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Oxidative burst and the activity of defense-related enzymes in compatible and incompatible tomato-*Alternaria solani* interactions

Explosão oxidativa e atividade de enzimas relacionadas à defesa em interações compatíveis e incompatíveis de tomateiro-*Alternaria solani*

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Abstract

The production of reactive oxygen species (ROS), hypersensitive response (HR), and the activity of the enzymes guaiacol peroxidase, catalase, polyphenol oxidase, β-1,3-glucanase and chitinase, were studied in leaves of resistant [CNPH 1287 (*Solanum habrochaites* syn. *Lycopersicon hirsutum*)] and susceptible [Santa Cruz Kada (*S. lycopersicum* syn. *L. esculentum*)] tomato genotypes inoculated with *Alternaria solani*. Leaves were collected at the time of inoculation and at 4, 8, 12, 24, 48, 72, 96 and 120 hours post inoculation. Conidia germination occurred equally onto the leaf surface in both genotypes and germination tubes grew without apparent orientation. Lesion frequency was lower in CNPH 1287, and it was the consequence of a lower number of appressoria formed in that genotype. ROS were observed in low frequency in both genotypes. HR was observed in penetrated epidermal host cells also in both genotypes. It seems that ROS and HR would not contribute to the resistance of *S. habrochaites* to *A. solani* in this study. The activity of guaiacol peroxidase, polyphenol oxidase, β-1,3-glucanase and chitinase was significantly increased in the resistant genotype. These results suggest that defense-related enzymes but no oxidative burst play a role in the defense response of *S. habrochaites* to *A. solani*.
Key words: Hydrolytic enzymes, reactive oxygen species, *Solanum habrochaites, Solanum lycopersicum,* tomato early blight

Resumo

A produção de espécies reativas de oxigênio (EROs), a resposta de hipersensibilidade (HR) e a atividade das enzimas peroxidase de guaiacol, catalase, polifenoloxidase, β-1,3-glucanase e quitinase foram estudados em folhas de tomateiro resistente [CNPH 1287 (*Solanum habrochaites* syn. *Lycopersicon hirsutum*)] e suscetível [Santa Cruz Kada (*S. lycopersicum* syn. *L. esculentum*)] inoculadas com *Alternaria solani*. Essas folhas foram coletadas no momento da inoculação e às 4, 8, 12, 24, 48, 72, 96 e 120 h pós-inoculação. Os conídios germinaram igualmente na superfície foliar de ambos os genótipos sem orientação definida no crescimento dos tubos germinativos. A frequência de lesões causadas por *A. solani* foi menor no genótipo CNPH 1287 como consequência do menor número de apressórios formados nesse genótipo. O acúmulo de EROs foi observado em baixa frequência tanto no genótipo suscetível quanto no resistente. A HR foi observada nas células epidérmicas onde ocorreu penetração em

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ambos os genótipos. Aumentos significativos na atividade de peroxidase de guaiacol, polifenoloxidase, β-1,3-glucanase e quitinase foram registrados no genótipo resistente. Estes resultados sugerem que, enquanto EROs e HR não parecem contribuir com a resistência de *S. habrochaites* frente a *A. solani,* as enzimas relacionadas à defesa cumprem um papel importante nas respostas de defesa deste genótipo.
Palavras-chave: Enzimas hidrolíticas, espécies reativas de oxigênio, pinta preta, *Solanum habrochaites, Solanum lycopersicum*

Introduction

Early blight is one of the most common and destructive diseases of the cultivated tomato [*Solanum lycopersicum* L. (PERALTA; KNAPP; SPOONER, 2005) syn. *Lycopersicon esculentum* Mill.] worldwide. Early blight has been traditionally attributed to the fungus *Alternaria solani* Sorauer (ROTEM, 1994). In 2000 was described a new specie, *Alternaria tomatophila* Simmons, whose individuals were commonly associated with early blight of tomato (SIMMONS, 2000; LOURENÇO et al., 2009). *A. tomatophila* has been detected in the United States, Australia, New Zealand, Venezuela and more recently, in Brazil (RODRIGUES et al., 2010). These authors also reported *A. grandis* causing early blight on tomato in Brazil.

Tomato early blight has high epidemic incidence in many regions of North, Central and South America, South Asia and Africa (FOOLAD; MERK; ASHRAFI, 2008). According with Chaerani and Voorrips (2006), sources for early blight resistance have been identified in wild relatives of tomato (*S. habrochaites* S. Knapp & D. Spooner [sin. *L. hirsutum* Dunal]*, S. pimpinellifolium* L. [syn. *L. pimpinellifolium* (L.) Mill.]*, S. peruvianum* L. [syn. *L. peruvianum* (L.) Mill.] and *S. chilense* (Dunal) Reiche [syn. *L. chilense* Dunal]). Some of these have been utilized by traditional breeding, but an increased level of resistance is negatively correlated with agronomic characteristics and yield. Therefore, early blight resistant cultivars with better agronomic characteristics are still needed.

Although passive or preformed defense mechanisms could prevent infection, more frequently plants show active responses to pathogenic infection including gene transcription and the formation of defensive products leading to delayed pathogen development or plant cell death (MIESLEROVÁ; LEBEDA; KENNEDY, 2004). Induced defenses include, among others, the production of reactive oxygen species (ROS), cell wall strengthening, phytoalexin synthesis and accumulation of defense-related proteins. The localized generation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^{-}) cytologically detectable is one of the first events following the recognition of the pathogen by the plant (BAKER; ORLANDI, 1995) and is probably involved with the hypersensitive response (HR) induction during the expression of plant disease resistance (LEVINE et al., 1994).

