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Mycelium homogenates from a virulent strain of *Phytophthora capsici* promote a defence-related response in cell suspensions from *Capsicum chinense*

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Abstract We analysed changes in the transcript population produced in habanero pepper (*Capsicum chinense*) cell suspensions by the addition of whole mycelium homogenates from a pathogenic isolate of *Phytophthora capsici*, to identify plant cellular processes modified by the oomycete effectors. The elicitation produced several defence-like cellular responses: alkalinisation of the medium, a two-step oxidative burst, induction of β-1,3-glucanases, and activation of mitogen-activated protein kinases. The elicitation modified the accumulation of transcripts representative of diverse metabolic pathways, including ethylene biosynthetic enzymes, MAP kinases and defence-related products, like PR proteins, but did not affect the expression of *C. chinense* NPR1 and WRKY orthologue genes, which are important modulators of plant defence responses. Interestingly, apart from some defence-related genes, inoculation of six-leaf-stage habanero pepper plantlets with the pathogenic isolate revealed few systemic modifications in the transcript patterns. All plantlets ultimately died, even though the *in planta* inoculation induced the strong accumulation of two MAPK transcripts. As few resistance-related genes were expressed in susceptible habanero pepper plantlets that died, either the extent or the timing of the defence response could be insufficient to establish a proper response against Phytophthora blight.

Keywords Defence response · Elicitation · Phytophthora blight · Gene expression · *Capsicum chinense* (habanero pepper).

Abbreviations

MAPK	Mitogen-activated protein kinase
DOC	Days of culture
MPI	Minutes post-induction
DAI	Days after inoculation
MyH	Mycelium homogenates from *Phytophthora capsici*

Introduction

Phytophthora blight is a devastating disease caused by *Phytophthora capsici* (Leonian 1922), an oomycete that infects several important crops, which include members of the Solanaceae and Cucurbitaceae families (Lee et al. 2001). In the genus Capsicum, where there are almost no true resistant species, the disease has been managed by cultural practices or the use of fungicides (Hausbeck and Lamour 2004); however, none of these strategies has been effective as they

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have only reduced the incidence (Hausbeck and Lamour 2004), and fungicide-resistant isolates have appeared (Parra and Ristaino 2001). Thus, significant attention has been focused on the development of stable Phytophthora-resistant pepper cultivars (Oelke et al. 2003). In this respect, numerous breeding programmes have attempted to find sources of resistance; nevertheless, none of the pepper 'resistant' cultivars currently available shows consistent high levels of resistance (Thabuis et al. 2004).

It has been proposed that the success of the oomycetes in colonising specific hosts rests on their ability to reprogramme the host defence response by the introduction of a range of effectors (Birch et al. 2006). There is evidence indicating that the effector secretome of most plant-pathogenic oomycetes is composed of hundreds of proteins (Kamoun 2006; Jiang et al. 2008). There is an increasing body of evidence supporting the concept that detection of specific effectors (which in turn are considered avirulent proteins, AVR) by specific R-gene products triggers a class of non-host defence responses in oomycete-resistant varieties (Vleeshouwers et al. 2000); however, apart from the data regarding suppression of plant defence exerted by oomycete RxLR-dEER effectors (Vleeshouwers et al. 2000), very little is known about the molecular mechanisms by which the host defence response is evaded, repressed or manipulated by oomycetes (Birch et al. 2006).

Habanero pepper is a promising alternative to the production of pepper for industrial uses, since it contains the highest capsaicin content among Capsicum species (Canto-Flick et al. 2008). However, it is susceptible to lethal Phytophthora blight, and only a few reports regarding defence mechanisms have appeared in this species (Hamada et al. 2005).

Thus, the aim of the present work was to analyse the alteration of the transcript population in cell suspensions of *C. chinense*, by elicitation with *P. capsici* mycelium homogenates, as an indirect way to obtain information on the modifications in pepper metabolism that are induced by the oomycete. Since it has been postulated that effectors of the oomycete contribute to the colonisation success by blocking the defence response in their hosts (Birch et al. 2006), it can be expected that the addition of *P. capsici* mycelium homogenates could activate defence-related responses, but could suppress the activation of signal transduction intermediates or other key signalling modulators of the defence response when added to the habanero pepper cell suspensions. Our findings indicated that, while several defence-related events were activated, the expression of some key regulators of the defence response was not evident.

