ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/45098182

Cell wall alterations in the leaves of fusariosis-resistant and susceptible pineapple cultivars

Article *in* Plant Cell Reports · October 2010

DOI: 10.1007/s00299-010-0894-9 · Source: PubMed

CITATIONS
15

READS
219

6 authors, including:

David S Buss
Keele University

21 PUBLICATIONS **375** CITATIONS

SEE PROFILE

José Aires Ventura
Instituto Capixaba De Pesquisa, Assistência Técnica E Extensão Rural

140 PUBLICATIONS **953** CITATIONS

SEE PROFILE

Patricia M B Fernandes
Universidade Federal do Espírito Santo

56 PUBLICATIONS **840** CITATIONS

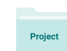
SEE PROFILE

A. A. R. Fernandes
Universidade Federal do Espírito Santo

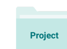
81 PUBLICATIONS **525** CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Bases moleculares biotecnológicas da interação planta-vírus gerando produtos inovadores para a melhoria da resistência e qualidade de frutos de mamão e abacaxi. View project



Increment of coconut hydrolysis View project

All content following this page was uploaded by José Aires Ventura on 01 June 2016.

The user has requested enhancement of the downloaded file.

Plant Cell Rep (2010) 29:1109–1117
DOI 10.1007/s00299-010-0894-9

ORIGINAL PAPER

Cell wall alterations in the leaves of fusariosis-resistant and susceptible pineapple cultivars

Glória Maria de Farias Viégas Aquije · Poliana Belisário Zorzal · David Shaun Buss · José Aires Ventura · Patricia Machado Bueno Fernandes · Antonio Alberto Ribeiro Fernandes

Received: 24 April 2010 / Revised: 21 June 2010 / Accepted: 26 June 2010 / Published online: 6 July 2010
© Springer-Verlag 2010

Abstract Fusariosis, caused by the fungus *Fusarium subglutinans* f. sp. *ananas* (Syn. *F. guttiforme*), is one of the main phytosanitary threats to pineapple (*Ananas comosus* var. *comosus*). Identification of plant cell responses to pathogens is important in understanding the plant–pathogen relationship and establishing strategies to improve and select resistant cultivars. Studies of the structural properties and phenolic content of cell walls in resistant (Vitoria) and susceptible (Perola) pineapple cultivars, related to resistance to the fungus, were performed. The non-chlorophyll base of physiologically mature leaves was inoculated with a conidia suspension. Analyses were performed post-inoculation by light, atomic force, scanning and transmission electron microscopy, and measurement of cell wall-bound phenolic compounds. Non-inoculated leaves were used as controls to define the constitutive tissue characteristics. Analyses indicated that morphological differences, such as cell wall thickness, cicatrization process and lignification, were related to resistance to the pathogen. Atomic force microscopy indicated a considerable difference in the mechanical properties of the resistant and susceptible cultivars, with more structural integrity, associated with higher levels of cell wall-bound phenolics, found in the resistant cultivar. *p*-Coumaric and ferulic acids were shown to be the major phenolics bound to the cell walls and were found in higher amounts in the resistant cultivar. Leaves of the resistant cultivar had reduced fungal penetration and a faster and more effective cicatrization response compared to the susceptible cultivar.

Keywords *Ananas comosus* var. *comosus* · Disease · Fungus · *Fusarium subglutinans* f. sp. *ananas* · *F. guttiforme* · Host–parasite interaction

Communicated by P. Puigdomenech.

G. M. de Farias Viégas Aquije (✉) · P. B. Zorzal · D. S. Buss · P. M. B. Fernandes · A. A. R. Fernandes
Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitoria, ES 29040-090, Brazil
e-mail: viegasaquije@yahoo.com.br

J. A. Ventura
Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Incaper, Vitoria, ES 29052-010, Brazil

Introduction

Pineapple is one of most produced tropical fruits worldwide and thus a symbolic fruit of tropical and subtropical regions, with great trade value in fresh or processed form.

Native to Central and South America, pineapple is now grown extensively in Hawaii, Philippines, Caribbean, Malaysia, Thailand, Australia, Mexico, Kenya, South Africa and China (Ma et al. 2007).

Brazil is a major pineapple producer, but this crop has serious phytosanitary issues, which, besides economic losses within the country, limit export of the fruits (Santos et al. 2002; Ventura and Zambolim 2002). Among these, fusariosis, caused by the fungus *Fusarium subglutinans* f. sp. *ananas* (Syn.: *F. guttiforme*), is the most serious disease, with losses estimated at 30–40% in marketable fruits and 20% of propagative material (Ventura and Zambolim 2002; Ventura et al. 2009). Fusariosis is also considered to be a quarantine disease for other countries and, therefore, is an organism of economic importance both for the area infected and areas where it has not yet been established.

Extensive application of chemical pesticides is the normal control practice in commercial plantations, but this has become highly questionable due to elevated costs,

deteriorating soil health and general environmental issues (Wuyts et al. 2007). Resistant cultivars are considered a more sustainable management option, which is equally accessible to subsistence pineapple growers as well as commercial producers (Ventura and Zambolim 2002).

In Brazil 'Perola' and 'Smooth Cayenne', both susceptible to fusariosis, are the most commonly planted cultivars. Recently, a new pineapple cultivar, Vitoria, resistant to fusariosis, with better fruit quality and agronomic characteristics similar or superior to those of cvs. Perola and Smooth Cayenne, has been released to growers and attracted interest in Brazil for export (Ventura et al. 2009). The resistance of cv. Vitoria to fusariosis eliminates the use of fungicides and reduces production costs and risks of negative environmental impact by pesticides. However, the mechanism of resistance is yet to be determined.

Plant defense mechanisms have been intensively studied and it has been recognized that a better knowledge of both the pathogen and the plant will allow the development of novel approaches to enhance the durability of resistance (Rodrigues et al. 2006). Such responses may be general or specific and detailed knowledge is valuable in implementing the appropriate preventative measures (Rodrigues et al. 2009), as well as providing a model for other cultivars that possess trade value, but are susceptible to phytopathogens (Silva et al. 2006).

