

Effect of different carbon sources on proteases secreted by the fungal pathogen *Sclerotinia sclerotiorum* during *Phaseolus vulgaris* infection

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ABSTRACT. *Sclerotinia sclerotiorum* (Sclerotiniaceae) is a plant pathogenic fungus that causes white mold disease in vegetable crops, including the common bean (*Phaseolus vulgaris*). Proteases produced by fungi are normally an important part of the pathogenic process in the host. We examined the effect of different carbon sources - pectin, glucose, and cell wall of *P. vulgaris* on the production of proteases in cultures of *S. sclerotiorum*. These proteases were also assayed in infected *P. vulgaris* plants. Enzyme activity was increased with all carbon sources, but the highest levels were found when pectin was added. Based on real-time quantitative reverse transcription-PCR analyses, protease induction in *S. sclerotiorum* was found to occur at the level of gene transcription. The finding of increased expression of acid phosphatase 1 and aspartyl

protease *in vivo* in infected *P. vulgaris* plants supports the role of these enzymes in the invasion process of *S. sclerotiorum*.

Key words: Protease; *Phaseolus vulgaris*; *Sclerotinia sclerotiorum*; White mold; *acp1*; *asp5*

INTRODUCTION

The phytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary causes stem rot disease in a vast range of plant species and families, making it one of the most economically important fungal pathogens (Boland and Hall, 1994). *S. sclerotiorum* uses an infection strategy based on the acidification of the environment via the production of oxalic acid (Magro et al., 1984) and the secretion of numerous cell-wall degrading enzymes. The action of these enzymes, such as endopolygalacturonases, cellulases, pectin lyase, xylanase, β -galactosidase, and proteases, is believed to facilitate intercellular fungal growth and destabilize host cell integrity (ten Have et al., 2004).

Proteases have been suggested to be involved in many plant-pathogen interactions (Murphy and Walton, 1996; Clark et al., 1997; Farley and Sullivan, 1998; Paris and Lamattina, 1999; Poussereau et al., 2001a). Proteases of various phytopathogenic fungi have been detected in infected host plant tissues, but their precise function has not been elucidated yet. In *S. sclerotiorum* and *Botrytis cinerea* the proteolytic activity seems to be correlated with the development of symptoms (Movahedi and Heale, 1990) and the enzymes are considered important virulence factors (Cotton et al., 2002; Rollins, 2003). Proteases are also regarded as antagonists of antifungal proteins secreted as part of the defense responses by the host (Poussereau et al., 2001a; ten Have et al., 2004).

During saprophytic growth and pathogenesis, *S. sclerotiorum* secretes large amounts of oxalic acid that accumulate in infected tissues and rapidly decrease the ambient pH (Magro et al., 1984). It has been shown that ambient pH regulates gene expression (Poussereau et al., 2001a,b; Rollins, 2003; Hegedus and Rimmer, 2005).

We focused our attention on acid proteases *in vitro* and *in planta* during the interaction of this phytopathogen with dry bean plants. In this study, we present the characterization of proteases secreted during *S. sclerotiorum* growth in culture medium with different carbon sources and analyze the regulation of both *acp1*, a gene that encodes a non-aspartyl acid protease expressed during sunflower cotyledon infection (Poussereau et al., 2001a), and *aspS*, a gene that encodes an aspartyl protease expressed since the beginning of sunflower cotyledon infection (Poussereau et al., 2001b).

MATERIAL AND METHODS

Organism and culture conditions

S. sclerotiorum, strain SPS, was collected from a naturally infected bean plant and grown on Petri dishes containing potato-dextrose-agar (PDA) culture medium for 5 days at 20°C. Conical flasks containing 20 mL minimal salt medium (2 g NH_4NO_3 , 1 g KH_2PO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g yeast extract, 3 g DL-malic acid, and 1 g NaOH per liter of ddH₂O) were inoculated with a single 3-mm mycelial plug excised from the margin of a 5-day-old *S. sclero-*

tiorum colony growing on PDA and shaken at 20°C for 48 h. The cultures were centrifuged at 2000 rpm, washed twice, and used as inoculum in minimal salt medium. The effect of different carbon sources was determined by adding 1% citrus pectin or 1% glucose or 1% cell wall of *Phaseolus vulgaris* (CWPv). The cultures were grown in conical flasks with constant shaking at 20°C for 24-96 h. The pH of the culture media was recorded daily. The mycelium was harvested by filtration and stored at -80°C until RNA or protein extraction. Culture filtrates were collected, sterilized through a 0.22-mm filter, freeze-dried, and used as the enzyme source.