Many enzyme systems are involved in plant defense reponses during pathogen attack. Peroxidases participate in various physiological processes related to defense, such as cross-linking of cell wall structural proteins and pectins by ferulic acid bridges and the polymerization of lignin precursors during lignin formation (FRY, 1986), production of H_2O_2 by cell wall peroxidases (WOJTASZEK, 1997) and inducing phytoalexins production (KRISTENSEN; BLOCH; RASMUSSEN, 1999). Catalase plays an important role in the ROS metabolism, protecting cells from the toxic effects of H_2O_2 (LEBEDA et al., 2001). Chitinases and β-1,3-glucanases are hydrolytic enzymes, associated with defense against disease, that are induced in several plant-pathogen interactions (VAN LOON, 1997). They can degrade cell wall components of many fungi and can also act by releasing cell wall fragments that induce the host active response (MAUCH; MAUCH-MANI; BOLLER, 1988; YOSHIKAWA; YAMAOKA; TAKEUCHI, 1993). Polyphenol oxidases are enzymes found in plastids that use molecular

oxygen for the oxidation of mono and *o*-diphenols into *o*-diquinones, which have antimicrobial activity (MOHAMMADI; KAZEMI, 2002). Polyphenol oxidases also participate in the lignification process during pathogen invasion (LI; STEFFENS, 2002) and in the production of ROS (RICHARD-FORGET; GAUILLARD, 1997; THIPYAPONG; HUNT; STEFFENS, 2004).

Previous histochemical and biochemical studies have shown that exist differences in the response of *S. lycopersicum* and *S. habrochaites* inoculated with the biotrophic fungus *Oidium neolycopersici* (BALBI-PEÑA; SCHWAN-ESTRADA; STANGARLIN, 2012).

The objective of this study was to compare the induced defense reponses against *A. solani* in resistant *S. habrochaites* and susceptible *S. lycopersicum* plants. To do that, we histochemically detected H_2O_2 and O_2^{-}, assessed the development of the hypersensitive response and studied the dynamics of some defense-related enzymes after inoculation with the necrotrophic fungus *A. solani.*

Material and Methods

Pathogen and plant material

Alternaria solani (Ellis and Martin) Sorauer isolate (1707 EH) was provided by Embrapa Hortaliças (Brasilia – DF, Brazil). For maintenance, the fungus was grown on a V8 medium at 25 ºC with a photoperiod of 8 h of light and 16 h of dark. For sporulation induction, when the colony reached 4/5 of the plate, the aerial mycelium was scratched and the plates washed under running tap water for 4 h in order to exhaust the medium. The plates were allowed to dry upside down for 24 h and then closed, sealed with a plastic film and incubated at the conditions previously described.

The tomato genotypes used were CNPH 1287 (PI 126445 – *S. habrochaites)* and Santa Cruz Kada (*S. lycopersicum),* which are resistant and susceptible to early blight, respectively. The two different genotypes were planted in trays containing commercial substrate for seedlings. After 18 days, seedlings were transferred to 0.6 L pots (one plant/pot) containing the same substrate with gradual release fertilizer (N 14%, P_2O_5 16%, K_2O 18%, S 8%, Mo 0.2%) and kept in a growth chamber at 20-25 ºC, 4000 lx and 12-h photoperiod (fluorescent light).

Inoculation and sample collection

At 38 days after transplantation, when Kada and CNPH 1287 plants had the five-seven and seven-nine leaves fully developed, respectively, inoculation of the second, third and fourth true leaves was performed. The upper side of each leaf was spray inoculated with a *A. solani* conidial suspension of 1,25 x 10^3 conidia mL^{-1}. High humidity conditions were maintained by placing the inoculated plants under plastic covers. At 0, 4, 8, 12, 24, 48, 72, 96 and 120 hours post-inoculation (hpi), inoculated leaves were collected, along with tomato leaves that had not been inoculated. Samples intended for biochemical analysis (third and fourth leaves) were weighed, frozen in liquid nitrogen and stored in a freezer for later analysis.

Localization of reactive oxygen species and development of A. solani

Histochemical detection of ROS was performed according to Romero-Puertas et al. (2004), with a few modifications. For H_2O_2 detection, three 11-mm leaf tissue discs were cut from the second leaf of each plant and submerged in diaminobenzidine (DAB) solution at 0.1 % (w/v) in 10 mM MES buffer (pH 6.5). The DAB stain was vacuum infiltrated until the entire leaf tissue was infiltrated. To verify the specificity the DAB reaction, some discs were infiltrated with 10 mM ascorbic acid (H_2O_2 detoxifier). Discs were incubated at room temperature for 1 h in light, then cleared by immersion in ethanol (92%) heated to 78ºC and

preserved in 50% glycerol until observation. Discs were analysed using light microscope, and lactophenol-cotton blue (TUITE, 1969) was used to visualise the structures of the pathogen. Fungal development was characterised by the porcentage of conidial germination, number of germination tubes/conidium, formation of appresoria and lesions. H_2O_2 detection was determined by the number of sites exhibiting brown precipitate under the penetration site of the fungus and by brown epidermal cells.

For O_2^{-} detection, three 11-mm leaf tissue discs were cut from the second leaf of each plant and submerged in nitroblue tetrazolium (NBT) solution 0.1% (w/v) in 10 mM potassium phosphate buffer (pH 7.8). Complete vacuum infiltration of the dye in the leaf tissue was performed. To verify the specificity of the NBT reaction, some discs were infiltrated with superoxide dismutase at 50 µg ml^{-1}. Infiltrated discs were incubated, cleared, preserved and analysed as previously described. O_2^{-} detection through the *in situ* reduction of NBT was performed by determining the number of sites with blue precipitate in the epidermal cells at the site of fungal infection. H_2O_2 and O_2^{-} assessments were performed in at least twenty infection sites in the three foliar discs of each plant (replication) at each time point.

