leaves may become completely dehydrated and/or yellowish and usually drop (ARAUJO et al., 2008).

Synthetic fungicides are the most common tactic used in GLS management (BECKER et al., 2000). However, as no fungicide with a curative effect is currently available for controlling the disease, the spraying of fungicides commences as soon as the early symptoms are observed and are repeated whenever a rainfall event of > 30 mm occurs (BECKER et al., 2000; CRUSIUS et al., 2002; KATSURAYAMA; BONETI, 2009). Although such an application program has proved to be effective, both environmental and health concerns have fostered the development of eco-friendly technologies for plant protection.

Genetic resistance is considered to be a key strategy and one of the most efficient methods of controlling plant diseases (DANTAS et al., 2009). The Gala cv., the consumer's favorite and the bestselling apple in the world, is highly susceptible to GLS, whereas the cultivars descended from the 'Delicious' group, such as cv. Fuji, are completely resistant to the disease (BECKER et al., 2000). The cultivar-specific resistance to GLS appears to be conferred by a recessive allele at a single gene locus (DANTAS et al., 2009).

Recently, we found that ulvan, a water-soluble polysaccharide extracted from green seaweed (*Ulva fasciata* Delile), has a high potential for use in GLS management. When sprayed six days prior to inoculation, ulvan conferred systemic protection against GLS and reduced the disease severity by half (ARAUJO et al., 2008). Because ulvan does not exhibit any antimicrobial activity, it has been assumed that the polysaccharide acts by inducing plant resistance (ARAUJO et al., 2008; PAULERT et al., 2009, 2010). Indeed, molecular studies have shown that ulvan can induce the expression of genes related to plant defense responses in *Arabidopsis* (JAULNEAU et al., 2010) and *Medicago truncatula* L. (CLUZET et al., 2004). Ulvan also exhibits priming activity, inducing an oxidative burst in plant cells that correlates with a decrease in disease severity (PAULERT et al., 2010).

Plants have evolved strategies to oppose attack by pathogens, and consequently, plant genomes encode resistance proteins (R proteins) that allow them to recognize specific pathogen-derived molecules known as avirulence (*avr*) factors (KIRÁLY et al., 2007). One of the most common and efficient defense mechanisms activated after the recognition of a pathogen is the synthesis of pathogenesis-related proteins (PR proteins) (METRAUX, 2001), such as peroxidases (POD) and β-1,3-glucanases (GLU) (VAN LOON et al., 2006).

Peroxidases catalyze the oxidation and polymerization of hydroxycinnamic alcohols in the presence of hydrogen peroxide (H_2O_2) to produce lignin, which, together with cellulose and other polysaccharides, can accumulate in the cell walls of plants, functioning as a physical barrier to fungal penetration (STADNIK; BUCHENAUER, 2000). Indeed, induced resistance has often been correlated with enhanced POD activity in apple plants (BAYSAL; ZELLER, 2004; BRISSET et al., 2000; HASSAN; BUCHENAUER, 2007). Glucanases are lytic enzymes that hydrolyze β-1,3-glucans, one of the major components of the cell walls of fungi and bacteria (VAN LOON et al., 2006), and an increase in GLU activity has been associated with apple resistance against fire blight (BRISSET et al., 2000) and ulvan-induced resistance against bean rust (BORSATO et al., 2010).

Although some information on the inheritance of the genetic resistance of apple to GLS and the potential of ulvan to control plant diseases is available (ARAUJO et al., 2008; PAULERT et al., 2009, 2010; BORSATO et al., 2010), the biochemical defense mechanisms involved during the infection and colonization processes of apple plants by *C. gloeosporioides* remain unknown. Thus, the aim of this study was to understand whether the defense mechanisms of cultivar-specific and ulvan-induced resistance to GLS are associated with changes in the activities of peroxidase and β-1,3-glucanase upon inoculation.

Material and methods

Seedlings were obtained from seeds according to methodology adapted from Brisset et al. (2000) and Hassan and Buchenauer (2007). ‘Gala’ apple seeds were distributed in plastic germination boxes between layers of moistened cotton and then stored at 5°C for 50 days to break dormancy. The germlings were transferred to polystyrene boxes containing soil and maintained under greenhouse conditions. When the true leaves appeared, the seedlings were transplanted into individual plastic pots containing 1 L of a mixture of organic compost and loamy soil (1:2, v v^{-1}) and were grown for 45 days until they had from 10 to 15 expanded leaves. The seedlings were irrigated as needed and individually fertilized with 0.25 g of monoammonium phosphate (12-61-0/N-P-K). Powdery mildew and aphids were controlled with sprays of 80% sulfur (3 g L^{-1}, BASF) and deltamethrin insecticide (3 mL L^{-1}, Agrevo),

respectively. Prior to starting the experiment, the residual sulfur was removed by washing the leaves. To identify the new leaves developing after the treatment, a tag was attached to the petiole of the last fully expanded leaf (designated as the 1st leaf).

