

# Interaction of *Moniliophthora perniciosa* biotypes with Micro-Tom tomato: a model system to investigate the witches' broom disease of *Theobroma cacao*

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The miniature tomato (*Solanum lycopersicum*) cultivar Micro-Tom (MT) has become an important platform to investigate plant–pathogen interactions. In the case of the witches' broom disease of *Theobroma cacao* (cacao), the existence of *Moniliophthora perniciosa* isolates pathogenic to Solanaceae (S-biotype) may enable the use of MT to circumvent limitations of the cacao host, whereas the availability of a non-infective cacao C-biotype allows the evaluation of contrasting responses of MT. Infection of MT by the S-biotype led to stem swelling and axillary shoot growth to form broom-like symptoms similar to the biotrophic phase in cacao, but the infected tissues did not progress to necrosis. Conversely, inoculation with the C-biotype did not cause typical symptoms, but reduced plant height, appearing as a non-host interaction. Histopathological characterization of the S-biotype infection of MT by light and electron microscopy indicated limited germ tube penetration, preferentially through wounds at the base of trichomes or actively through the epidermis. No intracellular mycelium was observed, corroborating the lack of the necrotrophic stage of the pathogen. The analysis of gene expression during the interaction between the S- or C-biotype with MT indicated that expression of plant defence-associated genes differs for kinetics and intensity between a compatible or incompatible *M. perniciosa*–MT interaction. The pattern of spore germination and low rate of mycelia penetration suggests that the S-biotype is not a fully adapted tomato pathogen, but possibly a case of broken non-host resistance, and evidence suggests the occurrence of a non-host MT response against the C-biotype.

**Keywords:** Agaricales, Marasmiaceae, non-host resistance, plant–pathogen interaction, ultrastructure

## Introduction

Tomato (*Solanum lycopersicum*) has been widely used as a suitable model system to study plant–pathogen interactions, as it is a crop species with a wide range of pathogens with significant agricultural importance (Carvalho *et al.*, 2011). In particular, the miniature cultivar Micro-Tom (MT) has become an important platform for genetic studies (Carvalho *et al.*, 2011). The small plant size (*c.* 15 cm tall), short life cycle (up to 80 days) enabling the production of three to four generations per year, the availability of isogenic mutants and genetic resources (Carvalho *et al.*, 2011), and an efficient genetic transformation system (Pino *et al.*, 2010) make MT highly attractive for investigating interactions with pathogens. The adoption of a model genetic system is crucial to investigate interactions between non-model hosts and/or unusual pathogens.

The witches' broom disease of *Theobroma cacao* (cacao) is caused by the Agaricales *Moniliophthora perniciosa* (Marasmiaceae *sensu stricto*; Aime & Phillips-Mora, 2005) (syn. *Crinipellis perniciosa*, Tricholomataceae). Currently, this cacao pathogen is distributed over all the South American and Caribbean producing countries, while threatening to reach west Africa, a region responsible for over 70% of the world cacao production, with potentially catastrophic social and economic consequences. *Moniliophthora perniciosa* infects actively growing meristematic tissues inducing a range of symptoms depending on organ infected and stage of development (Purdy & Schmidt, 1996). Hypertrophic and hyperplastic growth of infected buds, called brooms, are the most remarkable symptoms (Purdy & Schmidt, 1996).

Initially, *M. perniciosa* was believed to infect only cacao and *Theobroma* congeners (Malvaceae *sensu lato*), but it was later demonstrated to naturally infect various unrelated hosts. Griffith & Hedger (1994) proposed that *M. perniciosa* contains at least three discrete biotypes (C-, S- and L-biotypes), with some host specificity, differences in life history and somatic compatibility. Isolates infecting cacao and relatives are considered to be from

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the C-biotype (Griffith & Hedger, 1994). The L-biotype is represented by isolates that typically cause symptomless infection with production of basidiocarps in dead or living woody liana vines (*Arrabidaea verrucosa*, Bignoniaceae) in forests of western Ecuador (Griffith & Hedger, 1994). The S-biotype includes isolates that naturally infect some weedy solanaceous species (e.g. *Solanum rugosum*), but upon artificial inoculation can be pathogenic to species from the *Solanum* (e.g. tomato) and *Capsicum* (e.g. sweet pepper) genera (Bastos & Evans, 1985). Somatic compatibility and molecular analyses have suggested that the broom-forming C- and S-biotypes are more similar to each other than to the L-biotype, while exhibiting a minimal level of genetic diversity (Griffith & Hedger, 1994; Marelli *et al.*, 2009). There is a single report of an S-biotype isolate inducing hyperplastic symptoms in 10% of inoculated cacao plants (Lopes *et al.*, 2001).

The investigation of the *M. perniciosa* × *T. cacao* interaction and its underlying molecular aspects is limited by biological characteristics of the host in respect to the long reproductive cycle, limited genetic information, and recalcitrance to genetic transformation (Micheli *et al.*, 2010). As an alternative, tomato has been shown to be susceptible to isolates of the S-biotype of *M. perniciosa* (Bastos & Evans, 1985; Marelli *et al.*, 2009). The availability of a non-infective C-biotype might enable the evaluation of molecular aspects from contrasting responses of MT to compatible or incompatible interactions.

In the current study, the suitability of MT as a model system to investigate the *M. perniciosa* interaction with a susceptible host was tested. Symptom characterization and phenotyping was conducted for compatible (S-biotype) or incompatible (C-biotype) interactions with MT. Histopathological characterization was carried out to determine patterns of S-biotype spore germination, mycelia penetration and colonization of MT using light and electron (scanning and transmission) microscopy to interpret the biology of this interaction. Additionally, defence-related gene expression patterns were compared between a compatible and an incompatible interaction to investigate the mode of resistance.

## Materials and methods

### Plant and fungal material

Plants of the tomato cultivar Micro-Tom (MT) and the commercial cultivar MoneyMaker were used in the inoculation experiments. Isolates from S- and C-biotype were obtained from naturally infected shoots (brooms) of *Solanum lycocarpum* from Tiradentes, Minas Gerais, Brazil (21°11'9.4"S, 44°19'15.8"W) or cacao from Uruçuca, Bahia, Brazil (14°31'7.2"S, 39°18'26.8"W), respectively. Commercial bakers' yeast (Fleischmann) was used as an elicitor (negative control) in a MT inoculation experiment (Wulff & Pascholati, 1998).

### Inoculation experiments

Basidiocarps of *M. perniciosa* were induced by exposing dry brooms from either host to alternating wet–dry cycles (12 h).

Basidiospores were collected into a 16% glycerol solution in 10 mM 2-morpholinoethane sulphonic acid (MES), and stored in liquid nitrogen (Frias *et al.*, 1991). Seeds of MT and MoneyMaker were germinated in pots containing 250 mL of a mixture (1:1) of substrate PlantMax HT (Eucatex) and expanded vermiculite (Carvalho *et al.*, 2011). Shoot apices and leaf axillary buds of 17-day-old seedlings were inoculated with 60 µL of a *M. perniciosa* basidiospore suspension ( $10^5$  spores mL<sup>-1</sup>). After inoculation, seedlings were kept in a humid chamber for 48 h. For MT symptom characterization after inoculation with isolates from the S- or C-biotype, a complete randomized design was adopted, with 30 plants and five non-inoculated controls. Evaluation of symptom development began 5 days after inoculation (dai), and every 5 days for 35 days. Seedlings were evaluated for symptoms, such as the presence of terminal and/or axillary brooms, and/or seedlings exhibiting swollen shoot, measured with a steel pachymeter (Brasfort).

To obtain an elicitor from *Saccharomyces cerevisiae*, 200 g of a commercial yeast tablet was dissolved in 1 L water (Wulff & Pascholati, 1998) and autoclaved four times at 121°C for 1 h. Between each autoclaving, cell extracts were chilled on ice-water before the next autoclaving, followed by centrifugation at 15 000 g for 30 min at 4°C. The pellet was then discarded and the supernatant was autoclaved. For elicitation, 30 MT plants were sprayed with the autoclaved extract until run-off, and five plants were kept as untreated controls. Evaluations were conducted 5 days after treatment, and every 5 days for 35 days.

