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Induction of a defense response in strawberry mediated by an avirulent strain of *Colletotrichum*

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Abstract

In the strawberry crop area of Tucumán (north-west Argentina) the three species of *Colletotrichum* causing anthracnose disease (*C. acutatum*, *C. fragariae* and *C. gloeosporioides*) were detected. Among all isolates characterized, one of them identified as *C. acutatum* (M11) and another as *C. fragariae* (F7) were selected due to their conspicuous interaction with the strawberry cultivar Pájaro. Whereas isolate M11 produced a strong compatible interaction in cv. Pájaro with clear disease symptoms (DSR = 5.0), the isolate F7 brought about a typical incompatible interaction (DSR = 1.0). When plants of cv. Pájaro were inoculated with F7 prior to the inoculation with M11, the former avirulent strain prevented the growth of the latter virulent pathogen. Experimental evidence indicated that the time elapsed between the first inoculation with the avirulent pathogen and the second inoculation with the virulent one was crucial to inhibit the growth of the latter. The growth of F7 on the plant without provoking damage and the fact that there was no *in vitro* antagonistic effect between the pathogens, suggests that the avirulent strain triggers a plant defensive response against M11. The defense response was further confirmed by the detection of an early oxidative burst occurring within 4 h after the first inoculation and by the observation of anatomical changes associated with defense mechanisms that lasted 50 days after the inoculation with F7. Results obtained support the hypothesis that the plant resistance against the virulent strain M11 is elicited by one or more diffusible(s) compound(s) produced by the avirulent strain F7.

Abbreviations: AOS – Active Oxygen Species; dai – day after inoculation; DSR – Disease Severity Rating; hai – hour after inoculation; RH – Relative Humidity; SEM – Surface Electron Microscopy

Introduction

Strawberry (*Fragaria* × *ananassa*) anthracnose, caused by fungi of the genus *Colletotrichum* (Howard et al., 1992), is one of the major diseases that adversely affects the strawberry crop (Freeman and Katan, 1997). Three species of *Colletotrichum* have been identified as the casual agents of the anthracnose disease in strawberry: *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (teleomorph *Glomerella cingulata*) (Smith and Black, 1990; Adaskaveg and Hartin, 1997; Xiao et al., 2004). Previous studies carried out in the strawberry crop area of Tucumán (north-west Argentina) have revealed that the three species of *Colletotrichum* are present in different

proportions: approximately 85% *C. acutatum*, 10% *C. fragariae* and, to a lesser extent, 5% *C. gloeosporioides* (Mena et al., 1974; Mónaco et al., 2000; Ramallo et al., 2000).

The infection of strawberry by *Colletotrichum* spp. has been well documented and many compatible and incompatible interactions have been reported (Smith and Black, 1990; Denoyes and Baudy, 1995; Denoyes-Rothan et al., 1999, 2005). A large number of light and electron microscope studies have been published concerning the interaction of plants with fungal pathogens including *Colletotrichum* species (O'Connell et al., 2000; Wharton et al., 2001; Curry et al., 2002; Kim et al., 2004). Ultrastructural studies have been carried out to study the changes of the cell wall and other plant components in response to fungal infection, and the manner in which these structural modifications contribute to plant defense (Kim et al., 2004). Appositional cell wall thickening at points of fungal attack have been reported in several species of Gramineae (barley, wheat, oats, rye, sorghum, corn) associated with unsuccessful penetration attempts in incompatible plant/fungi interactions (Sherwood and Vance, 1980). Lignin-like compounds (Sherwood and Vance, 1976), callose (DeFosse, 1976; Yun et al., 2006), cellulose (Sherwood and Vance, 1976), phenolic compounds (Mayama and Shishiyama, 1978), silicon (Kunoh and Ishizaki, 1976) and other unidentified 'basic staining' material were also detected in these appositions, some of which may provide a passive or active mechanism of resistance to fungal attack. The occurrence of unusual crystals have also led to several hypotheses regarding their function in plants, including plant defense. Brown (1978) reported the *de novo* synthesis of the phenolic materials, lignin (Staples et al., 1989) and tannin (Robb et al., 1975) in fruits of sensitive Robinson tangerine in response to infection by *C. gloeosporioides*. Similar defense mechanisms induced by methyl jasmonate were reported by Hudgins et al. (2004) in stems of diverse conifers including oleoresin, phenolics, tannins, and static structures in secondary phloem such as lignified cells and calcium oxalate crystals.

Over the last century, it was reported that some plants previously infected with a pathogen became resistant to further infection suggesting the existence of an immune system. Induced resistance to a virus in virus-infected plants has been observed in numerous laboratories (Ross, 1961a, b; Kuc, 1982). Furthermore, plant protection mediated by attenuated viruses was successfully used to control disease in several agricultural systems making plants more resistant to subsequent pathogen attacks (Fulton, 1986; Kuc, 2001). Although based on different molecular mechanisms, similar phenomena were also observed in other plant species involving bacteria and fungi (Ryals et al., 1994, 1996; Kuc, 2001; Métraux, 2001; Shoresh et al., 2005). These defense responses have received different names (SAR, ISR, cross-protection) depending mainly on the microorganism involved (Métraux, 2001). The role and participation of fitohormones such as salicylic and jasmonic acids and ethylene in these defense mechanisms have been demonstrated and extensively discussed in a review published by Métraux (2001). Induction of defense responses mediated by (a)virulent pathogenic fungi has also been described. Kuc and Richmond (1976) have reported a plant protection effect in cucumber mediated by *Colletotrichum lagenarium* and Manandhar et al. (1998) reported suppression of rice blast when preinoculating with a non-rice pathogen *Bipolaris sorokiniana* or an avirulent rice pathogen *Pyricularia oryzae*. Shishido et al. (2005) used the strain Fo-B2 of *Fusarium oxysporum* as a biological control agent against the disease *Fusarium* wilt on tomato and Shetty et al. (2003) induced resistance on wheat by using a non-pathogenic strain of *Septoria tritici*. In the latter case, a positive correlation between the increment of hydrogen peroxide and restriction of pathogen growth was observed (Shetty et al., 2003).

In this work we present experimental evidence of the induction of a defense response in strawberry, mediated by the avirulent *C. fragariae* isolate F7 against a virulent isolate of *C. acutatum* isolate M11, halting its pathogenic process and impeding the development of the disease. The protection effect observed is accompanied by a rapid accumulation of the active oxygen species (AOS) hydrogen peroxide and superoxide anion (oxidative burst) and also with anatomical and morphological alterations in leaves. This finding represents the first report of induced resistance described in strawberry.