The seedlings originated from the seeds of 'Gala' pollinated by 'Fuji' (pollen donor) (HEGEDUS, 2006). The segregating individuals resistant and susceptible to GLS were identified through an adapted detached leaf test (LIU et al., 2007) in which the uppermost leaf was detached from 40-day-old seedlings and inoculated by spraying a conidial suspension of *C. gloeosporioides* (10^5 conidia mL^{-1}). After eight days of incubation in a moist chamber at 25°C and a 12h photoperiod (photon flux density of approx. 170 μmol m^{-2} s^{-1}), the leaves were assessed for the incidence and severity of GLS as described by Araujo et al. (2008). The susceptible and resistant individuals were detected by the presence and absence of symptoms, respectively.

Ulvan was obtained as previously described (PAULERT et al., 2009). Briefly, 100 g of dried *U. fasciata* seaweed was autoclaved in 1 L of distilled water at 110°C for 2h. The aqueous solution was filtered, and the polysaccharides were precipitated by adding three volumes of ethanol, followed by cooling to -20°C for 4h. The precipitates were collected, dried to a constant weight at 45°C and stored at 5°C until used in the experiments.

The concentration of ulvan used in the tests (10 mg mL^{-1}) was determined in previous studies (ARAUJO et al., 2008, PAULERT et al., 2009). The ulvan was completely dissolved in distilled water using a magnetic stirrer at room temperature. Six days before the fungal inoculation, the susceptible plants were sprayed with the ulvan solution until runoff (~4 mL $plant^{-1}$) using a manual sprayer (Griffin, Italy) coupled to an air compressor (25 psi, Schulz, Brazil). The resistant and control susceptible plants were sprayed with distilled water. All of the seedlings were kept on greenhouse benches until the inoculation.

A pathogenic strain, MANE147, of *C. gloeosporioides* was isolated from the necrotic spots of 'Gala' apple leaves and maintained on potato dextrose agar medium (PDA). To produce the inoculum, 8 mm discs of fungal cultures were transferred to Petri dishes with PDA culture medium. After 10 days at 25°C and a 12h photoperiod, the colonies were lightly scraped, and the concentration of the conidial suspension was determined using a Neubauer chamber.

Six days after the ulvan treatment, the resistant and treated and untreated susceptible apple seedlings were inoculated with the fungus by spraying a suspension of 3 x 10^5 conidia mL^{-1} until runoff; the inoculation was performed using a sprayer as previously described. The inoculated plants were maintained in the dark at 26°C and nearly 100% relative humidity for 24h. After this period, the plants were returned to the greenhouse benches until the disease evaluation.

The GLS severity was assessed daily from 6 to 10 days post inoculation (dpi) based on visual estimates of the percent necrotic leaf area, as described by Araujo et al. (2008). Linear regression models were fitted to the log-transformed severity data over time, and the slopes of the regression represented the apparent infection rate (CAMPBELL; MADDEN, 1990).

On the 10th dpi, all of the leaves were detached for image analyses in which the leaves were placed between two transparent glass plates (28 x 21 x 0.8 cm) in front of a black sheet of paper that functioned as a background, and the leaves were scanned. The percent necrotic area for each leaf image was determined using Quant® *software* (VALE et al., 2002) for two groups of leaves, the upper (from the 1st to 4th leaf) and lower leaves (from the 5th to 8th leaf), due to the influence of leaf age on the disease susceptibility, whereby the younger leaves are more susceptible than the older ones (ARAUJO et al., 2008).

Protein extracts were obtained from the 2nd and 3rd expanded leaves at 24, 48 and 72 hours after inoculation (HAI) according to methodology adapted from Brisset et al. (2000) and Hassan and Buchenauer (2007). The leaves were collected, wrapped in aluminum foil, snap-frozen in liquid nitrogen, and then stored at -20°C until the protein was extracted. The leaf samples were ground in liquid nitrogen using a mortar and pestle, and the powder was homogenized at a ratio of 1:4 (w v^{-1}) in sodium phosphate buffer (50 mM, pH 7.5) containing 0.01% Triton X-100 (Sigma-Aldrich, USA), 1% polyvinylpolypyrrolido-ne (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1 mM 2-mercaptoethanol (Sigma-Aldrich). After centrifugation at 20.000 x g for 30 min. at 4°C (model 5804R, Eppendorf), the supernatants were collected and divided into equal portions. One portion was stored at -20°C for the subsequent evaluation of the GLU activity, and another was kept on ice for the immediate determination of the POD activity. The protein content was determined using the method of Bradford (1976), with bovine serum albumin as the standard.