### Sample preparation and analysis using scanning electron microscopy (SEM), light microscopy (LM) and transmission electron microscopy (TEM)

Shoot apices from MT seedlings inoculated with the S-biotype isolate were collected at various periods to be analysed by electron and light microscopy. For SEM, shoot apices from MT seedlings were collected 6, 12, 24, 48, 72 and 96 h after inoculation, with the respective non-inoculated controls. Samples (three replicates from each period) were collected and immediately fixed in a modified Karnovsky (1965) solution (2% glutaraldehyde, 2% paraformaldehyde, 5 mM CaCl<sub>2</sub> in 0.05 M sodium cacodylate buffer, pH 7.2). Post-fixation was carried out in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h. The samples were then rinsed three times in distilled water and dehydrated through an ethanol series (30, 50, 70, 80%), followed by three washes in 100% ethanol. The samples were finally critical-point dried through liquid carbon dioxide (CPD/030 Baltec equipment), mounted on metal stubs, sputter coated with 20 nm gold (SCD 050 Baltec equipment), and examined under a scanning electron microscope (LEO 435 VP) at 20 kV, with images digitalized.

For LM, shoot apices were collected in triplicate from non-inoculated and inoculated seedlings at 48, 120, 240, 480, 720, and 1440 h after inoculation with the S-biotype isolate. Up to 240 h, tissues were collected at random as symptoms were not evident, but subsequently, only plants with symptoms were sampled. Samples were fixed and post-fixed as described for SEM. The samples were then dehydrated in a graded acetone series and embedded in Spurr's resin for 48 h. Semithin sections (120–200 nm) were prepared in a Porter-Blum MT-1 ultramicrotome (Dupont-Sorvall), and sections were collected on glass slides, stained with 2% toluidine blue in water for 5 min, rinsed in distilled water and air-dried. The sections were permanently mounted in Entellan resin, observed and documented using an upright light microscope (Zeiss Axioscop2 Jena). After examina-

tion, sections with more visible mycelia were selected for ultrathin sections (60–90 nm). Tissue samples 60 dai with pronounced hyperplasia were sectioned for TEM at the same ultramicrotome. Ultrathin sections (60–90 nm) were collected on copper grids (300 mesh) and stained with 2.5% uranyl acetate followed by 0.1% lead citrate. Sections were observed using a transmission electron microscope (EM 900; Zeiss) at 80 kV and the images digitalized at NAP/MEPA, ESALQ, USP (Piracicaba, SP, Brazil).

### Tomato RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from MT plants sampled at 48, 72, 120 and 240 h after inoculation with isolates from the S- or C-biotype, and from respective non-inoculated control plants. RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. RNA quality and concentration was evaluated by 1.2% agarose gel electrophoresis and by spectrophotometry using a NanoDrop 2000 (Thermo Scientific). Total RNA (2 µg) was treated with DNase I (Fermentas) at 1 U µL<sup>-1</sup>, 40 U Ribolock RNase inhibitor (Fermentas), in a final volume of 10 µL incubated at 37°C for 30 min, followed by inactivation by addition of 25 mM EDTA. The reaction was then incubated at 65°C for 10 min and cooled to 4°C. cDNA synthesis was performed using 1 µg RNA and adding 5 µM oligo-dT<sub>18</sub> and 1 mM each dNTP in a final volume of 10 µL, followed by incubation at 65°C for 5 min, and cooling at 4°C for 5 min. Reverse transcription was conducted in a 20 µL reaction containing 200 U RevertAid Premium Reverse (Fermentas), 20 U RNase OUT (Invitrogen) at 50°C for 30 min, followed by 5 min at 85°C, and afterwards kept at 4°C. Synthesis quality was verified by amplification using tomato tubulin-specific primers (Table S1). Amplification reactions contained 0.1 µL cDNA or 1 µL DNase I-treated RNA (negative control), 2 mM MgCl<sub>2</sub>, 200 µM dNTP, 0.2 µM each primer and 1 U *Taq* DNA polymerase in appropriate buffer, in 25 µL reactions. Amplifications were conducted starting with 2 min at 95°C, followed by 40 cycles of 95°C for 30 s; 60°C for 30 s and 72°C for 60 s, with a final extension at 72°C for 5 min. Amplification products were verified by 1% agarose gel electrophoresis.

### Detection of *Moniliophthora perniciosa* transcripts in inoculated plants

The presence of *M. perniciosa* mycelia in samples from inoculated MT plants was confirmed by quantitative reverse transcription (qRT) PCR. Detection was conducted based on amplified product dissociation using primers specific for *M. perniciosa* ribosomal protein gene (*RpL35*; Table S1; Leal *et al.*, 2007), between 72 and 95°C, using as reference cDNA derived from *in vitro* grown C-biotype *M. perniciosa* mycelia (positive control). Transcript detection was evaluated at 48, 72, 120 and 240 h after MT inoculations with isolates from the S- and C-biotypes.

### Evaluation of gene expression by quantitative amplification of reverse transcripts (qRT-PCR)

Primers specific for tomato reference and defence-related genes, previously described by Lovdal & Lillo (2009) and Balaji *et al.* (2008) (Tables S1 & S2), respectively, were used to analyse expression. qRT-PCRs were conducted in 10 µL reactions containing 1 µL cDNA 1:10 (v/v) dilution; 0.5 µM each transcript-

specific primer (Table S2) and 5 µL Platinum SYBR-Green qPCR SuperMix-UDG 2× (Invitrogen). Amplifications were performed in a RotorGene Q thermocycler (QIAGEN) in triplicate, with initial incubation at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, with signal detection at the end of each extension step. After cycling, a melting curve for each amplicon was determined between 72 and 95°C. Experiments included technical duplicates, a negative control (no template DNA) and a DNase I-treated RNA sample. Data acquisition was performed in a Rotor-Gene Q Real-Time Analysis 6.0 (QIAGEN). Amplification efficiency of each primer pair was determined by standard curve from three serial dilutions of cDNA (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>). Selection of reference genes was conducted using GENORM (Vandesompele *et al.*, 2002), while data analysis was performed using the software REST 2009 (Pfaffl *et al.*, 2002) using multiple gene references.