Hypersensitive response

The discs were cleared and conserved as previously described. The autofluorescence of the epidermal cells was observed under incident blue light (460-490 nm excitation) using a Zeiss Axioskop microscope. Hypersensitive response (necrosis of the attacked cells) was detected as autofluorescence of the cell wall or of the entire epidermal cell (KOGA et al., 1988). To visualise the fungal structures, plant material was treated with lactophenol-cotton blue (TUITE, 1969).

Preparation of enzymatic extracts

The third leaf of each plant (approximately 0.5 g) was macerated with liquid N_2 using a mortar in 4 ml of 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA and 1% (w/w) PVP (poly-vinyl-pyrrolidone). The homogenate was centrifuged at 15,000 g for 30 min at 4°C. The supernatant obtained was considered the enzymatic extract, and used for determining protein content and the activity of guaiacol peroxidase, catalase and polyphenol oxidase.

To determine chitinase and *β*-1,3-glucanase activity, enzymatic extract obtained from the fourth leaf with 100 mM sodium acetate buffer (pH 5.2) was used. Extraction and centrifugation procedures were the same as described above.

Total protein determination

Bradford test (1976) was used to quantify the total protein content in the samples. To each 50 µl of supernatant, 2.5 ml of Bradford reagent was added and mixed by vortex. After 5 min, readings were performed using a spectrophotometer (595 nm). Protein concentration, expressed in mg protein ml^{-1} of sample was determined using a bovine serum albumin (BSA) standard curve from 0 to 0.5 mg ml^{-1}.

Enzyme activity assays

Guaiacol peroxidase activity (EC 1.11.1.7) was determined at 30°C by measuring the conversion of guaiacol to tetraguaiacol at 470 nm (LUSSO; PASCHOLATI, 1999). Each reaction mixture contained 0.10 ml of enzyme extract and 2.9 ml of a solution, containing 250 µl of guaiacol (97%), 306 µl of hydrogen peroxide (34%) and 100 ml of 0.01 M phosphate buffer (pH 6.0). The reference cuvette contained only 3 ml of the solution. Peroxidase activity was determined over 2 min. The differential between 90 s and 30 s readings was used to determine activity. Results were expressed in absorbance min^{-1} mg^{-1} of protein.

Catalase activity (EC 1.11.1.6) was determined using the stable complex formed by ammonium molybdate and hydrogen peroxide (TOMÁNKOVÁ et al., 2006). The enzyme extract (0.1 ml) was incubated in 0.5 ml of the reaction mixture containing 60 mM hydrogen peroxide in 60 mM potassium phosphate buffer (pH 7.4) at 38ºC for 4 min. The consumption of hydrogen peroxide was stopped by adding 0.5 ml of 32.4 mM ammonium molybdate after the first 4 min of incubation. A blank was prepared for each sample by adding ammonium molybdate to the reaction mixture, omitting the incubation period. The yellow complex formed by molybdate and hydrogen peroxide was measured at 405 nm. The difference between the absorbance of the blank and the incubated sample indicated the amount of hydrogen peroxide used by the enzyme. H_2O_2 concentration was determined using the extinction coefficient $\epsilon = 0.0655\ mM^{-1}\ cm^{-1}$.

Polyphenol oxidase activity (EC 1.10.3.2) was measured by the method of Duangmal and Apenten (1999). The oxidation of catechol in quinone, a reaction mediated by polyphenol oxidase, was measured. The substrate used was 20 mM catechol dissolved in 100 mM potassium phosphate (pH 6.8). The reaction was performed by adding 900 µl of substrate to 100 µl of enzyme extract at 30°C and measured by a spectrophotometer at 420 nm for a 2-minute period. The difference between the reading in the first minute and the initial reading was used to determine activity. Results were expressed in absorbance $min^{-1}\ mg^{-1}$ of protein.

Chitinase activity (EC 3.2.1.14) was determined by the method of Wirth and Wolf (1990). Polymeric carboxy-methyl-substituted chitin, labelled covalently with Remazol Brilliant Violet (CM-Chitin-RBV, Loewe Biochemica GmbH, Germany) was used as substrate. Here, 600 µl of extraction buffer (100 mM sodium acetate pH 5.2) were mixed with 200 µl of enzyme extract and 200 µl of "CM-chitin-RBV" (2 mg l^{-1}). After a 20-minute incubation at 40ºC, the reaction was stopped with 200 µl of 1 M HCl, followed by cooling on ice and centrifugation at 10,000 g for 5 min. Supernatant absorbance at 550 nm was determined, and the results expressed in absorbance units $min^{-1}\ mg^{-1}$ of protein minus the blank absorbance, which was measured using 800 µl of extraction buffer + 200 µl of "CM-chitin-RBV".

To determine *β*-1,3-glucanase (EC 3.2.1.6) activity, 150 µl of enzyme extract was added to 150 µl of laminarin (1.5 mg ml^{-1}) in extraction buffer (100 mM sodium acetate pH 5.2). The reaction was performed at 40ºC for 60 min. After incubation, reduced sugars formed were quantified by the Lever method (LEVER, 1972). As a control, the same reaction was performed without incubation. To determine sugar content, an aliquot of 50 µl was taken from the incubated tubes and added to 1.5 ml of a solution containing 0.5% *p*-Hydroxy benzoic acid hydrazide (PAHBAH) in 0.5 M NaOH. The sample was kept in a boiling water bath for 10 min and then cooled in an ice bath. Absorbance was determined at 410 nm in a spectrophotometer. The absorbance of the blanks was subtracted from the reading. Sugar amounts were determined using a glucose standard curve ranging from 0 to 85 µg glucose ml^{-1}.