Bean tissue infection and sampling

Plants of dry bean cultivar Pérola were grown in 2-kg plastic pots containing soil fertilized with 4-30-16 NPK (1 g/kg soil) in a greenhouse at 24° ± 2°C temperature, 60 ± 5% relative humidity under a 16-h light/8-h dark period. Inoculation was performed 10 days after emergence using the toothpick method. Briefly, 1-cm long toothpick ends were poked onto Petri dishes containing PDA culture medium, inoculated with *S. sclerotiorum*, and grown for 5 days at 20°C. After this period, toothpick ends wrapped by the pathogen mycelia were introduced into the base of dry bean plant stems 1.0 cm above the soil surface for inoculation. All plants were kept at 20°C and 90% relative humidity for 48 h to provide adequate conditions for infection. Tissue samples were collected from the necrotic part of each lesion within 4 cm of the lesion edges 24, 48, 72, and 96 h post-inoculation (hpi) and immediately frozen in liquid nitrogen prior to RNA and protein extraction.

Protease assays

This analysis was based on the assay of MacDonald and Odds (1980). The assay mixture contained 0.9 mL 1% BSA in 50 mM sodium citrate buffer, pH 3.2, and 0.2 mL enzyme solution. After 2 h at 37°C, 0.4 mL 10% (w/v) trichloroacetic acid was added. The tubes were stored on ice for 10 min and then centrifuged (13,000 rpm for 10 min). The absorbance of the supernatant was read at 280 nm and corrected for background using a zero-time control in which trichloroacetic acid was added prior to the enzyme. One unit of enzyme was defined as the amount needed to increase A_{280} by 0.1 OD. Protein determinations were carried out using the method of Bradford (1976). Specific active-site inhibitors were incubated with the enzymes for 30 min prior to assaying protease activity. The final concentrations for protease inhibitors (Sigma) used were 0.04 M PMSF (serine protease inhibitor), 0.05 M EDTA (metalloprotease inhibitor), 1 M β-mercaptoethanol (cysteine protease inhibitor), and 0.1 M Pepstatin A (aspartyl protease inhibitor). All protease assays were run in triplicate and data are reported as means ± SEM.

qRT-PCR

RNA was extracted from plant and fungal tissue material using the standard protocol of the Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). RNA was treated with RNase-free-DNAse I (Invitrogen Corp.), followed by enzyme inactivation (EDTA 2.5 mM, 65°C/10 min) and ethanol precipitation to remove any genomic DNA contamination. The sequences of primers used were: acid protease (*acp1*) (GenBank/EMBL accession No. EU314718) [*acp1*-F: 5'-GCC ACC CAA AAC GGA GAA T-3' and *acp1*-R: 5'-GAG GTG AGG ACG GAG TTT TGT T-3']; aspartyl protease (*aspS*) (GenBank/EMBL accession No. DV643778) [*aspS*-F: 5'-

TGC TAC TGG GTC CAA CAT CGT-3' and aspS-R: 5'-TGC GCT TGA TGC ACT TGG T-3'], and the actin gene (*act*) (GenBank/EMBL accession No. Q11197) [act-F: 5'-CCC CAG CGT TCT ACG TCT-3', act-R: 5'-CAT GTC AAC ACG AGC AAT G-3']. Equal amounts of RNA (0.5 µg) were reverse-transcribed using oligo(dT)12-18 primer and submitted to real-time PCR. Amplification assays were carried out with a 7900HT Sequence Detection System ABI PRISM instrument (Applied Biosystems, Carlsbad, CA, USA) in 12-µL reaction mixtures containing 0.4 µM of each primer described above, 2X 6 µL SYBR Green PCR Master mix, and 0.2 µL template cDNA. After an initial denaturation step at 95°C for 10 min, amplifications were carried out for 40 cycles at 95°C for 15 s and 60°C for 1 min. The comparative crossing threshold method, employing the constitutive *S. sclerotiorum* actin gene expression data for normalization, was used to evaluate the fold-change of each gene of interest in the induction sample compared to the control. Each sample was examined in triplicate for all analyzed genes.