Knowledge of the pathogen–host interaction at a histological level allows understanding of the infection processes, evidencing possible structural mechanisms of resistance (Bentes and Matsuoka 2005). Cell wall-associated plant defense is an important component of structural resistance and may also be key to elucidating distinct kinds of resistance (Hückelhoven 2007). The cell wall not only strengthens the plant body, but also has key roles in plant growth, cell differentiation, intercellular communication, water movement and defense (Cosgrove 2005). Following pathogen recognition, apoplastic defenses are initiated at the cell wall for inhibition of pathogen enzymes, wall strengthening and toxicity to the pathogen (Hückelhoven 2007). Increased production of the lignin polymer creates a barrier that is physically difficult for pathogens to penetrate (Quiroga et al. 2000), and lignification has been associated with resistant cultivars (Eynck et al. 2009; Wally et al. 2009).

Atomic force microscopy (AFM) has proved to be a very useful technique in providing information on the physical structure and mechanical properties of the cell wall (Lesniewska et al. 2004) and of the lignin polymer (Micic et al. 2001).

A positive correlation has been established between host resistance and the production of secondary metabolites issued from the phenylpropanoid pathway. These metabolites, frequently referred to as phenolic compounds (Wuyts et al. 2007), are associated with lignification and so limit pathogen development at the site of infection (Hückelhoven 2007). An increase in cell wall-bound phenolics in *Musa acuminata* following *Fusarium* elicitor application, which is associated with increased lignin formation, has been found (de Ascensao and Dubery 2003). Additionally, inhibition of the phenylpropanoid pathway in barley and chickpea weakens resistance to the fungal pathogens, *Blumeria graminis* and *Ascochyta Rabiei* (Kruger et al. 2002; Kavousi et al. 2009).

The purpose of this research was to identify and compare the structural alterations, in the cell wall, of fusariosis-resistant pineapple cv. Vitoria and susceptible cv. Perola, before and post-inoculation with the fungus *F. subglutinans* f. sp. *ananas*.

Materials and methods

Plant material

Plant material (suckers and slips) of pineapple cultivars, Vitoria (resistant) and Perola (susceptible), was obtained from the Sooretama Research Experimental Station of Incaper (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural) and individually planted into plastic pots of 25-cm diameter and 30-cm height, containing a mixture of 2:1 w/w fertilized soil and river-washed sand. Plants were kept in a greenhouse with weekly irrigation until 4–6 months old. The basal non-chlorophyll portion of mature (D stage) leaves was used for all tests.

Fungal inoculum

The fungus used was *F. subglutinans* f. sp. *ananas* (E-203; NRRL25624), derived from single conidia from INCAPER Plant Pathology Laboratory. The *Fusarium* was maintained on potato dextrose agar (PDA) (Oxoid Unipath Ltd., Basingstoke, Hampshire, UK) slants. The isolates were identified using *Fusarium* taxonomic keys (Ventura 2000). Histological needles (15) were arranged so as to make an injury site of approximately 5-mm diameter and 1-mm depth in detached pineapple leaves. Injury sites were about 2 cm from the leaf base and inoculated with the fungal suspension (10^5 conidia ml^{-1}), according to Ventura (1994). Constitutive tissue features were determined from non-treated leaves. The analyses were carried out at 0, 24, 48 and 168 h post-inoculation (h.p.i.).

Light microscopy

Leaf samples were free-hand cut in transverse sections. Sections were stained with safranin or lactophenol-cotton blue for plant cell walls or hyphae, respectively. For

safranin staining, sections were soaked for 1 min in 0.5% aqueous safranin O (Sigma Chem. Co, MO, USA) (Johansen 1940). For lactophenol staining, sections were soaked for 1–2 min in 0.1% lactophenol-cotton blue (Sigma Chem. Co, MO, USA). Stained sections were mounted between slide and coverslip, using 50% glycerol as the mounting medium. Microscopic observation was performed with a light microscope (Leica Microsystems, Wetzlar, Germany). Photographic documentation and analysis were carried out using a digital camera (Moticam 2000) with Motic Images Plus software (Motic China Group Co., Xiamen, China). At least 20 fields ($\approx$1 mm^2) were analyzed for each sample.

Electron and atomic force microscopy

Squares of 1 cm^2 were excised from leaves using a sterile razor blade. The specimens were fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 10 mM cacodylate buffer (pH 7.4) at 4°C overnight and then rinsed in the same buffer for 10 min. The samples were then post-fixed in 1% osmium tetroxide (w/v) for 1 h at 25°C and dehydrated in a graded series of 30, 50, 70, 90 and 100% (v/v) acetone, with 30 min for each step. Samples were critical point dried in CO_2 for scanning electron microscopy and atomic force microscopy. For transmission electron microscopic (TEM) analysis, dehydrated samples were embedded in Spurr's low viscosity resin and ultrathin sections (60–70 nm) were prepared from the region of the lesion, with a glass knife using an ultramicrotome (Reichert Ultracut, Bio-Imaging). The sections were mounted on copper grids and stained with 2% uranyl acetate and lead citrate for 5 min. Observations were carried out using an EM-ZEISS 900 transmission electron microscope working at 80 kV. Three replicates were prepared for each of three leaves, and at least 10 ultrathin sections were examined for each cultivar and time point.

Scanning electron microscopy (SEM) samples were mounted on aluminum stubs, sputter coated with 20 nm gold, and examined using a Shimadzu SSX 550 (scanning electron microscope) operating at 12 kV. Three replicates were prepared from each of the three leaves for each cultivar and time point.

For atomic force microscopy (AFM), the critical point-dried samples were attached to a glass slide, using a small piece of double-faced adhesive tape. Force–distance measurements were recorded in contact mode at room temperature using a Shimadzu microscope (SPM-9600 series). Si_3N_4 cantilevers tips model OMCL-TR (Olympus, Tokyo, Japan) with a nominal spring constant of 0.57 N/m and a resonance frequency of $\approx$73 kHz were used. Non-functionalized tips were used to measure the adhesion forces. All reported images were made with typical scan rates of 0.3–1 Hz and a scan size of 1,000 nm. The settings used for data acquisition in the force mapping mode consisted for each force map 16 $\times$ 16 force curves over a lateral scan size of 1,000 nm. The force maps were analyzed using the force mapping software for the SPM-9600 series (Shimadzu Corporation, Kyoto, Japan).