Experimental design and statistical analysis

A completely randomized design with three replicates in a factorial arrangement 2 x 2 x 9 (two genotypes, presence or absence of pathogen and nine sampling times) was set up. The experimental unit was one pot containing a single plant. Analysis of variance was conducted at a 0.05 level of significance. When treatments effects were significant, the means were separated by the Scott-Knott test ($p \leq 0.05$).

Results

A. solani development on tomato plants

At the first observation (4 hpi), the majority of conidia had germinated and the average germination

percentage was 83.7% and 84.9% on Kada and CNPH 1287, respectively. The highest germination percentage was registered at 12 hpi, with 93.3% and 93.2% in susceptible and resistant genotype, respectively. Germ tubes were formed from cells situated at the basis, in the middle or at the end of the conidium (Fig. 1A). On average, at 12 hpi, each conidium formed 3.7 and 2.8 germ tubes on Kada and CNPH 1287, respectively. Germ tubes showed high variation in size and grew without orientation onto the leaf surface. The majority of them grew and formed hypha onto epidermal cells and some of them formed appressoria (Fig. 1A and 1B). Appressoria were characterized by prominent dilations at the end of the germ tubes or sidelong the hypha. Most appressoria were formed at the cell wall junctions of the epidermal cells (Fig. 1B and 1C) and rarely at the stomatal complex. The number of appressoria in Kada cv. was significantly higher than in CNPH 1287 in all post-inoculation times (Table 1).

Tissue lesions began to appear at 96 hpi in Kada and at 120 hpi in CNPH 1287 (Fig. 1D). The number of lesions were significantly lower in resistant genotype (Table 1).

Table 1. Frequency of appressoria and lesions (% of observed conidia) at different post-inoculation times (hpi) in two tomato genotypes after inoculation with *Alternaria solani*.

Hpi	Appressoria (%)		Lesions (%)	
	Kada	CNPH 1287	Kada	CNPH 1287
12	29,1 a[1]	1,3 b	0,0	0,0
24	20,6 a	6,6 b	0,0	0,0
48	28,0 a	5,3 b	0,0	0,0
72	28,6 a	6,8 b	0,0	0,0
96	36,5 a	8,8 b	7,0 a	0,0 b
120	23,2 a	11,8 b	16,6 a	2,2 b

[1]At each time interval, values followed by different letters in the rows are significantly different at 5%.
Source: Elaboration of the authors.

Localization of ROS

Sites of fungal-host interaction showing accumulation of H_2O_2 (visualised as brown precipitate) were assessed in both plant genotypes at all sampling times after inoculation. However in low frequency, epidermal cells penetrated by the fungus showed H_2O_2 accumulation in both susceptible and resistant genotypes after 12 hpi (Fig. 1E-G).

Sites of penetration that accumulated $O_2^{\cdot-}$ were detected using NBT. Starting at 72 hpi, epidermal cells with a weak blue precipitation around dead cells became visible in both genotypes (Fig. 1H and 1I). With lower frequency, superoxide accumulation was detected in epidermal living cells under appressoria, with no traces of dead cells. When lesions were observed, penetrated epidermal cells and neighbouring cells showed a yellow colour at a regular light microscope.

Hypersensitive response

In the evaluations performed after 72 hpi, the cells with yellowish colour were observed under fluorescence microscopy and autofluorescence were detected (Fig. 1J and 1K). This happened in both resistant and susceptible genotypes. In more advanced pathogenesis stages, was observed fluorescence in mesophyll cells under injured areas.

Enzyme dynamics during tomato – A. solani interaction

Guaiacol peroxidase was significantly higher in CNPH 1287 when compared to Kada plants (at time 0, 4, 8, 24, 48 and 120 hpi) (Fig. 2A). The peroxidase activity was statistically different in inoculated CNPH 1287 plants when compared to healthy plants at certain time points (at 24, 72 and 120 hpi).

Only at 48 hpi, catalase activity was significantly higher in CNPH 1287 plants than in Kada plants (Fig. 2B). At 4 and 96 hpi, catalase activity was higher in inoculated CNPH 1287 plants when compared to healthy plants of the same genotype. In Kada plants, at 8 and 96 hpi, catalase activity was higher in inoculated plants than healthy plants, while at 24 hpi healthy plants showed higher activity.

Polyphenol oxidase activity was significantly higher in CNPH 1287 plants than in Kada plants until 72 hpi (Fig. 2C). Significant differences in enzyme activity was detected when inoculated CNPH 1287 plants were compared to healthy CNPH 1287 plants at 72 and 96 hpi. In Kada plants, a significant difference was detected between inoculated and non-inoculated plants only at 120 hpi.

At 0, 24 and 72 hours post-inoculation, chitinase activity was significantly higher in CNPH 1287 plants when compared to Kada plants (Fig. 2D). Chitinase activity was greater at 96 hpi in inoculated CNPH 1287 plants when compared to healthy plants of the same genotype.