Materials and methods

Plant material and treatments

Cell suspension cultures from the local cv. Naranja of *Capsicum chinense* were kindly donated by Dr. Vazquez-Flota. For maintenance, cell suspension cultures were grown in MS medium supplemented with 2,4-D, and sub-cultured every 11 days. Quantification of fresh and dry weights, pH and conductivity, were conducted every 12 h during a 36-h period. For the elicitation, 10-ml aliquots of the cell suspension from day ten after sub-culturing were transferred to 50 ml of fresh medium and, after 24 h of acclimatisation, different concentrations (60 μg, 90 μg or 120 μg of glucose eq ml^{-1}) of *P. capsici* mycelium homogenates (MyH) were added. Samples were collected at different intervals after MyH addition to make the following measurements: medium alkalinisation (at 0 h, 12 h, 24 h and 48 h); H_2O_2 production (extracellular at 0 min, 15 min, 30 min, 45 min, 60 min and 120 min; and intracellular at 0 h, 2 h, 6 h and 8 h); measure of β-1,3-glucanase activities (at 0 h, 12 h, 24 h, 48 h and 72 h). MAPK activation and gene expression were measured as described in the corresponding sections. As negative controls for mechanical and chemical stress, parallel cell suspension aliquots were incubated with sterile distilled deionized water (ddH_2O) or with different concentrations of a glucose solution (60 μg ml^{-1}, 90 μg ml^{-1} and 120 μg ml^{-1}), respectively.

For plant inoculation, 0.2 cm^2 mycelium discs from *P. capsici*, grown for 6 days in solid PDA agar were deposited on the third and fourth true leaves of six-leaf-stage plantlets cultivated in vitro from surface-sterilised seeds. Disease symptoms were recorded every 24 h. After 48 h, total RNA was extracted from the first and second leaves with the Trizol reagent method (Invitrogen).

Elicitor preparation

The *Phytophthora capsici* compatible strain was obtained from the Colegio de Postgraduados, where it was characterised at the molecular level (EMBL AY726623). It was stored in PDA, from which an aliquot was transferred to 1.5 l of liquid V8 medium supplemented with 0.025% $CaCO_3$, and cultivated at 25°C until mycelium covered the entire medium surface. Mycelium was collected, washed with sterile ddH_2O and ground with liquid nitrogen. The resultant powder was thawed in 50 ml of ddH_2O, sonicated and homogenised with a polytron for 10 s. Finally, the homogenate was autoclaved. The concentrations of MyH prepared in this way were defined as µg of glucose eq ml^{-1}. Concentration of total sugars was determined by the method described by Dubois et al. (1956).

Preparation of cell extracts

Cell cultures subjected to the different treatments described above were quickly frozen with liquid nitrogen and ground in a mortar. Ground powder was homogenised with 0.5 ml of extraction buffer g^{-1} of tissue (50 mM HEPES-Tris, pH7.5, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 µM aprotinin, 1 µM leupeptin, 5 mM DTT). Cell extracts were centrifuged at 23,700×*g* for 45 min at 4°C. The supernatant was recovered, quickly frozen in liquid nitrogen and stored at −80°C until used. The protein concentration of the samples was measured by the method of Bradford (1976), using bovine serum albumin as standard (SIGMA).

Detection of β-1,3-glucanase activities

Capsicum chinense cell suspensions were exposed to different concentrations of MyH, and β-1,3-glucanase activities in cell extracts were determined at the time periods described above, as follows. Thirty-five µg of protein from cell extracts were fractionated by native 15% polyacrylamide gel electrophoresis. After a 5-min wash with 0.05 M sodium acetate, gels were incubated for 1 h at 40°C in the same solution supplemented with 13.3 mg ml^{-1} laminarin (SIGMA); then 0.075% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 1 M NaOH was added and enzymatic activity revealed by heating the gels in a microwave oven for 10 s. Gels were photographed and software from a Kodak EDAS 290 system was used to determine the relative band intensities.

