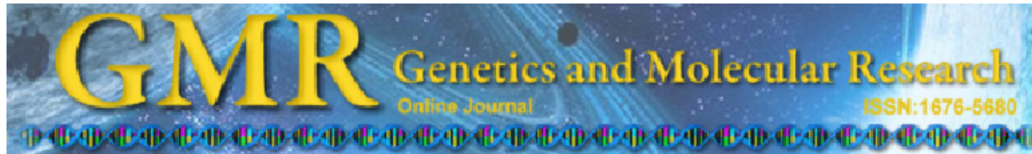

Peroxidase is involved in Pepper yellow mosaic virus resistance in *Capsicum baccatum* var. *pendulum*

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ABSTRACT. Pathogenesis-related proteins (PRs) are among the defense mechanisms of plants that work as an important barrier to the development of pathogens. These proteins are classified into 17 families according to their amino acid sequences, serology, and/or biological or enzyme activity. The present study aimed to identify PRs associated with the pathosystem of *Capsicum baccatum* var. *pendulum*: Pepper yellow mosaic virus (PepYMV). Forty-five-day-old plants from accession UENF 1624, previously identified as resistant to PepYMV, were inoculated with the virus. Control and infected leaves were collected for analysis after 24, 48, 72, and 96 h. The inoculated and control plants were grown in cages covered with anti-aphid screens. Proteins were extracted from leaf tissue and the presence of β-1,3-glucanase, chitinase, peroxidase, and lipid transport protein was verified.

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No difference was observed between the protein pattern of control and infected plants when β-1,3-glucanase, chitinase, and lipid transport protein were compared. However, increased peroxidase expression was observed in infected plants at 48 and 72 h after inoculation, indicating that this PR is involved in the response of resistance to PepYMV in *C. baccatum* var. *pendulum*.

Key words: Pathogenesis-related proteins; Resistance to diseases; Biotic stress; Biochemical mechanisms

INTRODUCTION

Plants are subject to a number of diseases caused by viruses, bacteria, fungi, and nematodes. These pathogens are responsible for crop losses and may have contributed to hunger and malnutrition of humanity since early agricultural practice. Therefore, disease control through genetic resistance is a major goal of plant breeding, and the association between plants and microorganisms has become the focus of many interdisciplinary studies.

Resistance against diseases is associated with preformed and induced mechanisms (Hammond-Kosack and Jones, 1997). The preformed mechanisms include physical barriers such as papillae, haloes, trichomes, and cuticles, which hinder the penetration of a pathogen in plant tissue, and secondary metabolites such as alkaloids, cyanogenic compounds, phenolics, glucosinolates, and terpenoids, which can prevent pathogen colonization, reproduction, and development. On the other hand, induced defense is a set of responses activated by hosts after pathogens are detected. The activation of these responses in plants depends on the efficiency of hosts in recognizing the presence of pathogens by means of perception and signal transduction (Jones and Dangl, 2006; Van Loon et al., 2006).

Induced resistance is often manifested as a hypersensitivity response, i.e., the death of host cells within hours after contact with the pathogen. Other resistance responses may include structural changes, accumulation of reactive oxygen species, synthesis of secondary metabolites, and the production of a wide variety of defense molecules, including pathogenesis-related proteins (PRs) (Van Loon et al., 2006; Elvira et al., 2008).

PRs were first described in tobacco that expressed hypersensitivity response after infection by tobacco mosaic virus (TMV). Later, it was identified in at least 13 plant families during infection by oomycetes, fungi, bacteria, viruses, viroids, nematodes, or insect attack (Van Loon et al., 2006). These proteins are classified into families according to their amino acid sequences, serological relationships, and/or biological or enzymatic activity (Van Loon and Van Strien, 1999; Van Loon et al., 2006).

