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Recovery of active pathogenesis-related enzymes from the apoplast of *Musa acuminata* infected by *Mycosphaerella fijiensis*

Ignacio Islas-Flores[1]*, Carmela Alcocer-Alvarez[1], Yasmín Abril Sánchez-Rodríguez[1] and Blondy Canto-Canché[2]

[1]Unidad de Bioquímica y Biología Molecular de Plantas, Calle 43 No. 130, Colonia Chuburná de Hidalgo, 97200 Mérida, Yucatán, México.
[2]Unidad de Biotecnología, Centro de Investigación Científica de Yucatán A.C., Calle 43 No. 130, Colonia Chuburná de Hidalgo, 97200 Mérida, Yucatán, México.

The fungus *Mycosphaerella fijiensis* causes black Sigatoka (BS) disease, a major pathogen in the banana industry worldwide. Numerous molecular and biochemical studies have been done for the *M. fijiensis*, *Musa acuminata* interaction, but this is the first study describing the zymographic behavior of β-1,3-glucanase, chitinase and protease in the apoplast and symplast of healthy, BS-infected but asymptomatic and BS-diseased banana leaves. In BS-infected tissues, β-1,3-glucanase enzymatic activity was associated with two polypeptides with retention index (R_i) values of 0.43 and 0.56. These were more notable in the apoplast than in the symplast. Chitinase activity in BS-infected tissue in both the apoplast and symplast was mainly associated with a single polypeptide (R_i = 0.89). Both β-1,3-glucanase and chitinase activities were apparently more intense in BS-infected leaves than in healthy leaves. Protease activity was associated with two polypeptides (R_i = 0.04 and 0.14). In both the apoplast and symplast, the R_i 0.04 polypeptide increased in intensity with disease progression, whereas R_i 0.14 polypeptide intensity decreased. Overall protease activity intensity was higher in the symplast. Maximum symplast contamination of the apoplast was 2% as estimated by glucose 6-phosphate dehydrogenase activity, a biochemical marker for symplast. Accumulation of pathogenesis-related enzymatic activities in the apoplast of *M. acuminata* leaf tissue was caused by host-controlled enzyme downloading in response to *M. fijiensis* infection. Clear differences were identified in the electrophoretic profiles of healthy and diseased banana plants. The results further support a putative role of these enzymes in the extracellular defense repertoire of banana and, more importantly, suggest that *M. fijiensis* possesses a mechanism for suppression and delay of defense response in *M. acuminata*.

Key words: Black Sigatoka, glucose 6-phosphate dehydrogenase, pathogenesis-related (PR) proteins, polyacrylamide gel electrophoresis (PAGE), retention index (Ri), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

The apoplast is a continuous space surrounding plant cells, and is comprised of the cell wall matrix and intercellular spaces. Constituting 5 to 10% of plant mass (Gau et al., 2004), it plays a major role in a wide range of physiological processes. Its chemical composition is mostly water, ions and proteins, and it may be dependent on tissue distribution (Gau et al., 2004; Sattelmacher, 2001). The apoplast functions in cell signaling and plant

defense suggest it plays a key role during plant-pathogen interactions (Gau et al., 2004; Joosten and De Wit, 1989). During defense responses, the apoplast may contain over 200 proteins (Robertson et al., 1997). These are exported from the cell interior, mostly via the endoplasmic reticulum and the plasma membrane, but also via the plasma membrane through different, still incompletely understood routes (Gau et al., 2004; Hoson, 1998;=Floerl et al., 2008; Shabab et al., 2008). When bacteria and fungi attempt to gain access to the cell apoplast through the stomata or cuticle, the plant cell must respond to this challenge to protect itself. This can be accomplished via secretion of proteins that exert protective functions (Floerl et al., 2008), including a vital group known as pathogenesis-related (PR) proteins (van Loon and Kammen, 1970; van Loon and Strien, 1999; Blein et al., 2002; Brito-Argáez et al., 2010). Several research groups have used different approaches to analyze apoplast content during plant-pathogen interaction (Floerl et al., 2008; Shabab et al., 2008). Proteomics is increasingly popular, but has serious disadvantages in recovery of proteins with associated enzymatic or structural functions (Haslam et al., 2003; Boudart et al., 2005; Zhou et al., 2011). Extraction and characterization of the apoplastic proteins involved in pathogenesis or plant defense is an important strategy for study of specific pathosystems. This method is known to be feasible in diverse plant-pathogen interactions for example *Cladosporium fulvum-Lycopersicon esculentum* (Joosten and De Wit, 1989); *Leptosphaeria maculans-Brassica napus* (Brownfield and Howlett, 2001); non-pathogenic bacteria-*Malus domestica* (Kürkcüoglu et al., 2004); *Verticillium longisporum-Brassica napus* var. napus (Hoson, 1998); and *Septoria tritici-Triticum aestivum* (Shetty et al., 2009). Although, they are biologically significant, research into apoplastic proteins is hampered by their low abundance relative to overall intracellular protein concentration (Haslam et al., 2003; Mendoza-Rodríguez et al., 2006).

The fungus *Mycosphaerella fijiensis* is the most significant threat to banana and plantain production worldwide. This pathogen causes the disease known as black Sigatoka (BS) or black leaf streak (Churchill, 2011). It affects leaf tissues, thus reducing photosynthetic area, and leads to premature fruit ripening and important production losses (De Bellaire et al., 2010). It is hemibiotrophic, with an initial biotrophic mode of nutrition, a long asymptomatic period, and finally necrotrophy, which produces visible symptoms (hence the term black Sigatoka). During biotrophy, hyphae grow through the mesophyll layers, colonizing intercellular space (De Bellaire et al., 2010; Churchill, 2011). During this stage, the fungus is believed not to cause any host damage because it uses the available host resources to support its own growth. When host resources are insufficient to support continued hyphae growth, the fungus transitions to the necrotrophic stage and begins to secreting effectors (that is, secondary metabolites or proteins) to manipulate host defensive responses and continue to successfully colonize host tissues. The first extracellular effectors of *M. fijiensis*, MfAvr4 and MfEcp2, were recently identified (Stergiopoulos et al., 2010). MfAvr4 binds fungal chitin and protects the fungal cell wall against plant chitinases. The function of MfEcp2 is not entirely clear, but it probably functions as a necrotrophic factor (Stergiopoulos et al., 2010). These preliminary findings only highlight how very little is known about the apoplastic scenario of the *M. fijiensis-Musa* spp. Interaction. Better understanding, this scenario is vital since it is a potential area for isolation of proteins essential to pathogen establishment, and identification of the plant proteins involved in fungus rejection.

An initial attempt to analyze intercellular proteins from healthy and *M. fijiensis*-infected leaves of *Musa acuminata* 'Grande Naine' (AAA) produced only scarce and denatured proteins, undermining any claim of their biological importance (Mendoza-Rodríguez et al., 2006). The present study objective was to recover and analyze native proteins from the apoplast and symplast of healthy, asymptomatic (but infection-positive) and symptomatic diseased *M. acuminata* 'Grande Naine' leaves. Electrophoresis analyses were done of enzymatic activities (that is, β-1,3-glucanases, chitinases and proteases) associated with plant defense in this system.

MATERIALS AND METHODS

Plant material

M. acuminata 'Grande Naine' plants were grown in nurseries. At pre-fructification stages (approximately 8 months of age), visual criteria (Foure, 1987) were used to identify them as BS-negative (healthy) or BS-positive. *M. fijiensis* infection was confirmed using polymerase chain reaction (PCR) by amplification of the β-tubulin gene of *M. fijiensis*. With these criteria, plants were classified as healthy (absence of *M. fijiensis*; that is, neither visible symptoms of *M. fijiensis* induced lesions, nor PCR amplification of the *M. fijiensis* β-tubulin gene); BS-asymptomatic (early stage with no visible BS symptoms, but positive for the *M. fijiensis* β-tubulin gene by PCR); and BS-diseased plants (visible lesions from BS disease, including stages 1, 2, 3 and 4, and positive for the *M. fijiensis* β-tubulin gene

*Corresponding author. E-mail: islasign@cicy.mx. Tel: +529999428330. Fax: +529999813900.

by PCR) (Foure, 1987). The selected leaves (sixth or seventh) from healthy or BS-diseased plants were individually sanitized *in planta*, using the method described for recovering of apoplast and symplast, immediately excised from plants, and then processed for each one of the specific analysis.