RESULTS AND DISCUSSION

Characterization of protease activity secreted by *S. sclerotiorum*

The effect of carbon sources on protease activity

In order to determine the pattern of protease production, *S. sclerotiorum* grown in a minimal glucose medium was transferred to culture media containing 1% pectin or 1% CWPv or 1% glucose. Protease activity was detected in cell-free culture filtrates of *S. sclerotiorum* in the presence of all carbon sources tested, but the levels of activity varied depending on the source used (Figure 1A). The activity of these enzymes reached maximum levels 48-72 h after being transferred to the medium supplemented with pectin or CWPv and remained stable for several hours (Figure 1A). The highest specific activity was obtained with pectin (0.69 U/mg) and CWPv (0.49 U/mg). No significant levels of protease-specific activity were found in the presence of glucose (0.065 U/mg). The enzymatic activity was higher in the medium containing pectin, probably due to the release of pectin polymers (oligogalacturonides) in the culture medium resulting from the secretion of hydrolytic enzymes by *S. sclerotiorum* (Riou et al., 1992).

In culture medium supplemented with 1% glucose, we observed a reduction in enzyme activity. Hydrolytic enzymes induced when the fungi is subjected to an extra source of carbon undergo a catabolic repression, a behavior commonly found in *Aspergillus nidulans*. As described by Mathieu and Felenbok (1994) and Panozzo et al. (1998), since this fungus preferentially uses glucose as an energy source, the biosynthesis of enzymes involved in protein degradation is only observed in the absence of easily metabolized carbon, such as glucose. Vautard-Mey and Fevre (2003) showed that when *S. sclerotiorum* grows in the presence of glucose, it increases the expression of the gene *cre1* and, consequently, this may contribute to the repression of genes that encode enzymes that degrade the host cell wall, such as proteases.

After the transfer, the culture pH dropped rapidly from 3.5 (12 hpi) to 2.8 (72 hpi) in the medium containing 1% pectin (Figure 1B). In the culture medium supplemented with 1% CWPv, pH decreased from 4.2 (12 hpi) to 3.9 (48 hpi) and remained constant. Interestingly, the nature of the proteases produced by the pathogen correlates with the acidification of the

culture medium: when the pH of the medium fell below 4.0 (48–96 h), the acid protease activity noticeably increased (Figure 1A).

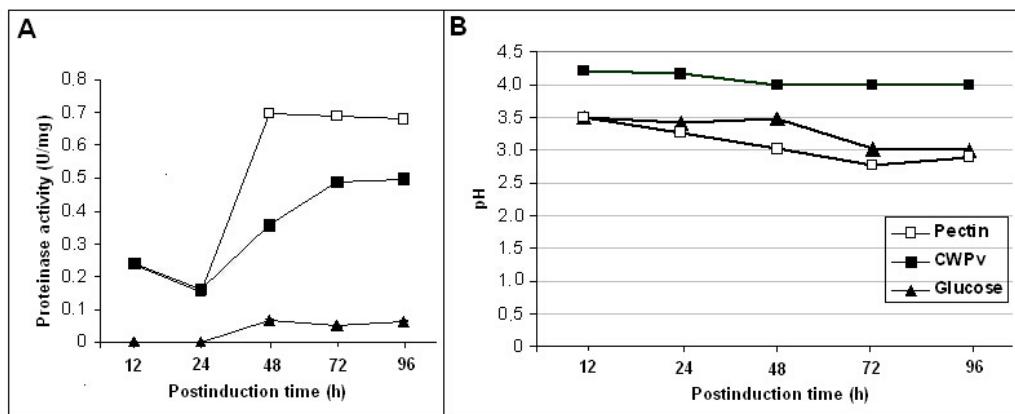


Figure 1. Kinetics of proteolytic activity of *Sclerotinia sclerotiorum* culture in different carbon sources (pectin, or *Phaseolus vulgaris* L. cell wall - CWPv, or glucose). **A.** Proteolytic activity of *S. sclerotiorum* cultivated in minimal salt media supplemented with 1% pectin or 1% CWPv or 1% glucose. The enzyme kinetics was performed in citrate buffer at pH 3.2 using BSA as substrate, incubated at 37°C (see Material and Methods). Tests on the proteolytic activity were carried out in triplicate. Values are reported as means \pm SEM. **B.** Comparison of pH profile evolution in culture media containing different carbon sources.