The POD (EC 1.11.1.7) activity was determined spectrophotometrically according to the methodology adapted from Brisset et al. (2000) and Hassan and Buchenauer (2007). In brief, 100 μL of protein extract was added to 2.9 mL of sodium acetate

buffer (50 mM, pH 5.5) containing 0.25% guaiacol (v v^{-1}) (Sigma) and 100 mM H_2O_2 (Merck). The absorbance values of the reaction were recorded every 30 s for 3 min., at 30°C using a spectrophotometer at 470 nm (700 Plus, Femto). The enzyme activity was expressed as units of optical density at 470 nm mg^{-1} protein $min.^{-1}$

The GLU (EC 3.2.1.39) activity was determined according to the method adapted from Wirth and Wolf (1996) and Brisset et al. (2000). Carboxymethyl-curdlan-Remazol Brilliant Blue (CM-curdlan-RBB, Loewe Biochemica, Germany) was used as substrate. An aliquot of 200 μL CM-curdlan-RBB (4.0 mg mL^{-1}) and 200 μL of sodium acetate buffer (200 mM, pH 5.0) were pipetted into 2 mL microtubes, vortexed for 5 s and incubated in a water bath at 40°C for 10 min. Aliquots of 400 μL of the crude extracts were added, and the mixture was shaken again and incubated in a 40°C water bath for 2h. After stopping the reaction with 200 μL of 2 N HCl and keeping the microtubes on ice for 10 min., the samples were centrifuged at 10.000 x g for 10 min. The absorbance of the supernatant samples was then determined at 600 nm. The GLU activity was expressed as the increase of absorbance per min and per mg of protein.

The experimental design was completely randomized, with five replicates per treatment. For assessing the disease severity, an experimental unit consisted of one plant/pot, and each unit was composed of two leaves/plant for the enzyme activity determination. Two independent experiments were performed at different times with similar results, and one of them was chosen for representing the results.

A factorial analysis of variance (ANOVA) was used to study the single and interaction effects of the leaf position and ulvan treatment on the disease severity, as digitally estimated at ten days after inoculation. A Tukey test ($p \leq 0.05$) was used to discriminate the treatment means whenever significant differences were detected by the ANOVA. The single and interaction effects of the treatment and time on the enzyme activity data were also analyzed by ANOVA. Whenever significance of the factors was detected, a regression analysis was used to model the enzyme activity at different times. The statistical analysis was performed using the *software* Statistica 6.0 (STATSOFT, 2001).

Results and discussion

The resistant and susceptible individuals previously selected by the detached leaf test confirmed the same resistance reactions during the experiments with the seedlings under greenhouse conditions. No GLS symptoms were observed on the resistant seedlings, whereas the inoculated susceptible plants exhibited the typical GLS symptoms (Figure 1).

Figure 1. Glomerella leaf spot symptoms in resistant (R), ulvan-treated (U) and mock-treated control (C) apple seedlings at 10 days after the inoculation with *Colletotrichum gloeosporioides* inocula. Plants were sprayed with water (R,C) or ulvan (U) six days prior to the inoculation.

In this study, we found that resistance to GLS can be easily screened using the detached leaf test: approximately half of the seedlings obtained from the cross between 'Gala' and 'Fuji' displayed complete resistance to the disease under greenhouse conditions. Taking into account the fact that 'Gala' is self-incompatible (HEGEDUS, 2006) and the resistance to GLS is conferred by a major gene (DANTAS et al., 2009), the introduction of resistance genes by traditional breeding methods may not be difficult. Nevertheless, efforts should be made to identify polygenic sources of resistance to GLS for future breeding programs because they are more durable and offer fewer risks that the pathogen will overcome the resistance (KIRÁLY et al., 2007). Equally important are the studies identifying the biotypes and races of the pathogen for a better understanding of their population dynamics in apple orchards. However, most of the basic information is not yet readily available for this plant-pathogen interaction with respect to the influence of both the leaf position and treatment.