DNA extraction

Extraction of genomic DNA (gDNA) from healthy, BS-asymptomatic and BS-diseased banana leaves was carried out according to Weising et al. (1991), using the cetyltrimethyl ammonium bromide (CTAB) method. Briefly, pellets were suspended in 50 µL sterile distilled water, the gDNA (1 µg) treated with 50 ng/µL RNAse A, and the quality and quantity ratio of DNA determined by electrophoresis on 1% agarose gels and at 260/280 nm absorbance using a spectrophotometer (Genesys 10 UV).

Amplification of the *M. fijiensis* β-tubulin gene

The PCR reactions were run using gDNA (25 ng) from the banana leaves as template. Each reaction tube contained 3 µL 5x PCR buffer; 0.6 µL 50 mM MgCl$_2$; 0.12 µL Taq Tango; 0.3 µL 10 mM dNTPs; 0.5 µL 10 µM primer β-tubMfF 5'-cgacacagcaagagcagcttc-3'; 0.5 µL 10 µM primer β-tubMfR 5'-ttcgaaagccttggcacttcaa-3'; and sterile distilled water. Total reaction volume was 25 µL. Conditions for PCR were 94°C for 5 min and 35 cycles x [94°C for 40 s, 60°C for 45 s, 72°C for 40 s]; and 72°C for 8 min. The resulting PCR products were separated by electrophoresis on 1% agarose-ethidium bromide gel and viewed on a Gel Doc documentation system (Bio Rad) under UV light.

Apoplast and symplast recovery

The sixth or seventh leaf of selected plants (five leaves per condition) was individually sanitized using a piece of cotton previously soaked with 70% ethanol, and then rinsed with distilled water. Sanitized leaves were excised from the plants and immediately transported to the laboratory. Central veins were removed with a scalpel and leaves cut into homogenous sections. Each condition group (healthy, BS-asymptomatic and BS-diseased) was individually vacuum infiltrated for 1 h under negative pressure at 25 mm Hg using apoplast buffer (AB; 100 mM Tris-HCl [pH 8.0] with added 10 mM ascorbic acid, 5 mM dithiotreitol, 5 mM phenylmethylsulfonyl fluoride, and 3% polyvinyl polypyrrolidone [PVPP]). Apoplastic proteins were recovered by centrifuging at 10,000 x *g* and 4°C; the eluate was called "apoplast". The apoplast-free tissues were then extracted by maceration in a pre-chilled mortar and pestle in the same buffer. The resulting homogenates were centrifuged at 16,000 x g for 10 min; the supernatants were called "symplast". Symplast samples were stored at -20°C until use.

Protein precipitation

For protein enrichment, the apoplasts were independently precipitated with acetone at a final concentration of 8%, incubated at -20°C for 2 h and then centrifuged at 16000 x g for 10 min at 25°C. The supernatants were discarded and the pellets dried at 25°C for 20 min. Pellets from ten tubes were suspended in 10 µL AB buffer supplemented with 1% glycerol and stored at -20°C until use.

Protein quantification

Protein concentration was determined according to Peterson (1977) at 750 nm absorbance, using bovine serum albumin and fraction V as a protein standard.

Protein electrophoresis

Protein preparations (20 µg each) were separated on 12% denaturing gels (SDS-PAGE) according to Laemmli (1970), and on 12% native gels (PAGE). Polypeptides were viewed by staining the gels for total protein using silver or for enzymatic activity (described below).

Zymography of β-1,3-glucanases, chitinases and proteases

For zymographic analyses, native 12% polyacrylamide gels were loaded with samples recovered from apoplastic fluids of healthy (1), BS-asymptomatic (2), and BS-diseased (3) leaves or with symplastic fluids from healthy (5), BS-asymptomatic (6), and BS-diseased (7) leaves. Gels were electrophoresed for 3 h at 100 V, and 25°C. The β-1,3-glucanase activity was assayed according to Pan et al. (1991). Briefly, the gels were individually incubated in sodium acetate (50 mM; pH 5.0) for 5 min immediately after the PAGE, and the solution discarded. The gels were then incubated at 40°C for 60 min in 25 mL 50 mM sodium acetate (pH 5.0) with addition of 0.15 g laminarin previously dissolved in 25 mL H$_2$O. Finally, they were submerged in methanol:water:acetic acid (5:5:2; V/V/V) for 5 min and washed in deionized water for 30 s. The activity of β-1,3-glucanase was viewed by incubating the gels in 30 mL triphenyl tetrazolium chloride (0.15 g/50 mL 1.0 M NaOH) and warming in a microwave oven five times, 10 s each time. Activity bands appeared as purple bands on a pink background. Beta-1,3-glucanase from *Helyx pomatia* (Sigma) was included in the gel as a positive control.

Chitinase was identified by co-polymerizing 12% native gels in the presence of 0.01% glycol-chitin (Sigma). Gels were loaded with protein from each sample, and after electrophoresis were washed for 30 min in 100 mM sodium acetate (pH 5.0). The washing buffer was exchanged with 50 mL sodium acetate (100 mM [pH 5.0], with added 1% Triton X-100) and incubated for 22 h at 37°C. The gels were then transferred to 50 mL 500 mM Tris-HCl (pH 8.9) with added 0.05% Brightener 28 (5 min), and washed 5 times in distilled water. Chitinase activity was viewed as dark bands on a white background by using UV light in a Gel Doc device. Commercial chitinase from *Streptomyces griseus* (Sigma) was used as a positive control.

Protease identification was done according to Distefano et al. (1997). Briefly, native gels were co-polymerized in the presence of 2% gelatin, and incubated for 60 min at 37°C in 1% Triton X-100. This solution was exchanged with 2.5% Triton X-100 solution and incubated at 37°C for 60 min, followed by an overnight incubation at 37°C in 50 mL 250 mM Tris-HCl (pH 7.5). Enzymatic activity was viewed by staining the gels with Coomassie blue to produce clear bands on a blue background. Trypsin II-6 (10 µg) from porcine pancreas (Sigma) was used as a positive control. Retention index (Ri) values for all enzyme migrations were calculated using the ratio: Ri = distance (mm) migrated by enzyme in gel / total distance (mm) migrated in gel by front of samples.