Adhesion force maps and histograms were generated by the force curves, resulting from the interactions between the AFM tip and the sample surface. The force of repulsion of the tip interacting with the sample was the main force used to measure the mechanical properties of the cell wall. For force measurements, a 16 $\times$ 16 array map of 256 force curves was made for each cultivar and each time point.

A microscope connected to the instrument allows visualization of the region examined and the connection of measurements to particular cellular features such as cell walls. As in previous tests by light microscopy and electron microscopy, AFM studies focused on cells in the region of the lesion.

Extraction and quantification of cell wall-bound phenolic compounds

Extraction of total phenolics was performed using the method of Moore et al. (2005) with modifications. Leaf material (0.5 g) was ground in a pestle and mortar with liquid nitrogen and then resuspended in MeOH/H_2O (7:3, v/v). After incubation for 5 min, the suspension was centrifuged (6,000*g*, 5 min). The pellet was re-suspended twice using three volumes of solvent per mass of sample and re-centrifuged. The supernatants, containing extracted soluble phenolics, were pooled and stored.

For the saponification of the cell wall-bound phenolics, 2.0 ml of 0.5 M NaOH was added to the pellet for 24 h. The mixture was acidified to pH 2 with 2 M HCl, and the extract was centrifuged at 6,000*g* for 20 min. To 150 µl of supernatant (cell wall-bound phenolics), 3 ml of Na_2CO_3 (2% m/v) and 150 µl of Folin-Ciocalteu reagent diluted in water (1:1 v/v) were added. Absorbance was read at 750 nm. The concentration of phenolics was expressed as chlorogenic acid equivalents (µg/mg of fresh tissue), calculated from data of three plants from each cultivar, and evaluated in triplicate.

A 20-µl aliquot of the extracted cell wall-bound phenolics was subjected to analytical high-performance liquid chromatography (HPLC), performed on a Shimadzu Prominence with UV/visible LC-20A detector (Shimadzu Corporation, Kyoto, Japan). Fractions were eluted from a Shimadzu Shim Pack CLC (M) C18 (4.6 mm $\times$ 250 mm) column using a linear gradient between TFA/H_2O (1:1,000 v/v) and TFA/acetonitrile (1:1,000 v/v) over 50 min at a flow rate of 0.7 ml/min and absorbance was read at

280 nm. Results were analyzed using LC Solution-Release 1.21 SP1 Software.

To identify the peaks of HPLC profiles, *p*-coumaric and ferulic acids (Sigma–Aldrich, St. Louis, USA) were run alone on HPLC as standards (20 μg in 20 μl) or co-chromatographed (100 μg added) with the samples.

Results

Plant–pathogen interaction

Visual observation of leaves 7 days after inoculation showed an absence of any disease symptoms on cv. Vitoria (Fig. 1a–c). However, leaf samples from cv. Perola showed severe fusariosis symptoms (Fig. 1d–f). These results are comparable with disease severity on plants and fruits observed under field conditions (Fig. 1a, d) reported by Ventura and Zambolim (2002) and Ventura et al. (2009).

Scanning micrographs of pineapple leaves allowed close examination of the leaf surface of Vitoria (Fig. 2a–c) and Perola (Fig. 2d–f). Micrographs immediately after injury failed to show any contaminating epiphytic microorganisms (Fig. 2a, d).

Pathogen hyphae and conidia germ tubes typical of *F. subglutinans* f. sp. *ananas* were detected 24 h post-inoculation (h.p.i.) (Fig. 2b, e). It was observed that hyphal growth was restricted to the surface of the resistant cv. Vitoria compared to the susceptible cv. Perola, where a dense mycelial network was formed at the injury sites. Cicatrization of the wound sites was more developed in the resistant cultivar, as can be seen in Fig. 2c, f and in light microscopy in Fig. 3.

The inter- and intra-cell features of the infection process caused by the fungus were also observed in leaf transverse sections. After inoculation, the fungus first colonized the parenchyma cells of the aerenchyma canals, and then the parenchyma as a whole. No hyphae were observed in the xylem and phloem (Fig. 3a, b 48 h; c, d 168 h post-inoculation). After 168 h post-inoculation, the inoculation sites in Perola (susceptible cultivar) were characterized by collapsed cells in the mesophyll and a large quantity of hyphae (Fig. 3d), including hyphae invading other mesophyll tissues (data not shown) demonstrating the necrotrophic lifestyle of the fungus.

Analysis of cv. Perola samples (Fig. 3b, d) showed not only fungus hyphae formation, but also a degraded mesophyll throughout the injury and inoculation site, thus