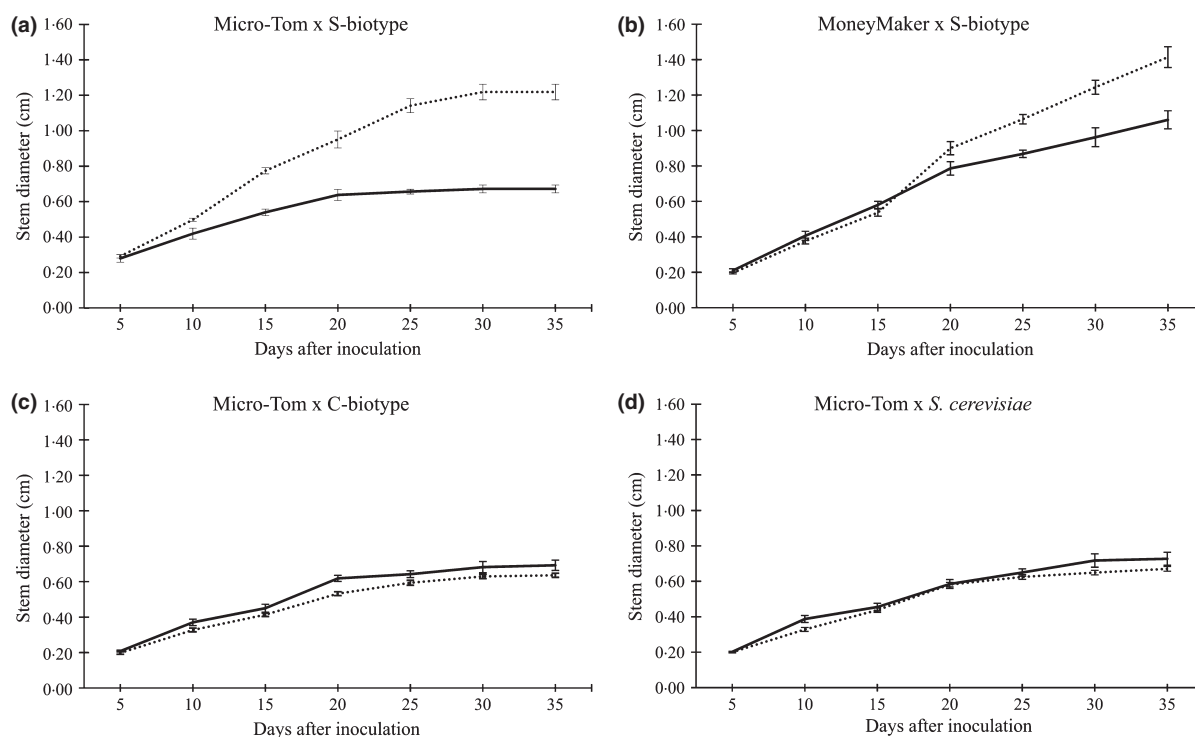
## Results

### Micro-Tom as a model system to characterize *Moniliophthora perniciosa* infection

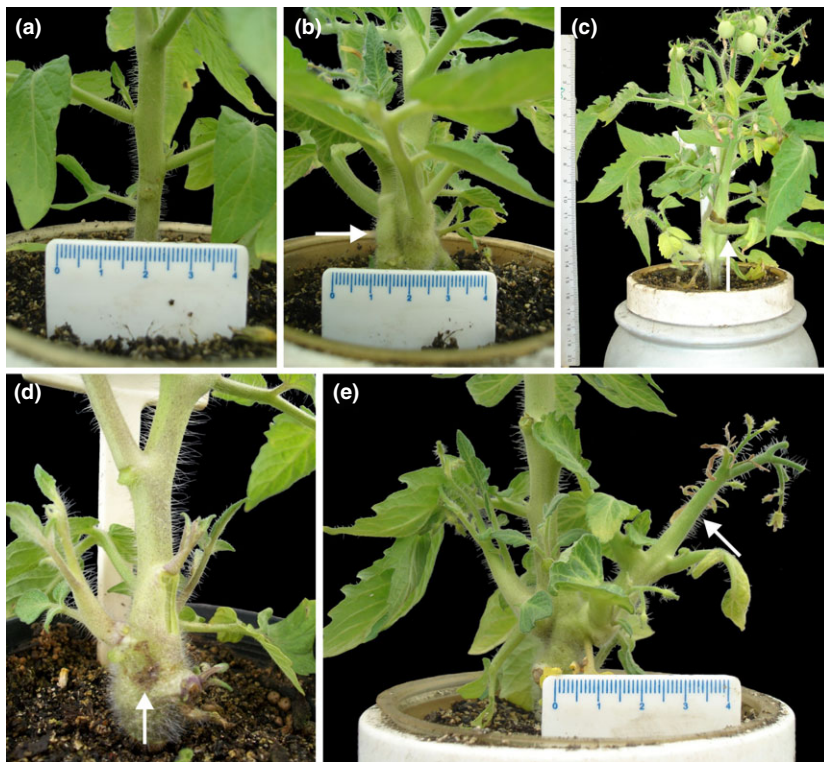
MT was evaluated in inoculations with an isolate of the S-biotype to study pathogenesis in comparison to the witches' broom disease of cacao. In controlled inoculations, 24 of 30 MT plants (80%) presented swollen stems at 35 dai. The first stem swelling symptoms were observed 10 dai for 23 plants, which, after 35 days, reached an average diameter of 1.22 cm, in comparison to 0.67 cm for non-inoculated controls (Fig. 1a). In addition to stem swelling (Fig. 2b), leaf petioles near the site of inoculation were also thickened (Fig. 2c), together with some leaf wrinkling or lack of full leaf expansion. Initially, small axillary bud growth (<0.5 cm) was detected, which were confirmed as brooms at 20 dai, when shoots were larger (>4 cm). The maximum number of axillary shoots per broom detected for each inoculated plant was two. Small necrotic lesions were observed in some tissues between 20 and 30 dai, predominantly at regions of stem swelling (Fig. 2d). Eventually, leaf vein necrosis was observed. During this period, death of a few shoot apices was detected (Fig. 2e), but the infected plants with symptoms completed their life cycles, bearing fruits similar to the non-inoculated control plants.

To evaluate if the mutation or additional alleles present in MT affected *M. perniciosa* infection, the commercial cultivar MoneyMaker was also inoculated. From 30 MoneyMaker plants inoculated, 22 (73.4%) exhibited symptoms. The average stem swelling of inoculated plants was 1.41 cm compared to 1.06 cm in non-inoculated controls, 35 dai (Fig. 1b). Similar to MT, MoneyMaker plants showed lateral shoot outgrowths (brooms) and a pronounced stem swelling when compared to controls. Again, the MoneyMaker plants successfully completed their life cycle, producing fruits, and without developing necrosed tissues (dry brooms). In summary, infection of MT and MoneyMaker led to the expression of typical symptoms, with hypertrophy of organ and tissues, typical of the witches' broom disease of *T. cacao*. However, for both cultivars, infected tissue did not





**Figure 1** Mean stem diameter of inoculated (dashed line) and non-inoculated (solid line) tomato seedlings evaluated every 5 days. (a) Micro-Tom (MT) seedlings inoculated with spores from S-biotype isolate; (b) MoneyMaker seedlings inoculated with spores from S-biotype isolate; (c) MT seedlings inoculated with spores from C-biotype isolate; (d) MT seedlings treated with autoclaved extract from *Saccharomyces cerevisiae* cells.



**Figure 2** Symptoms in Micro-Tom (MT) seedlings inoculated or not with spores from S-biotype isolate of *Moniliophthora perniciosa*, 30 days after inoculation. (a) Non-inoculated MT seedling (control); (b) MT seedling inoculated with *M. perniciosa* displaying stem swelling (arrow); (c) MT seedlings inoculated with *M. perniciosa* displaying petiole swelling (arrow); (d) stem swelling of MT inoculated with *M. perniciosa* displaying necrotic region (arrow); (e) MT seedling inoculated with *M. perniciosa* with axillary outgrowth (broom), exhibiting die-back from shoot tip.

progress to conspicuous necrosis, as commonly observed for cacao.

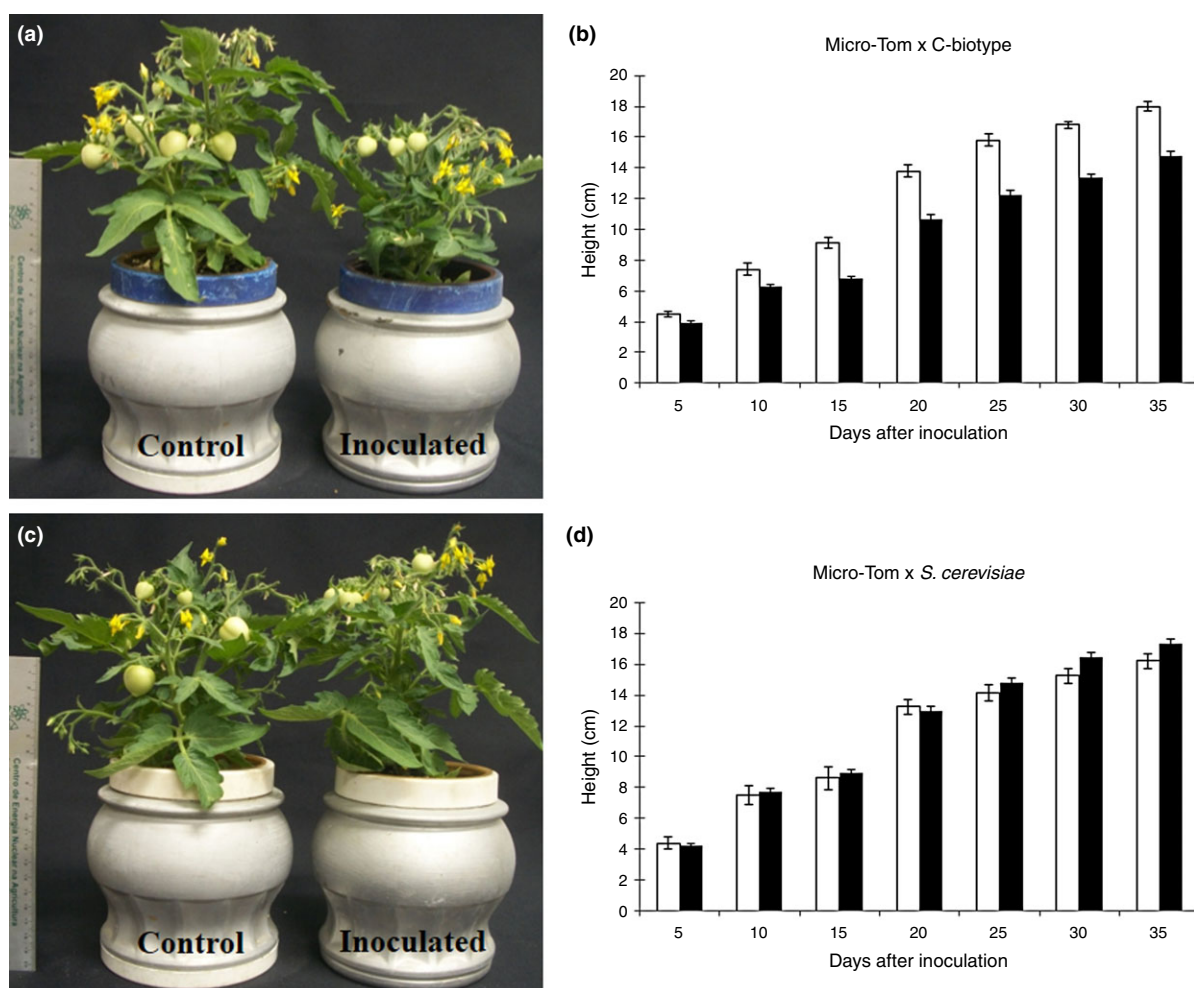
### Micro-Tom plants inoculated with a C-biotype isolate