The analysis of variance revealed that both the leaf position and ulvan treatment significantly influenced the GLS severity, though their interaction was not significant ($p > 0.05$) (Figure 2). The upper leaves were consistently more susceptible than the lower leaves, and the ulvan treatment reduced the disease severity by 58% and 75% on the upper and lower leaves, respectively (Figure 2). The results of this study are in agreement with those of Araujo et al. (2008), who also reported a severity reduction of 65% by ulvan treatment and the greater susceptibility of the younger apple leaves to GLS. Ulvan does not have any antimicrobial effect against

Colletotrichum spp. (ARAUJO et al., 2008; PAULERT et al., 2009), but it can reduce disease by inducing resistance and stimulating defense responses both locally and distant from its application site (CLUZET et al., 2004; JAULNEAU et al., 2010; PAULERT et al., 2009, 2010).

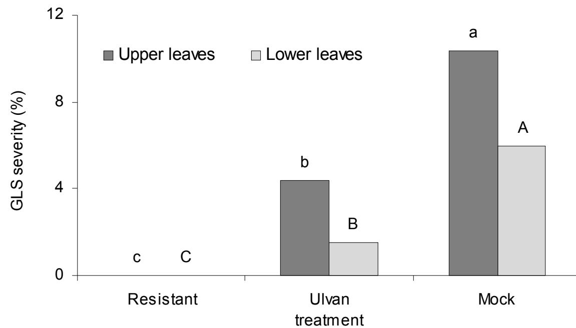

Figure 2. Glomerella leaf spot (GLS) severity (%) at 10 days after inoculation with *Colletotrichum gloeosporioides* in the upper and lower leaves of resistant and susceptible apple seedlings treated or untreated (mock control) with ulvan. For the statistical analysis, the data were transformed to $\sqrt{(x)} + 1$, and the columns with the same uppercase and lowercase letters indicate no significant difference ($p \leq 0.05$).

The linear equations fitted to the log-transformed severity progress data showed a coefficient of determination varying from 0.93 to 0.98 (data not shown). The results for the apparent infection rates estimated from the linear equations corresponded to the maximum severity estimated digitally when comparing the treatments. For instance, the infection rate was significantly reduced ($p < 0.05$) with the application of ulvan for both the lower and upper leaves compared to the mock control. The highest rates were found for the upper untreated leaves (0.38 day^{-1}) and lower untreated leaves (0.24 day^{-1}). The application of ulvan reduced the infection rate in the upper and lower leaves to 0.22 and 0.02 day^{-1}, respectively. As demonstrated in other pathosystems, it is known that the induced resistance of the host generally operates by reducing the number and size of lesions (KIRÁLY et al., 2007; BORSATO et al., 2010).

The ANOVA for the POD activity revealed a significant effect for the interaction of treatment x time. In resistant plants, the POD activity was the highest (3x higher than in the ulvan-treated and control plants) at 24 HAI and was reduced linearly from 24 HAI to 72 HAI (Figure 3A). The induction of such defensive enzymes as POD has been recognized as the initial resistance response in plants (METRAUX, 2001). In general, resistant plants recognize the presence of the pathogen quickly and activate effective defenses against the invader; in contrast, this recognition often occurs later in susceptible plants, thus the infection is not prevented (METRAUX, 2001; WHARTON et al., 2001). For instance, sugar cane plants resistant to red rot have higher peroxidase activities compared to susceptible plants at earlier stages of infection (*i.e.*, 24 HAI), thereby controlling the infection of *Colletotrichum falcatum* (ASTHIR et al., 2009).