Materials and methods

Fungal cultures

Strains of *C. acutatum* (M11) and *C. fragariae* (F7) used in this work correspond to local isolates characterized in our laboratory. Isolates were single-spore propagated to obtain pure cultures on potato dextrose agar (PDA) supplemented with streptomycin (300 μg ml^{-1}) and maintained on PDA slants at 4 °C. Liquid cultures were carried out in PD (potato dextrose) medium. Evaluation of antagonistic growth effect was carried out on PDA plates inoculated with 10 μl of a suspension (10^6 conidia ml^{-1}) of each isolate separated by 5 cm and incubated at 28 °C under continuous white fluorescent light (200 μmol m^{-2} s^{-1}) for 8 days. M11 growth inhibition tests were carried out with conidial extracts (SC, see Figure 5) and axenic filtrate of 3-week-old supernatant of *C. fragariae* F7 liquid culture (SN, see Figure 5). Euparen as the antifungal agent (0.015 %, EU) and methanolic leaf extract of uninoculated strawberry (1% v/v, LE) were used as controls (see Figure 5).

Inoculum and inoculation

Fungal isolates were grown on PDA for 10 days under continuous fluorescent light a 28°C to induce conidial formation (Smith and Black, 1990). The culture surface was gently scraped with a Pasteur pipette to remove conidia and suspended in sterile distilled water (SDW). The conidial suspensions obtained were filtered through sterile gauze to remove mycelial debris under axenic conditions. Suspensions were then diluted with SDW (containing two drops of Tween 20 l^{-1}) to a final concentration of 1.5×10^6 conidia ml^{-1} and applied to plants as a spray to run-off (Smith and Black, 1990). Immediately after inoculation, plants were placed in a dew chamber at 100% relative humidity (RH), 28 °C and for 48 h in the dark (infection chamber). Then plants were returned to a growth chamber at 70% RH, 28 °C and a light (white fluorescent, 450 μmol m^{-2} s^{-1}) cycle of 16 h day^{-1}.

Strawberry plants

Plants of cv. Pájaro were used in experiments. Mother plants were purchased as dormant crowns from commercial nurseries, planted in 8 cm pots with sterilized (1 h at 120 °C) substrate (humus: perlome, 2:1), and grown in a greenhouse for 6 weeks or more to confirm that they were free of anthracnose. Plants for phytopathological studies were obtained from runners of this cultivar and rooted in sterile substrate under axenic conditions. Daughter plants were grown from 14 to 16 weeks and maintained in growth cabinets at 28 °C, 70% RH with a light cycle of 16 h day^{-1}. Plants were watered every other day with 50 ml of distilled water. All senescent leaves and petioles were removed periodically until 10 days before the inoculation experiments, leaving only three to four young healthy leaves.

Phytopathological tests

Susceptibility was evaluated by spraying leaves and petioles of four plants of the cv. Pájaro with a 1.5×10^6 conidia ml^{-1} suspension. Four uninoculated plants were used as control. Plants were placed in the infection chamber for 48 h and then transferred to a growth cabinet. Disease Severity Rating (DSR) was assessed using the following scale: 1, healthy petiole without lesions; 2, petiole with lesions < 3 mm; 3, petiole with lesions from 3 to 10 mm; 4, petiole with lesions from 10 to 20 mm and girdling of petiole; 5 entirely necrotic petiole and dead plant (Delp and Milholland, 1980). The DSR was evaluated 9, 30 and 50 days after inoculation (dai). After 50 days of incubation, pathogens were isolated from crowns and leaves of plants that developed the disease and compared with the strains used in the inoculation to check pathogen identity. Identity of isolates were routinely checked by reisolation from the plants followed by microscopic and phytopathological tests.

Induced resistance experiments

These were carried out following the procedure described above except that plants received a double inoculation, firstly with the avirulent isolate (F7) and then, at different times with the virulent one (M11). In the first experiment the batch of plants was inoculated with a suspension mixture (1:1) of both pathogens (final concentration 1.5×10^6 conidia ml^{-1}) and in subsequent experiments the inoculation with M11 was carried out at increasing times (e.g. 48, 72, 96 and 120 h) from

the first inoculation with F7. DSR values were evaluated at 9, 30 and 50 days after M11 inoculation (in Figure 1 we report results evaluated 50 dai and in Figure 6, at 9 and 30 dai).

Anatomical studies

Leaf and petiole samples for anatomical studies were obtained from selected plants challenged with the virulent (M11), the avirulent (F7), both isolates (in induced resistance experiments) or none (sterile water) in control experiments. Central leaflets or petioles were fixed in FAA (formalin-acetic acid-alcohol) and made transparent according to Dizeo de Strittmater (1986). Free-hand cross-sections and cuts with a Minot Microtome (Leitz, Germany) of leaflets and petioles (10–15 μm section) were firstly dehydrated with increasing concentrations of ethanol and then embedded in liquid paraffin. For visualizing lignified structures staining was made with safranin, cresyl violet dye, cotton blue in lactophenol and trypan blue dye for visualizing the hyphae. All prepared material was observed and photographed with an Olympus BH-2 light microscope. The calcium oxalate crystals were observed with a Zeiss polarized light microscope.

Samples for Scanning Electronic Microscopy (SEM) examination were fixed with 3% glutaraldehyde for 6 h at 4 °C and then post-fixed overnight with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). The samples were then rinsed with distilled water and treated for 40 min with uranyl acetate (2%) in aqueous solution. After fixation, samples were stepwise dehydrated with increasing concentrations of ethanol (10–90%), followed with acetone (100%), critical-point dried and gold-sputter-coated. Samples were observed and photographed in a JEOL JSM35 CF scanning electron microscope.