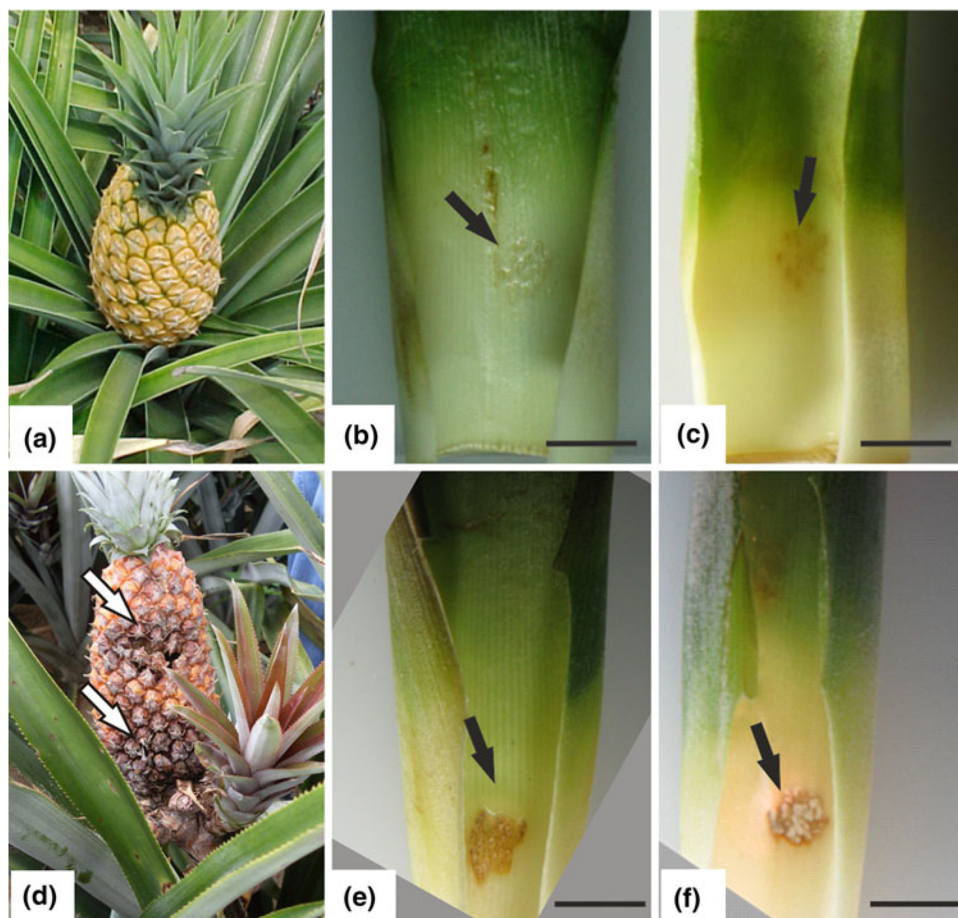


Fig. 1 Pineapple susceptibility to *F. subglutinans* f. sp. *ananas*. **a**, **b**, **c** Cv. Vitoria; **a** healthy uninfected plant; detached leaves **b** 48 h after injury and inoculation; and **c** 168 h after injury and inoculation. **d**, **e**, **f** Cv. Perola; **d** plant with symptoms of fusariosis (*arrows*); detached leaves **e** 48 h after injury and inoculation; and **f** 168 h after injury and inoculation. *Bar* 1.0 cm

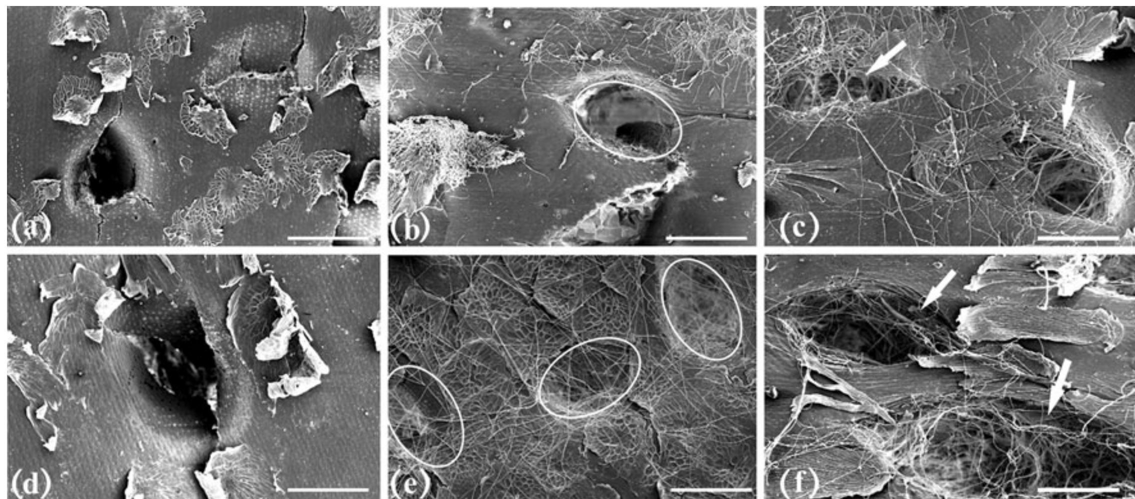


Fig. 2 Scanning electron microscopy of the adaxial view of the basal non-chlorophyll portion of the pineapple leaf after injury and inoculation with *Fusarium subglutinans* f. sp. *ananas*. **a**, **b**, **c** Cv. Vitoria (resistant) and **d**, **e**, **f** cv. Perola (susceptible); **a**, **d** immediately after injury showing absence of pathogens on the leaf surface; **b**, **e** injury sites 24 h post-inoculation (*white ovals*); **c**, **f** fungal hyphae on the leaf surface and tissue colonization 48 h post-inoculation (*arrows*). *Bar* 200 μm

demonstrating the susceptibility of this tissue. In contrast, at 168 h.p.i. cv. Vitoria (Fig. 3c) had completed its cicatrization process and cell regeneration throughout the injury and inoculation site, principally through periclinal cell division around the lesion, suggesting formation of wound periderm through the cicatrization process.

Images from cells next to the lesion in cv. Vitoria leaves at 48 h.p.i. showed that cell walls were thick (0.55 ± 0.06 μm) and intact with alternating light and dark layers (inset of Fig. 3a). In susceptible cv. Perola, the cell walls were thinner (0.2 ± 0.02 μm), layering was less evident and breaks were observed in the cell layers (inset of Fig. 3b).

Mechanical properties of the cell wall

Using atomic force microscopy, adhesion force maps and histograms were obtained for cvs. Vitoria and Perola. This provided more details on the mechanical properties of the cell wall. The level of the interaction force is associated with the structure of the cell wall, so that less interaction force suggests lower adhesion of the tip and surface.

The frequency of each adhesion force detected in a 1,000-nm lateral scan of 256 force curves was given as the percentage. Significant differences were found in the mechanical characteristics of the cell walls, such that the cell walls of cv. Vitoria were more rigid than those of cv. Perola. The histograms for the constitutive characteristics of the cell wall indicated that the average and maximum adhesion 'pull-off' values observed from an individual retraction curve were 0.33/1.98 nN for the cv. Vitoria (Fig. 4a) and 10.94/83.06 nN for cv. Perola (Fig. 4c). The average and maximum adhesion 'pull-off' at samples 48 h.p.i. were 1.546/2.795 nN for cv. Vitoria (Fig. 4b) and 16.12/82.01 nN for cv. Perola (Fig. 4d).

Biochemical analysis of cell wall-bound phenolics showed a significant difference in the mean cell wall polyphenol content between cultivars and in the accumulation kinetics upon inoculation. Cultivar Vitoria showed higher constitutive concentration of cell wall-bound polyphenols than Perola. However, at 48 h.p.i., Perola showed a higher concentration of polyphenols (Table 1).