Van Loon and Van Strien (1999) initially proposed a nomenclature for PRs, grouping them into 14 classes. The first five classes were observed in tobacco plants infected by TMV, and identified as PR-1, PR-2 (β-1,3-glucanase), PR-3, PR-4 (chitinase), and PR-5 (thaumatin-like). Subsequently, other classes of PRs were identified, resulting in a new classification containing 17 groups, namely PR-6 (proteinase inhibitor), PR-7 (endoproteinase), PR-8 (chitinase), PR-9 (peroxidase), PR-10 (ribonuclease-like), PR-11 (chitinase), PR-12 (defensin), PR-13 (thionin), PR-14 (lipid carrier protein), PR-15 (oxalate oxidase), PR-16 (oxalate oxidase-like), and PR-17 (unknown) (Van Loon et al., 2006).

Yellow mosaic, which is caused by the potyvirus Pepper yellow mosaic virus

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(PepYMV), is considered one of the most important diseases for solanaceous crops, such as chili pepper, sweet pepper, and tomato in Brazil (Maciel-Zambolim et al., 2004; Nascimento et al., 2007; Bento et al., 2009). The symptoms caused by PepYMV in *Capsicum* spp include curling leaves, development of yellowish-green mosaic, general reduction in the size of plants and fruits, and fruit deformation (Carmo et al., 2006).

Genetic resistance is the main form of PepYMV control, which has many advantages, especially regarding economic, ecological, and practical aspects. In *Capsicum* spp, the primary sources of resistance to PepYMV were found in *C. annuum*, 'Criollo de Morelos' (monogenic dominant resistance), and *C. chinense* PI 159236 (monogenic recessive resistance) (Boiteux and Pessoa, 1994). Other sources of resistance to PepYMV were detected in *C. chinense* and *C. baccatum* var. *pendulum* (Bento et al., 2009). Despite the identification of resistance in different species of *Capsicum*, the defense mechanisms involved in these resistances remain unclear.

The present study aimed to analyze the induction of PRs, focusing on β-1,3-glucanase, chitinase, lipid transport proteins (LTPs), and peroxidase in *C. baccatum* var. *pendulum* plants, accession UENF 1624, which is resistant to PepYMV.

MATERIAL AND METHODS

Plant materials

Seeds of *C. baccatum* var. *pendulum* from accession UENF 1624, resistant to PepYMV (Bento et al., 2009), were sown in 128-cell polystyrene trays with a commercial substrate. After the development of two pairs of definitive leaves, 160 seedlings were transferred individually to plastic pots containing a mixture of soil and substrate (2:1). The experiment was conducted in cages covered with anti-aphid screens in a greenhouse.

Inoculation procedure

Nicotiana debneyi plants infected with isolate 3 of PepYMV were used as the source of inoculum. This isolate was obtained from sweet pepper plants collected in a field of the Igarapé municipality, Minas Gerais State and was donated by Prof. Murilo Zerbini from Universidade Federal de Viçosa. Inoculation was performed via plant extract buffered in 0.05 M potassium phosphate, pH 7.2, containing 0.01% sodium sulfite using 600-mesh carborundum abrasive (Truta et al., 2004). The seedlings were inoculated at the stage of three definite leaves. The youngest fully expanded leaves were inoculated. Sixteen seedlings inoculated only with buffer solution and abrasive were used as control. Leaves of seedlings from the control plants and the accession UENF 1624 infected with PepYMV were collected at four different times (24, 48, 72, and 96 h) after inoculation.

Protein extraction and quantification

The leaves of infected and control seedlings were weighed and then macerated in liquid N_2. The macerated material was used for the different extraction methods.

For assays using β-1,3-glucanase and chitinase, extraction was performed using 20 mM sodium acetate, pH 5.5, 1% polyvinylpolypyrrolidone (w/v), and 1 mM phenylmethyl-

sulfonyl fluoride (PMSF) at a ratio of 1 g fresh weight of leaf for 1 mL buffer, which remained in agitation in microtubes for 60 min at 4°C. Then, the material was centrifuged at 10,000 *g* at 4°C for 20 min. The supernatant was collected and the precipitate was discarded (Caruso et al., 1999; Vieira et al., 2010).