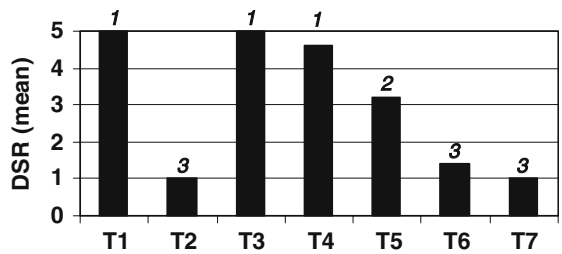

Figure 1. Influence of time elapsed between first inoculation with isolate F7 and second with M11 on susceptibility (DSR) of cv. Pájaro to M11. T1 and T2 indicate control plants inoculated only with M11 (virulent) or F7 (avirulent) strains, respectively; T3, plants treated with a mixture (1:1) of both pathogens (simultaneous inoculation); T4–T7, when M11 was inoculated 48, 72, 96 and 120 h after prior inoculation with F7, respectively. Results are for plants evaluated 50 dai with M11. DSR values with different numbers represent statistically different values (Tukey test, $P = 0.05$).

Oxidative burst

The generation of hydrogen peroxide was detected by a peroxidase-dependent *in situ* histochemical staining procedure using 3,3′-diaminobenzidine (DAB; Thordal-Christensen et al., 1997), and superoxide according to Doke (1983) using a superoxide-dependent reduction of nitroblue tetrazolium (NBT). Analyses were performed on inoculated plant leaves using one of the following methods: (i) spraying plants to run-off with a conidial suspension of each isolate (F7 or M11, see Figure 4) or (ii) by infiltrating 20 μl of conidial extracts (10 μg protein ml^{-1} of F7, see Figure 6) in two sites on the abaxial surface of one leaflet per plant using a 1 ml syringe without a needle. At different times from 0 to 120 hai 10 leaves (or proximal leaflets in the case of infiltration) from equally treated plants (per experiment) were excised and incubated in a solution of 0.1% (w/v) DAB, 10 mM MES, pH 3.8 (for hydrogen peroxide detection) or in 0.1% (w/v) NBT, 10 mM sodium azide, and 10 mM potassium phosphate (pH 7.8) without the addition of NADPH (for the detection of superoxide). Leaves were then incubated for 8 h in the dark under pressure of nitrogen gas, fixed and decolourized in boiling 95 % (v/v) ethanol, cleared in lactic acid/glycerol/H_2O [3:3:4] for 24 h and mounted on slides in 60 % glycerol. Microscopic observations and photos were obtained with an Olympus BH-2 microscope. Water and sterile PD medium were used as control.

Conidial extracts

The avirulent strain F7 was cultivated in 100 ml of PD medium for 3 weeks at 28 °C under continuous fluorescent light without agitation.

Culture supernatant containing conidia were harvested and filtered through sterile gauze as indicated earlier, washed three times with SDW, centrifuged ($5000 \times g$) and suspended in SDW to a concentration of 10^9 conida ml^{-1}. One milliliter of this suspension was disrupted by sonication (30 min with intervals of 20 s at 60% output power and 4 °C with a Branson sonicator), filter-sterilized (0.21 μm), diluted with SDW and used in *in vitro* inhibition experiments (SC, see Figure 5b). The rest of the suspension was frozen at −20 °C before passing four times through a French press at 13,000 psi. The conidial extract was collected on ice and centrifuged for 10 min at $10{,}000 \times g$ and 4 °C. Supernatant was filter-sterilized and diluted with SDW for evaluating the defense elicitation activity as mentioned above (extract CE, see Figure 6). Cellular debris was recovered, washed and centrifuged three times with water, sonicated until dissolution, filter-sterilized and diluted with SDW to test elicitation activity (extract CP, see Figure 6). Defense response was evaluated as for the induced resistance experiment, except that the first inoculation was carried out with the F7 conidial extract (supernatant or centrifuged, in separate experiments) instead of intact F7 conidia, prior to the infection with the virulent strain M11 (see Figure 6). The rest of the experiments were conducted and evaluated as described above. In order to rule out any possible contamination of intact F7 conidia, sterility controls of extracts were carried out on PDA and cultured at 28 °C.

Statistical analyses of data

The experimental design was randomized with 8 plants per genotype and per experimental unit, with 4 plants corresponding to the inoculated and 4 to the control plants (see above). Experimental data obtained from phytopathological tests were analyzed with the programme Statistix (Analytical Software, 1996). LSD test was used for determining the arithmetic mean of the DSR value (significance level, 0.05) of plants inoculated with each isolate and the Analysis of Variance test (ANOVA) was used for evaluating the data dispersion with respect to the mean value. Experiments were repeated three times to diminish the dispersion of DSR values.

Results

Phytopathological tests

Plant-pathogen interaction studies of cv. Pájaro exposed to isolates M11 and F7 of *C. acutatum* and *C. fragariae*, respectively, showed that whereas the former strain produced a strong compatible interaction with a DSR = 5 (T1 in Figure 1), the latter produced a totally incompatible interaction with a DSR = 1 (T2 in Figure 1). Plants inoculated with the avirulent isolate (F7) showed no symptoms of the disease (even many months after the inoculation) like the control plants treated with water, whereas the virulent pathogen (M11) caused the death of all plants used in the experimental batch, within 2 weeks after inoculation. Since the strain F7 is a virulent pathogen showing typical anthracnose symptoms in other cultivars of strawberry (results not shown), we decided to investigate whether the lack of disease symptoms observed in cv. Pájaro was due to an interaction that would not support the growth the pathogen. Consequently, we conducted controlled inoculation experiments with the aim to observe and compare the germination of conidia, the growth of hyphae coming from the virulent (M11) and avirulent (F7) strains and their effect on strawberry leaflets and petioles.

Microscopic studies of the compatible interaction

Strawberry leaf tissues infected with the virulent pathogen M11 of *C. acutatum* were examined using electron and light microscopy (Figure 2). We have observed a typical hemibiotrophic growth. In the first biotrophic stage, conidia germinated during the first 24–72 hai and the hyphae (h) grew on the surface of the leaf above the cuticle. Shortly after, the hyphae infected the inner plant tissues utilizing three alternative ways: (i) by producing appressoria (ap) that attached and bored through the cuticle of leaves penetrating the epidermal cells of leaflets or petioles (Figure 2a), (ii) through stomata and (iii) through trichomes (results not shown). We have observed as a conspicuous pathogenic feature, that hyphal growth and appressorial development caused the detachment of the leaf cuticle as the fungus invaded internal tissues (Figure 2a). Later, in the second necrotrophic stage, hyphae grew throughout the rest of the