High-performance liquid chromatography (HPLC) analysis of extracted polyphenols bound to the cell wall revealed that the qualitative profiles of Vitoria (Fig. 5a, c) and Perola (Fig. 5b, d) cultivars in uninoculated leaves (Fig. 5a, b) and at 48 h.p.i. (Fig. 5c, d) are quite similar. Both cultivars consistently presented a double peak at 20 min retention time.

Two well-known cell wall constituents, *p*-coumaric and ferulic acids, were run as standards and found to be co-chromatographed with peak 1 and 2 of the extracts, respectively. Both compounds were present in higher amounts in healthy leaves of the resistant cultivar and increased strongly after infection of the two pineapple cultivars. The higher accumulation was recorded for ferulic acid in the infected Perola cultivar (Fig. 5d).

Discussion

The fungus *F. subglutinans* f. sp. *ananas* can survive epiphytically on the pineapple surface, without causing any disease (Dianese 1981). However, wounding of the

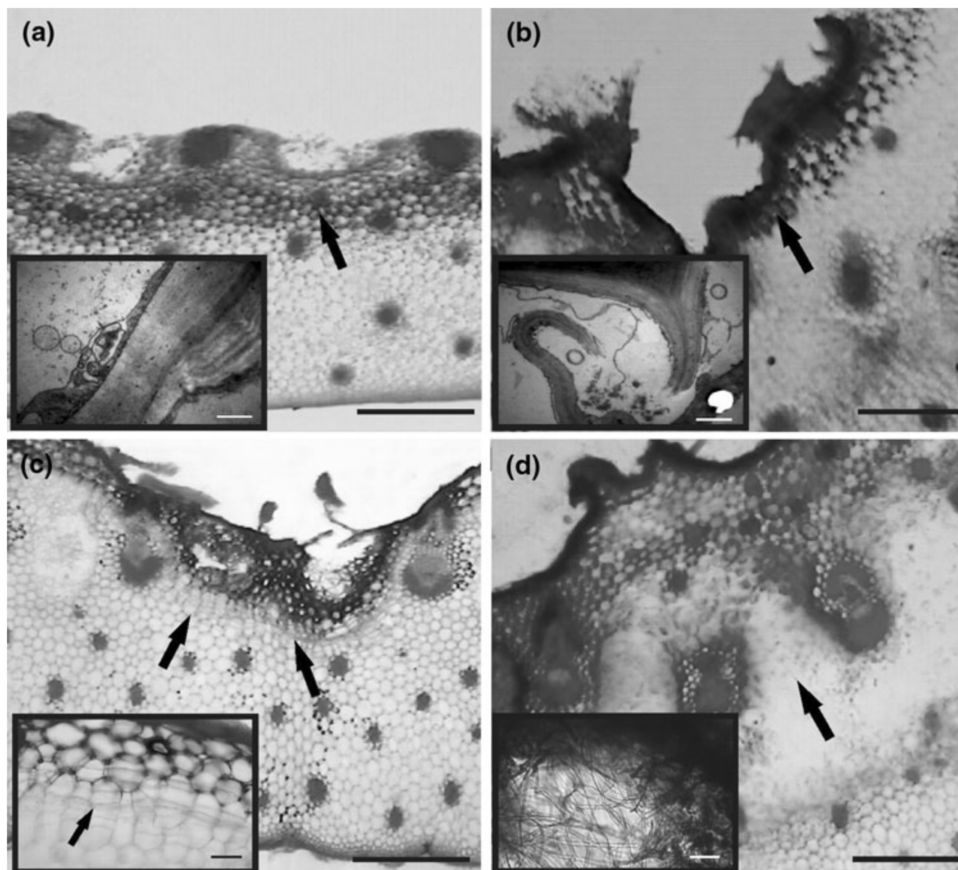


Fig. 3 Transverse sections of pineapple leaves after injury and inoculation with *Fusarium subglutinans* f. sp. *ananas*. Light microscopy (**a**, **b**, **c**, **d**, *insets* of **c**, **d**) and transmission electron microscopy (*insets* of **a**, **b**). **a**, **c** Cv. Vitoria (resistant) and **b**, **d** cv. Perola (susceptible); **a** 48 h post-inoculation, complete cicatrization (*arrow*) and intact mesophyll; *inset* of **a** transverse sections showing thick cell walls and alternate light and dark layers; **b** 48 h post-inoculation, cicatrization (*arrow*) and partially degraded mesophyll; *inset* of **b** transverse sections showing thinner and broken cell walls; **c** 168 h post-inoculation, intact mesophyll; *inset* of **c**, details of figure, showing cell regeneration along the injury (*arrow*); **d** 168 h post-inoculation, damaged mesophyll (*arrows*); *inset* of **d** transverse section stained with lactophenol-cotton blue showing hyphae in mesophyll. Light microscopy *bar* 500 μm; *insets* of **c** and **d** *bar* 50 μm; transmission electron microscopy *bar* 0.25 μm

non-chlorophyll parts of the leaves allow the fungus to penetrate the epidermis and for pathogenesis to take place (Ventura 1994; Ventura and Zambolim 2002).

We inoculated two cultivars of pineapple with *F. subglutinans* f. sp. *ananas*, Vitoria (resistant cultivar) and Perola (susceptible cultivar), and measured responses within the leaf over time. After 48 h post-inoculation (h.p.i.), both cultivars exhibited fungal growth on the surface of the leaf; however, only cv. Perola exhibited symptoms of disease. After 168 h, cv. Perola leaves were severely infected, but the leaves of cv. Vitoria were symptomless.

The thicker cell wall in the resistant cultivar Vitoria appeared to maintain the intact mesophyll after injury and inoculation, and Vitoria also had a more efficient local cicatrization response (Fig. 3a, c). After 48 h cicatrization was already evident, and after 168 h the pathogen was completely restricted to the inoculation site. However, in the susceptible cv. Perola, which presents major disease severity, cell walls were thinner and several broken walls were observed 48 h.p.i, so that cell colonization by the pathogen was less restricted (Fig. 3d). Similar results to those of cv. Perola were observed by Kang et al. (2005) for *Fusarium avenaceum* infection of wheat spikes with hyphae penetration accompanied by cell wall disintegration.