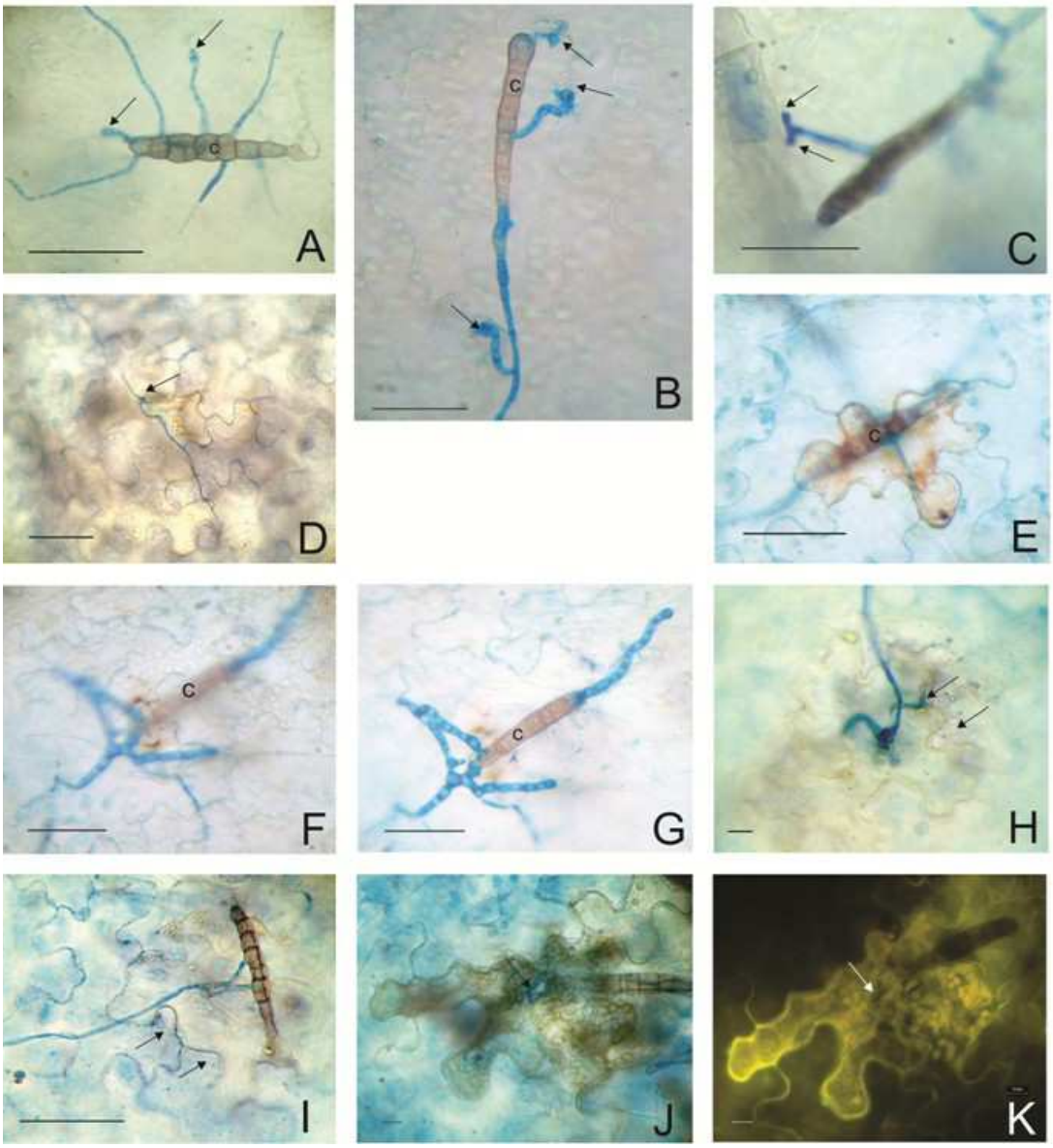
β-1,3-glucanase activity was significantly higher in CNPH 1287 plants than Kada plants at 0, 4, 8, 24, 72 and 96 hpi (Fig. 2E). *β*-1,3-glucanase activity was higher in inoculated CNPH 1287 plants when compared to healthy plants only at 96 hpi.

Discussion

The fungal germination on the plant surface of both genotypes was equal. In general, the spores of *Alternaria* spp. germinate on the leaves of the resistant cultivars and nonhost plants as well as host plants (ROTEM, 1994). Araújo and Matsuoka (2004) reported the same conidial germination of *A. solani* on leaves of *S. lycopersicon* and *S. habrochaites*. Spores of *A. brassicae, A. brassicicola*, *A. raphani* and *A. solani* germinated almost at the same rate on foliages of both host and nonhost plants, including oilseed rape, poppy, tomato and wheat (McROBERTS; LENNARD, 1996).