Production of hydrogen peroxide

Capsicum chinense cell suspension cultures were elicited with MyH as described above. Cell samples collected at different periods were ground in liquid nitrogen. After homogenisation with 1 ml of 10 mM 2-morpholinoethanesulphonic acid (MES), pH 6.5, g^{-1} of tissue, samples were centrifuged at 14,000×*g* for 5 min at RT. Total H_2O_2 was determined in the supernatant according to the following procedure. Two-hundred µl of supernatant were quickly mixed at RT with freshly prepared reaction buffer (10 mM MES, pH6.5; 20 µM phenol red; 10 ng ml^{-1} horseradish peroxidase (SIGMA)) in a final volume of 1 ml. The reaction was stopped after 3 min by addition of 20 µl of NaOH (0.5 N). H_2O_2 molar concentration in samples was determined by measuring the absorbance decrement at 558 nm resulting from the H_2O_2-dependent peroxidase-catalysed oxidation of phenol red (SIGMA). As negative control, production of H_2O_2 was evaluated in cell suspensions incubated with glucose (90 µg ml^{-1}). Every experimental point was repeated three times, each with three independent beakers.

In-gel kinase assay

Determination of active MAPK through the entire culture cycle and in cell suspensions elicited with MyH was performed by in-gel kinase assays as follows. Twenty-five µg of sample proteins from either different points of the culture cycle (every other day from 0 day to 27 days) or different times after elicitation (0 min, 5 min, 10 min, 15 min, 20 min and 30 min) were fractionated in a 12% SDS-PAGE containing 0.35 mg ml^{-1} myelin basic protein (MBP, Invitrogen Life Technologies), which was added to the gel just before polymerisation. After electrophoresis, SDS was removed by washing the gel four times with washing buffer (buffer A: 50 mM Tris–HCl pH8.0, and 20% 2-propanol) for 30 min each at room temperature. The gel was then equilibrated by washing it in buffer B (50 mM Tris–HCl pH8.0, and 5 mM β-mercaptoethanol) for 90 min. Proteins were

denatured with 8 M urea in buffer A for 1 h at room temperature, followed by an overnight re-naturation at 4°C in buffer B containing 0.04% Tween 20 (five changes). Subsequently the gel was incubated for 60 min at room temperature in 15 ml of reaction buffer (40 mM HEPES pH7.5, 1.5 mM EGTA, 1.5 mM EDTA, 5 mM DTT, 12 mM $MgSO_4$ and 5 μM ATP) supplemented with 2.96 MBq of [γ-^{32}P]ATP (110 TBq mmol^{-1}, Amersham Biosciences). Reaction was terminated by washing the gel with a solution containing 5% trichloroacetic acid and 1% sodium pyrophosphate. The unincorporated [γ-^{32}P]ATP was removed by washing the gel with this solution until the washing solution was determined to contain no more radioactivity. Finally, the gel was stained with Coomassie Brilliant Blue, dried, and exposed to a film. Incubation of cell suspensions with the equivalent concentration of glucose was used as a negative control. To corroborate the specificity of the MBP-kinase activity, casein and histone H1 were used in place of MBP as substrates in the in-gel kinase assay.

Immunoblotting

Levels of activated MAPK-like enzymes in protein extracts from elicited or control *C. chinense* cell suspensions were determined immunologically using an anti-pERK 1/2 (Thr 202/Tyr 204) antibody (Santa Cruz Biotechnology), according to the following procedure: protein extracts were mixed with an equal volume of running buffer containing 0.125 mM Tris–HCl (pH6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.05% (w/v) bromophenol blue, boiled and fractionated in 15% SDS-PAGE. Then, proteins were electroblotted onto a PVDF membrane. The membrane was blocked for 1 h at room temperature in TBST buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.6% Tween 20) containing 5% semi-skimmed milk, and then incubated for 2 h in the same buffer with 1:5000 diluted primary antibodies. The buffer was poured and the membrane was washed three times in TBST, for 10 min each; it was then incubated with an alkaline phosphatase-conjugated secondary antibody diluted 1:10000 with TBST. After three washes with TBST buffer, substrate for alkaline phosphatase (ECL, Amersham Biosciences) was added and positive bands were detected using a chemiluminescence method (ECL, Amersham) and exposure of the membrane to a light-sensitive film.