Inoculations of MT plants with an *M. perniciosa* isolate from the C-biotype were conducted to evaluate the potential occurrence of cross-infection, with the development of typical symptoms. Inoculated MT plants exhibited similar development to the non-inoculated controls, with only a subtle reduction in stem diameter detected in inoculated plants (Fig. 1c). A drastic effect of inoculation with a C-biotype isolate was the reduction in plant height from an average 18 cm in control plants to 14.8 cm in inoculated plants (around 18%; Fig. 3). However, no stem swelling or development of axillary shoot outgrowth (brooms) was observed. The inoculated plants were able to complete their life cycles.

To evaluate if the reduction in plant height of C-biotype inoculated plants was a specific response to this fungus, simply represented a general defence response, or a response to a nonspecific component common to distinct fungi, MT plants were treated with autoclaved crude extract from cells of *S. cerevisiae*. Spraying the yeast extracts did not cause any visible change in morphology (Fig. 3), and plant height and stem diameter of treated plants remained identical to untreated controls for all periods evaluated (Fig. 1d). These results suggested that the reduction in height caused by the C-biotype was related to a specific defence response.

### Histopathological characterization of the MT–*Monilophthora perniciosa* S-biotype interaction

To propose and develop a new study model, it is critical to obtain basic information to compare with the target



**Figure 3** Micro-Tom (MT) seedlings inoculated or not with spores from a C-biotype isolate of *Monilophthora perniciosa* (a, b) or autoclaved extracts of *Saccharomyces cerevisiae* cells (c, d), and mean height (b, d) of non-inoculated (white bar) or inoculated (black bar) Micro-Tom (MT) plants. (a) Non-inoculated control and MT seedlings inoculated with spores from a C-biotype isolate, exhibiting reduction in plant height in the inoculated plant; (b) mean height for MT plants inoculated with spores from a C-biotype isolate of *M. perniciosa*, indicating significant reduction in height between treatments; (c) MT non-inoculated control and MT seedlings inoculated with extracts of *S. cerevisiae* cells, without change in plant height; (d) mean height for MT plant inoculated with autoclaved extracts from *S. cerevisiae*, indicating no significant difference in plant height between treatments.

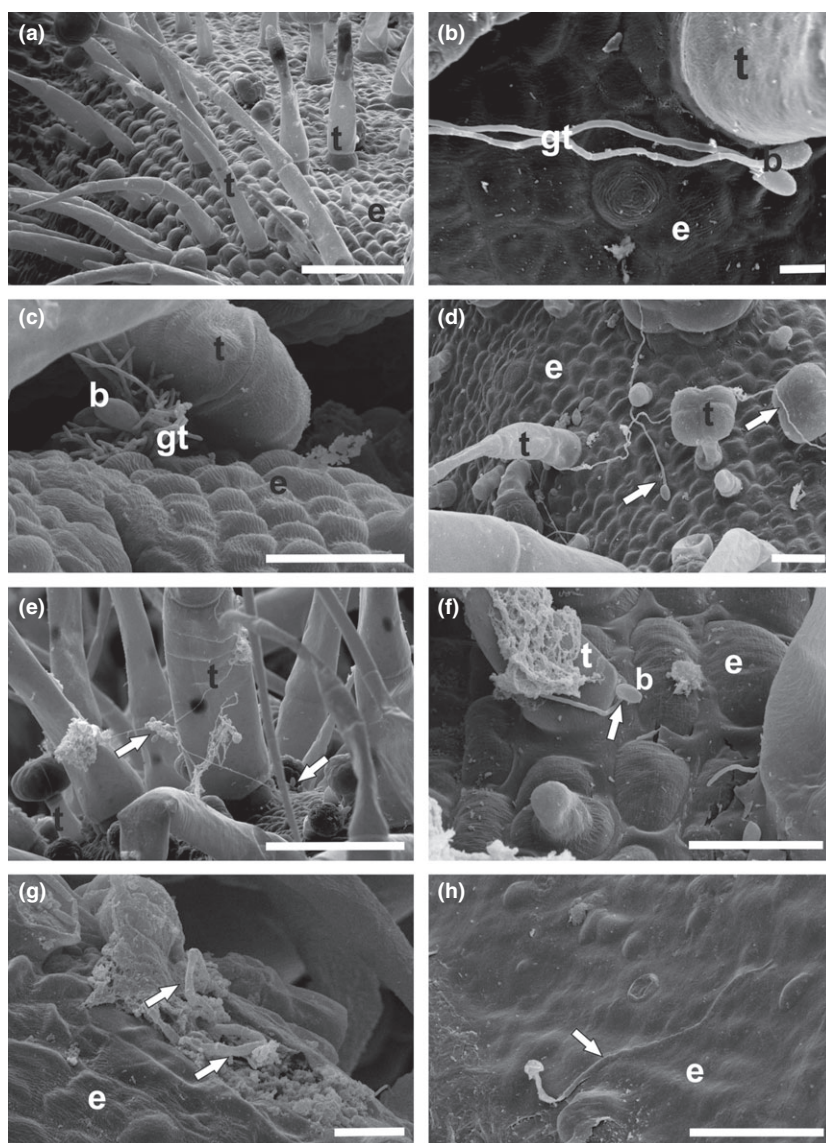


system, in this case the witches' broom disease of *T. cacao*. Basidiospore germination, hyphae penetration and pattern of colonization of *M. perniciosus* S-biotype in MT was analysed by SEM, whilst mycelia colonization was observed through LM and TEM.