Determination of enzymatic activity of glucose 6-phosphate dehydrogenase

Glucose 6-phosphate dehydrogenase (G6PD) activity was assayed in 750 µL of buffer containing 100 mM Tris-HCl (pH 8.0), with added 10 mM MgCl$_2$, 6 mM glucose 6-phosphate and 4 mM NADP (200 µL). The mixture was incubated at 25°C for 5 min and the reaction initiated by adding 10 µL protein sample (3 µg protein) in 950 µL
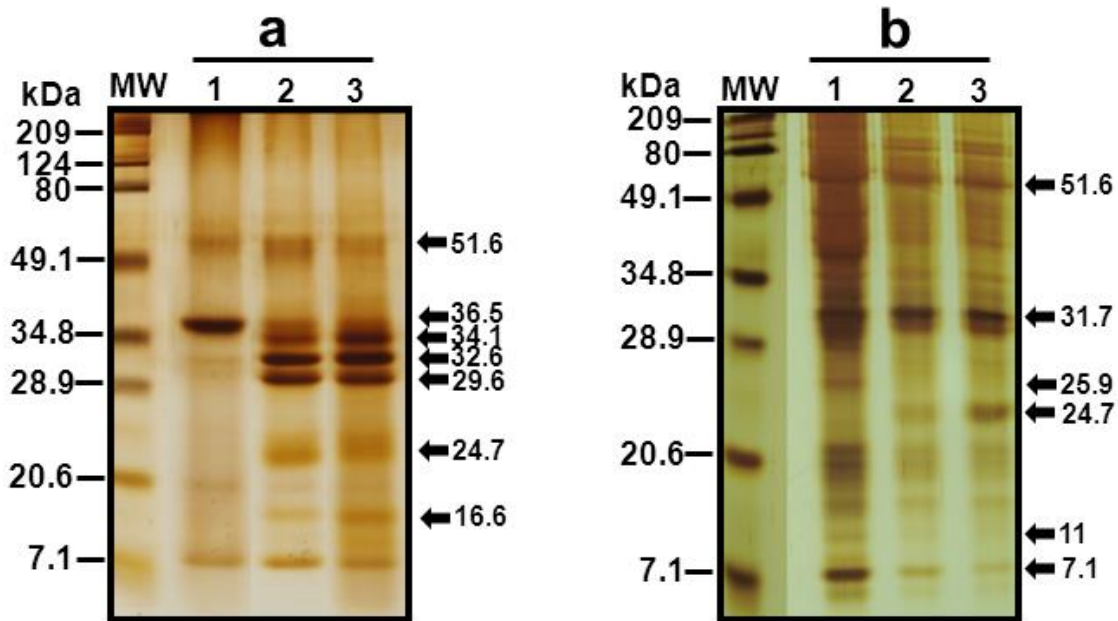
Figure 1. Apoplastic and symplastic polypeptides from *Musa acuminata* and the *Musa-acuminata-Mycosphaerella fijiensis* interaction. Protein samples were resolved on 12% SDS-PAGE gels. Fluids were from healthy (a1), BS-asymptomatic (a2), and BS-diseased (a3) apoplast, or from healthy (b1), BS-asymptomatic (b2), and BS-diseased (b3) symplast. Arrows to the right indicate polypeptides that remained unchanged, appear, or disappear as disease symptoms progressed. MW corresponds to sizes of protein molecular weight standards.

total volume. Enzyme activity was monitored at 25°C by following increases in absorbance at 340 nm for 15 min. Specific activity was calculated using the extinction coefficient of NADP (6.18 mM cm^{-1}), and expressed as µmol substrate reduced per min^{-1} g of tissue^{-1}.

Evaluation of banana tissue integrity

Leaf disks from healthy, BS-asymptomatic and BS-diseased leaves (three replicates each), were infiltrated with extraction buffer and then bleached at 8°C in ethanol-chloroform (4V/1V) for 18 days. Bleached samples were transferred to 70% ethanol and incubated overnight at 4°C. Samples were then transferred to 50% glycerol-ethanol and incubated for an additional two days at 4°C. After incubation, the samples were covered with a 0.01% trypan blue solution for 20 min and observed with a normal transmitted light microscope (Carl Zeiss). Images were taken using a digital camera (Kodak).

RESULTS

Apoplast and symplast analyses

SDS-PAGE analysis identified few polypeptides in the apoplastic fluid from healthy banana plants. The most abundant had a molecular weight of 36.5 kDa (Figure 1a, lane 1), and was notably less abundant in apoplastic fluids from BS-asymptomatic and BS-diseased plants (Figure 1a, lanes 2 and 3, respectively). Three polypeptides (molecular weights = 34.1, 24.7 and 16.6

kDa) were present in apoplastic fluid from BS-asymptomatic and BS-diseased plants, but absent in fluid from healthy plants (Figure 1a, lanes 2 and 3). A third group of polypeptides (MW = 32.6 and 29.6 kDa) was barely present in the healthy tissue, but quite notable in the BS-asymptomatic and BS-diseased tissues (Figure 1a, lanes 1, 2 and 3). In the symplastic fluid, a complex pattern of polypeptides ranging from 2.1 to 209 kDa was observed in healthy plants (Figure 1b, lane 1). Levels of these polypeptides were generally lower in BS-asymptomatic and BS-diseased plants. The 32.6, 25.9, 20.6, 11 and 7.1 kDa polypeptides decreased in abundance in comparison with levels in the healthy symplast (Figure 1b, lanes 2 and 3). In contrast, a small number of polypeptides (24.7 kDa) increased in the BS-diseased symplastic fluid (Figure 1b, lanes 2 and 3), while some polypeptides (51.6 kDa) remained relatively unchanged in all conditions (Figure 1b). Protein profiles dynamics are summarized in Figure 6.

Zymography of β-1,3-glucanase, chitinase and protease

Plants use β-1,3-glucanase and chitinase enzymes to efficiently control fungal pathogens (Floerl et al., 2008; Shetty et al., 2009; Pan et al., 1991). The possible presence of enzymatic defensive weapons in *M.*
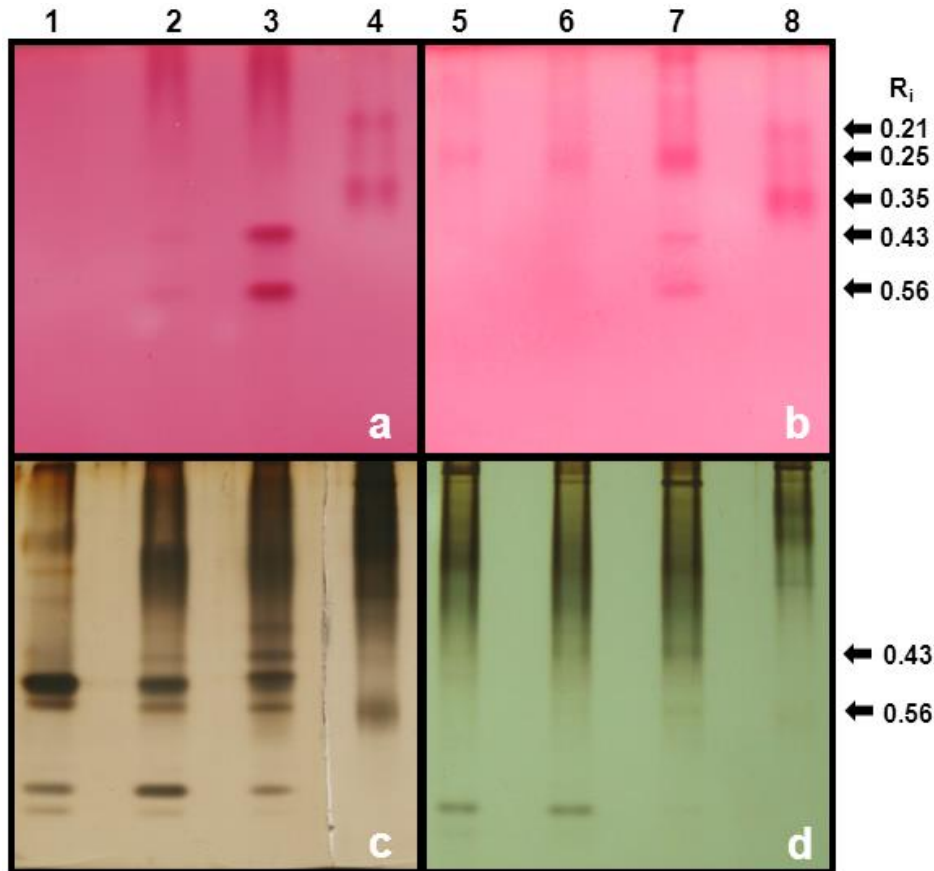
Figure 2. Zymography in non-reducing 12% polyacrylamide gels of β-1,3-glucanase from apoplastic and symplastic fluids of *Musa acuminata* and the *M. acuminata-M. fijiensis* interaction. Beta-1,3-glucanase was analyzed in apoplastic fluids recovered from healthy (a1), BS-asymptomatic (a2), and BS-diseased (a3) leaves, or in symplastic fluids from healthy (b5), BS-asymptomatic (b6), and BS-diseased (b7) leaves. Commercial (Sigma) β-1,3-glucanase from *Helix pomatia* (lanes a4 and b8) was used as a positive control. Arrows to the right indicate R_i of bands with enzymatic activity (a and b), and silver-stained polypeptides (c and d) that co-migrated at the same position where enzymatic activity was detected.