Peroxidase extraction was performed using 10 mM sodium borate, pH 9.0, 0.125 M NaCl, and 1 mM PMSF at a ratio of 1 g fresh weight for 4 mL buffer, which remained under agitation in tubes for 3 h at 4°C. The material was centrifuged at 10,000 *g* at 4°C for 10 min. Next, the supernatant was collected and the pellet discarded. We added 14% polyethylene glycol and 8.5% potassium phosphate to the supernatant to separate the pigments. After phase separation, the lower phase was collected (Leon et al., 2002).

The quantitative determinations of proteins were carried out by the Bradford method (1976), using ovalbumin (Sigma, USA) as the standard protein.

Enzyme detection

Chitinase activity (EC 3.2.1.14) was determined in a Shimadzu spectrofluorometer according to O'Brien and Colwell (1987). The amount of methylumbelliferone released was measured by a primary filter with excitation at 320 nm and a secondary emission filter at 460 nm. One unit of activity (u.a.) was defined as 1 nmol methylumbelliferone released per minute. Data are reported as u.a. RV (reaction volume)/SV (sample volume) per µg protein/mL.

The determination of β-1,3-glucanase activity (EC 3.2.1.39) was performed according to the method described by Fink et al. (1988). One unit of β-1,3-glucanase activity was defined as the concentration of the enzyme that yields 0.001 absorbance when read at 500 nm in a spectrophotometer (Shimadzu UVVIS-1203). Data are reported as u.a. RV/SV per µg protein/mL.

Peroxidase activity (EC 1.11.1.7) was determined by spectrophotometry (Shimadzu UVVIS-1203) according to Leon et al. (2002), with some modifications suggested by Vieira et al. (2010). The enzyme u.a. was defined as the increase of 0.01 in absorbance in the reaction mixture for 3 min/mg protein.

Gel electrophoresis

Gel electrophoresis containing sodium dodecyl sulfate (SDS)-tricine was performed according to Schägger and von Jagow (1987). The SDS polyacrylamide gel electrophoresis (PAGE) was prepared according to Laemmli (1970).

Western blotting

The extraction of plant tissues for the detection of LTP was performed according to Granier (1988). Polyclonal antibodies produced against purified LTP of *Capsicum* spp seeds were prepared as described by Diz (2007). After electrophoresis, samples (10 mg crude extract of proteins from infected and uninfected leaves) were transferred to nitrocellulose membranes at 24, 48, 72, and 96 h after inoculation as described by Towbin et al. (1979). The Western blots were revealed using a chemiluminescence kit (ECL Reagent, Amersham Pharmacia Biotech., USA) according to manufacturer instructions.

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Detection of peroxidase activity on polyacrylamide gel

Peroxidase activity was detected on 14% polyacrylamide gel after electrophoresis of leaf tissue extract under non-denaturing conditions. Samples from different extractions containing 40 µg protein were mixed with sample buffer without β-mercaptoethanol and applied on the gel. After the race, the gel was washed three times for 20 min in 25 mL 0.05 M phosphate citrate buffer, pH 5.4, with slow agitation. Peroxidase activity was carried out by soaking the gel in 25 mL citrate-phosphate buffer, pH 5.4, containing 3% H_2O_2 and 8 mL guaiacol. After 2 min, brown bands indicated peroxidase activity (Shimoni, 1994).

RESULTS AND DISCUSSION

The SDS-PAGE gel analysis revealed similar protein profiles for seedlings infected with PepYMV and control at different times after inoculation for the chitinase, β-1,3-glucanase (Figure 1), and LTP (Figure 2) extractions.

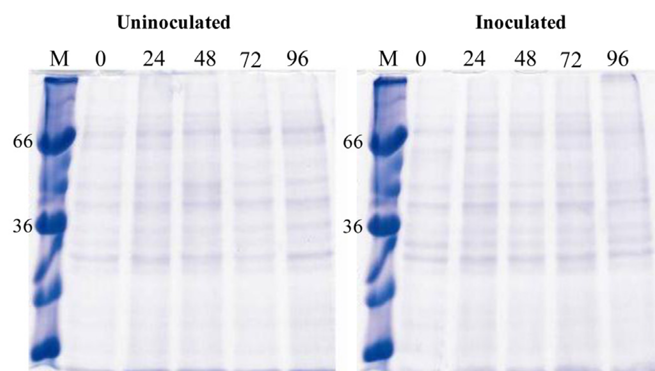

Figure 1. Polyacrylamide gel electrophoresis in 15% of inoculated and control (non-inoculated) samples extracted for chitinase and glucanase, at different times after inoculation with Pepper yellow mosaic virus (0, 24, 48, 72, and 96 h) in *Capsicum baccatum* var. *pendulum*. *Lane M* = molecular weight marker in kDa.