Gene expression analysis

To analyse the changes in transcript populations, a cDNA array containing 46 genes (representing different metabolic pathways) was hybridised differentially with either control or induced DIG-labelled total single-strand cDNA probes. Control and induced cDNA probes were obtained by reverse transcription of total RNA isolated from cell suspensions treated with glucose (control cells) or MyH (induced cells), or from plantlets inoculated with discs of PDA (control plantlets) or *P. capsici* mycelium (infected plantlets), using oligo-dT and SuperScript™ III reverse transcriptase (Invitrogen) in the presence of digoxigenin-11-dUTP (alkali labile, Roche). Target cDNAs cloned in pGEM-T-Easy plasmid (actin, MAPK1, 2 and 3, NPR1, and WRKY) or from a *C. chinense* cDNA library (Smart® cDNA library construction kit, Clontech) synthesised expressly from a cell suspension culture (41 genes in the pTriplEx2 plasmid), were amplified from their corresponding plasmids by PCR (94°C, 5 min., 1 cycle; 94°C, 1 min., 60°C, 1 min., 72°C, 2 min., 30 cycles; 72°C, 12 min., 1 cycle), using insert-flanking primers (sequencing primers in pTriplEx2, and M13 forward and reverse primers, respectively). PCR products were diluted with 0.5 M NaOH/1.5 M NaCl and equal amounts (~200 ng) were vacuum-blotted onto nylon membranes (Zeta-Probe, BIO-RAD) using a bio-dot® microfiltration system (BIO-RAD). Duplicate blots were hybridised separately with the corresponding digoxigenin-labelled probe, and positive hybridisations were detected by enhanced chemiluminescence (CSPD®, Roche Diagnostics). Hybridisations were repeated three times.

All target cDNAs were classified based on the alignments of their deduced amino acid sequences with the Munich Information Centre for Protein Sequences database (MIPS; http://mips.gsf.de/proj/funcatDB/) (Frishman et al. 2001).

Results

Inoculation with *P. capsici* MyH induces a series of defence-related responses in *C. chinense* cell cultures

Under non-elicited conditions, the cell culture cycle was completed within 20–24 days, with a rapid

growing phase that lasted from day 6 to day 16–20. Conductivity of the culture medium decreased, and the pH increased gradually until 20 days of culture (DOC); after that, both values remained without significant changes until 30 DOC (Fig. 1).

The incubation with 90 μg of glucose eq ml^{-1} of MyH induced an alkalinisation that was observed from 12 h after elicitation (Fig. 2a). This alkalinisation was not likely caused by chemical or osmotic effects induced by the carbohydrate content of the MyH, as incubation with 90 μg ml^{-1} glucose did not alter the pH to the same extent, even after longer incubation periods.

A well defined characteristic of a host early defence response against pathogens is the oxidative burst (Davies et al. 2006). Quantification of hydrogen peroxide (H_2O_2) at different times after elicitation showed an increment of extracellular H_2O_2, with highest values found from 1 to 2 h after treatment (Fig. 2b). Production of intracellular H_2O_2 also increased, with a peak at 2 h, then diminished by 6 h, but did not reach normal values (Fig. 2c). In contrast, incubation of the cell suspensions with equivalent concentrations of glucose induced a more modest production of H_2O_2 (Fig. 2b and c).

Zymography performed under native conditions revealed the activation of at least two β-1,3-glucanase isoenzymes with Rf values of 0.05 and 1.1, respectively. The activities of these two enzymes were absent from cell cultures at the beginning of the treatment, but were induced by at least 12 h after treatment. Both activities decreased after 48 h (Fig. 3). A β-1,3-glucanase with an Rf value of 0.16 was also detected, but it exhibited inconsistent results, that could not be attributed to the elicitation treatment.

Even though *C. chinense*, as well as other species in the genus Capsicum, is lethally susceptible to Phytophthora blight, the above results showed that incubation with MyH activated several defence-related responses when added to cell suspensions.

The addition of MyH to cell cultures of *C. chinense* activated two MAPK

From in-gel kinase assays using Myelin Basic Protein (MBP) as substrate, a faint band of activity was found between 15 and 27 DOC, with an apparent molecular mass of ~44-kDa (Fig. 4a, KA panel). The anti-activated-MAPK antibody cross-reacted extremely poorly with few bands; however, as in the in-gel kinase assay, the intensity of a 44-kDa band was slightly more evident during the 15–27 DOC period (Fig. 4a, pTEpY panel). Immunoblotting also revealed the presence of a ~42-kDa band, with identical kinetics of detection.