acuminata versus *M. fijiensis* attack was tested by analyzing β-1,3-glucanase enzymatic activity in apoplastic and symplastic fluids, with and without fungal challenge. Enzymatic activity was scarce in apoplastic fluid from healthy plants but more detectable as bands of variable intensity with Ri = 0.43 and 0.56, in apoplastic fluids from BS-asymptomatic plants (Figure 2a, lane 2) and BS-diseased plants (Figure 2a, lane 3). In general, symplastic fluids exhibited similar results with strong β-1,3-glucanase activity detected in BS-diseased tissues (Figure 2b, lanes 3 and 7, respectively). A band with a Ri = 0.25 was observed in the symplast of healthy and BS-diseased plants but with the strong signal in the last (Figure 2b, lanes 5, 6 and 7). The positive control, a commercial β-1,3-glucanase from *H. pomatia* (Sigma), exhibited two polypeptides (R_i = 0.21 and 0.35) that produced clear activity signals (Figure 2a and b, lanes 4

and 8, respectively). In all samples, β-1,3-glucanase enzymatic activity bands correlated with protein bands in the PAGE gels (Figure 2c and 2d).

Chitinases in apoplastic fluids (Figure 3a, lanes 1 to 3), exhibited activity associated with two bands of R_i = 0.63 and 0.89. The 0.63 band decreased in activity in fluids from BS-asymptomatic and BS-diseased plants, while the 0.89 band increased in intensity in BS-asymptomatic and BS-diseased plants (Figure 3a, lanes 1, 2, 3). Chitinase activity in symplastic fluids was associated with three bands showing R_i = 0.58, 0.63 and 0.89. Unlike in apoplastic fluids, the 0.63 band remained constant in healthy and BS-asymptomatic symplastic fluids, and was negligible in BS-diseased fluids (Figure 3b, lanes 5, 6, 7). The 0.89 band progressively increased intensity from healthy to BS-diseased samples (Figure 3b, lanes 5, 6, 7). The 0.58 band was visible only in symplast from BS-
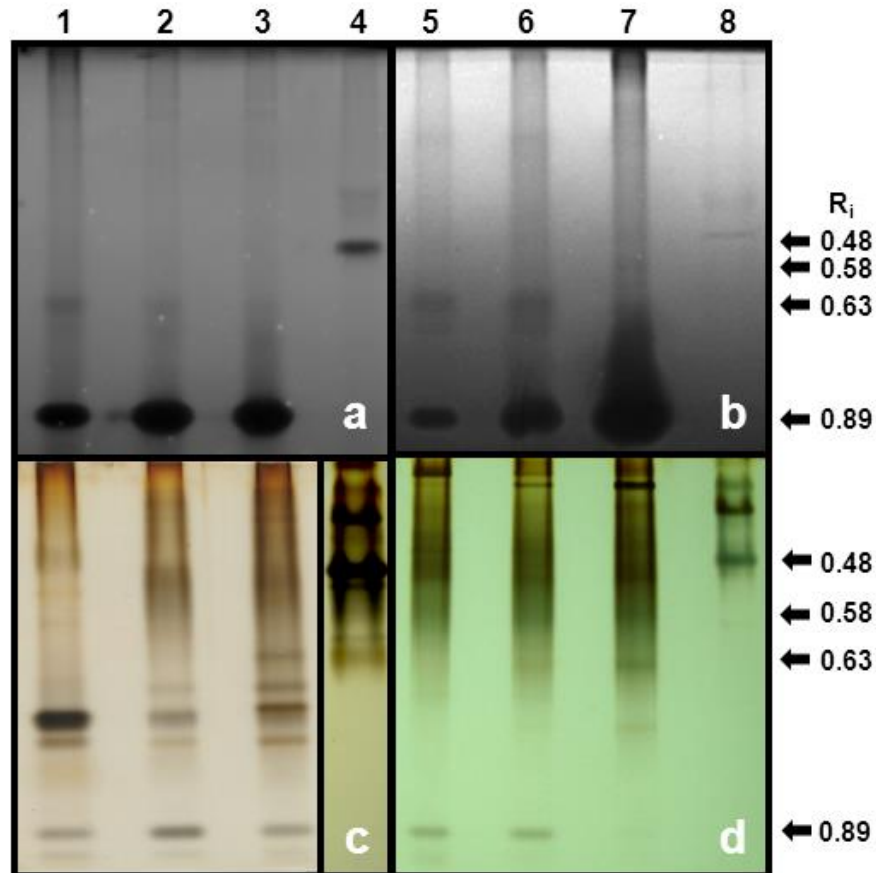
Figure 3. Zymography in non-reducing 12% polyacrylamide gels of chitinase enzymatic activity in apoplastic and symplastic fluids of *Musa acuminata* and the *M. acuminata-M. fijiensis* interaction. Lane order is the same as in Figure 2. Commercial (Sigma) chitinase from *Streptomyces griseus* (a4 and b8) was used as a positive control.

diseased samples (Figure 3b, lane 7). The chitinase activity bands of the apoplastic or symplastic fluids coincided with polypeptides in the equivalent silver-stained gels (Figure 3c and d). The 0.63 polypeptide was most abundant in healthy apoplast, decreased in asymptomatic and diseased apoplast (Figure 3c, lanes 1, 2, 3), and was faint in the symplast samples (Figure 3d, lanes 5, 6 and 7). The 0.89 polypeptide remained constant in all three apoplast conditions (healthy, BS-asymptomatic and BS-diseased) (Figure 3c, lanes 1, 2 and 3), as well as in the healthy and BS-asymptomatic symplast (Figure 3d, lanes 5, 6); it was barely detectable in the BS-diseased symplast (Figure 3d, lane 7). One band with $R_i = 0.48$ representing chitinase activity was visible in both the positive control (commercial chitinase from *S. griseus*, Sigma) (Figure 3a and b, lanes 4 and 8), and the equivalent silver-stained gel (Figure 3c and d, lanes 4 and 8).

Proteolytic activity was evaluated because it is prominent in the interaction between plants and pathogens (Shabab et al., 2008; Song et al., 2009; Bozkurt et al.,

2011; Dixelius, 1994). In the apoplast samples, protease activity was slight and mainly associated with two polypeptides showing $R_i = 0.04$ and 0.14 (Figure 4a). The 0.14 band was more intense in healthy plant apoplast (Figure 4a, lane 1) than in the BS-asymptomatic and BS-diseased tissues (Figure 4a, lanes 2 and 3, respectively). The contrary occurred with the 0.04 band, which was faint in healthy plant apoplast (Figure 4a, lane 1), but more intense in the BS-asymptomatic and BS-diseased apoplast (Figure 4a, lanes 2 and 3). In the symplastic fluids, proteolytic activity was stronger than in the apoplastic fluids, and was again associated with two bands ($R_i = 0.04$ and 0.14).