The mechanical properties of the cell walls were investigated by AFM. The resulting force versus displacement curves provide information on the mechanical properties of the material, resulting from the interactions between the AFM tip and the surface under examination (Gaboriaud and Dufrêne 2007). The AFM tip indentations may originate from different sources, such as physical structures or pressure differences between the cell interior and exterior (Butt et al. 2005); however, we eliminated tip indentations due to pressure differences by using dried samples. It was observed that for a given sample, the

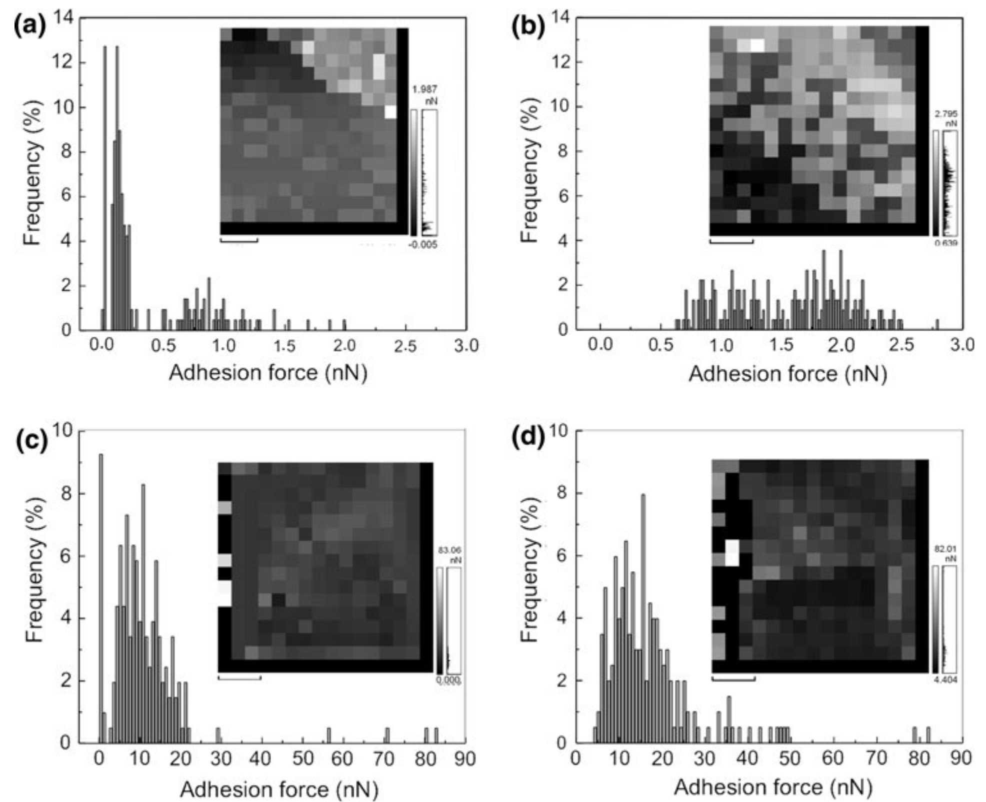


Fig. 4 Atomic force microscopy map and histograms of adhesion force, resulting from the interactions between the tip and the sample surface. Frequency of each adhesion force detected in a 1,000-nm lateral scan of 256 force curves given as a percentage. **a**, **c** Constitutive adhesion force; **b**, **d** 48 h post-inoculation; **a**, **b** cv. Vitoria (resistant) and **c**, **d** cv. Perola (susceptible). *Insets* within each histogram are force maps of the tissue studied. Cv. Vitoria displayed a higher frequency of low adhesion force measurements than cv. Perola constitutively and 48 h post-inoculation

Table 1 Cell wall-bound phenolic compounds from fresh leaves of pineapple cultivars

Cultivars	Phenolic compounds (μg/mg)	
	Constitutive	Inoculated
Vitoria (resistant)	1.70 ± 0.09aA	1.98 ± 0.08aB
Perola (susceptible)	0.51 ± 0.05bA	2.32 ± 0.03bB

Constitutive = intact leaf, without injury and inoculation. Inoculated = leaf 48 h after injury and inoculation with *Fusarium subglutinans* f. sp. *ananas*. Means of three replications in triplicate. Means followed by the same letter in the same row (capitals) or column are not significantly different ($P < 0.05$) by Tukey's test

images and force data obtained in air were reproducible with repeated AFM operations.

A dramatic disparity was observed in cell wall properties of the cv. Vitoria compared to cv. Perola constitutively and at 48 h.p.i. (Fig. 4). The frequency of low adhesion force measurements from force mapping was higher for cv. Vitoria, indicating a higher rigidity.

These data indicate that the morphological differences, the thickness of cell walls and increased cicatrization, observed by electronic and light microscopy, were accompanied by differences in force mapping. These results suggest that it is not only the structure of the cell wall, but also its mechanical properties that are involved in the resistance to pathogenesis. Similar cell wall modifications were found in grapevines exposed to a fungal elicitor (Lesniewska et al. 2004).

The process of cell wall thickening and increased leaf stratification was presumably a result of lignification. Lignification not only makes the wall more resistant to mechanical intrusion, but also more water resistant, thus reducing the potency of wall degrading enzymes and inhibiting pathogen penetration and development (Hückelhoven 2007). Studies on the histology of pathogenesis in *Myrtus communis* showed lignification of the parenchyma cells of the resulting knot, finally leading to its hardening and cell death (Temsah et al. 2007).

The plant defense mechanism to fungi is related to the production of a strong physical barrier around the wound, which prevents hyphae penetration into the leaf tissue. The presence of this "cicatrization process" in pineapple suggests the formation of ligno-suberized tissues in the wound periderm, as has been observed for parasite–host interactions in fruits and leaves of citrus infected with *Elsinoe fawcettii*, where the ligno-suberized cork layers in the wound periderm act as a protective barrier leading to restricted growth of *E. fawcettii* (Kim et al. 2004). The pine tree fungus, *Fusarium circinatum,* appears to be almost completely inhibited by high levels of ligno-suberized wound periderm in stems of *Pinus densiflora* (Kim et al. 2009).