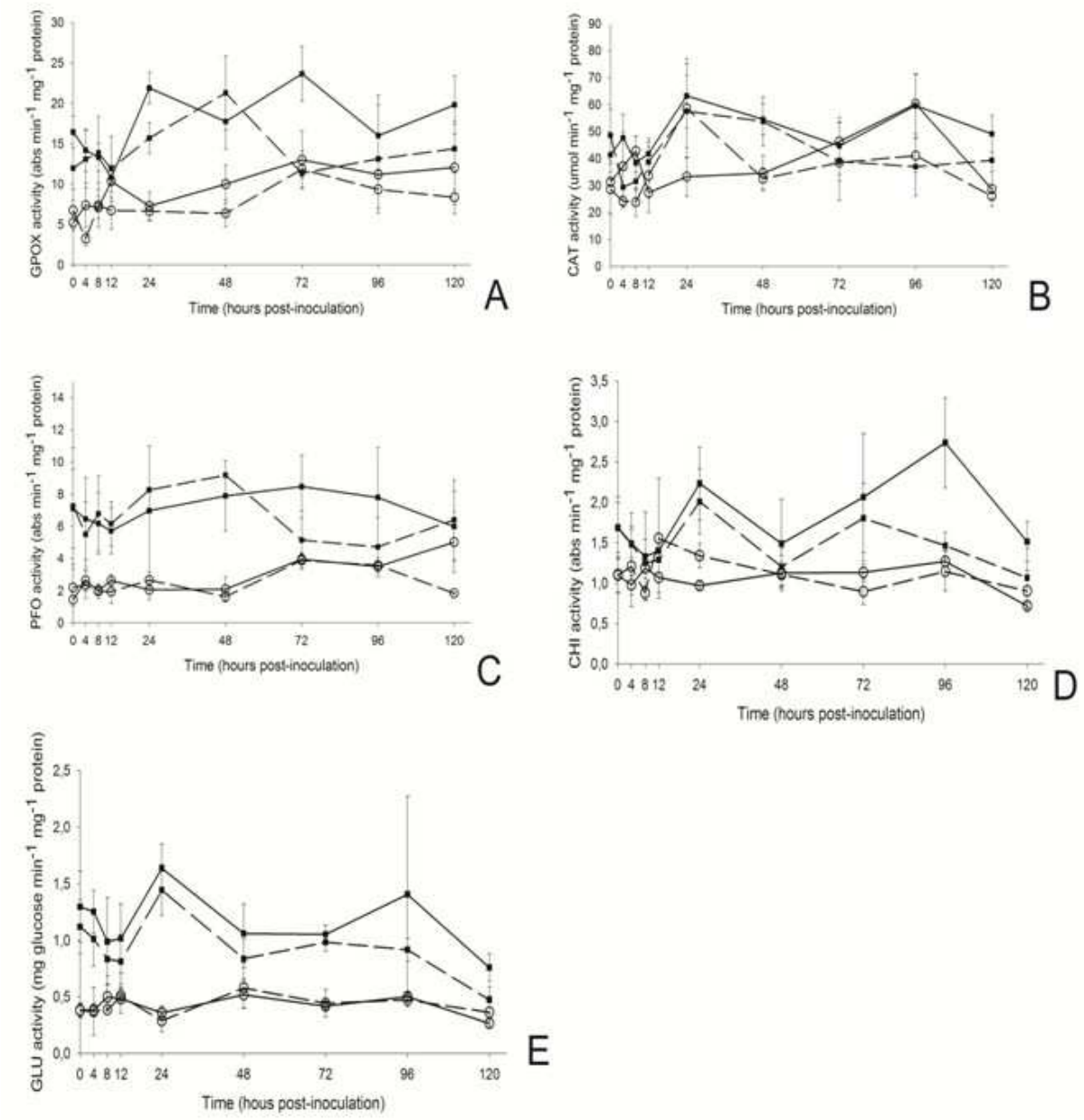
The absence of apparent orientation of the germ tubes on the leaf surfaces found in our work, was also reported by Araújo and Matsuoka (2004) in *A. solani – S. lycopersicon* and *S. habrochaites*, by Dita et al. (2007) in *A.solani* – potato, by Aveling, Snyman and Rijkenberg (1994) in *A. porri* – onion, by McRoberts and Lennard (1996) in *A. brassicae*, *A. brassicicola* and *A. raphani* – cruciferous plants, by Quayyum, Dobinson and Traquair (2005) in *A. panax* – American ginseng and by Van der Berg, Aveling and Venter (2003) in *A. cassiae – Vigna unguiculata.* In spite of the apparent random pattern in the growth of the germ tubes, the majority of the appressoria were formed at the junctions between the epidermal cells. This has been observed by some authors in *A. solani* (ARAÚJO; MATSUOKA, 2004; DITA et al., 2007) and in other *Alternaria* species (AVELING; SNYMAN; RIJKENBERG, 1994; McROBERTS; LENNARD, 1996; BOEDO et al., 2008) infecting a variety of hosts. Hancock and Huisman (1981) suggested that the general features of leaf topography and fluctuations in the nutrient microclimate around the cell junctions could stimulate the formation of appresoria at these sites.

Figure 1. Infection structures and growth of *A. solani* on tomato leaf discs and localization of H_2O_2 (DAB staining) and O_2^{-} (NBT staining) at interaction sites in resistant (CNPH 1287) and susceptible (cv. Kada) tomato plants after inoculation with *A. solani* at different post-inoculation times (hpi). A. Conidium with germ tubes and appressoria (arrows) at the end of two germ tubes. B. Appressoria (arrows) at the cell wall junctions of the epidermal cells at 48 hpi. C. Two appressoria (arrows) on the same epidermal cell at 48 hpi. D. Penetration site (arrow) and lesion developed from it at 96 hpi. E. CNPH 1287 epidermal cell with H_2O_2 accumulation at 72 hpi. F. Stomatal complex under appressoria with H_2O_2 accumulation in Kada at 12 hpi. G. The same as F but in upper focal plane. H. Dead epidermal cells at the site of penetration and neighboring cell showing weak accumulation of O_2^{-} (arrows) in Kada at 72 hpi. I. Dead epidermal cells at the site of penetration and neighboring cell showing weak accumulation of O_2^{-} (arrows) in CNPH 1287 at 96 hpi. J. Penetration site under appressorium (arrow) and reaction of epidermal cells in CNPH 1287 at 96 hpi; K. Same as J under fluorescent light: epidermal cells showing HR. Abbreviations: C = conidium. Bar A-G, and I: 50 µm Bar H, J and K: 10 µm.



Source: Elaboration of the authors.

Figure 2. Activity of guaiacol peroxidase – GPOX (A), catalase – CAT(B), polyphenol oxidase – PFO (C), chitinase – QUI (D) and *β*-1,3-glucanase – GLU (E) in leaves of tomato plants inoculated with *A. solani* (—) and healthy control plants (- – –) of resistant *S. habrochaites* CNPH 1287 (■) and susceptible *S. lycopersicum* cv. Kada (○) during 120 hours post- inoculation. Bars indicate mean ± standard deviation.



Source: Elaboration of the authors.

The susceptible Kada cultivar showed higher percentage of appressoria than the resistant CNPH 1287 cultivar. In the analysis of the infection process of *A. solani* on susceptible cv. Miller (*S. lycopersicon*) and on resistant cv. CNPH 417 (*S. habrochaites* var. *glabratum*), the formation of appressoria was a necessary step for the infection success, showing a clear relationship between low

level of appressoria development and low levels of lesions in the resistant genotype (ARAÚJO; MATSUOKA, 2004).