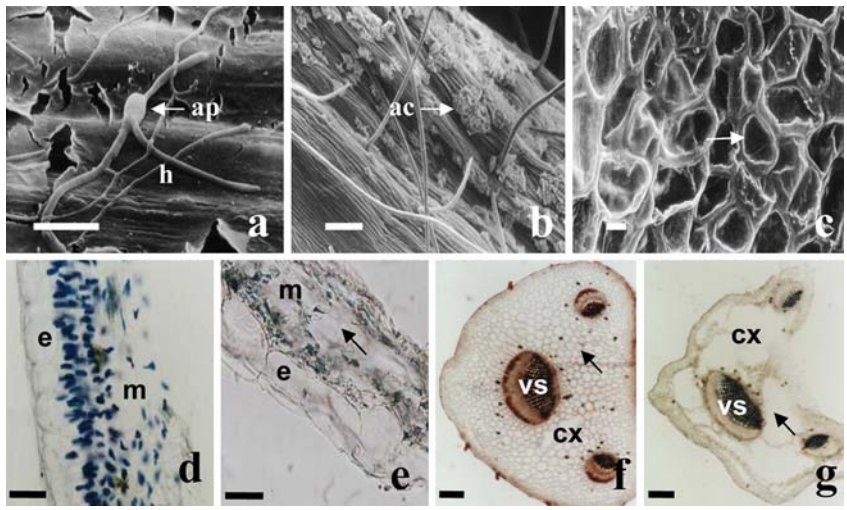

Figure 2. Growth of virulent isolate M11 and effect of anthracnose disease on leaflets and petioles of strawberry cv. Pájaro. SEM of infected strawberry leaves: a) appressorium (ap), growing hyphae (h) detached cuticle from growth of strain M11, b) acervuli (ac) formed on the petiole surface, c) cellulosic matrix after cell disruption caused by pathogen invasion. Leaflet cross-sections showing mesophyll (m) and epidermis (e) of: d) uninfected leaflet, e) leaflet infected with virulent strain M11 of *C. acutatum*. Petiole cross-section showing cortex (cx) and vascular (vs) tissues of: f) uninfected petiole, g) petiole infected with virulent strain M11 of *C. acutatum*. Arrows in e) and g) indicate areas where severe alterations of mesophyll and cortical leaf tissues were observed, and arrow in f) indicates normal uninfected cortical tissue. Leaflets and petioles were analyzed 10 dai. Scale bars indicate 10 μm, in a–b), 100 μm in c), and 20 μm in d–g).

plant tissue invading intracellular and intercellular spaces and after 10 days, acervuli (ac) attached to the remains of cellulosic matrix deprived of cells were observed (Figure 2b and c). Figure 2c shows a microscopic view of a leaflet surface in an advanced stage of the disease symptoms. In Figure 2 we also compare, normal (Figure 2d and f) and infected (Figure 2e and g) leaflet and petiole tissues, respectively, 10 dai. Transverse sections of infected petioles show that although the epidermal and subepidermal cellulosic cell walls remained apparently intact at an early stage, as the infection progressed and the invasion of vascular tissue occured, cell walls became increasingly affected as the cortical cells died (Figure 2e and g) and all the cellular content is removed from the cellulosic cell walls, as shown in Figure 2g. Comparison of cross-sections of normal and infected leaves (Figure 2d and e, respectively), revealed that in an advanced stage of the disease, the hyphal growth caused the lysis of mesophyll (m) and epidermal (e) cells, leaving voids in places originally occupied by cellular tissues. The same occurred in the petiole tissues. In Figure 2f and g we show cross-sections of an uninfected healthy petiole and a petiole with advanced symptoms of anthracnose disease (10 dai), respectively. Alterations in cortex (cx) tissue surrounding the vascular bundles (vs) was observed in infected petioles in comparison with normal healthy tissue (Figure 2d and f). Although hyphal growth was also observed in vascular tissues, surprisingly the vascular bundles were not so severely affected as mesophyll and cortex tissues (Figure 2e and g), compared with uninfected healthy tissues (Figure 2d and f).

Microscopic studies of the incompatible interaction

Microscopic observations carried out on leaflets and petioles of plants infected with the avirulent pathogen (F7) did not show any disease symptoms or tissue damage during 50 dai. SEM observations showed that conidia (co) of isolate F7 of *C. fragariae* germinated and hyphae (h) spread out on the surface of leaves (Figure 3a and b). Similarly as described for the virulent pathogen M11, we observed the production of appressoria (ap) penetrating the cuticle and penetration hyphae growing underneath the cuticle within the first 10 dai (Figure 3c and d). Nonetheless, in contrast to what was observed

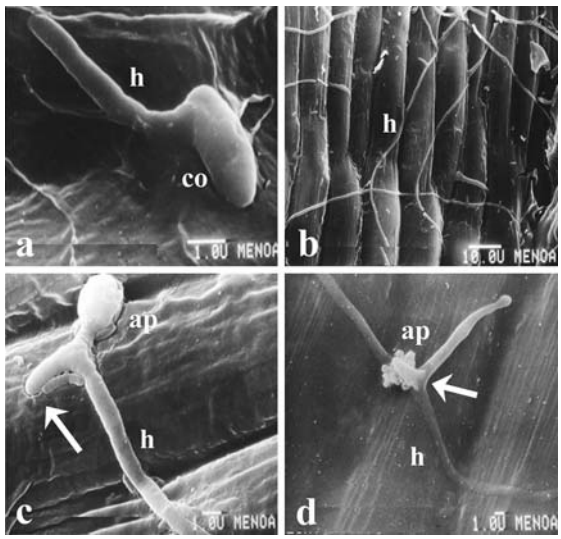

Figure 3. SEM of leaflets and petioles of cv. Pájaro inoculated with avirulent isolate F7 of *C. fragariae*: a) germinating conidium (co) showing a growing hypha (h) on the surface of a leaflet, b) vegetatively growing fungus forming a hyphal (h) matrix on the petiole surface, c) hypha (h) with appressorium (ap) attached to the surface of a petiole, d) view of an external hypha (h) forming an appressorium (ap) and a penetrating hypha growing out of the apressorium, boring through and extending underneath the cuticle. Arrows indicate the penetration points of invading hyphae. The growth of both types of hyphae (external and internal) did not cause detachment of the cuticle or damage the plant tissue. Scale bar in μm as indicated in each photograph.

with isolate M11, strain F7 grew without penetrating through stomata or trichomes (as observed in M11), and without inner tissue invasion, cellular damage or cuticle detachment (Figure 3b, d). The latter observation rules out that the cuticle detachment observed in the compatible interaction (Figure 2a) was due to a technical artifact during sample preparation (e.g. sample dehydration, critical-point drying or gold coating). We also observed a biphasic hyphal growth in the avirulent isolate. In the first stage, a rapid growth of thick superficial hyphae occurred during the first 24–72 hai (Figure 5c), followed by thinner subcuticular hyphal growth in the second stage (Figure 3b and d). Since the conidia and hyphae of isolate F7 actively grew on the surface of the plant tissue, we could confirm that the interaction established was of the host type (e.g. plants supporting the growth of the avirulent isolate F7).