Ventura (1994) found that inoculation of pineapple cultivars Smooth Cayenne and Queen by *Fusarium* 72 h

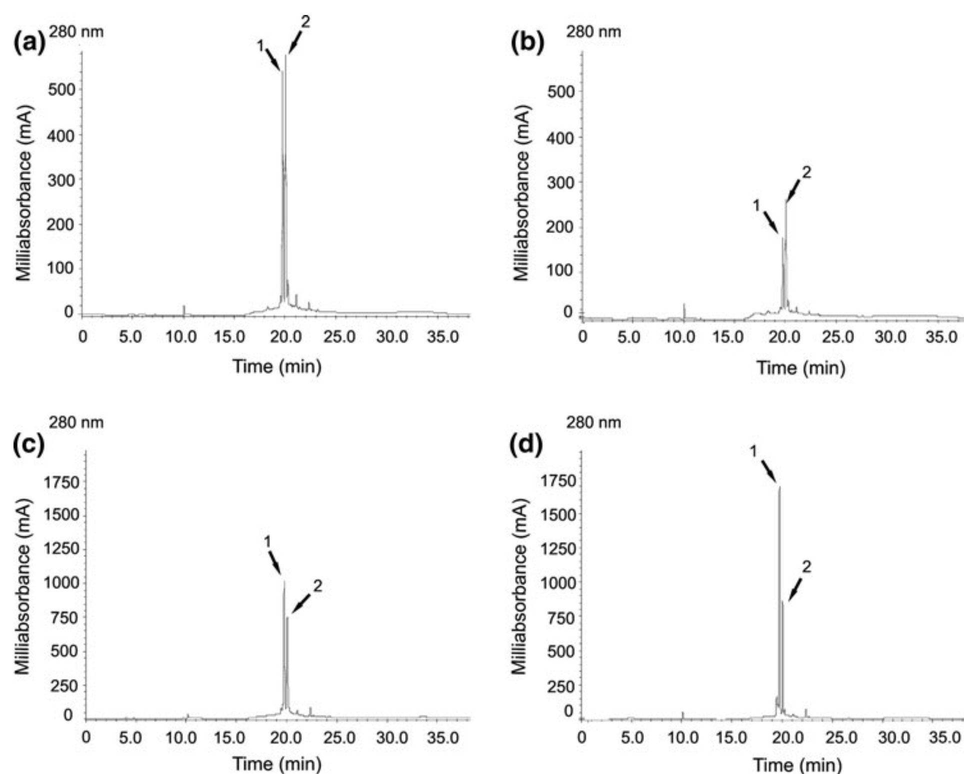


Fig. 5 High-performance liquid chromatography chromatograms of cell wall-bound phenolic compounds extracted from leaves of pineapple. **a**, **b** Constitutive feature; **c**, **d** 48 h post-inoculation; **a**, **c** cv. Vitoria (resistant) and **b**, **d** cv. Perola (susceptible). The same chromatogram profile was observed for each cultivar at both time points; the peaks 1 and 2 corresponded to the retention times for *p*-coumaric acid and ferulic acid, respectively, but the relative quantities varied

post-injury was severely restricted by a local physical barrier formed from regenerating parenchyma cells, probably by lignin deposition and later cicatrization. This is supported by our data from two other cultivars. In cultivars other than Vitoria, cicatrization is not rapid enough to inhibit *Fusarium* infection.

Biochemical analysis of total cell wall-bound phenolics (Table 1) and HPLC chromatography (Fig. 5) showed similar trends. Two peaks, identified as *p*-coumaric acid and ferulic acid, were constitutively present in the control samples and also at 48 h.p.i. Both compounds are phenylpropanoids derived from phenylalanine (de Ascensao and Dubery 2003). The phenylpropanoid pathway also provides lignin monomers, *p*-coumaryl and coniferyl alcohols that give rise to hydroxyphenyl (H) and guaiacyl (G) units of lignin. The H unit is particularly abundant in lignin of monocots such as pineapple. Changes in the relative amounts of *p*-coumaric and ferulic acids bound to infected cell walls indicate that the phenylpropanoid pathway is activated upon infection. It has been suggested that esterification of phenolic acids to the cell wall provides attachment sites for lignin and so promotes lignification (Lewis and Yamamoto 1990). Kováčik and Klejdus (2008) found a similar result in camomile under metallic stress, with elevated levels of cell wall *p*-coumaric acid and ferulic acid associated with lignification.

The resistant cultivar Vitoria showed higher baseline levels of phenolics bound to the cell wall than the susceptible cv. Perola. However, concentrations measured 48 h.p.i. in both infected cultivars were similar.

The key difference between a resistant plant and a susceptible one is defined by Yang et al. (1997) as the time spent by the plant in prompt and effective activation of the defense mechanisms by the host plant. Our results demonstrated that the resistant pineapple, cv. Vitoria, was able to quickly develop a defense response that could prevent pathogen invasion and tissue colonization. Constitutively high levels of cell wall polyphenols and lignification appeared to enable initial resistance to the pathogen and rapid cicatrization. In contrast, the susceptible cultivar, Perola, presented a delayed defense response that could not restrict the advance of pathogen into plant tissues.

Acknowledgments The authors thank Giovana A. de Moraes and Beatriz F. Ribeiro for technical assistance with the electron microscopy and Paulo Moscon for technical assistance with the atomic force microscopy. This work was supported by Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Banco do Nordeste do Brasil (BNB) and Fundação de Amparo à Pesquisa do Estado do Espírito Santo (FAPES).

References

Bentes JLS, Matsuoka K (2005) Histology of *Stennphylium solani* and tomato interaction. Fitopatol Bras 30:224–231

Butt H-J, Cappella B, Kappl M (2005) Force measurements with the atomic force microscope: technique, interpretation and

applications. Surface Sci Rep 59:1–15. doi:10.1016/j.surfrep.2005.08.003

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6:850–861. doi:10.1038/nrm1746

de Ascensao ARFDC, Dubery IA (2003) Soluble and wall-bound phenolics polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f. sp. *cubense*. Phytochemistry 63:679–686. doi:10.1016/S0031-9422(03)00286-3

Dianese JC (1981) Pathogenicity of epiphytic population of *Fusarium moniliforme* var. *subglutinans* pathogenic to pineapple. Phytopathology 71:1145–1149. doi:10.1094/Phyto-71-1145

Eynck C, Koopmann B, Karlovsky P, von Tiedemann A (2009) Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. Phytopathology 99(7):802–811. doi:10.1094/PHYTO-99-7-0802