The effect of pH and inhibitors on protease activity

The optimal pH for protease activity was determined. The highest level of activity was detected at pH 3.0 in cell-free culture filtrates of *S. sclerotiorum* grown in minimal salt medium, supplemented with either 1% pectin or 1% CWPv extract (Figure 2A). However, enzymes responsible for the protease activity 12–24 hpi seem to belong to different families of proteases, probably acid and neutral and were subjected to temporal influences. No significant differences were observed in protease activity profiles between cell-free culture filtrates of *S. sclerotiorum* grown in minimal salt medium supplemented with pectin or CWPv.

Different protease inhibitors were tested in order to identify the type of protease responsible for this acidic enzyme activity (Figure 2B). PMSF, EDTA, β -mercaptoethanol, and Pepstatin A, inhibitors of serine, metalloproteases, cysteine and aspartyl proteases, respectively, did not affect this activity completely, indicating that the acidic activity secreted in the medium containing pectin or CWPv extract consists of overlapping activities of different classes of proteases.

Previous studies have shown that aspartyl proteases and acid proteases are secreted by *S. sclerotiorum* (Poussereau et al., 2001a; Billon-Grand et al., 2002). However, in contrast to what was observed in our study, Billon-Grand et al. (2002) did not observe activity of cysteine protease or metalloprotease secreted by *S. sclerotiorum* in culture medium containing sunflower extracts.

Extracellular proteases are produced by many pathogenic fungi but the profiles of proteases that these organisms produce have often not been fully understood. It has been proposed

that the range of proteases produced by a fungus reflects the adaptation of saprophytic or pathogenic fungi to the requirements of their ecological niches. In this sense, one can imagine that in response to environmental signals, protease production during pathogenesis must be regulated by the structural cell-wall proteins, nitrogen and/or nutrient limitation, and the ambient pH (Jarai and Buxton, 1994; Poussereau et al., 2001a,b; Billon-Grand et al., 2002; Rolland and Bruel, 2008; Rolland et al., 2009). In *S. sclerotiorum* for example, secreted proteases with an acidic optimum pH can be detected when the environment has been acidified via oxalic acid production by this phytopathogenic fungus (Marciano et al., 1983; Magro et al., 1984; Poussereau et al., 2001a; Cotton et al., 2002).