Induced resistance response

In order to provide evidence that would explain the behaviour observed with isolate F7, morphological, physiological and phytopathological analyses were carried out. Cross-section analysis of leaflets exposed to F7 showed a significant thickening of the cell wall compared to control uninfected plants, 72 hai (Figure 4a and b). We observed that the thickening of the semi-lamina was not due to an increase in the number of layers of the mesophyll, but rather to the enlargement of the parenchyma cells and the intercellular space. The average intercellular distance determined in uninoculated petiole tissues or inoculated with M11 was 1.8 ± 0.5 μm, whereas it was 4.5 ± 0.8 μm in plants inoculated with F7. Figure 4b also shows a heavily stained area that clearly indicates the accumulation of pigments (pi). Pigment accumulation was only observed in plants infected with the isolate F7.

Another anatomical feature observed during the incompatible interaction that implicates chemical changes induced by biotic stress was the appearance and accumulation of a new type of amorphous crystals (cr) mainly in the intracellular mesophyll cells. These crystals were completely different to the rombic crystals of calcium oxalate (ox, Figure 4c) already reported and normally found in strawberry tissues (Arias et al., 2004). The new crystals were brown, highly irregular or amorphous, only observed in leaves of plants infected with the avirulent pathogen (F7) 48 hai (cr, Figure 4c) and persisted for more than 50 dai. These amorphous crystals became darker, larger and more numerous 120 hai and were not seen in control plants and those infected with the M11 isolate.

Since plants infected with F7 showed visible anatomical alterations that suggested a defense response occurred, we investigated the development of an oxidative burst. Consequently we analyzed the production of hydrogen peroxide (Figure 4d–f) and anion superoxide (Figure 4g–i) in plants exposed to water (Figure 4d and g), M11 (Figure 4e and h) and F7 (Figure 4f and i) at different times after infection. Results revealed that plants induced a strong AOS production in localized areas of the mesophyll and vascular bundles (Figure 4f and i) within the first hai with F7, with a maximum production of hydrogen peroxide and

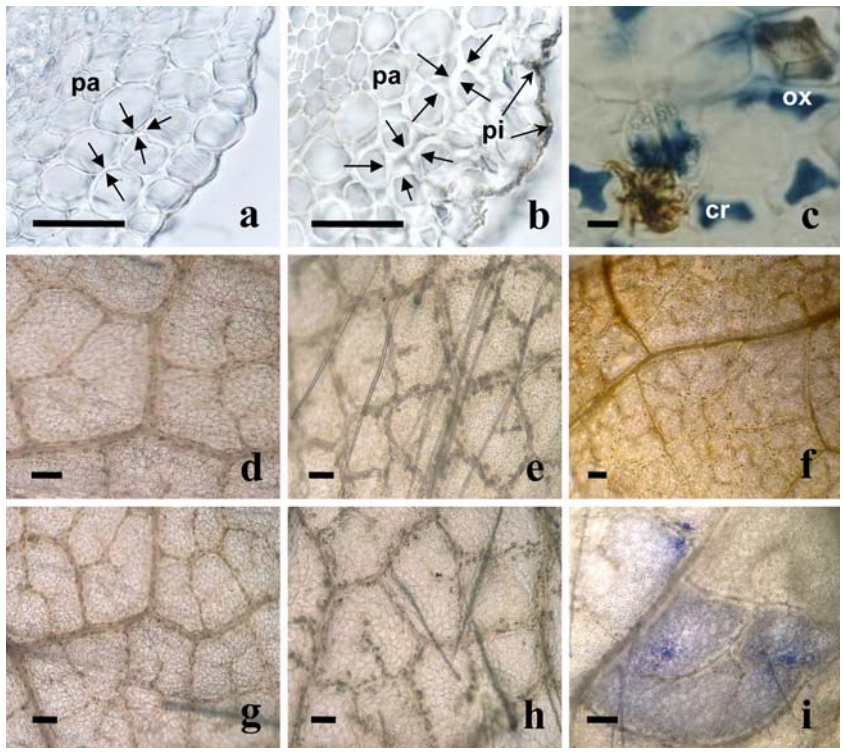

Figure 4. Anatomical effects and oxidative burst induced in leaflets of strawberry cv. Pájaro during incompatible interaction with avirulent isolate F7: a) petiole cross-section of control inoculated with water, b) inoculated with avirulent isolate F7 of *C. fragariae*. Arrows indicate parenchyma (pa) intercellular separation and pigment (pi) accumulated in outer layer of parenchyma cells, c) cross-section of leaflet showing oxalate (ox) and amorphous (cr) crystals formed in parenchyma cells only in the incompatible interaction. Hydrogen peroxide (DAB) detection on leaflet tissue of plants treated with: d) water (control), e) suspension (10^6 conidia ml^{-1}) of virulent isolate M11 of *C. acutatum*, f) suspension (10^6 conidia ml^{-1}) of avirulent isolate F7 of *C. fragariae*. Superoxide anion (NBT) detection on leaflet tissue of plants treated with: g) water (control), h) suspension (10^6 conidia ml^{-1}) of virulent isolate M11 of *C. acutatum,* i) suspension (10^6 conidia ml^{-1}) of avirulent isolate F7 of *C. fragariae*. a–c) were analyzed 72 hai and d–i) 4 hai. Scale bar in a–b) and d–i) equals 100 μm, in c) equals 10 μm.

superoxide anion 4 hai. Although AOS accumulated rapidly, superoxide exhibited a faster decay rate than hydrogen peroxide. Whereas at 6 hai superoxide was barely detected hydrogen peroxide was still detectable 8 hai. AOS were not produced at detectable levels either in control plants or plants inoculated with the virulent isolate M11 (Figure 4e and h).