Both in-gel kinase assay and the MAPK-specific antibody revealed the activation of a 44-kDa MAPK that was apparently not dependent on the MyH concentrations tested (Fig. 4b, KA and pTEpY panels). We performed all subsequent elicitations with 90 μg of glucose eq ml^{-1}. Immunoblotting also revealed the presence of the ~42-kDa band, only under elicitation conditions (Fig. 4b, pTEpY panel). A band of the same size was not detected clearly by the in-gel kinase assay (even at longer exposure times),

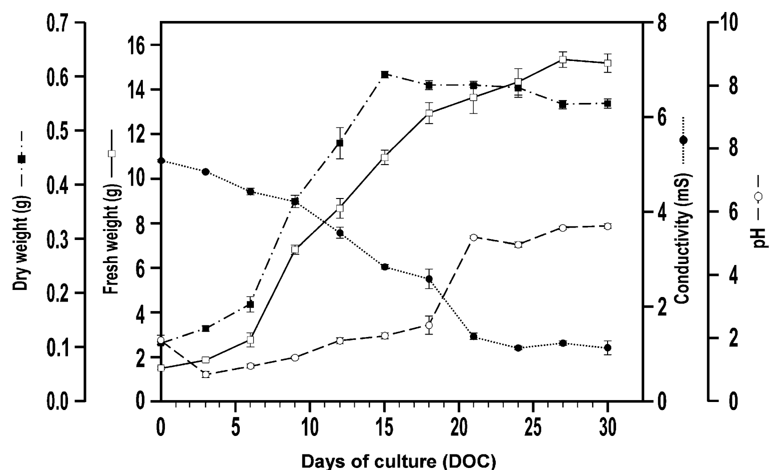

Fig. 1 *Capsicum chinense* cell culture cycle. Cell suspension cultures from the Naranja cultivar of habanero pepper, established from calli generated from plantlet leaves, were propagated in MS medium with 2,4-D. Values of dry (■) and fresh (□) weight, pH (○) and conductivity (●) were measured every 3 days. Data represent mean values ± SD of three independent experiments

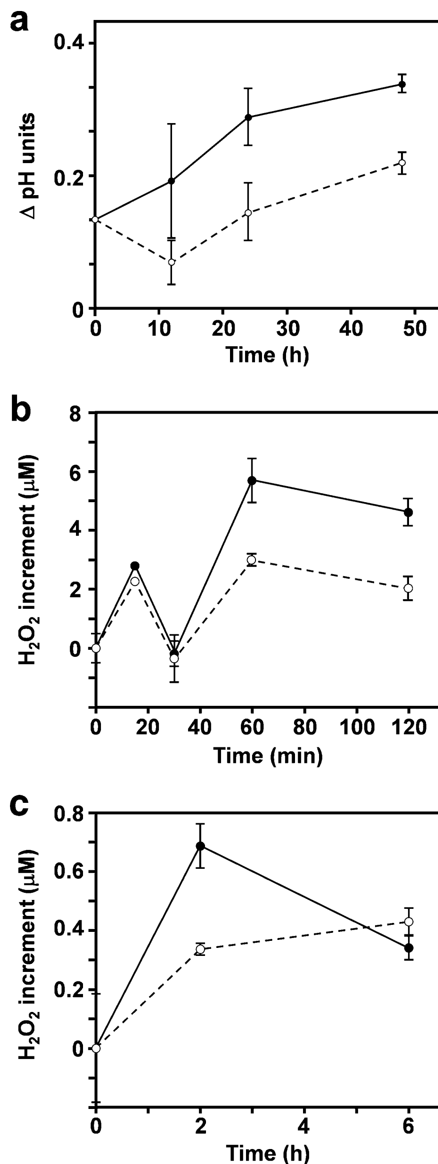

Fig. 2 Elicitation induces alkalinisation of the medium and production of hydrogen peroxide in the cell suspensions. Aliquots of 10 ml of the cell suspension culture were incubated with glucose (90 μg glucose ml^{-1}, ○) or with an equivalent concentration of MyH (90 μg glucose eq ml^{-1}, ●). pH values (**a**), and concentrations of extracellular (**b**) and intracellular hydrogen peroxide (**c**) were measured as described in the "Materials and methods" section. Data represent mean values of three independent experiments, ±SD

most probably because it was masked by the darker signal of the 44-kDa band.

In-gel kinase and immunoblotting assays revealed that the MAPK-like proteins were activated 5 min after MyH addition, they showed constant activation up to 20 min, and returned to basal levels at 30 min (Fig. 4c, KA and pTEpY panels). Again, the 42-kDa light band was poorly detected by the in-gel kinase assay.