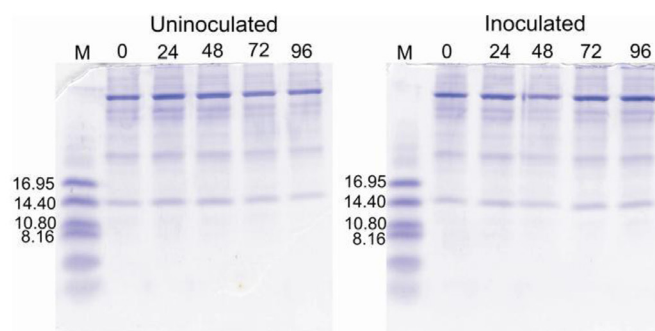

Figure 2. Polyacrylamide-tricine gel electrophoresis of control (non-inoculated) and inoculated samples extracted for lipid transport protein, at different times (0, 24, 48, 72, and 96 h) after inoculation with Pepper yellow mosaic virus in *Capsicum baccatum* var. *pendulum*. *Lane M* = molecular weight marker in kDa.

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No chitinase protein activity was observed in infected leaves at different times. Using the same methodology, Vieira et al. (2010) evaluated defense proteins expressed in cowpea seedlings infected by the fungi *Fusarium oxysporum f.* sp *phaseoli* and *F. oxysporum f.* sp *ubense*, and did not verify chitinase activity after inoculation either. Plant chitinases may be constitutively present or induced after biotic and abiotic stresses. Along with β-1,3-glucanase, they are directly related to defense against fungi. These enzymes degrade the linear homo-polymer β-1,4-N-acetylglucosamine, which is an abundant component of fungus cell walls (El-Katatny et al., 2001; Van Loon et al., 2006).

In relation to β-1,3-glucanase activity and compared to the control, the highest values for activities in the inoculated leaves were observed at 0 and 48 h after inoculation (Figure 3). Several studies demonstrate a positive correlation between expression of the β-1,3-glucanase gene and virus invasion (Gorovits et al., 2007; Elvira et al., 2008; Benitez-Alfonso et al., 2010). According to Boevink and Oparka (2005) and Benitez-Alfonso et al. (2010), callose production is regulated by a large family of callose synthases and β-1,3-glucanase. Thus, the increased induction of glucanase favors callose accumulation and decreased permeability of plasmodesmata, which limits the cell-cell movement of viruses.

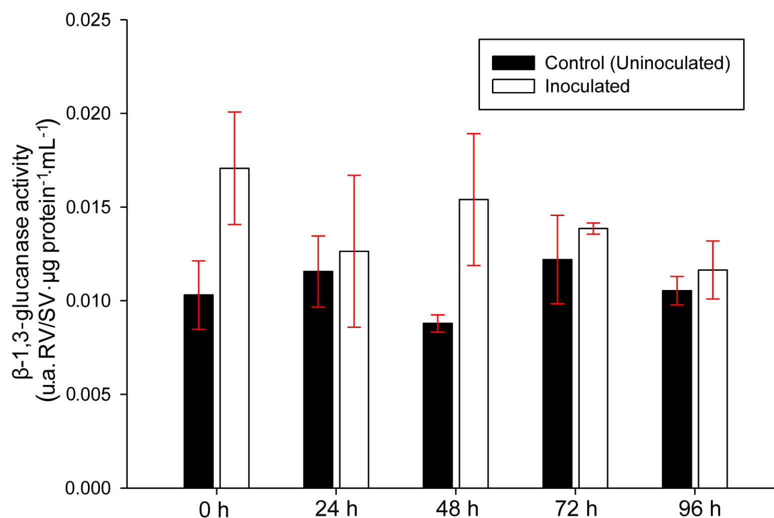

Figure 3. β-1,3-glucanase activity on leaves of *Capsicum baccatum* var. *pendulum* (UENF 1624) non-inoculated and inoculated with Pepper yellow mosaic virus from 0 to 96 h after inoculation.