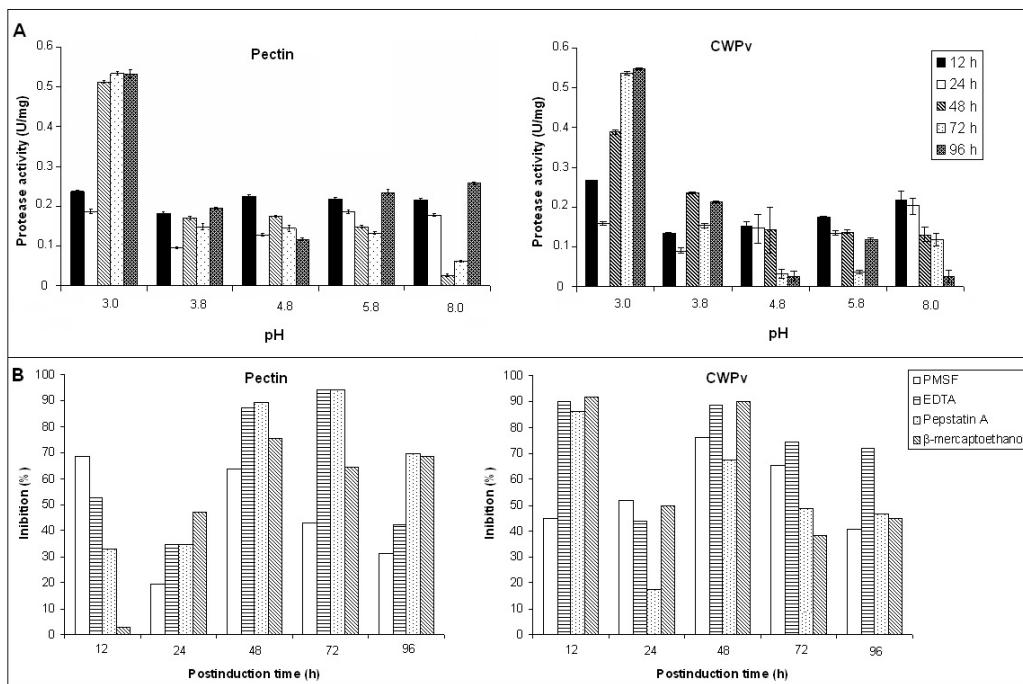


Figure 2. Effect of pH and inhibitors on the proteinase activity in the culture medium of *Sclerotinia sclerotiorum* after inoculation with pectin and *Phaseolus vulgaris* L. cell wall (CWPv). **A.** Profile of proteolytic activity at different levels of pH. **B.** Effect of protease inhibitors on proteolytic activity. Classification of inhibitors: PMSF (0.04 M) - serine protease inhibitor; EDTA (0.05 M) - metalloproteinase inhibitor; β -mercaptoethanol (1 M) - cysteine protease inhibitor; Pepstatin A (0.1 M) - aspartyl protease inhibitor. Tests on the proteolytic activity were made in triplicate.

Based on the expressed sequence tag (EST) approach proteases have been identified from *S. sclerotiorum* libraries. cDNAs encoding an acid protease and two aspartic proteases were identified in the infected *Brassica napus* stem library (Li et al., 2004). Also, cDNAs encoding an aspartyl protease was found only in the infection cushions and a serine protease in libraries prepared from mycelia grown on agar. CaaX prenyl protease, a metalloprotease, was found only in the infected *B. napus* leaf libraries (Sexton et al., 2006).

Protease activity during dry bean infection

Proteolytic activity during the interaction of *S. sclerotiorum* in bean plants was detected 24 h after inoculation and increased after that. This increased proteolytic activity was correlated with increased intensity of colonization and maceration of host tissue by *S. sclerotiorum* (Figure 3A and B). However, it is not possible to determine whether the observed activity is derived from the fungus or the plant.

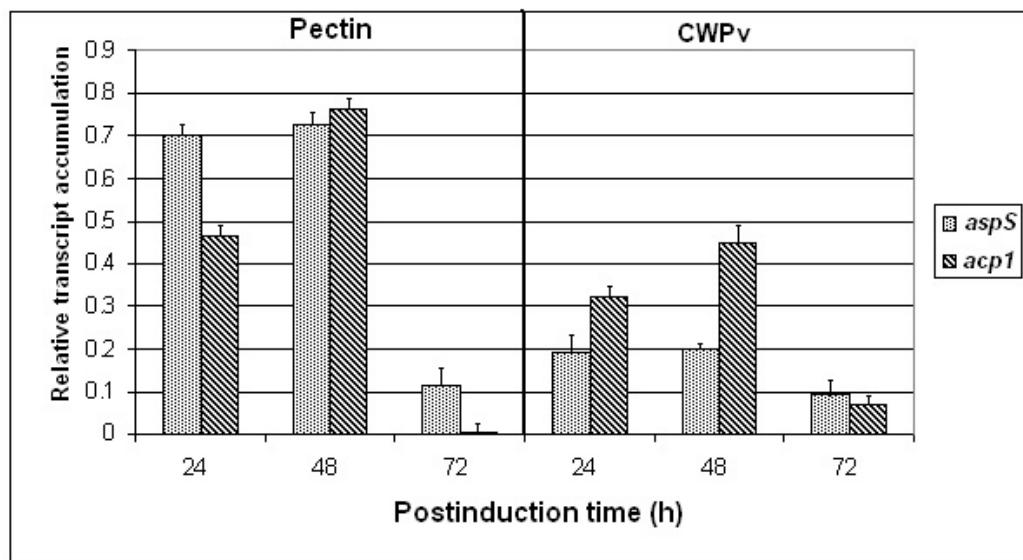


Figure 3. Real-time analysis of transcription patterns of active *Sclerotinia sclerotiorum* proteinase genes (*aspS* and *acp1*) during cultivation of the fungus in culture medium with pectin and *Phaseolus vulgaris* L. cell wall (CWPv).