Six hours after inoculation, it was possible to visualize the emission of vigorous germ tubes without the formation of appressoria (Fig. 4b) or any specialized penetration structure, with germination of basidiospores on cuticle and trichome bases (Fig. 4c). In samples collected 12 h after inoculation, the germinated basidiospores were dispersed over the epidermis, without a determined colonization pattern (Fig. 4d). The same was noticed for samples collected 24 h after inoculation (Fig. 4e). At 96 h after inoculation, there were no more spores germinating on the plant surface in the samples analysed. Germ tube penetration on the base of the trichomes was observed 12 h after inoculation (Fig. 4f) and through

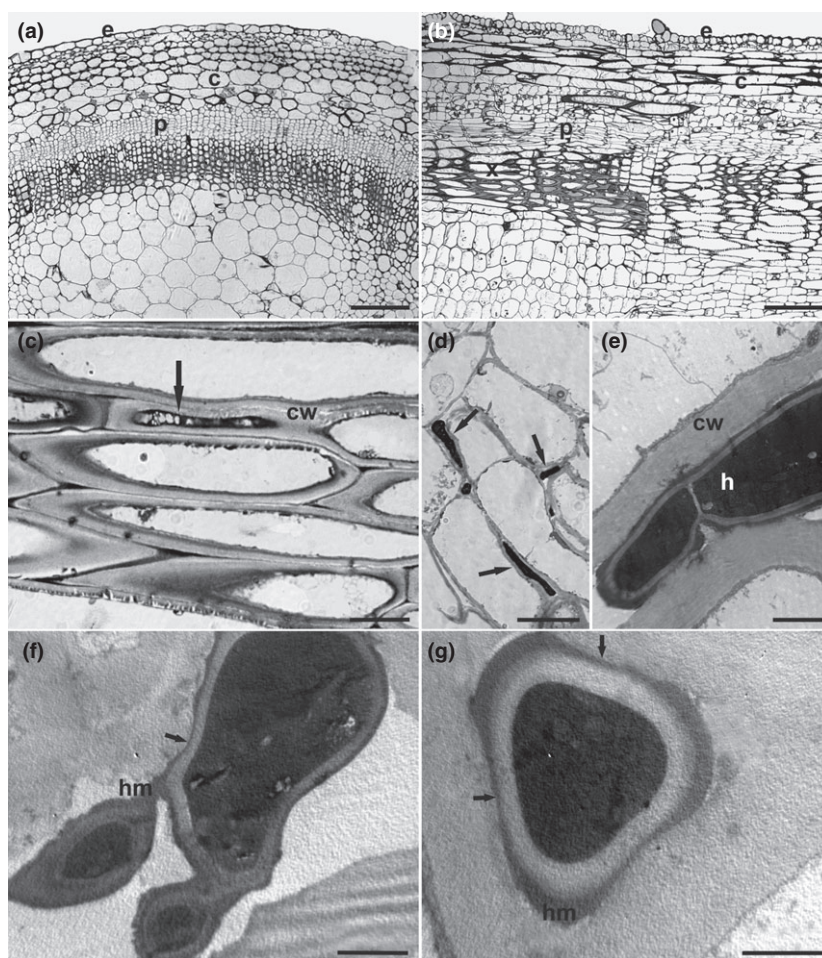
wounds on the epidermis at 24 h (Fig. 4g). Furthermore, 72 h after inoculation, it was possible to observe some direct penetration of the fungal hyphae through the epidermis (Fig. 4h).

Using light microscopy, infected MT plants showed tissue hypertrophy, with noticeable increase in cellular volume and disorganized growth (Fig. 5b) when compared to non-inoculated control plants (Fig. 5a). Fungal mycelium was only detectable 30 dai, but with a limited volume of hyphae, occurring intercellularly in the cortical region (Fig. 5d). When in contact with the host cells, mycelia apparently induced plant cell wall thickening, while well-developed vesicles were observed in the hyphal cytoplasm (Fig. 5c). Colonization of tissues with a reduced volume of hyphae, essentially occurring intercellularly, was detected by TEM 60 dai. Ultrathin sections were obtained from the cortical area of MT stems, and the observed hyphae displayed electron-dense



**Figure 4** Shoot tips of Micro-Tom plants observed by scanning electron microscopy. (a) Shoot tip from non-inoculated Micro-Tom plant (control); (b) shoot tip from Micro-Tom plant 6 h after inoculation with *Moniliophthora perniciosus*, indicating basidiospores and germ tube; (c) *M. perniciosus* basidiospores, emitting germ tube at the base of a nonglandular trichome, 6 h after inoculation; (d, e) germinated basidiospores, 24 and 48 h after inoculation respectively, dispersed over the epidermis and trichomes, with a regular pattern of colonization; (f) possible penetration of *M. perniciosus* hyphae at the base of a trichome, 12 h after inoculation; (g) fungal penetration through a wound at the epidermis, 24 h after inoculation; (h) possible penetration of *M. perniciosus* hyphae directly through the epidermis, 72 h after inoculation. t, trichome; b, basidiospore; gt, germ tube (arrows); e, epidermis. Bars: a, e = 100  $\mu$ m; b, g = 10  $\mu$ m; c, f, h = 20  $\mu$ m; d = 30  $\mu$ m.

**Figure 5** Stem sections of Micro-Tom observed by light microscopy (LM) and transmission electron microscopy (TEM). (a) Non-inoculated control, normal conformation of cells; (b) inoculated with spores from S-biotype of *Monilophthora pernicioso*. Hypertrophic and disorganized development of cells; (c, d) hyphae from *M. pernicioso* grown intercellularly 30 days after inoculation, indicating vesicles inside the hyphae; (e) detail from hypha of *M. pernicioso*, with dense cytoplasmic content (TEM); (f, g) stem of Micro-Tom plants, 60 days after inoculation with spores from S-biotype of *M. pernicioso*. Interaction of intercellular hyphae with the junction of hyphal matrix and the host cell wall (arrow). e, epidermis; c, cortex; p, phloem; x, xylem; h, hypha; cw, cell wall; hm, hyphal matrix. Bars: a, b = 200  $\mu$ m; c = 20  $\mu$ m; d = 50  $\mu$ m; e, f = 2  $\mu$ m; g = 1  $\mu$ m.



cytoplasm with dispersed vesicles (Fig. 5e). Under the same conditions, the presence of an extracellular matrix, besides the increased thickness of the host cell wall, were detected, together with the flattening of the hyphal extracellular matrix at the sites of contact (Fig. 5f,g).

#### Confirmation of *Monilophthora pernicioso* presence in infected MT plants

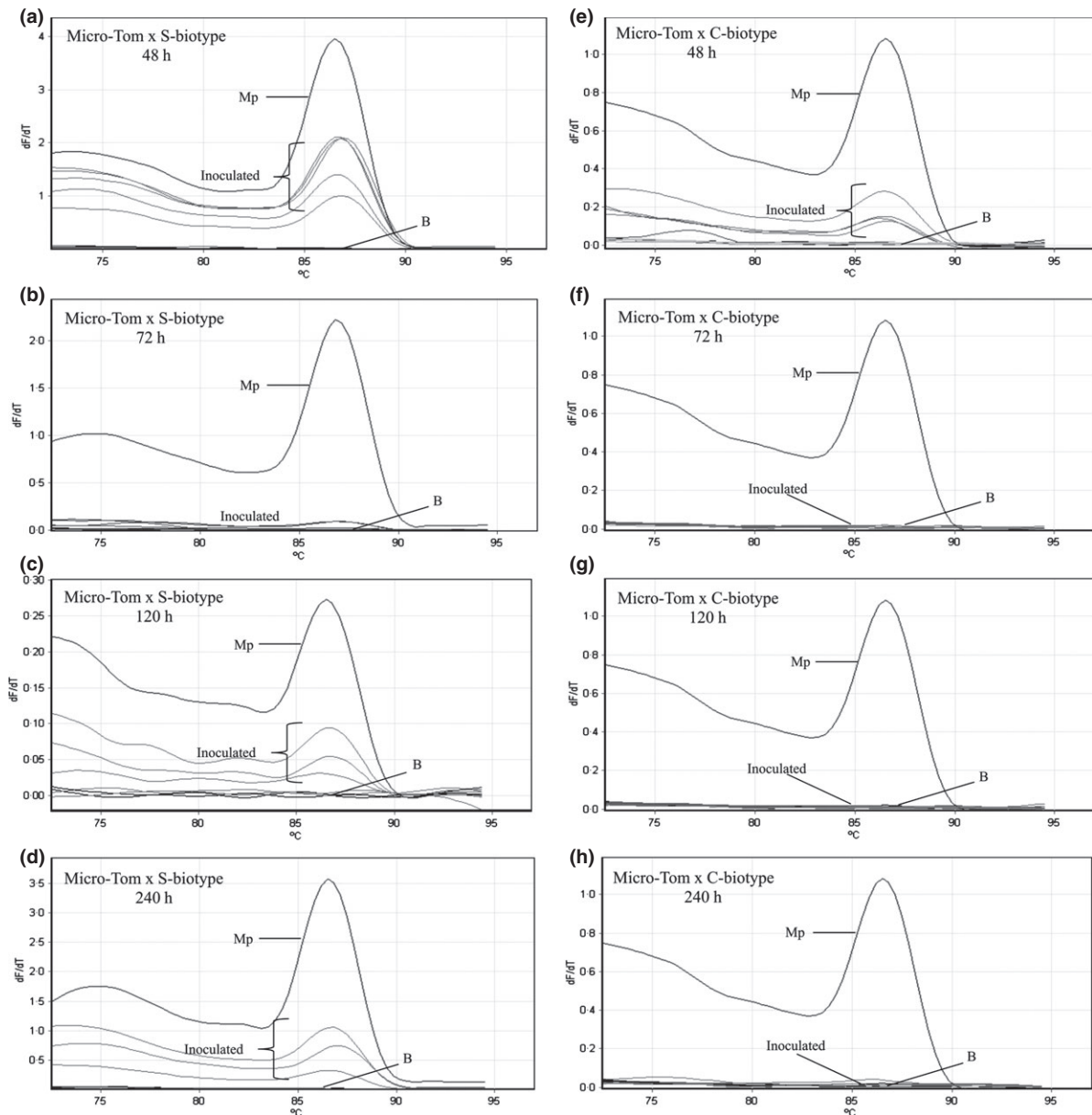
To confirm that the symptomless MT tissues sampled for defence-related gene expression were in fact infected, the presence of fungal transcripts were first detected using quantitative amplification of reverse transcripts (qRT-PCR). The detection of the *M. pernicioso* *RpL35* gene in inoculated and non-inoculated control plants was based on the melting curve of the amplified product, including cDNA from *in vitro* cultivated mycelia from the C-biotype as a positive control. The presence of the fungus was confirmed by the detection of an amplicon with mean  $T_m$  of 87.6°C, similar to the positive control, observed only in inoculated plants (Fig. 6). In MT plants inoculated with the isolate from the S-biotype, the pathogen was detected in all analysed periods (48, 72, 120 and 240 h; Fig. 6a–d), with the largest number of