In order to verify whether plants previously inoculated with the isolate F7 can resist the attack of M11, we conducted double infection experiments analyzing the disease symptoms under different experimental conditions. Results of the simultaneous inoculation with both pathogens (T3 in Figure 1) showed no difference in DSR values compared with those obtained in a typically compatible interaction (plants treated only with M11, T1 in Figure 1). However, in experiments with delayed second-infection (with M11), we observed a significant decrease in DSR values compared to the control experiment as the elapsed time from the first to the second infection increased (T4–7 in Figure 1). Therefore, when the second inoculation with M11 was carried out 96 or 120 h after the first inoculation (with F7) the DSR values obtained showed no statistical difference compared to the incompatible interaction (cv. Pájaro/F7, DSR = 1.0, see T6 and T7 in Figure 1). Analyses of cell wall thickening, amorphous crystal production, pigment accumulation and AOS production confirmed that plants exposed previously to F7 induced a defensive response shortly after the infection. However, although AOS production was detectable only during the first hours after infection (2–8 hai), the anatomic features associated with the defense response mentioned above

(Figure 4a–c) were clearly visible even 120 hai (results not shown). In contrast, plants infected with both pathogens simultaneously (Figure 1, T3) did not show AOS accumulation or the anatomic features associated with a defense response. Controls carried out with water and PD medium showed no morphological, physiological or phytopathological effects (DSR = 5.0).

Fungal interaction

In order to investigate whether the plant protection effect observed was due to a microbial antagonistic effect among the fungal strains or an effective defense response induced in the plant by F7, we carried out two types of experiments. The first one consisted of analyzing *in vitro* the behaviour of M11 growing on a PDA plate in the presence of intact and inactivated F7 conidia. The second experiment consisted of *in situ* microscopic observation of conidial growth of both strains on strawberry leaves, to determine conidial behaviour when induced resistance is activated. Figure 5a shows that colonies of isolates F7 and M11 grew on the same PDA plate. However, colony morphology did not permit us to clearly distinguish whether a weak antagonistic or a nutrient depletion effect was taking place. In order to test whether a diffusible compound produced by F7 and responsible for the assumed antagonistic effect on M11 may be present, we carried out similar experiments with inactivated sterile F7 conidial extracts (SC) and the supernatant of liquid culture from F7 after 21 days of growth (SN). A commercial fungicide Euparen (EU) and strawberry methanolic leaf extract (LE) were used as controls. The latter was included to test whether any preformed compound present in strawberry leaves was responsible for M11 inhibition (Filippone et al., 1999, 2001). In Figure 5b we show that M11 grew to confluence displaying no inhibition halo, regardless of the concentration of conidial extracts and supernatant of culture medium. On the other hand controls with the antifungal agent (EU) showed a clear inhibition halo (as expected) and methanolic leaf extract from uninoculated plants caused no inhibition halo. These results clearly indicate that the isolate F7 produced no diffusible or intracellular compound able to inhibit directly the growth of the isolate

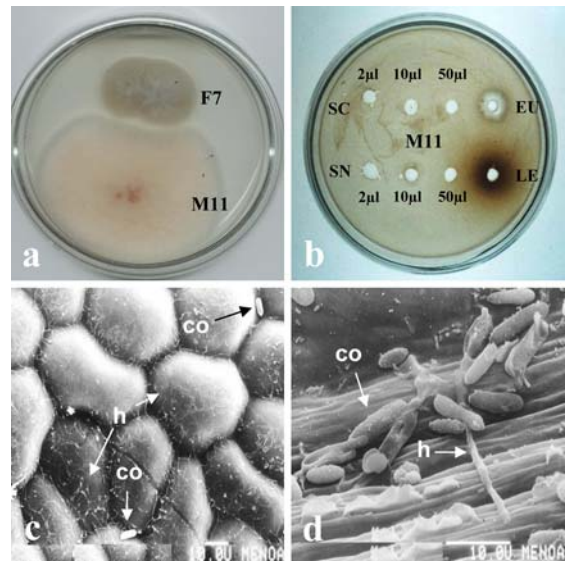

Figure 5. *In vitro* and *in situ* growth of isolate M11 in the presence of isolate F7: a) PDA plate showing colony growth of both strains after 8 days of culture at 28 °C, b) growth of M11 isolate on PDA plate after 8 days in culture at 28 °C in the presence of: SC, different amounts of sterile conidial extracts obtained by sonication of a suspension of avirulent isolate F7 of *C. fragariae,* corresponding to 10^8 conidia (10 μg protein μl^{-1}), SN, different amounts of concentrated supernatant of exhausted PD culture medium (10 μg protein μl^{-1}), EU, 10 μl of antifungal Euparen (0.015%) LE, 10 μl of methanolic strawberry leaf extract (1%), c) SEM photography of adaxial side of cv. Pájaro leaflet completely covered with F7 primary hyphae showing un-germinated M11 conidia (arrows), d) SEM photography of a petiole surface of cv. Pájaro pre-treated with F7 showing un-germinated M11 conidia. Micrographs shown in c) and d) were obtained 9 dai with M11. Scale bar in μm indicated in each photograph.

M11, suggesting therefore that the result presented in Figure 5a was due to a nutrient competition effect. This outcome also suggested that there was no inhibitory component present in strawberry leaves extract that would inhibit M11, at least under these experimental conditions.

In contrast, when analyzing the induced resistance experiments *in situ*, SEM observations showed that conidia of the isolate F7 germinated and grew, spreading rapidly throughout the surface of leaflets (Figure 5c) and petioles (Figure 5d) forming a compact matrix of crossing superficial primary hyphae (Figure 5c), whereas M11 conidia did not germinate and remained arrested as long as the experiment lasted (Figure 5c and d). Considering that M11 was not inhibited by any

hypothetical antagonistic (secreted or intracellular) factor produced by F7 when grown in culture media or by preformed compounds present in strawberry leaves at inhibitory concentrations, these experiments suggest that isolate M11 was inhibited by an unidentified factor that did not come from F7 or the plant separately, but when F7 is in contact with the plant.