Protease expression

Effects of ambient pH and carbon sources on *aspS* and *acp1* expression

In order to determine the pattern of *aspS* and *acp1* expression, *S. sclerotiorum* grown in minimal glucose medium was transferred to culture media containing 1% CWPv extracts and 1% pectin, respectively. Both *aspS* and *acp1* genes were expressed during glucose medium pre-culture (data not shown) and also expressed 24-72 h after transfer to 1% CWPv or 1% pectin media (Figure 3). The transcript accumulations were higher in the culture medium containing pectin, indicating that the expression of *aspS* and *acp1* genes is probably controlled by this carbon source. We presume that these variable differences between the two carbon sources used - CWPv and pectin - occurred because the degradation of pectin polymers may create an environment where low molecular weight products (oligogalacturonides) are released. These compounds serve to induce the expression of a second wave of degradative enzymes that collectively bring about the total dissolution of

the plant tissue (Hegedus and Rimmer, 2005). In this sense, Pereira et al. (2006) reported that chitin could induce the expression of two different proteases in *Aspergillus fumigatus* during the process of insect colonization.

During the course of the present *S. sclerotiorum* cultures in unbuffered medium, pH progressively decreased from 3.5 to 2.8 (as indicated in Figure 1), due to oxalic acid secretion, allowing a strong expression of *aspS* and *acp1* during this decrease. And interestingly, *aspS* and *acp1* transcript accumulation patterns were down-regulated 72 hpi in both media. As observed in previous studies, the ambient pH controls the acid protease gene (*acp1*) expression and may affect *aspS* expression (Poussereau et al., 2001a,b; Rollins and Dickman, 2001; Rollins, 2003; Kim et al., 2007).

In vitro expression of *acp1* is subject to several transcriptional regulatory mechanisms. Expression induced by plant cell-wall proteins is controlled by both carbon and nitrogen catabolite repression. Ambient pH higher than 5.0 overrides induction, resulting in full repression of *acp1*. These transcriptional regulatory mechanisms and the presence of several motifs in the promoter of *acp1*, which may encode binding sites for the regulators CREA, AREA, and PacC, suggest the involvement of these regulators in the control of *acp1* expression (Poussereau et al., 2001a; Kim et al., 2007).

Incongruence in the expected transcript accumulation pattern was observed in the *aspS* expressed gene. Interestingly, our study indicates that *aspS* expression was modulated during the fungal growth period in culture medium supplemented with CWPv and/or pectin media. In order to determine the effect of carbon and nitrogen sources on *aspS* expression, Poussereau et al. (2001a) transferred *S. sclerotiorum* mycelia cultivated in a minimal glucose/ NH_4^+ medium to unbuffered media containing sunflower extracts and glucose, glycerol, and/or NH_4^+ as carbon and nitrogen sources. Their study showed that *in vitro* expression of *aspS* is constitutive and not pH-regulated.

aspS and *acp1* expression during dry bean infection

In order to compare the temporal expression of *aspS* and *acp1*, qRT-PCR experiments were performed on total RNA extracted from *P. vulgaris* stems at different times after inoculation with *S. sclerotiorum*. The transcript putatively corresponding to the aspartyl protease (*aspS*) increased from the beginning of infection and remained high 72 hpi (Figure 4C). *acp1* transcripts, putatively corresponding to an acidic protease, were detected 24 hpi and increased 72 hpi. The increase of the transcripts coincided with the phase of symptom development, in which intensive mycelia colonization of the stems occurred (Figure 4A and C). Poussereau et al. (2001a,b) also showed that *aspS* and *acp1* were expressed *in planta* during sunflower cotyledon infection. The authors affirmed that the expression is low at the beginning of infection but increases suddenly at the stage of necrosis spreading.

A dynamic among oxalic acid production, environment pH acidification, enzyme activity, enzyme gene regulation, and ambient pH-signal transduction has been postulated to play a significant role in *Sclerotinia* disease development (Rollins, 2003; Hegedus and Rimmer, 2005). In summary, this report provides new information with respect to protease production during *S. sclerotiorum* host infection. Nevertheless, it is necessary to carry out more studies to further investigate the possible role of proteases in *S. sclerotiorum* during infection of different hosts.