infected plants detected at 48 h, and positive detection in five out of the six plants analysed. However, when MT plants were inoculated with an isolate of the C-biotype (Fig. 6e–g), the amplifications were only positive for the presence of the fungus up to 48 h after inoculation, becoming undetectable after this.

#### Expression of genes associated with plant defence in compatible and incompatible *Monilophthora pernicioso*–MT interactions

To determine the pattern of gene expression during a compatible or incompatible interaction, 12 defence-related genes were evaluated by qRT-PCR. The level of expression varied among genes for the analysed periods of 48, 72, 120 and 240 h after inoculation, chosen to encompass initial levels of infection with the presence of biotrophic mycelia from both biotypes. Among the target defence-associated genes, there were ones related to pathogenesis and stress response, such as: *PIN-II2x* (proteinase inhibitor II protein); *EIX1* (EIX receptor 1); *PIN2* (wound-induced protease inhibitor II); *PR1a* (pathogenesis-related protein P4); *PR1b* (pathogenesis-related protein P6); *Chi* (chitinase); *ChiII* (chitinase class 2); *OSM*



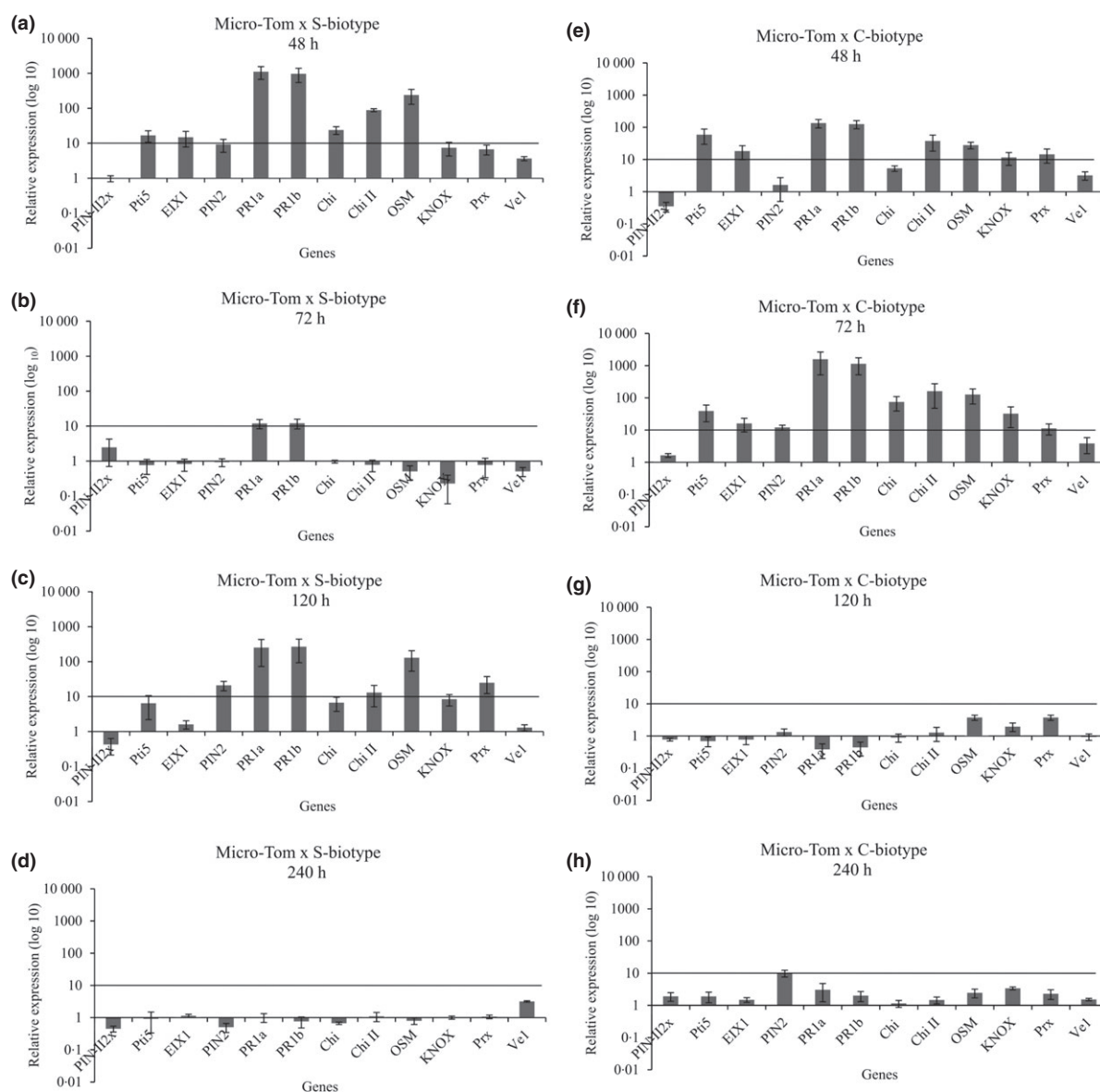


**Figure 6** Dissociation curves for amplification products of the *Moniliophthora perniciosa* RpL35 gene obtained from Micro-Tom samples inoculated with spores of the S-biotype (a–d) or the C-biotype (e–h) of *M. perniciosa* from samples collected at 48 h (a, e); 72 h (b, f); 120 h (c, g); and 240 h (d, h) after inoculation. Mp = positive control (cDNA from *M. perniciosa* mycelia grown *in vitro*); B = negative control (non-inoculated sample and sample without cDNA).

(osmotin-like protein); *Prx* (peroxidase); and *Ve1* (Verticillium wilt disease resistance protein); and transcription factors, such as: *Pti5* (ERF/AP2 transcription factor family); and *KNOX* (transcription factor, homeobox, KNOTTED-1), previously described by Balaji *et al.* (2008). Under the given experimental conditions, the analysis conducted using GeNorm (Vandesompele *et al.*, 2002) indicated that the most stable reference genes were *PPA2Acs*, *GAPDH* and *tubulin* (Table S1), which were adopted as multiple references in the subsequent analysis based on the software REST 2009 (Pfaffl *et al.*, 2002).