Both immunoblotting and in-gel kinase assays showed the absence of any band in cell extracts when ddH_2O or glucose were added (Fig. 4d-MBP, lanes 3 and 4, respectively), while the 44-kDa band was clearly observed when MyH was added (Fig. 4d-MBP, lane 5); this time, a very light, diffused 42-kDa band seemed to be detected (Fig. 4d-MBP, lane 5).

Neither the 42 nor the 44 kDa kinases were able to phosphorylate casein or histone, since no single band of activity was detected irrespective of the substrate or the treatment applied (Fig. 4d-CASEIN and 4d-HISTONE H1, all lanes), despite the anti-pTEpY antibody corroborating the presence of the 42, and the 44-kDa activated MAPKs in the same cell extracts (Fig. 4d-pTEpY).

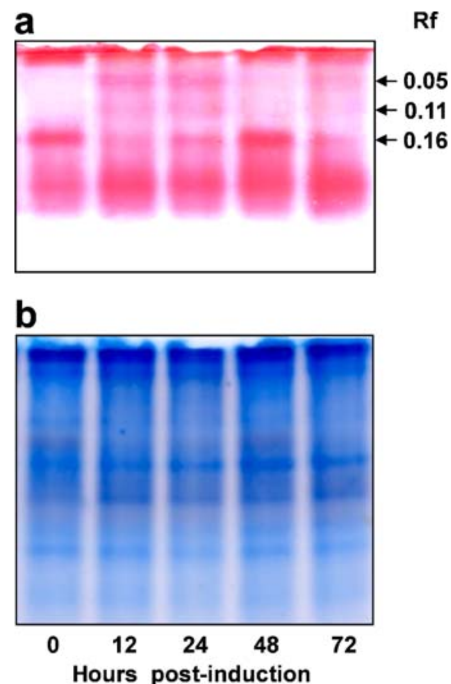

Fig. 3 Elicitation induces β-1,3-glucanase activities in the cell suspensions. Protein extracts (25 μg) from cell suspensions incubated for different periods with MyH (90 μg glucose eq ml^{-1}) were fractionated by native electrophoresis and then β-1,3-glucanase activities were measured as described in the "Materials and methods" section. (**a**) Representative image of the activity gels; (**b**) Coomassie staining of the gel. The experiment was repeated three times with reproducible results. A representative image is presented