In a pattern similar to that observed in the apoplastic fluids, the 0.14 band progressively decreased in intensity from the healthy tissues to the BS-asymptomatic (Figure 4b, lane 6) and BS-diseased fluids (Figure 4b, lane 7). In contrast, the 0.04 band increased in intensity from the healthy to the BS-asymptomatic and BS-diseased tissues (Figure 4b, lanes 6 and 7, respectively). These data suggest that proteolysis behaves differentially and
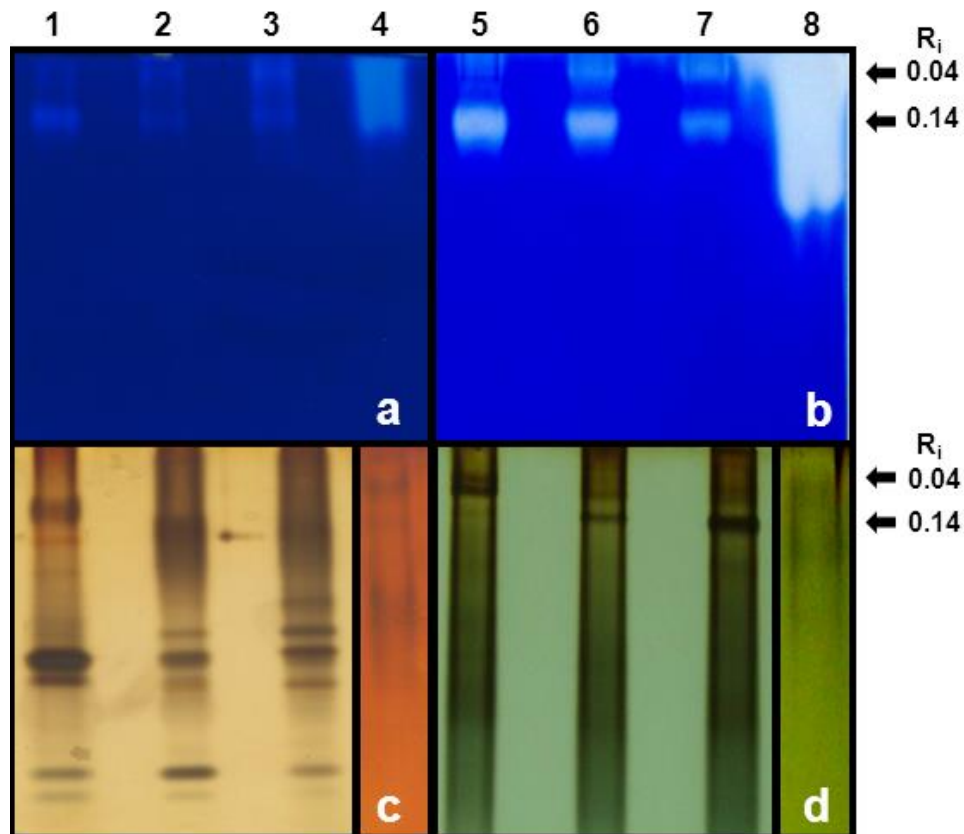
Figure 4. Zymography in non-reducing 12% polyacrylamide gels of protease activity in apoplastic and symplastic fluids from *Musa acuminata* and the *M. fijiensis-Musa acuminata* interaction. Lane order is the same as in Figures 2 and 3. Commercial bovine trypsin II-6 (lanes a4 and b8) was used as a positive control.

dynamically in apoplastic and symplastic fluids during BS disease progression. Commercial Trypsin II-6 from porcine pancreas (Sigma) was used as a positive control for protease activity, and exhibited high proteolytic activity in the upper portion of the lane (Figure 4a and 4b, lane 4 and 8, respectively).

The silver-stained gels exhibited a complex pattern of polypeptides in the apoplastic fluids, with some bands having the same R_i as the putative proteases (Figure 4c, lanes, 1, 2, 3). Two polypeptides co-migrated to the same positions where protease activity bands were observed (Figure 4d, lanes 5, 6, 7).

Glucose 6-phosphate dehydrogenase (G6PD) activity

Since β-1,3-glucanase, chitinase and protease activities were present in both apoplastic and symplastic fluids from all the samples, the specific cytosolic marker, glucose 6-phoshate dehydrogenase (G6PD), was measured to test if apoplast enzymatic activities were caused by loss of cell integrity and/or contamination with cytosolic content. Comparison of specific G6PD activity

(μkat gPF^{-1}) in the symplastic and apoplastic fluids identified minimal contamination of apoplast samples with symplast content (Table 1). Therefore, the enzymatic activities detected in the apoplast, especially of β-1,3-glucanase and chitinase, cannot be explained by contamination with symplastic fluid.

Banana leaf tissue integrity

Macro-histology of the banana tissues showed color differences after buffer infiltration and bleaching and trypan blue staining. Healthy tissue remained slightly green (Figure 5a, lane 1), while BS-asymptomatic tissues were slightly brown and BS-diseased tissues were clearly brown (Figure 5a, lanes 2 and 3), suggesting tissue phenolyzation. Micro-histology of the healthy tissue confirmed tissue integrity and the absence of fungal mycelium (Figure 5b, lane 1), while the BS-asymptomatic and BS-diseased tissues clearly exhibited affectation. In addition, mycelium was observed in the host extracellular space in the BS-diseased tissue (Figure 5b, lane 3). Tissue directly stained with trypan blue for 20 min

Table 1. Comparison of glucose 6-phosphate dehydrogenase activity in apoplastic and symplastic fluids of *Musa acuminata* and the *Musa acuminata-M. fijiensis* interaction.

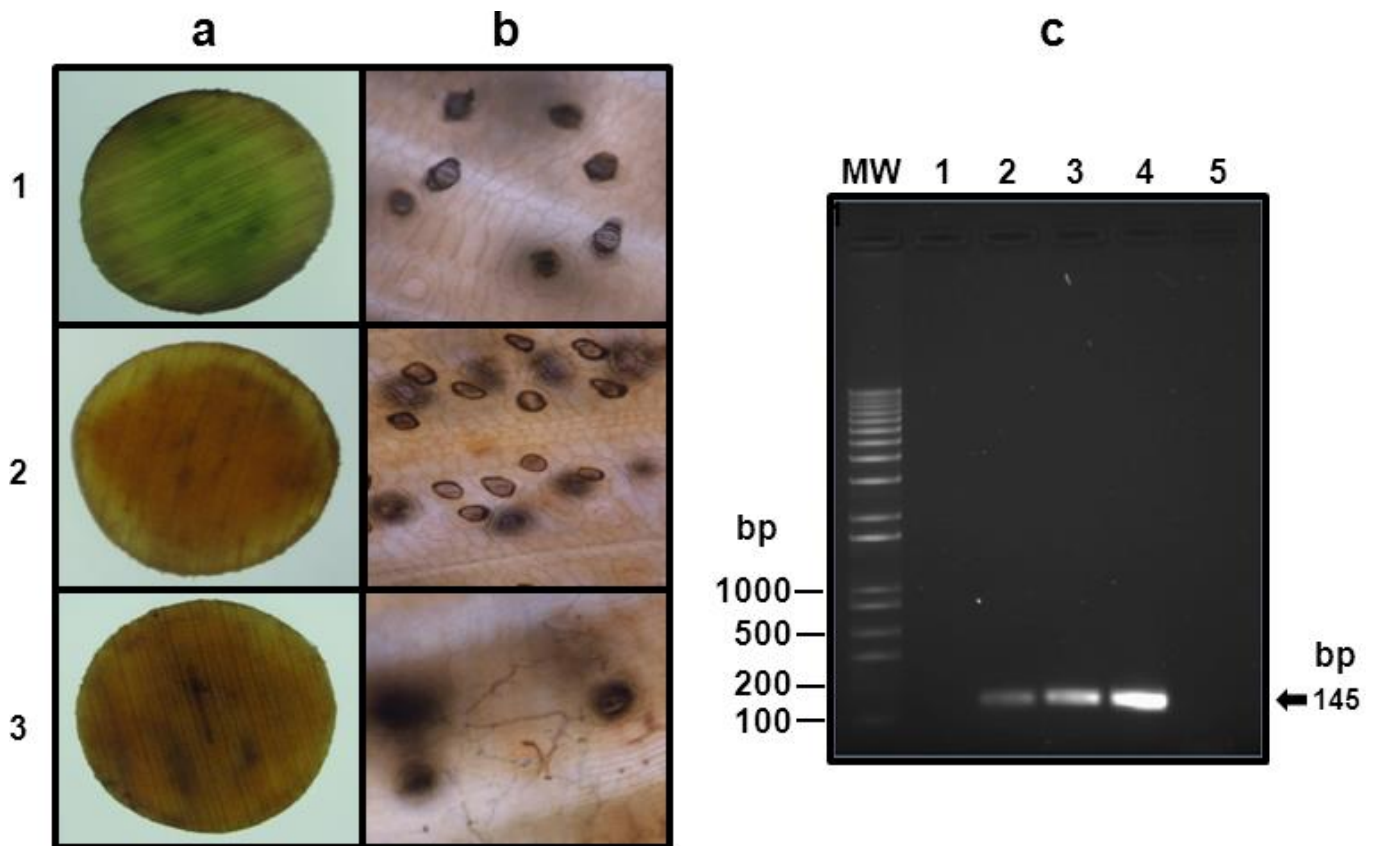| Sample	Enzymatic activity (µkat/g fresh weight)	Times of enzymatic activity symplast/apoplast
Healthy symplast	2.5056	57.75
Healthy apoplast	0.04338	
Asymptomatic symplast	0.3791	51.6
Asymptomatic apoplast	0.00734	
Diseased symplast	0.2562	34.9
Diseased apoplast	0.00734	