It was not possible to detect the presence of LTP in the plant tissues of infected and control seedlings (data not shown). The main function of LTP is to transfer phospholipids between membranes. However, regarding plant defense, several studies have demonstrated that LTPs have antimicrobial activities against *in vitro* fungi and bacteria (Cammue et al., 1995; Wang et al., 2004). Wang et al. (2004) observed LTP isolates from mung bean seeds exhibiting antifungal activity against *F. solani*, *F. oxysporum*, *Pythium aphanidermatum*, and *Sclerotium rolfsii*, and antibacterial activity against *Staphylococcus aureus*. Park et al. (2002) found the expression of LTP cDNA in viruses during the response of resistance to TMV in *Capsicum*, suggesting possible LTP activity in the defense against viruses.

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For peroxidase, analysis of the SDS-PAGE gels (Figure 4A) revealed increased band expression of approximately 45 kDa between 48 and 72 h after inoculation. Subsequently, peroxidase activity was confirmed at this period on 14% polyacrylamide gel under non-denaturing conditions, where brown bands determined peroxidase activity (Figure 4B).

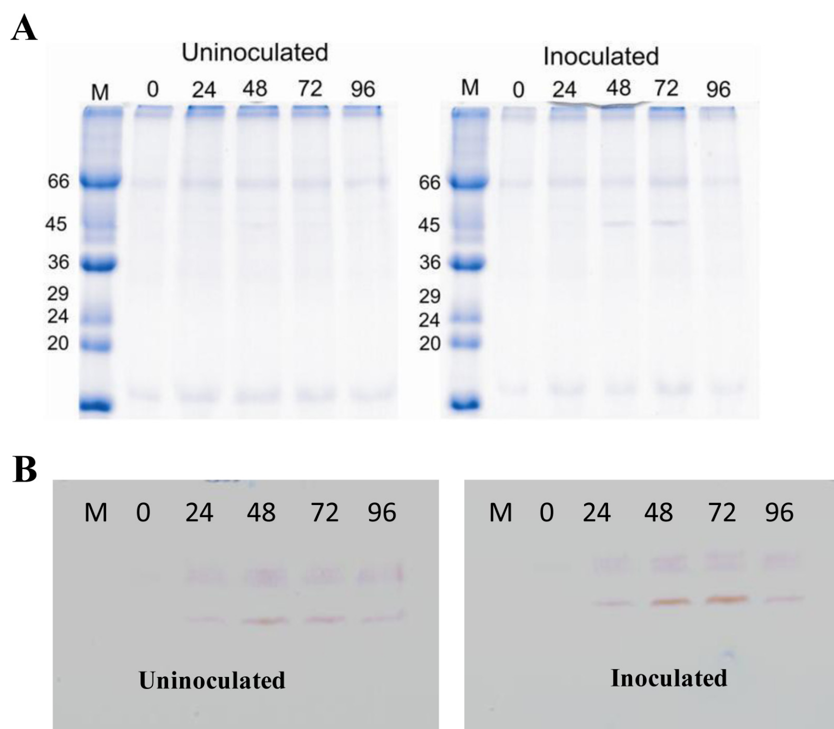

Figure 4. A. Polyacrylamide gel electrophoresis in 15%. **B.** Peroxidase activity gel electrophoresis of control (uninoculated) and inoculated samples, at different times (0, 24, 48, 72, and 96 h) after inoculation with Pepper yellow mosaic virus in plants of *Capsicum baccatum* var. *pendulum* (UENF 1624). *Lane M* = molecular weight marker in kDa.