Gaboriaud F, Dufrêne YF (2007) Atomic force microscopy of microbial cells: application to nanomechanical properties, surface forces and molecular recognition forces. Colloids Surf B Biointerfaces 54:10–19. doi:10.1016/j.colsurfb.2006.09.014

Hückelhoven R (2007) Cell wall-associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol 45:101–127. doi:10.1146/annurev.phyto.45.062806.094325

Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York

Kang Z, Zingen-Sell I, Buchenauer H (2005) Infection of wheat spikes by *Fusarium avenaceum* and alterations of cell wall components in the infected tissue. Eur J Plant Pathol 111(1):19–28. doi:10.1007/s10658-004-19839

Kavousi HR, Marashi H, Mozafari J, Bagheri AR (2009) Expression of phenylpropanoid pathway genes in chickpea defense against race 3 of *Ascochyta rabiei*. Plant Pathol J 8:127–132. doi:10.3923/ppj.2009.127.132

Kim KW, Hyun J-W, Park EW (2004) Cytology of cork layer formation of citrus and limited growth of *Elsinoe fawcettii* in scab lesions. Eur J Plant Pathol 110(2):129–138. doi:10.1023/B:EJPP.0000015330.21280.4c

Kim KW, Lee IJ, Thoungchaleun V, Kim CS, Lee DK, Park EW (2009) Visualization of wound periderm and hyphal profiles in pine stems inoculated with the pitch canker fungus *Fusarium circinatum*. Microsc Res Tech 72:965–973. doi:10.1002/jemt.20744

Kováčik J, Klejdus B (2008) Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. Plant Cell Rep 27:605–615. doi:10.1007/s00299-007-0490-9

Kruger WM, Carver TLW, Zeyen RJT (2002) Effects of inhibiting phenolic biosynthesis on penetration resistance of barley isolines containing seven powdery mildew resistance genes or alleles. Physiol Mol Plant Pathol 61:41–51. doi:10.1006/pmpp.2002.0415

Lesniewska E, Adrian M, Klinguerc A, Pugin A (2004) Cell wall modification in grapevine cells in response to UV stress investigated by atomic force microscopy. Ultramicroscopy 100:171–178. doi:10.1016/j.ultramic.2003.11.004

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41:455–496

Ma C, Xiao SY, Li ZG, Wang W, Du LJ (2007) Characterization of active phenolic components in the ethanolic extract of *Ananas comosus* L. leaves using high performance liquid chromatography with diode array detection and tandem mass spectrometry. J Chromatogr A 1165:39–44. doi:10.1016/j.chroma.2007.07.060

Micic M, Benitez I, Ruano MM, Jeremic M, Radotic K, Moy V, Leblanc RM (2001) Probing the lignin nanomechanical properties and lignin–lignin interactions using the atomic force microscopy. Chem Phys Lett 347:41–45. doi:10.1016/S0009-2614(01)01022-3

Moore JP, Farrant JM, Lindsey GG, Brandt WF (2005) The South African and Namibian populations of the resurrection plant *Myrothamnus flabellifolius* are genetically distinct and display variation in their galloylquinic acid composition. J Chem Ecol 31(12):2823–2834. doi:10.1007/s10886-005-8396-x

Quiroga M, Guerrero C, Botella MA, Bacelo A, Amaya I, Medina MI (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol 122:1119–1127

Rodrigues SP, Lindsey G, Fernandes PMB (2006) Biotechnological approaches to the control of plant viruses, chap 6. In: Thangadurai D et al (eds) Plant stress and biotechnology. Oxford Book Company, Jaipur

Rodrigues SP, Andrade JS, Ventura JA, Lindsey G, Fernandes PMB (2009) Biotechnological approaches for plant viruses resistance. Braz Arch Biol Technol 52(4):795–808

Santos BA, Zambolim L, Ventura JA, Vale FXR (2002) Severidade de isolados de *Fusarium subglutinans* f. sp. *ananas* sensíveis e resistentes ao benomyl em abacaxizeiro. Fitopatol Bras 27:101–103. doi:10.1590/S0100-41582002000100018

Silva MC, Várzea V, Guerra-Guimarães L, Azinheira HG, Fernandez D, Petitot A-S, Bertrand B, Lashermes P, Nicole M (2006) Coffee resistance to the main diseases: leaf rust and coffee berry disease. Braz J Plant Physiol 18:119–147. doi:10.1590/S1677-04202006000100010

Temsah M, Hanna L, Saad AT (2007) Histology of pathogenesis of *pseudomonas savastanoi* on *Myrtus communis*. J Plant Pathol 89(2):241–249

Ventura JA (1994) Pineapple fusariosis: characterization of the pathogen, epidemiology of disease, resistance and micropropagation of host in vitro. Ph.D. thesis, Federal University of Viçosa. Viçosa, Minas Gerais

Ventura JA (2000) Taxonomia de *Fusarium* e seus segredos. Parte II: Chaves para identificação. Rev Annu Patol Plantas 102:303–338

Ventura JA, Zambolim L (2002) Controle das Doenças do Abacaxizeiro. In: Zambolim L, Vale FXR, Monteiro AJA, Costa H (eds) Controle de Doenças de Plantas Fruteiras. UFV, Viçosa, pp 445–510

Ventura JA, Cabral JRS, Matos AP, Costa H (2009) 'Vitoria': new pineapple cultivar resistant to fusariose. Acta Hortic Leuven 882:51–56

Wally O, Jayaraj J, Punja Z (2009) Comparative resistance to foliar fungal pathogens in transgenic carrot plants expressing genes encoding for chitinase, β-1,3-glucanase and peroxidise. Eur J Plant Pathol 123:331–342. doi:10.1007/s10658-008-9370-6

Wuyts N, Lognay G, Verscheure M, Marlier M, de Waele D, Swennen R (2007) Potential physical and chemical barriers to infection by the burrowing nematode *Radopholus similis* in roots of susceptible and resistant banana (*Musa* spp.). Plant Pathol 56:878–890. doi:10.1111/j.1365-3059.2007.01607

Yang Y, Shah J, Klessig DF (1997) Signal perception and transduction in plant defense responses. Genes Dev 11:1621–1639. doi:10.1101/gad.11.13.1621

View publication stats