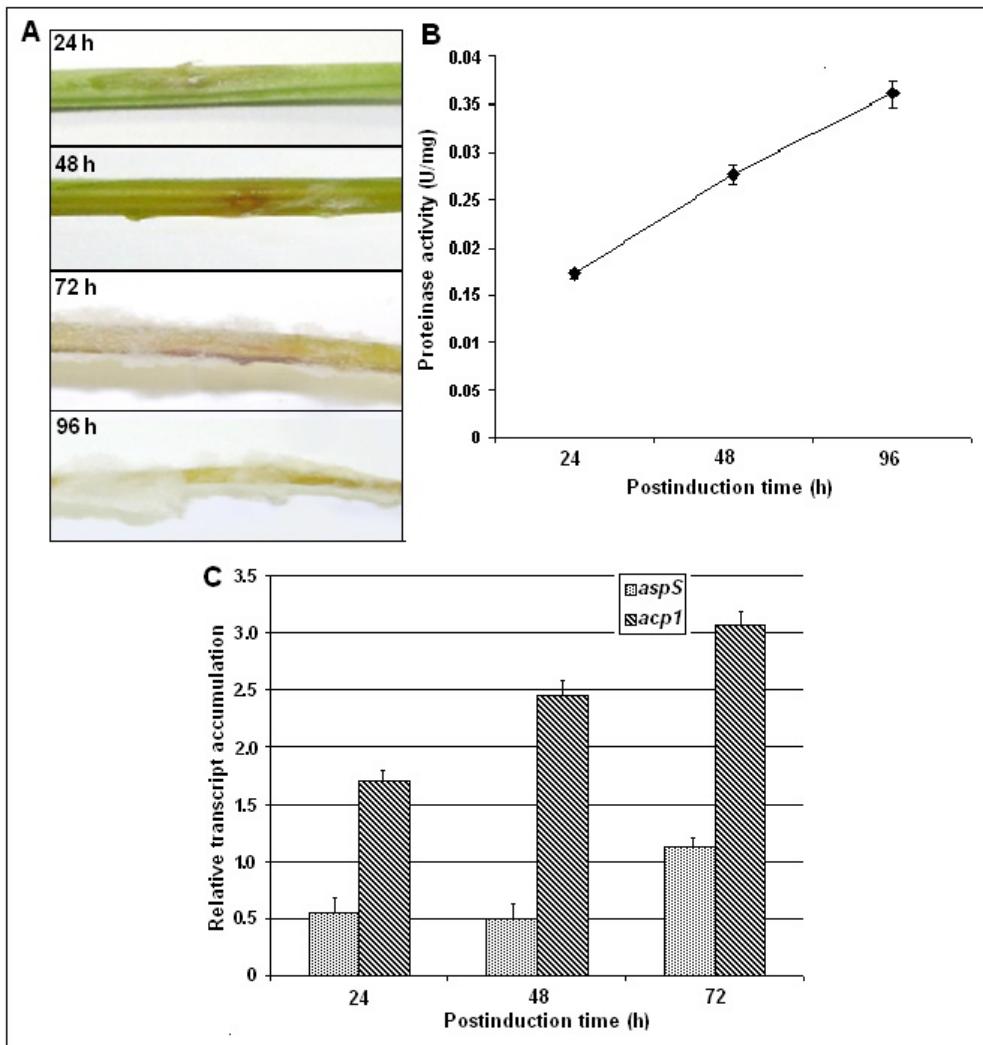


Figure 4. Expression of *Sclerotinia sclerotiorum* proteinases during pathogenesis. **A.** *S. sclerotiorum*-infected bean stem at different times after inoculation. Inoculation performed 10 days after emergence by introducing mycelium-colonized toothpick ends into the base of the stems 1.0 cm above the soil surface, which were removed to take the photograph in order to show the lesions at the following times: 24, 48, 72, and 96 hpi. **B.** Kinetics of proteolytic activity of *S. sclerotiorum*-infected bean stem (see Material and Methods). The enzymatic assay was performed in triplicate and values are reported as means \pm SEM. **C.** Real-time analysis of transcription patterns of *aspS* and *acp1* genes of *S. sclerotiorum*-infected bean stem. Transcript levels estimated using a standard curve for each gene and levels normalized against the amount of fungal actin transcripts in each sample.

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