Figure 5. Macro (panel a) and micro-histological (panel b) observations of *Musa acuminata* 'Grande Naine' leaf tissues and analysis on 1% agarose gels of the PCR amplified β-tubulin gene from *M. fijiensis* (c). Leaf disks were from infiltrated healthy (a1), BS-asymptomatic (a2), or BS-diseased (a3) leaf tissues. Microhistology images are of infiltrated healthy (b1), BS-asymptomatic (b2), or BS-diseased (b3) leaf tissues. The specific PCR and separation of its products on a 1% agarose gel show results for DNA samples from healthy (c1), BS-asymptomatic (c2), and BS-diseased (c3) leaves. Genomic DNA (gDNA) of *M. fijiensis* was used as a positive control (c4), and water as a negative control (c5). MW = 1 kb DNA ladder.

showed that all samples (that is, healthy, BS-asymptomatic and BS-diseased leaves) excluded the dye. This indicates that at the time the cells still had sufficient integrity to prevent trypan blue uptake, suggesting the possibility of cell controlled protein uploading into the apoplastic space.

PCR amplification of the *M. fijiensis* β-tubulin gene

A PCR diagnosis was run to support visual classification of the banana leaf tissue samples into healthy, BS-asymptomatic and BS-diseased conditions. Lack of amplification of the 145 bp-fragment of the *M. fijiensis* β-

	Polypeptides Presence and Dynamics						
	Symplast			Apoplast			
kDa	1	2	3	1	2	3	
81	M	M	M				
51.6	H	H	H	L	M	M	←
37	M	L	L				
36.5				M	L	L	
34.8	M	L	L				
34.1				L	M	M	
32.6	H	M	M		M	M	←
31.7	H	H	H				
30.3	H	M	M				
29.6					H		
28.3	M	L	L				
25.9	L						
24.7		L	M		M		
21.5	M	L	L				
20.6	M	L	L	M	L	L	←
16.6	L	L	L		L	M	
11	M	L				L	
8.3	M						
7.1	H	L	L	M	M	M	←
4.1	L	L					

Figure 6. Schematic representation of polypeptides from the apoplastic and symplastic fluids of *Musa acuminata* 'Grande Naine' and the *M. acuminata-M. fijiensis* interaction. Polypeptides were obtained from apoplastic or symplastic fluids collected from healthy (1), BS-asymptomatic (2), and BS-diseased (3) banana leaves. Tone intensity represents relative polypeptide abundance: black = high abundance; dark gray = moderate abundance; light gray = low abundance; white = absent. Arrows indicate polypeptide bands present in all samples.

tubulin gene confirmed absence of the pathogen in the healthy sample (Figure 5c, lane 1). This product was readily amplified in the BS-asymptomatic and BS-diseased tissues (Figure 5c, lanes 2 and 3, respectively). The analysis included a *M. fijiensis* gDNA template as positive control (Figure 5c, lane 4), and no template as a negative control (Figure 5c, lane 5).

DISCUSSION

Protein patterns in apoplast and symplast fluids

Plant apoplast is a dynamic compartment inside the organism with a broad range of physiological functions; for example, nutrition, growth, signaling, transport, plant defense, and plant-pathogen interaction, among others (Alves et al., 2006). The present study follows up on pilot research by Mendoza-Rodríguez et al. (2006). They began focusing on isolation of apoplastic proteins from the *M. fijiensis-M. acuminata* interaction, but obtained very low amounts of denatured proteins. The present results represent the first time proteins have been recovered from apoplastic compartment in an active form. Proteins in the apoplastic fluid were suitable for zymography of β-1,3-glucanases, chitinases and proteases, demonstrating that they retained their functional structure. This clearly illustrates this method's effectiveness in recovering native proteins from the *M. acuminata-M. fijiensis* interaction. Protein analysis by SDS-PAGE and PAGE identified clear differences in polypeptide profiles and zymographies between the apoplastic and symplastic preparations. The SDS-PAGE results showed accumulation of specific polypeptides (that is, 34.1, 24.7 and 16.6 kDa) in the BS-infected apoplast (Figures 1 and 6), strongly suggesting that these proteins originated in the host. These proteins were probably the result of *de novo* expression triggered by the

plant's pathogenic response since they were absent in healthy tissues. Under microscopic examination, the BS-asymptomatic samples contained no observable fungal tissue, indicating that fungal material was scarce (Figure 5, panel 2b).

Zymography of β-1,3-glucanase, chitinase and protease activity

Observation of putative pathogen-inducible β-1,3-glucanases with Ri = 0.43 and 0.56 in the apoplastic fluids of BS-infected tissues supports a putative role of these enzymes in the response of *M. acuminata* to *M. fijiensis*. Dixelius (1994) described a β-1,3-glucanase in *Brassica napus* which was uploaded in the apoplastic space of leaves after infection with *L. maculans*. He demonstrated that β-1,3-glucanase in the apoplast was not associated with cellular damage during fungal growth or with contamination of apoplastic fluid. Also, in *Brassica rapa*, β-1,3-glucanase gene expression was rapidly triggered after inoculation with an avirulent strain of *Xanthomonas campestris*, with maximum production at 24 h after inoculation (Newman et al., 1994). Apoplastic localization of β-1,3-glucanase has also been reported in other plant-fungal interactions (*C. fulvum*-tomato (Joosten and De Wit, 1989); *Venturia inaequalis*-apple 'Remo' (Gau et al., 2004). The present study addresses presence of β-1,3-glucanase in healthy, BS-asymptomatic (biotrophic stage), and BS-symptomatic (necrotrophic stage) leaf tissue in a compatible interaction of *M. acuminata* with *M. fijiensis*. Study results are consistent with similar reports in other pathosystems. Beta-1,3-glucanase accumulated in the necrotrophic phase since no enzymatic activity was observed in healthy tissues and was represented by only faint bands in the BS-asymptomatic tissues. This suggests that the compatible interaction plant response in the form of β-1,3-glucanase expression, is insufficient and thus unable to contend with the fungal infection. Torres et al. (2012) recently reported induction of different pathogenesis-related proteins in banana in response to *M. fijiensis* infection. They identified two peaks in β-1,3-glucanase activity in both the compatible interaction with *M. acuminata* 'Williams', and the incompatible interaction with *M. acuminata* 'Calcutta 4'. However, the time required to trigger the response was shorter in the 'Calcutta 4' (first peak at 6 h post-infection) than in the 'Williams' (18 h post-infection). Maximum β-1,3-glucanase activity was similar in both cultivars, but appeared earlier in 'Calcutta 4'. In both cultivars maximum activity peaks were triggered quickly (that is, <72 h post infection). Despite these similarities, the 'Williams' displayed symptoms nine days after infection, while the 'Calcutta 4' exhibited only small necrotic lesions after 72 h, and successfully arrested the pathogen. Given these results, it can be expected that differences in the

responses of susceptible and resistant hosts could be both temporal and spatial. However, these authors assayed β-1,3-glucanase activity in total leaf homogenates, making it impossible to associate the activity with symplastic or apoplastic compartments, or with local leaf zones. Further research assessing these differences in β-1,3-glucanase expression will help to build a more complete picture of β-1,3-glucanase's role in the banana-*M. fijiensis* interaction.