Peroxidase activity was also determined by spectrophotometry (Figure 5), and corroborated the results obtained from the SDS-PAGE and denaturing gels. The inoculated plants demonstrated some peroxidase activity (0.0113 u.a./t·µg protein^{-1}·mL^{-1}) soon after inoculation, which remained almost constant at 24 h after inoculation. Increased peroxidase activity was observed at 48 h after inoculation both for plants inoculated with the virus and for control plants, but greater activity expression was observed in the virus-inoculated plants (Figure 5). The peak of peroxidase activity was observed for inoculated plants 72 h after inoculation, reaching a value of 0.0453 u.a./t·µg protein^{-1}·mL^{-1}. Peroxidase activity decreased significantly in the inoculated plants 96 h after inoculation.Thus, there was high peroxidase activity at 48 and 72 h in the inoculated plants. In the control plants, peroxidase activity was detected from 48 h after inoculation mimicking buffer solution.

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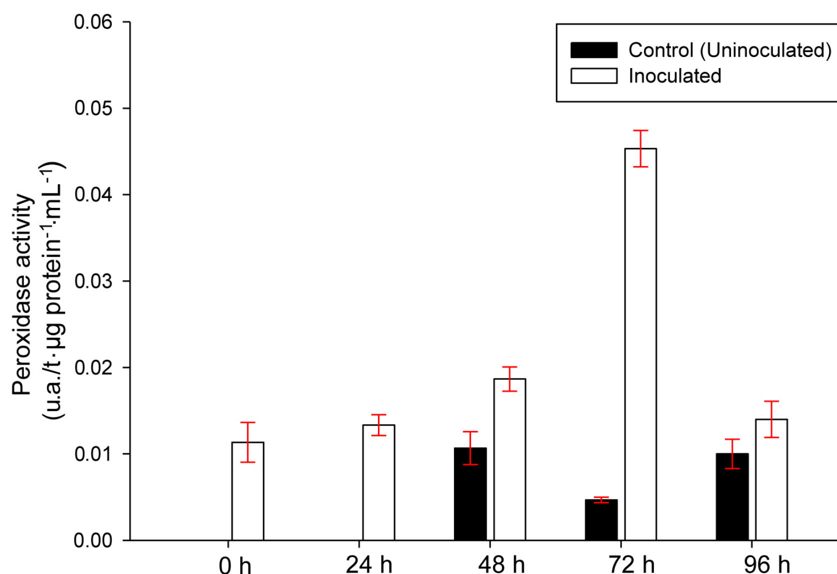

Figure 5. Peroxidase activity in leaves of *Capsicum baccatum* var. *pendulum* (UENF 1624) non-inoculated and inoculated with Pepper yellow mosaic virus, from 0 to 96 h.

Peroxidase is an important enzyme in plants that is involved in various reactions such as polysaccharide bonds, indole-3-acetic acid oxidation, monomer bonds, lignification, wound healing, phenol oxidation, defense against pathogens, and regulation of cell elongation, among others (Passardi et al., 2004). Several studies have demonstrated the role of peroxidase in the expression of genes related to defense, cell wall plasticity, and plant cell elongation (Quiroga et al., 2000). Besides the degradation of reactive oxygen species, peroxidases can also generate reactive oxygen species (H_2O_2, OH^-, O_2^-), which are toxic to pathogens and are produced by the oxidative activity of extracellular peroxidase during the strengthening of the cell wall, and by the synthesis of salicylic acid, aromatic monoamines, and chitooligosaccharides (Hiraga et al., 2001; Kawano, 2003; Vieira et al., 2010).

The induction or accumulation of peroxidase in response to pathogen inoculation has been demonstrated in the pathosystems of rice: *Pyricularia oryzae* and *Bipolaris sorokiniana*, pumpkin: *Colletotrichum lagenarium*, and cassava: *Xanthomonas axonopodis pv. manihotis* (Pereira et al., 2000; Houterman et al., 2007). In plant-virus interactions, peroxidase activity was also associated with tobacco - TMV (Lagrimini and Rothstein, 1987; Ye et al., 1990); in beans - White clover mosaic potexvirus (Clarke et al., 2002), and pumpkin - Cucumber mosaic virus and Zucchini yellow mosaic virus (Tecsi et al., 1996; Radwan et al., 2006, 2007).

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