Inoculation of MT plants with the isolate of the S-biotype induced a significant accumulation of the transcripts (more than 10-fold increase) of seven genes (*Pti5*, *EIX1*, *PR1a*, *PR1b*, *Chi*, *ChiII*, *OSM*), 48 h after inoculation compared to non-inoculated control plants (Fig. 7a). In the subsequent period (72 h), only transcripts of *PR1a* and *PR1b* maintained significant levels of accumulation, but at values below those observed at 48 h (Fig. 7b). At 120 h, six genes (*PIN2*, *PR1a*, *PR1b*, *ChiII*, *OSM*, *Prx*) exhibited significant accumulation of transcripts. At the last period analysed (240 h), no important accumulation





**Figure 7** Relative differential expression of 12 tomato genes (Table S2) in shoot apices of Micro-Tom plants inoculated with spores from the S-biotype (a–d) or the C-biotype (e–h) of *Monilophthora perniciosa* from samples collected at 48 h (a, e); 72 h (b, f); 120 h (c, g); and 240 h (d, h) after inoculation. Gene relative expression was estimated using the  $\Delta\Delta C_t$  program from  $C_q$  values determined by qRT-PCR using as reference genes *PPA2Acs*, *GAPDH* and *tubulin*.

of transcripts was observed, except for *Ve1*, but at much lower values (Fig. 7d).

When the same analysis was carried out for plant samples inoculated with the isolate of the C-biotype, significant accumulation of transcripts from genes *Pti5*, *EIX1*, *PR1a*, *PR1b*, *ChiI*, *OSM*, *KNOX* and *Prx* occurred at 48 h compared to non-inoculated plants (Fig. 7e). However, unlike the inoculation with the S-biotype, significant accumulation of transcripts was more intense at 72 h after inoculation, with an increase of transcript accumulation for all evaluated genes (Fig. 7f). A striking accumulation of transcripts of *PR1a* and *PR1b* at levels

1000 times higher than the non-inoculated controls was detected (Fig. 7f). In the later stages (120 and 240 h after inoculation), there was no significant accumulation of transcripts or levels were reduced (Fig. 7g,h).

Differences in gene expression kinetics and intensities were therefore observed between compatible and incompatible interactions, with the activation of the same response genes and signalling shared by the same transcription factors. However, inoculation with the S-biotype isolate led to a maximum accumulation of transcripts at 48 h, followed by another peak at 120 h after inoculation. In contrast, in inoculations with the

C-biotype isolate, accumulation of transcripts occurred at 48 h, but the maximum response happened 72 h after inoculation.

## Discussion

*Moniliophthora perniciosa* is a unique aggressive *T. cacao* pathogen, and both components of the interaction present biological limitations to adopt reverse genetic or complementation approaches, particularly the cacao host. However, the fungus contains biotypes that infect either *T. cacao* or Solanaceae species, which allows the establishment of a model system using *S. lycopersicum*, a species more amenable to genetic manipulations.

The early events of infection and colonization in the interaction of *M. perniciosa* with MT were compared with those previously described for cacao (Dabydeen & Sreenivasan, 1989; Silva & Matsuoka, 1999; Ceita *et al.*, 2007). It was demonstrated that MT can be a suitable genetic model to investigate the interaction with the pathogenic S-biotype during the biotrophic stage of infection. Similar to cacao, the infection of MT led to stem swelling and axillary shoot growth to form broom-like symptoms, typical of witches' broom disease (Purdy & Schmidt, 1996). However, unlike cacao, the MT green brooms did not progress to produce necrotic tissues (dry brooms). Previous work had shown that MT could reach the necrotrophic phase of the disease, with additional symptoms, such as blister formation (Marelli *et al.*, 2009), but the current work failed to detect both symptoms, even when the plants completed their life cycle (not shown). The report of the occurrence of a necrotrophic phase in tomato by Marelli *et al.* (2009) might have derived from a difference in isolate virulence or an opportunistic infection by another necrotrophic agent during those experiments. Inoculated MoneyMaker tomato, which does not harbour the same mutations as MT, developed identical symptoms as MT, without reaching the necrotrophic stage.

A large number of germinating basidiospores of the S-biotype were observed by SEM, but few germ tubes penetrated the host (Fig 4d). In general, non-pathogens exhibit a reduced rate of non-host penetration (Zhang *et al.*, 2011), and in this study, few germinating spores from the S-biotype penetrated the MT tissues. The penetration events observed were through wounds at the base of trichomes and cuticle. In cacao, penetration of the germ tubes appears to happen preferentially through stomata in young leaves and pods, but it can also occur in natural wounds, such as collapsed trichomes (Sreenivasan & Dabydeen, 1989; Frias *et al.*, 1991). The occurrence of direct penetration has been reported in cacao (Sreenivasan & Dabydeen, 1989), but not confirmed (Frias *et al.*, 1991), corroborated by the lack of specialized structures in *M. perniciosa*. A possible direct penetration through MT epidermis was observed 72 h after inoculation, such as described in cacao (Sreenivasan & Dabydeen, 1989). Comparing the patterns

of penetration between the S-biotype in MT and those described for the C-biotype in cacao suggest a better adaptation of the fungus to the cacao host. The C-biotype presented a broad strategy of germ tube growth toward the stomata and initial colonization of the substomatal chamber (Frias *et al.*, 1991). The interaction between the S-biotype and MT appeared to be not fully developed, with a lack of directional germ tube growth, and penetration through wounds and directly via the cuticle, as observed for non-host interactions (Zhang *et al.*, 2011).

A typical symptom of *M. perniciosa* infection of cacao and MT is tissue swelling, and the histopathological analysis revealed a pattern of tissue colonization similar to the ones reported for the C-biotype in cacao. Orchard *et al.* (1994) demonstrated that tissue swelling in cacao was related to the increase of cellular volume, with disorganized tissue development even without mycelia visualization. In MT, a clear increase in cell volume (hypertrophy) was observed in infected tissues compared to non-inoculated controls, but an increase in cell number (hyperplasia) by cell division, as demonstrated by Marelli *et al.* (2009), was not visualized. The low colonization of infected tissue was also noticed for MT, with detection of hyphae by light microscopy only 30 dai, similar to that described for *T. cacao* (Silva & Matsuoka, 1999). The presence of the fungus in infected tissues could also be detected by expression analysis of the *RpL35* gene at the initial symptomless stage of infection or by TEM at a later stage.

The absence of the typical intracellular mycelia in any of the periods analysed, associated with the lack of necrotrophic stage of the disease in tomato, was a major discrepancy from the cacao infection. In susceptible cacao plants intracellular mycelia can be detected 80 dai, with fungal colonization of host cells associated with the death of infected tissues (Silva & Matsuoka, 1999; Ceita *et al.*, 2007). The absence of intracellular mycelia in tomato could derive from the sensitivity of *M. perniciosa* to tomatin (A. Figueira, G. A. Leal, L. Gomes, S. Lira and F. Andrino, Universidade de São Paulo, Piracicaba, Brazil, unpublished observations) present inside the tomato cells (Ökmen *et al.*, 2013). The low concentration of  $\alpha$ -tomatin in the tomato apoplastic fluid ( $<0.02 \mu\text{mol g}^{-1}$  fresh tissue; Ökmen *et al.*, 2013) might not be effective against *M. perniciosa*. Therefore, the elevated concentration and the toxicity of  $\alpha$ -tomatin might justify the lack of cellular penetration in MT, with the completion of the fungal life cycle occurring only after natural tissue senescence, when levels of  $\alpha$ -tomatin decrease (Ökmen *et al.*, 2013). This peculiar condition of tomato might explain the development and specialization of a more aggressive pathogenic C-biotype in cacao, where hyphae can invade cells and finalize the life cycle before the tissues senesce.