Chitinase is found frequently in xylem sap and apoplastic fluids during plant-pathogen interactions. Apparently, it acts either independently against fungal attack, or in a concerted way with β-1,3-glucanases, by weakening the pathogen cell wall. Chitinases have been described in the interactions of *Verticillum longisporum-Arabidopsis thaliana* (Johansson et al., 2006); *V. longisporum-B. napus* (Floerl et al., 2008); *S. tritici-Triticum aestivum* L. (Shetty et al., 2009; Segarra et al., 2003); and *C. fulvum-Lycopersicon esculentum* (Joosten and De Wit, 1989), among others. In the present results, a band with $R_i = 0.89$, associated with chitinase activity was more intense (that is, active) in apoplastic and symplastic fluids from BS-diseased *M. acuminata* leaves and fainter (weaker) in fluids from healthy leaf tissue.

Sánchez-García et al. (2012) recently reported the presence of chitinase activity from leaf homogenates of infected banana 'Grande Naine' and 'Calcutta 4'. Response was faster and stronger in 'Calcutta 4' than in 'Grande Naine'. They evaluated chitinase activity only during the first 6 days post-infection, when leaves in the compatible interaction are still asymptomatic. In contrast, Torres et al. (2012) measured chitinases throughout the entire interaction (in the compatible interaction in 'Williams') in leaf homogenates. Their results were similar to those of Sánchez-García et al. (2012) in that strong chitinase activity peaks were apparent at an early interaction stage. At a later stage, a second large, broad peak in activity was observed; in this case, chitinase activity was higher during the asymptomatic stage than in the necrotrophic stage. The present results also support a slow or delayed response of the susceptible 'Grande Naine' host but, unlike in Torres et al. (2012), chitinase activity was highest during necrotrophy. This discrepancy may be due to use of a different susceptible host, or that the kinetics of BS disease progress is not identical in all cases. All the above results suggest that response timing is key to determining host capability to cope with or limit pathogen attack (Mendoza-Rodríguez et al., 2006).

No previous reports exist of proteolytic activity during the banana-*M. fijiensis* interaction. Our group has previously described detection of strong protease activity in the *in vitro* secretome of *M. fijiensis* (Chuc-Uc et al., 2011). In addition, proteolysis and proteases are reported to have important roles in other pathosystems (*S. tritici-Triticum aestivum* (Shetty et al., 2009); *Phytophthora infestans-Carica papaya* (Odani et al., 1996); *Phytophthora infestans-Lycopersicon esculentum*

(Valueva et al., 1998); and *Phytophthora infestans-Nicotiana benthamiana* (Bozkurt et al., 2011), particularly by regulating host serine protease activity (Segarra et al., 2003). In the present study, a dual behavior was observed for polypeptides displaying protease activity. On the one hand, the $R_i = 0.04$ polypeptide increased its activity in the apoplast and symplast of BS-infected samples. Our group has reported that proteases may participate in development of foliar lesions (Chuc-Uc et al., 2011), and therefore this polypeptide may be involved in the progress of BS disease symptoms. On the other hand, the $R_i = 0.14$ polypeptide displayed a high level of activity in apoplastic and symplastic fluids from healthy plants, with declining activity in infected tissues with disease progression. This suggests negative regulation which may or may not be due to presence of *M. fijiensis*. Inhibition of host proteases has been described as a pathogen strategy to inactivate host defenses. For example, in the host *Solanum lycopersicum*, *C. fulvum* inhibits a defense-related apoplastic Cys protease (Shabab et al., 2008), as well as the Rcr3 protease (Song et al., 2009). *Phytophthora infestans* also blocks a host protease to infect *Nicotiana benthamiana*, and in papaya it inhibits apoplast secretion of a papain-like cysteine protease C14, causing its peripheral accumulation inside the symplast (Song et al., 2009). In the present case, therefore, *M. acuminata* proteases may also be targeted by *M. fijiensis* during infection.

Glucose 6-phosphate dehydrogenase (G6PD) enzymatic activity

Apoplastic preparation quality is estimated by enzymatic measurement of specific proteins such as malate dehydrogenase (Stergiopoulos et al., 2010; Tasgin et al., 2006), or glucose-6-phosphate dehydrogenase (Tasgin et al., 2006). In the present study, glucose-6-phosphate dehydrogenase was used as a marker for apoplastic purity because it is generally accepted that this enzyme has an exclusively cytosolic localization. Its detection in apoplastic fluid is therefore indicative of apoplastic contamination with cell cytoplasmic contents. Based on this criterion, the apoplastic fluid preparations had only a 2 to 3% symplastic fluid contamination level (Table 1), meaning the majority of recovered fluid was apoplastic. Contamination with symplastic fluid occurs frequently due to damage caused during tissue sampling; this is supported by detection of trypan blue-stained cells surrounding the sampled area at tissue sampling sites (data not shown). Considering the high purity of the analyzed apoplastic fluid, it can be confidently stated that β-1,3-glucanase, chitinase, and protease activities identified here occurred in the extracellular space of the sampled banana leaves. In other words, apoplastic proteins were successfully recovered in their native state. This procedure opens the door for new and interesting

research possibilities in this pathosystem. The present results on black Sigatoka disease in banana will prove valuable in developing control strategies against *M. fijiensis* in addition to development of resistant plants. Proteins occurring in the apoplastic space during the plant-pathogen interaction dynamic play important roles and are specifically secreted by the host for plant defense, or by the pathogen to elude plant defenses. For instance, in *Malus domestica* 'Remo', pathogen resistance is associated with occurrence of a number of pathogenesis-related proteins in the apoplast (Gau et al., 2004). Knowledge of these protease activities suggests development of a black Sigatoka disease control strategy in *M. acuminata* 'Grande Naine' in which plants could be stimulated to constantly produce PR proteins, resulting in secretion in the apoplastic space at levels similar to those found in resistant varieties. This could reduce dependence on fungicides, with consequent financial advantages for producers.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Alves M, Francisco R, Martins I, Ricardo CPP (2006). Analysis of *Lupinus albus* leaf apoplastic proteins in response to boron deficiency. Plant Soil 279:1-11.

Blein JP, Coutos-Thévenot P, Marion D, Ponchet M (2002). From elicitins to lipid transfer proteins: A new insight in cell signalling involved in plant defence mechanisms. Trends Plant Sci. 7:293-296.

Boudart G, Jamet E, Rossignol M, Lafitte C, Borderies G, Jauneau A, Esquerré-Tugayé MT, Pont-Lezica R (2005). Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: identification by mass spectrometry and bioinformatics. Proteomics 5:212-221.

Bozkurt TO, Schornack S, Win J, Shindo T, Ilyas M, Oliva R, Cano LM, Jones AME, Huitema E, van der Horn RAL, Kamoun S (2011). *Phytophthora infestans* effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. Proc. Natl. Acad. Sci. USA 108:20832-20837.