Conidial extract defense elicitation

In order to test whether growth inhibition exerted by F7 against M11 on the plant required the participation of intact F7, we used sterile fractions of conidial extracts of isolate F7 obtained from a liquid culture (with and without plant extract addition) to treat plants before inoculating with M11. In Figure 6 we show that both fractions, cytosolic (CE) and cellular wall-membrane bound components (CP), elicited resistance against the virulent pathogen M11, accompanied by the induction of an oxidative burst. Although plants presented few disease symptoms during the first 9 dai, they recovered completely showing no symptoms 30 dai. Controls carried out with water (see Figure 6, C9 and C30), with PD medium and with the supernatant of the virulent isolate (M11, not shown) did not confer resistance to M11. Analyses of AOS production confirmed that the infiltration of both extracts (CE and CP) brought about the

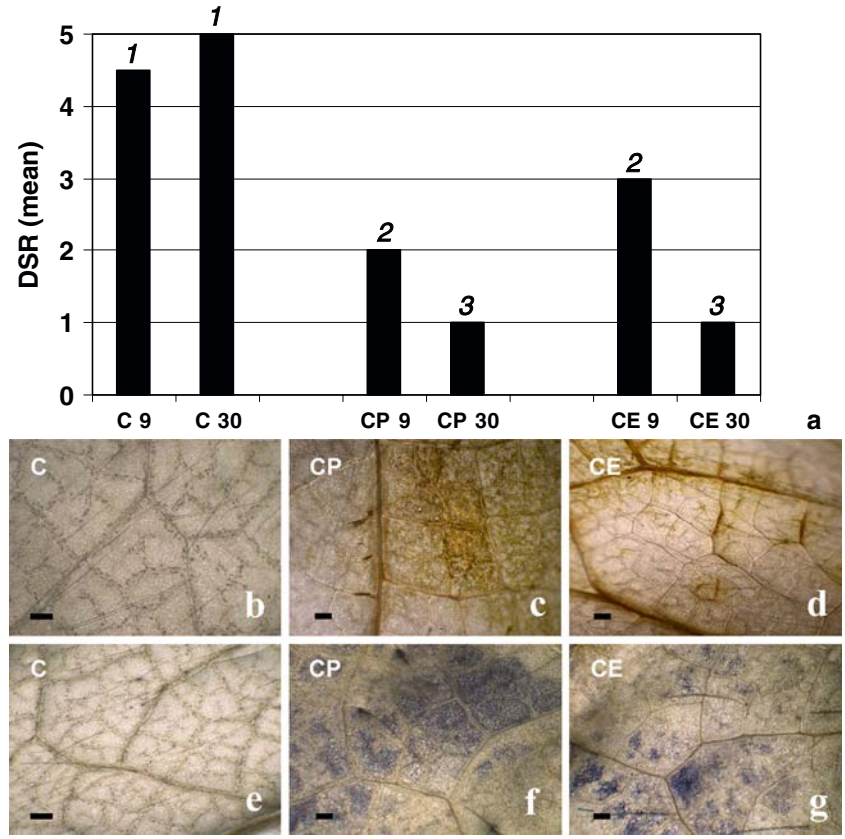

Figure 6. Effect of conidial fractions of avirulent isolate F7 of *C. fragariae* on elicitation of defense response in strawberry cv. Pájaro against virulent isolate M11 of *C. acutatum*. DSR values were evaluated on plants infected with a suspension (10^6 conidia ml^{-1}) of virulent isolate M11 of *C. acutatum*, previously treated with water (C, control), resuspended pellet (CP, wall-membrane fraction) and supernatant (CE, cytosolic fraction) of F7 conidia disrupted by French Press. Both fractions were applied at 10 μg protein ml^{-1}. DSR values were evaluated 9 dai and 30 dai with M11. DSR values with different numbers represent statistically different values (Tukey test, $P = 0.05$). Hydrogen peroxide (DAB) detection on leaflet tissue of plants treated with: b) water (control), c) CP fraction, d) CE fraction. Superoxide anion (NBT) detection on leaflet tissue of plants treated with: e) water (control), f) CP fraction, g) CE fraction. AOS in b–g) were analyzed 4 hai. Scale bar indicates 100 μm.

induction of the transient accumulation of hydrogen peroxide (Figure 6c and d) and superoxide anion (Figure 6f and g), with a maximum production at 4 hai. AOS production in these experiments exhibited an identical profile as observed in F7 conidial infections (Figure 1 T2) and induced resistance experiments (Figure 1 T4–T7), with a maximum at 4 hai followed by a decrease 8 hai (with a shorter accumulation period in the case of superoxide anion). Likewise, the leaf anatomical features used as a diagnostic tool for defense responses (cell wall thickening, amorphous crystal and pigment accumulation) showed a significant accumulation 72 hai in experiments with both extracts (CP and CE), persisting as long as the experiments lasted (not shown). These results were confirmed with conidial extract (CP and CE) spray inoculation experiments. In conclusion, these experiments show that: (i) the inhibition of M11 observed on the plant does not require the participation of intact F7 conidia and (ii) plants inoculated with conidial extracts also induce the oxidative burst and morpho-anatomical features associated with a defense response.

Discussion

Phytopathological experiments with the virulent (M11) and avirulent (F7) pathogens showed that whereas the former causes a strong and typical compatible interaction with clear and severe disease symptoms, the isolate F7 on the contrary, brings about an incompatible interaction and disease symptoms were never observed even many months after the inoculation. This divergent phytopathological behaviour stimulated us to investigate the physiological and structural changes that may occur and to test whether the incompatible interaction established between F7 and the cv. Pájaro could be attributed to the induction of some type of immune response in the plant.

Taking into account that both isolates are strawberry pathogens and that the interaction cv. Pájaro/M11 isolate was undoubtedly compatible (Figure 2), the first task was to show whether or not cv. Pájaro can support the growth of isolate F7. Results shown in Figure 3 confirmed that, although F7 apparently does not produce disease symptoms in cv. Pájaro, it can grow vigorously on leaflets and petiole surfaces. This and the fact that F7 was never observed to infect inner plant tissue (as observed with M11), suggested that F7 may be inducing a defensive response in the plant preventing infective F7 hyphae from penetrating deeply into the plant tissues. In order to support the latter, we have evaluated other parameters associated with the plant defense response.

Earlier studies carried out with *Colletotrichum* and other fungal species have reported changes in the cell wall and other plant components (lignin-like, phenolic, basic-staining, crystals, etc.) in response to the plant/pathogen interaction, speculating how these changes may be associated to plant defense (Sziráki et al., 1984; Lee et al., 2000; O'Connell et al., 2000; Wharton et al., 2001; Curry et al., 2002; Kim et al., 2004). Another important reaction of plant tissues induced by pathogens and/or elicitors is the oxidative burst that causes a rapid generation and release of AOS superoxide (O_2^-), hydroxyl radical (OH^-) and mainly hydrogen peroxide (H_2O_2) (Lamb and Dixon, 1997). These AOS can directly affect the growth of the pathogen by their intrinsic antibiotic activities and indirectly through complex signalling mechanisms involved in plant-pathogen recognition events (Shetty et al. 2003). The latter constitutes the first step in the activation of the defensive response cascade that brings about the modification of the cell wall, lipid peroxidation, phytoalexin production and the activation of genes related to the defense response (Lamb and Dixon, 1997).