C-biotype isolates do not usually cross-infect solanaceous hosts, but previous work had demonstrated that an isolate from *T. cacao* could induce light symptoms in *Solanum paniculatum*, with reduced hypertrophy and

hyperplasia (Lopes *et al.*, 2001). Conversely, the experiments here inoculating MT with the C-biotype did not cause any stem swelling or production of green brooms, but the inoculated plants exhibited a significant reduction in height and a subtle decrease in stem diameter. C-biotype isolates, when inoculated onto *Theobroma*, *Herrania* and various solanaceous species, were only pathogenic to *T. cacao*, *Theobroma speciosum* and *Herrania* sp., with no symptom development in *S. lycopersicum*, *Solanum melongena* and *Solanum gilo* (Bastos & Evans, 1985). The defence response was effective, as it was not possible to detect fungal transcripts after 48 h by qRT-PCR, suggesting a restriction of fungal growth limiting colonization. In cacao, similar observations were made when the same fungal transcript was only detected in resistant genotypes (CAB208 and CAB214) at the initial period of the interaction (24–72 h after inoculation), while in the susceptible ICS39, the presence of the fungus was detected up to the final point of analysis, 240 h after inoculation (Leal *et al.*, 2007).

The unexpected reduction of MT growth with the inoculation with the C-biotype was demonstrated to be specific to the pathogen and not due to response to some microbial molecular pattern. Experiments using mock-inoculation with water (not shown) or spraying an *S. cerevisiae* extract, a recognized resistance inducer (Wulff & Pascholati, 1998), confirmed that the changes in plant height were specifically caused by C-biotype inoculation, as no morphological alterations were observed for both inoculation controls. When challenged by a pathogen, plants need to activate defence mechanisms to resist infection, probably at a high energetic cost, which might decrease plant growth. The plant hormone gibberellic acid (GA) has been associated with plant growth, whereas jasmonic acid (JA) is connected with plant defence responses and might be associated with growth repression, with signalling between GA and JA regulated antagonistically in defence response against pathogens (Navarro *et al.*, 2008). While GA tends to promote growth by degrading the DELLA protein, JA acts in growth suppression and activation of defence-related genes by degrading the JAZ (*Jasmonate-Zim domain*) protein, with the integration of both pathways occurring by the interaction of both proteins (JAZ and DELLA; Hou *et al.*, 2010). Thus, the growth reduction in MT plants inoculated with the C-biotype might be associated with activation of the JA signalling pathway, compromising plant growth by reducing GA signalling. Non-host resistance of *Arabidopsis thaliana* to *Blumeria graminis* f. sp. *hordei* was characterized by a 30% reduction in growth, and the response was related to the activation of genes associated with ethylene and JA signalling pathways (Zimmerli *et al.*, 2004).

The expression of genes related to host response was evaluated during the early interaction between the S- and C-biotype with MT by qRT-PCR. After inoculation with the S-biotype, a large number of the genes tested presented an accumulation of transcripts 48 h after inoculation, with a second peak at 120 h after inoculation. In

contrast, after inoculation with the C-biotype, accumulation of defence-related transcripts occurred at 48 h after inoculation, but peaked at 72 h. In cacao, genotypes resistant to *M. perniciosa* displayed significant accumulation of defence-related transcripts at the initial period of infection, between 48 and 72 h (Leal *et al.*, 2007) similar to that observed here for MT inoculated with the C-biotype. Considering that the C-biotype is not a pathogen of tomato, the defence response should be categorized as non-host resistance. The molecular response to the S-biotype would represent a broken non-host resistance, which would be characterized by a host attempting to respond to a non-pathogen, followed by some mechanism acquired by the S-biotype that overcomes this reaction, neutralizing further host defence responses. Thus, the S-biotype might have either lost some molecular pattern that enables the recognition by the host as a non-pathogen or, most probably, gained a molecular function that enables the neutralization of the host defence response.

Among the genes analysed by qRT-PCR, there were those encoding pathogenesis-related (PR) proteins, which are commonly induced either at the site of infection or as a plant systemic response associated with the development of acquired resistance (SAR; van Loon & van Strien, 1999). In the evaluation of gene expression at the initial period of infection (48–120 h), high levels of transcripts associated with PRs were detected. The highest level of PR transcript accumulation was represented by *PR1a* and *PR1b* transcripts, which can be induced by pathogens or SA, and both are usually considered response markers of SAR (van Loon & van Strien, 1999). Accumulation of *PR-1* transcripts occurred in both compatible and incompatible interactions, demonstrating that these proteins might not act effectively in resistance against the pathogen or might be required at high levels for efficient defence against pathogens. Mycelium of *M. perniciosa* was shown to produce salicylic acid (SA) and to be tolerant to this plant hormone (Kilaru *et al.*, 2007). Furthermore, cacao plants infected with *M. perniciosa* presented high levels of SA when compared to healthy ones (Chaves & Gianfagna, 2006), suggesting that SA accumulation has a function in the pathogenicity, such as inhibiting the JA signalling pathway, which is usually active in defence response against necrotrophic pathogens (Glazebrook, 2005). Elevated accumulation of *PR-1* transcripts corroborated the induction of the transcription factor *Pti5* gene, which belongs to the ERF (Ethylene Response Factor) class, at the initial period of infection of compatible and incompatible interactions. *Pti5* transcription factor regulates the expression of PR genes, mainly those regulated by SA, such as *PR-1* and *PR-2* genes (Gu *et al.*, 2002). Besides the *PR-1* family, transcripts from other pathogenesis-related protein genes, such as chitinases and osmotin, presented high accumulation levels in both types of interaction.

Transcripts encoding peroxidases also displayed high accumulation during the initial stage of infection, at a



slower rate in the compatible interaction, with a significant peak only 120 h after inoculation with the S-biotype. In the inoculation with the C-biotype, peroxidase transcripts accumulated during the earlier periods (48 and 72 h after inoculation). The involvement of peroxidases in a specific defence response is commonly observed, especially for non-host resistance (Huitema *et al.*, 2003). The contribution of peroxidases in the interactions between *M. perniciosa*–*T. cacao* (Leal *et al.*, 2007) and between the S- and C-biotypes with *S. lycopersicum* confirmed their importance in the distinct defence mechanisms effective against this pathogen.

The fungal 22 kDa Ethylene-Inducing Xylanase protein promotes defence response in tobacco (*Nicotiana tabacum*) and tomato, inducing the biosynthesis of ethylene, expression of PR proteins, phytoalexin production, and hypersensitive response induction in certain plant species (Ron & Avni, 2004). This fungal protein binds to EIX receptor 1, homologue to Leucine-Rich Repeat Receptor-like protein (Ron & Avni, 2004). In the expression analysis, transcripts of *EIX1* accumulated during the evaluated period, suggesting a possible role in recognition of a *M. perniciosa* effector. This transcript accumulation occurred during the compatible interaction with the S-biotype, and at an early stage of the incompatible reaction (48 h). However, in the incompatible interaction with the C-biotype, the accumulation of this gene transcript remained at high levels at 72 h after inoculation, corroborating the potential role in recognition of a presumed *M. perniciosa* effector, with lasting effects. The differential expression of the *EIX1* gene corroborated the implication that a general non-host defence mechanism is occurring in this interaction, as the receptor recognizes a xylanase, a molecular pattern that activates a non-host resistance (Nürnberg & Lipka, 2005).

In conclusion, the use of the plant model system MT for investigation of the interaction with *M. perniciosa* has been demonstrated here. Micro-Tom developed similar symptoms and tissue colonization to cacao, but differed in the lack of a necrotrophic stage of the disease in MT. The pattern of spore germination and low rate of mycelia penetration suggests that the S-biotype is not a fully adapted tomato pathogen, and is possibly a case of broken non-host resistance. In the C-biotype–MT interaction, evidence based on expression of *Prx* and *EIX1* suggests the occurrence of a non-host response. Furthermore, the reduction in height in MT plants challenged by the C-biotype suggested a potential hormonal imbalance involving JA and GA. To confirm the involvement of these two plant hormones, future work will evaluate responses in MT hormonal mutants (Carvalho *et al.*, 2011) and investigate expression patterns of genes associated with GA- and JA-signalling and biosynthesis. Finally, defence-related gene expression in response to the S- and C-biotype demonstrated that, despite the induction of similar genes, there was a considerable difference in the intensity of transcript accumulation and kinetics.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Table S1.** Primer sequences for the tomato reference genes *tubulin*, *PPA2acs* and *GAPDH* and *Moniliophthora perniciosa* ribosomal protein gene *RpL35*.

**Table S2.** Primer sequences specific for defence-related genes from tomato (*Solanum lycopersicum*).