Brito-Argáez L, Canché-Fuentes R, Carballo-Bautista M, Cristóbal-Alejo J, Zúñiga-Aguilar JJ, Islas-Flores I (2010). Pathogenesis related proteins in pathogen challenged plants: chitinase and β-1,3-glucanase activities in the *Capsicum chinense-Phytophthora capsici* interaction. In: Martínez-Estévez M, editor. *Recent Research in Plant Science*, Kerala, India Trivandrum -695 023: Transworld Research Network; p. 57-76. http://www.ressign.com/UserBookDetail.aspx?bkid=1081&catid=236

Brownfield L, Howlett B (2001). An abundant 39 kDa protein from *Brassica napus* (canola) is present in the apoplast of leaves infected by the blackleg fungus, *Leptosphaeria maculans*. *Australas.* Plant Pathol. 30:7-9.

Chuc-Uc J, Brito-Argáez L, Canto-Canché B, Tzec-Simá M, Rodríguez-García C, Peraza-Echeverría L, Peraza-Echeverría S, James-Kay A, Cruz-Cruz CA, Peña-Rodríguez LM, Islas-Flores I (2011). The *in vitro* secretome of *Mycosphaerella fijiensis* induces cell death in banana leaves. Plant Physiol. Biochem. 49:572-578.

Churchill ACL (2010). *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and challenges of control. Mol. Plant Pathol. 12:307-328.

De Bellaire LE, Fouré de L, Abadie C, Carlier J (2010). Black leaf streak is challenging the banana industry. Fruits 65:327-342.

Distefano D, Palma JM, Gómez M, Rio LA (1997). Characterization of endoproteases from plant peroxisomes. Biochem. J. 327:399-405.

Dixelius C (1994). Presence of the pathogenesis-related proteins 2, Q and S in stressed *Brassica napus* and *B. nigra* plantlets. Physiol. Mol. Plant Pathol. 44:1-8.

Floerl S, Druebert C, Majcherczyk A, Karlovsky P, Kües U, Polle A (2008). Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. napus) attenuate *Verticillium longisporum* growth but not disease symptoms. BMC Plant Biol. 8:129-144.

Foure E (1987). Varietal reactions of bananas and plantains to black leaf streak disease. In: banana and plantain breeding strategies: Proceedings of an international workshop, Cairns, Australia, 13-17. October 1987 (Persley, GJ, and De Langhe EA, eds), pp. 110-113. ACIAR, Canberra, Australia.

Gau AEM, Piotrowski K, Kloppsteck K (2004). Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. Eur. J. Plant Pathol. 110:703-711.

Haslam RP, Downie AL, Raveton M, Gallardo K, Job D, Pallett KE, John P, Coleman JOD (2003). The assessment of enriched apoplastic extracts using proteomic approaches. Annu. Appl. Biol. 143:81-89.

Hoson T (1998). Apoplast as the site of response to environmental signals. J. Plant Res. 111:167-177.

Johansson A, Staal J, Dixelius C (2006). Early responses in the *Arabidopsis-Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. Mol. Plant-Microbe Interact. 19:958-969.

Joosten HAJM, De Wit JGM (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. Plant Physiol. 89:945-951.

Kürkcüoglu S, Piotrowski M, Gau AE (2004). Up-regulation of pathogenesis-related proteins in the apoplast of *Malus domestica* after application of non-pathogenic bacterium. Z. Naturforsch. 59:843-848.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophague T4. Nature 227:680-685.

Mendoza-Rodríguez M, Sánchez-Rodríguez A, Leiva-Mora M, Acosta-Suarez M, Rojas L, Jiménez E, Portal O (2006). Extracción de proteínas a partir del fluido intercelular en hojas de banano, cultivar Grande naine (*Musa* AAA). Biotecnol. Veg. 6:59-62.

Newman MA, Conrads-Strauch J, Scofield G, Daniels MJ, Dow JM (1994). Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. Mol. Plant-Microbe Interact. 7:553-563.

Odani S, Yokokawa Y, Takeda H, Abe S, Odani S (1996). The primary structure and characterization of carbohydrate chains of the extracellular glycoprotein proteinase inhibitor from latex of *Carica papaya*. Eur. J. Biochem. 241:77-82.

Pan S, Xiang Q, Kuc JA (1991). Technique for detection of chitinase β-1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing. Phytopathology 81:970-974.

Peterson GL (1977). A simplification of the protein assay method of Lowry. Anal. Biochem. 83:346-356.

Robertson D, Palma JM, Gilroy JS, Gerrish C, Bolwell GP, Slabas A (1997). Differential extraction and protein sequencing reveals major differences in pattern of primary cell wall proteins from plants. J. Biol. Chem. 272:15841-15848.

Sánchez-García C, Cruz-Martín M, Alvarado-Capo Y, Rojas L, Leiva-Mora M, Acosta-Suarez M, Roque B (2012). Detección y cuantificación de quitinasas en hojas de banano (*Musa* spp.) inoculadas con *Mycosphaerella fijiensis*. Biotecnol. Veg. 12:119-124.

Sattelmacher B (2001). The apoplast and its significance for plant mineral nutrition. New Phytol. 149:167-192.

Segarra CI, Casalongué CA, Pinedo ML, Ronchi VP, Conde RD (2003). A germin-like protein of wheat apoplast inhibits serine proteases. *J. Exp. Bot.* 54:1335-1341.

Shabab M, Shindo T, Gu C, Kaschani F, Pansuriya T, Chintha R, Harzen A, Colby T, Kamoun S, van der Hoorn RAL (2008). Fungal effector protein AVR2 targets diversifying defense-related Cys proteases of tomato. Plant Cell 20:1169-1183.

Shetty N, Jensen JD, Knudsen A, Finnie C, Geshi N, Blennow A, Collinge DB, Jorgensen HJL (2009). Effects of β-1,3-glucan from *septoria tritici* on structural defence responses in wheat. J. Exp. Bot. 60:4287-4300.

Song J, Win J, Tian M, Schornack S, Kaschani F, Ilyas M, van der Hoorn RAL, Kamoun S (2009). Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. Proc. Natl. Acad. Sci. USA 106:1654-1659.

Stergiopoulos I, van den Burg HA, Okmen B, Beenen HG, van Liere S, Kema GHJ, de Wit PJGM (2010). Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. Proc. Natl. Acad. Sci. USA 107:7610-7615.

Tasgin E, Atici O, Nalbantoglou B, Popova LP (2006). Effect of salicylic acid and cold treatment on protein levels and on the activities of antioxidant enzymes in the apoplast of winter wheat leaves. Phytochemistry 67:710-715.

Torres JM, Calderón H, Rodríguez-Arango E, Morales JG, Arango R (2012). Differential induction of pathogenesis-related proteins in banana in response to *Mycosphaerella fijiensis* infection. Eur. J. Plant Pathol. 133:887-898.

Valueva TA, Kladnitskaya GB, Ilínskaya LI, Gerasimova NG, Ozeretskovskaya OL, Mosolov VV (1998). Chymotrypsin inhibitors in potato tubers infected with *Phytophthora infestans*. Russ. J. Bioorgan. Chem. 24:305-308.

van Loon LC, van Kammen A (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var ¨Samsun¨ and Samsun NN. II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 40:199-211.

van Loon LC, van Strien EA (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55:85-97.

Weising K, Kaemmer D, Epplen T, Weigand F, Saxena C, Kahl G (1991). DNA fingerprinting of *Ascochyta rabiei* with synthetic oligonucleotides. Curr. Genet. 19:483-489.

Zhou J, Bokhari SA, Chun-Juan D, Jin-Yuan L (2011). Comparative proteomic analysis of the root apoplast of rice seedlings in response to hydrogen peroxide. Plos ONE 6:e16723,1-11.