The lesions were developed from penetrations that occurred after the formation of the appressorium. Penetration performed by *Alternaria* species is commonly done from appressorium, which proves the essentiality of this structure for the pathogenesis (AVELING; SNYMAN; RIJKENBERG, 1994; MCROBERTS; LENNARD, 1996; ARAÚJO; MATSUOKA, 2004; DITA et al., 2007). Consequently, a lower frequency of lesions was the result of a lower number of appressoria formed in resistant plants of CNPH 1287.

The accumulation of H_2O_2 was detected earlier (at 12 hpi) than the O_2^{-} accumulation (at 72 hpi), although at low frequency. Both ROS were observed in the susceptible genotype as much as in the resistant one. Hypersensitive reaction was observed in the penetrated epidermal cells at 72 hpi in both genotypes. Despite this, since penetrations and lesions were more frequent in cv. Kada, the frequency of HR in epidermal cells of this genotype was higher.

The generation of ROS as well as HR were reported in others pathosystems involving *Alternaria* species. The inoculation of *Arabidopsis thaliana* with *A. brassicicola* determined the development of small necrotic lesions on the leaves. *Pad3-1* mutant plants (defective in the production of the indole-type phytoalexin, camalexin) inoculated with the same pathogen produced spreading lesions although both production of H_2O_2 and localized cell death were similar in normal and mutant plants after the inoculation (NARUSAKA et al., 2003). Based on the changes in the expression patterns of ca. 7,000 genes by cDNA microarray analysis after inoculation with *A. brassicicola*, the authors suggested that the *pad3-1* mutation altered not only the accumulation of camalexin but also the timing of expression of many defense-related genes in response to the challenge with *A. brassicicola*.

Reactive oxygen species generation was examined in the interaction of *Alternaria alternata* Japanese pear pathotype and host plants (SHINOGI et al., 2003). ROS generation was induced at cell walls of appressoria and penetration pegs in susceptible and resistant leaves, with the difference being in the volumes generated. Generation of ROS was detected in plant cells only during the compatible interaction which would be associated with cell death that facilitated the fungus colonization.

Sharma et al. (2007) analysed proteome-level changes in two *Brassica* lines derived from an interspecific cross between *B. napus* and *B. carinata* inoculated with *A. brassicae* using two-dimensional electrophoresis. Levels of 48 proteins were significantly affected in the tolerant line, whereas in the susceptible line, only the levels of 23 proteins were affected. Among the proteins identified from the tolerant line, were enzymes involved in the generation of ROS, ROS mediating signaling, auxin signal transduction and metabolic pathways. Yakimova et al. (2009) verified development of necrotic lesions as well as cell death, increased levels of hydrogen peroxide, malondialdehyde, free proline and enhanced total protease activity in detached tobacco leaves infiltrated with an AT toxin preparation from the foliar pathogen *A. alternata* tobacco pathotype. Govrin and Levine (2000) examined the induced oxidative burst and hypersensitive cell death in *Arabidopsis* after inoculation with the necrotrophic fungus *Botrytis cinerea*. Growth of *B. cinerea* was suppressed in the HR-deficient mutant *dnd1*, and enhanced by HR caused by simultaneous infection with an avirulent strain of the bacterium *Pseudomonas syringae*. HR had an opposite (inhibitory) effect on a virulent (biotrophic) strain of *P. syringae*. Moreover, H_2O_2 levels during HR correlated positively with *B. cinerea* growth but negatively with growth of virulent *P. syringae*. Based on these results, the authors concluded that although hypersensitive cell death is efficient against biotrophic pathogens, it does not protect plants against infection by the

necrotrophic pathogens. On the contrary, Dita et al. (2007) verified that the number of infection sites exhibiting HR was higher in potato plants resistant against *A. solani* than in the susceptible genotype after inoculation with that pathogen. They also found a relationship between the number of sites with HR and the leaf age, being the number of the penetration sites with HR higher in leaves in the upper part of the plant.

The results of this study indicate that ROS and HR do not contribute with the resistance against *A. solani*, whereas, the higher frequency of HR in susceptible genotype, as consequence of higher number of lesions in that genotype, probably facilitated the colonization of host tissues. Therefore, the *A. solani*-tomato interaction would follows the model proposed for necrotrophic pathogens where gene-for-gene resistance characterized by a rapid oxidative burst and hypersensitive cell death should not be observed (GLAZEBROOK, 2005). The production of phytoalexin, which according to Thomma et al. (1999) plays an important role in the resistance of *Arabidopsis* against *A. brassicicola*, was not evaluated in this study and it could be involved in the resistance against *A. solani* in *S. habrochaites*.

Peroxidases participate in various physiological processes related to defense, such as cross-linking of cell wall structural proteins and pectins and the polymerization of lignin precursors. Peroxidases also have a multifunctional role, acting as a "peroxidase", reducing the levels of ROS by metabolising H_2O_2, but also as an "oxidase", generating H_2O_2 (HAMMOND-KOSACK; JONES, 1996).

In this study, increased guaiacol peroxidase activity was detected mainly in inoculated resistant plants. Fernández et al. (1996) and Capote et al. (2006) verified significant peroxidase increased activity in resistant tomato plants after inoculation with *A. solani*.

The presence of ROS is normal in cellular metabolism, but these can become toxic to the cell when accumulated, especially when converted to even more reactive species such as hydroxyl radicals (OH^-). Due to the harmful nature of ROS, plants use enzymatic (superoxide dismutase, peroxidases, peroxiredoxins, catalase, monodehydroascorbate reductase, glutathione reductase, thiol enzymes) and nonenzymatic antioxidative systems (glutathione, ascorbate, tocopherols and phenolic compounds) to prevent damage to host cellular components (SEDLÁROVÁ et al., 2007). Catalase catalyses the dismutation reaction of hydrogen peroxide into water and oxygen, thereby reducing excess ROS during oxidative stress.