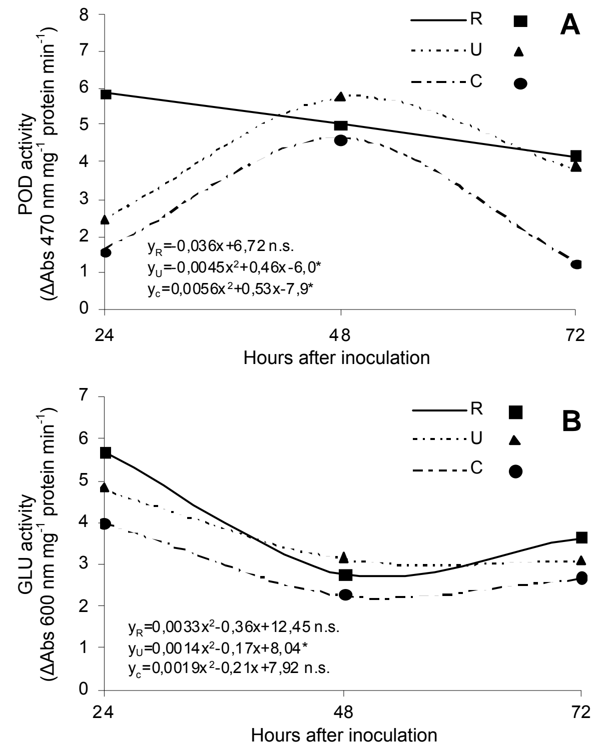

Figure 3. A) Activity of peroxidase (POD) and B) β-1,3-glucanase (GLU) in resistant (R), ulvan-treated (U) and mock-control (C) apple seedlings at 24, 48 and 72h after inoculation with *Colletotrichum gloeosporioides*. The data were transformed to $\sqrt{(x)}$ for the statistical analysis. *Indicates a significant difference by regression analysis for the equations of first or second order. n.s. = Not significant.

However, the role of PODs in plants is not fully understood, though these enzymes have often been associated with cell wall biosynthesis, responses to injury, disease resistance and wound repair (SLATNAR et al., 2010). PODs are responsible for the formation of lignin, which, together with cellulose and other polysaccharides that accumulate in the cell walls of plant cells, functions as a physical barrier to fungal penetration (STADNIK; BUCHENAUER, 2000). This could help to explain the higher susceptibility of younger leaves, which generally contain less lignin in their tissues than older leaves. Indeed, the rapid lignification of the cell wall of resistant plants under the appressoria of *Colletotrichum* spp. is one of the primary defense mechanisms to prevent the penetration of the

fungus into their tissues. For example, the induced resistance of cucumber plants that prevents the infection by *C. lagenarium* has been associated with a rapid accumulation of lignin in the cell walls (HAMMERSCHMIDT; KUC, 1982). Specific host peroxidase isogenes are induced at very early stages of the interaction of *C. gloeosporioides* with *Stylosanthes humilis*, and the host recognition of the pathogen appears to occur prior to the physical penetration of the epidermal cell wall (HARRISON et al., 1995). Therefore, it would be interesting to investigate the changes in the POD activity of resistant apple plants at *C. gloeosporioides* infection stages earlier than 24 HAI and to determine the content of lignin and phenolics in the leaves.

The POD activity for the ulvan-treated and mock-control susceptible seedlings showed a curvilinear pattern, with a peak in the activity at 48 HAI, reaching levels similar to the resistant genotype (Figure 3A). The POD activity in the ulvan-treated seedlings maintained higher levels of POD activity at 72 HAI compared to the mock-control plants. In accordance, the genes expressed in ulvan-treated *Arabidopsis* have been related to various functional classes, notably some well-known jasmonic acid-responsive genes belonging to defense responses, but also other genes, such as peroxidase (JAULNEAU et al., 2010). However, significant increases of this enzyme most likely would not have contributed to the inhibition of *C. gloeosporioides* penetration because GLS has a short incubation period, less than 48 h (ARAUJO et al., 2008). On the other hand, the increase in the POD activity at 48 and 72 HAI may have interfered with the colonization of the apple tissue by *C. gloeosporioides*, as in other pathosystems (BRISSET et al., 2000; HASSAN; BUCHENAUER, 2007).

With regards to GLU, the treatments had no significant effect on the activity levels, which was observed only for the time factor. The GLU activity was the highest at 24 HAI and tended to decrease at later time points (Figure 3B). Previous studies in apple have shown that the maximum GLU activity related to other pathosystems may occur earlier than the first time assessment in our work (BRISSET et al., 2000). Thus, one cannot exclude the possibility that the peak of GLU may have occurred before 24 HAI, as these enzymes have an immediate function in plant defense, with direct actions on the invading hyphae (BAYSAL; ZELLER, 2004; VAN LOON et al., 2006). It must also be taken into account that defense mechanisms are genotype dependent (KIRÁLY et al., 2007; METRAUX, 2001). For example, Borsato et al. (2010) observed a significant increase in the GLU activity in ulvan-treated plants that were moderately susceptible but not in those resistant to rust. The results of our study suggest that ulvan treatment does not affect the GLU activity in apple seedlings for the range of time studied. In contrast, a microarray analysis using *Medicago truncatula* has provided evidence that GLU is upregulated in infected plants previously treated with ulvan (CLUZET et al., 2004). However, the genetic studies performed by Jaulneau et al. (2010) did not list GLU as a gene upregulated by ulvan.

Conclusion

The cultivar-specific and ulvan-induced resistances to GLS in apple plants, which completely suppressed or reduced the development of the disease, respectively, are associated with the enhanced activity of peroxidases, but not β-1,3-glucanases, as monitored over time up to 72 hours after inoculation.

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