Our results show that the interaction between the avirulent strain (F7) and the plant produces cell parenchyma modification, accumulation of pigments and amorphous crystals and additionally, a strong oxidative burst 4 hai. Although we did not observe the appearance of HR, as reported by Shetty et al. (2003), all these events together suggest that F7 induces a defensive response in the cv. Pájaro. In contrast, we did not observe any conspicuous anatomical alteration and physiological response in control experiments or when the plants were inoculated with the strain M11 (Figure 1, T1 and Figure 4). We have also found other features such as starch accumulation in mesophyll cells and chloroplast alterations normally associated with a defense response (Heath, 1974; Mlodzianowski and Siwecki, 1975), observed only in the incompatible interaction with F7 (results not shown).

Figure 1 shows that when plants are exposed to F7, the disease symptoms produced by M11 decrease as the time between the first (with F7) and the second (with M11) inoculations increase, yielding complete plant protection when the time between inoculations is 96 h or more (Figure 1, T5–T7). The early and transient accumulation of AOS (with a maximum 4 hai) followed by a slower but sustained change of anatomical features associated with a defense response (observed 50 dai) in plants previously infected with F7, suggest that the effect of plant protection exerted by F7 against M11 depends on early events that take place during the interaction between the plant and F7. In contrast, we did not observe these changes in plants exposed exclusively to M11. Interestingly, when both pathogens were inoculated simultaneously (and to a lesser extent in the 48 h delayed second infection experiment), the interaction caused infection and disease manifestation (Figure 1, T3). It seems that the strength of the pathogenic mechanism produced by M11 can overcome the inhibition or defense response induced by F7 against M11. This result reveals that M11 may be interfering with some important early events involved in the defense or inhibition mechanism that are necessary for complete development of resistance. It is worthwhile to mention that this breakdown of the plant protection mechanism mediated by M11 (Figure 1, T3) takes place without oxidative burst and other morphological changes associated with the defense reaction as observed with the interaction M11/plant (Figure 1, T1 and Figure 4). The latter suggests that blockage of the M11 resistance is associated with inhibition of the oxidative burst and other plant defense mechanisms. Results obtained with the induced resistance and conidial extract infiltration experiments support the hypothesis that isolate F7 or its conidial extracts can induce a defense response more than an F7 plant-mediated antagonistic effect.

In Figure 5 we show that isolate F7 does not produce any compound causing an antagonistic effect on M11 *in vitro* (in solid or liquid cultures). The latter was also investigated with conidia and culture supernatant obtained from liquid cultures of F7 where strawberry leaf extract was added at different concentrations. In no case was the inhibition of M11 observed (results not shown). On the plant, in contrast, the germination of M11 conidia were completely arrested in the presence of F7 (Figure 5d), but according to the result shown in Figure 5b, the effect should not be attributed to the presence of preformed plant compounds such as phytoanticipins present at inhibitory concentrations (Filippone et al., 1999, 2001). Hence, taking into account that: (i) intact F7 does not inhibit the growth of M11 *in vitro*, (ii) F7 conidial extracts and strawberry leaf extract do not affect the growth of M11 *in vitro*, (iii) M11 can actively grow on the plant in the absence of F7 but is completely arrested by F7 on the plant, we may conclude that whatever inhibits M11 on the plant could be an inhibitory factor produced by F7 or by the plant only after (or during) their interaction.

Results presented in Figure 6 show that plants pretreated with sterile inactivated F7 conidial extracts (CP or CE) resisted the attack of M11 with clear evidence of oxidative burst induction 4 hai (Figure 6b–g). The most striking outcome of these experiments is that M11 can be inhibited on the plant without the direct participation of intact F7 conidia. Therefore, since conidial extracts proved to be inactive against M11 when used directly in *in vitro* experiments (Figure 5b), we may conclude that the M11 inhibition factor does not come from F7 conidia, but from another component (elicitor) that appears after their interaction. On the other hand, since the analyses of physiological, morphological and phytopathological tests gave similar results in experiments with F7 conidia and conidial extracts (see Figures 4 and 6), we may speculate that intact F7 affects M11 growth by using the same mechanisms as the elicitor(s) present in the conidial extract. Manandhar et al. (1998) arrived at the same conclusion when treating rice with non-rice and rice avirulent pathogens to suppress rice blast disease caused by *Pyricularia oryzae*. In a first attempt to explain the disease suppression observed, these authors assumed that some type of antagonism or nutrient competition was the cause of the disease symptom reduction.

Although results presented here show that the inhibitory factor is not present in F7 conidial or plant extracts, as mentioned above, we cannot completely rule out the possibility that the assumed antagonistic factor against M11 would be present or be inactive and would require the F7/plant interaction to induce or activate it. Nonetheless, whereas the participation of this hypothetical F7 plant-induced antagonistic factor would be difficult to test,

most of the experimental evidence points to the development of a defense response elicited by F7. Shetty et al. (2003) have clearly demonstrated that the accumulation of hydrogen peroxide induced on wheat after the interaction with an avirulent pathovar of *Septoria tritici* provoked an induced resistance response against a virulent strain. Accordingly, we may speculate that such an M11 inhibitory mechanism would include the deleterious effect of AOS on M11, and some structural and/or chemical changes on the plant surface that would modify or remove factors necessary for the induction of M11 germination.

Our results also show that the elapsed time from the first to the second inoculation is crucial to the defense response. Therefore, we may further speculate that the molecular mechanism involved in the development of the defense response, so far unknown, does not rely only on the early recognition events that take place between host and pathogen during the first contact (*avr* product/R gene), but also on delayed events that require time for eliciting a strong defense response (Hammond and Jones, 1996; Manandhar et al., 1998). Furthermore, our data agree with results reported by Manandhar et al. (1998) with rice, suggesting that acquired or induced resistance may be responsible for the disease reductions observed. Experiments are being conducted in our laboratory to investigate the nature of the fungal elicitor and to characterize the defense mechanism involved.

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