In this study, catalase activity was similar in susceptible and resistant plants and did not show significant increases during the evaluated period. This suggests that catalase was not operating as H_2O_2-scavenging enzyme as a consequence of low ROS accumulation. Catalase activity was stable or decreased in calli of *Brassica napus* and *B. juncea* cultured in medium with fungal culture filtrate of *A. brassicae* (DHINGRA; KIRAN; SANGWAN, 2004). Inoculation with *A. brassicae* did not affect catalase activity in resistant and susceptible plants of *B. juncea* (CHAWLA; GUPTA; SAHARAN, 2001). Peroxidase and superoxide dismutase activity were increased and catalase activity was slightly decreased in safflower (*Carthamus tinctorius* L.) tolerant plants regenerated via organogenesis and somatic embryogenesis from *in vitro* cultures treated with fungal culture filtrates of *A. carthami* when compared with control (susceptible) plants (VIJAYA KUMAR et al., 2008).

In our study, polyphenol oxidase activity was significantly higher in CNPH 1287 plants than in Kada plants at almost all sampling times. Solórzano et al. (1996) also verified the polyphenol oxidase involvement in the defense responses of resistant tomato plants after *A. solani* inoculation when detected higher activity of phenylalanine ammonia lyase and polyphenol oxidase in the resistant variety when compared with the susceptible ones.

In this study, β-1,3-glucanases and chitinases activities were detected in both genotypes but the levels were higher in CNPH 1287. The activity of both enzymes showed a significant increase in inoculated resistant plants at 96 hpi. The activity of β-1,3-glucanase and chitinase in control plants can be attributed to the constitutive expression of these enzymes in both genotypes. The constitutive level of chitinase and β-1,3-glucanase in resistant CNPH 1287 plants was 1.5 and 3 times higher than in the susceptible Kada plants. The higher pre-inoculation level of hydrolytic enzymes and the post-inoculation activity increase in inoculated plants of resistant genotype could have contributed to the lower development of the fungus. Lawrence, Joosten and Tuzun (1996) observed higher constitutive levels and greater induction of chitinase and β-1,3-glucanase in highly resistant tomato genotypes to early blight after *A. solani* inoculation when compared to susceptible genotypes. Solórzano et al. (1999) studied the chitinase isoenzymatic patterns in leaves of two varieties of tomato plants with different degrees of resistance to *A. solani* after inoculation with *A. solani*. They observed two acid isoenzymes in both varieties, but the induction of one of them in the resistant variety (NC EBR-1) was 24 hours earlier than in the susceptible variety (HC 3880). Lawrence et al. (2000) found constitutive levels of chitinase and β-1,3-glucanase five times and two times higher, respectively, in tomatoes resistant to *A. solani*.

The molecular mechanisms underlying activation of plant defense responses are very complex. Frequently, defense reponses begin with gene-for-gene recognition of the pathogen. The gene-for-gene concept (FLOR, 1971) implies that the production of certain virulence effectors by the pathogen leads to their recognition by plants that carry corresponding resistance gene (R). Recognition results in rapid activation of defense responses that limit the pathogen development in host tissues. Generally, R gene-mediated resistance is accompanied by a rapid production of ROS and HR which limit the access of the pathogen to nutrients.

Basically, plant pathogens are divided into biotrophs and necrotrophs, according to their lifestyles. Biotrophs feed on living host tissue while necrotrophs feed on dead host tissues after killing them. In the case of biotrophs, the HR prevent the pathogen of a food source, however, in the case of necrotrophs, that programmed cell death in the host would facilitate the pathogen colonization. R gene-mediated resistance is an important resistance response against biotrophic pathogens and is associated with activation of a salicylic acid-dependent signaling pathway. Gene-for-gene resistance should not be observed in interactions with necrotrophs, because host cell death do not limit the pathogen growth. In this kind of pathogens, defense responses depend on jasmonate and/or ethylene signaling pathway (GLAZEBROOK, 2005). Results from experiments in which *Arabidopsis* mutants with defects in various defense-related signaling pathways were tested for defects in resistance to *Peronospora parasitica, Erysiphe* spp. and *Pseudomonas syringae* led to the suggestion that SA-dependent defenses play an important role against biotrophs. On the other hand, results from the same mutants tested against *A. brassicicola* showed the absence of gene-for-gene resistance. Resistance to *A. brassicicola* depended on JA signaling and camalexin production, but R gene-mediated resistance, SA signaling, and ET signaling do not appear to play important roles (GLAZEBROOK, 2005).

Our results indicate that guaiacol peroxidase, hydrolytic enzimes (chitinase and β-1,3-glucanase) and enzymes related to phenol metabolism like polyphenol oxidase are some defense responses activated in *S. habrochaites – A. solani* interaction. Conversely, the magnitude of the ROS accumulation and HR observed, do not appear to play important roles in the resistance against *A. solani.*

In an analogous experiment where resistant (Kada) and susceptible (CNPH 1287) tomato genotypes were inoculated with the biotroph fungus *Oidium neolycopersici*, Balbi-Peña, Schwan-

Estrada e Stangarlin (2012) verified gene-for-gene resistance with expression of oxidative burst and HR in the incompatible interaction. Besides the increase in guaiacol peroxidase, polyphenol oxidase, chitinase and β-1,3-glucanase activity, they reported higher activity of catalase that was in agreement with the expressive amount of ROS produced in resistant plants.

While only a select group of defense responses were investigated in this study, many others such as phytoalexins and others enzymes related to phenol and ROS metabolisms could be involved in these interactions. We are beginning to understand the spatiotemporal dynamics of defense related enzymes, and which role ROS and HR play against necrotrophic pathogens.

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