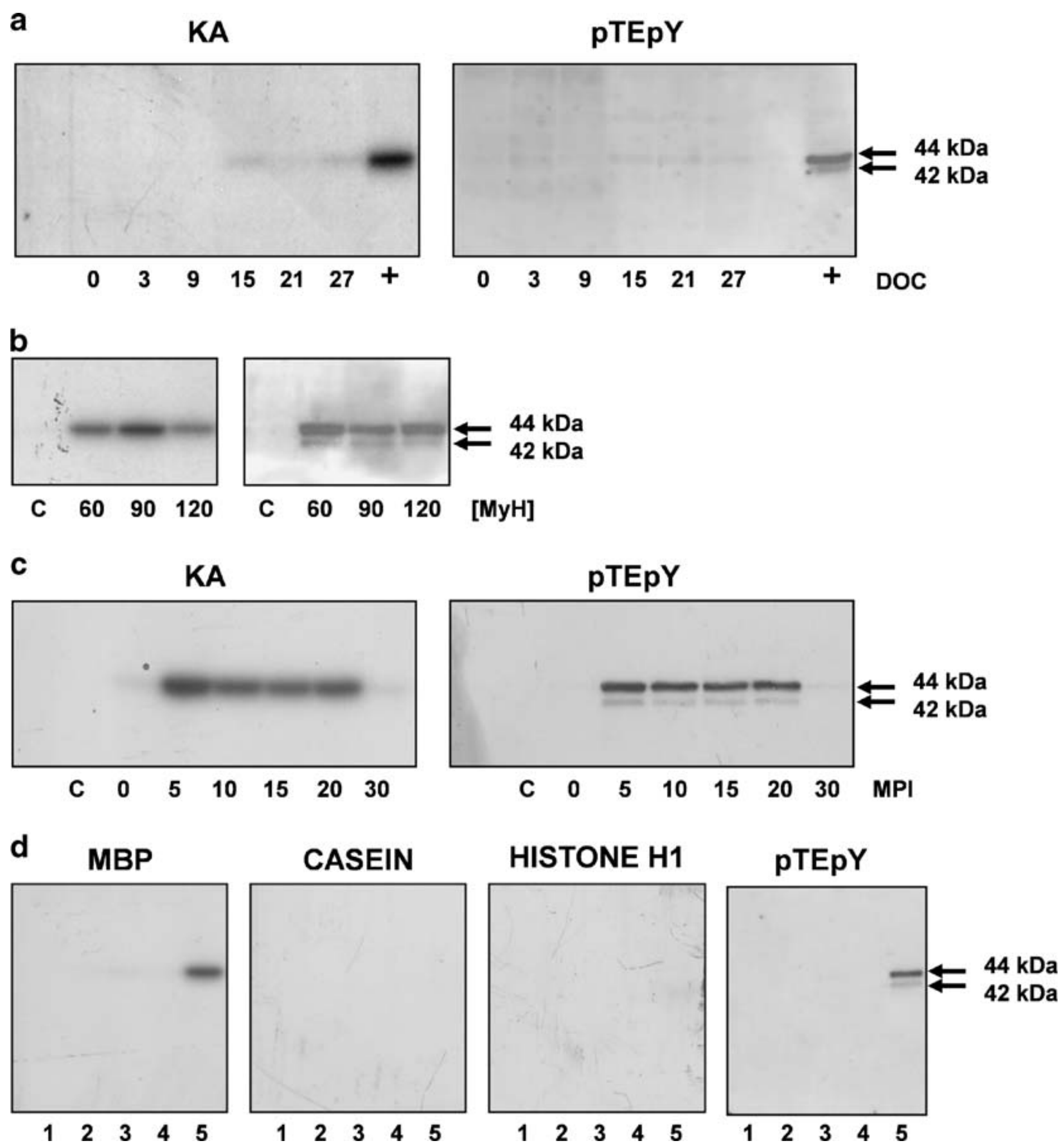

Fig. 4 Incubation with MyH activates 44, and 42-kDa MAPK-like enzymes in cell suspensions. In all determinations, 25 μg of protein extracts were fractionated by SDS-PAGE in gels containing (**KA**) or not MBP (**pTEpY**). MAPK-like activity in cell extracts was evaluated by in-gel kinase assay (**KA**), and bis-phosphorylated MAPK was detected by immunodetection (**pTEpY**), as described in the "Materials and methods" section. (**a**) Detection of MAPK-like enzymes in the culture cycle under normal conditions. Lanes labelled with the+symbol were loaded with protein extracts obtained from cell suspensions incubated with MyH (90 μg glucose eq ml^{-1}). (**b**) Presence of MAPK-like enzymes in cell suspensions incubated with different concentrations of MyH (expressed as μg glucose eq ml^{-1}). (**c**) Cell suspensions from day 10 of the culture were incubated with MyH (90 μg glucose eq ml^{-1}), and protein extracts were obtained at different periods. (**d**) Cell suspensions were incubated with ddH_2O (lanes 3), glucose (90 μg glucose ml^{-1}, lanes 4), MyH (90 μg glucose eq ml^{-1}, lanes 5) or without any treatment (lanes 2). Protein molecular mass-marker was loaded in lane 1 of each panel. Cell extracts were obtained and either fractionated by SDS-PAGE in a gel containing different substrates (**MBP**, **casein** or **histone H1**) or blotted to a PVDF membrane (**pTEpY**). All experiments were repeated more than three times with reproducible results, and representative images are presented

Addition of MyH to cell cultures of *C. chinense* modified the transcript population

Twenty-six target cDNAs were differentially modified under the elicitation conditions (Fig. 5a and Table 1). Transcript abundance of seventeen cDNAs was strongly or moderately increased, with predominance of those encoding pathogenesis-related (PR) proteins (EMBL AJ878871 and others not yet reported) and signal transduction intermediates (MAPK1, EMBL AJ608158; MAPK2, EMBL 698159). From the ten cDNAs whose transcript levels were reduced, we found a 1-aminocyclopropane-1-carboxylate oxidase (ACO) homologue (EMBL AJ879117), which encodes the last enzyme in the ethylene-biosynthesis pathway; also, glutathione-S-transferase (EMBL AJ879121) and glutathione peroxidase homologues (EMBL AJ973135), which are ROS-scavenging enzymes. The cDNAs whose transcript levels were not modified by the elicitation include energy-producing enzymes (ATPase subunit, EMBL AJ879064), ROS-scavenging enzymes (anionic peroxidase, EMBL AJ879066; glutathione-S-transferase/peroxidase, EMBL AJ879067; glutathione-S-

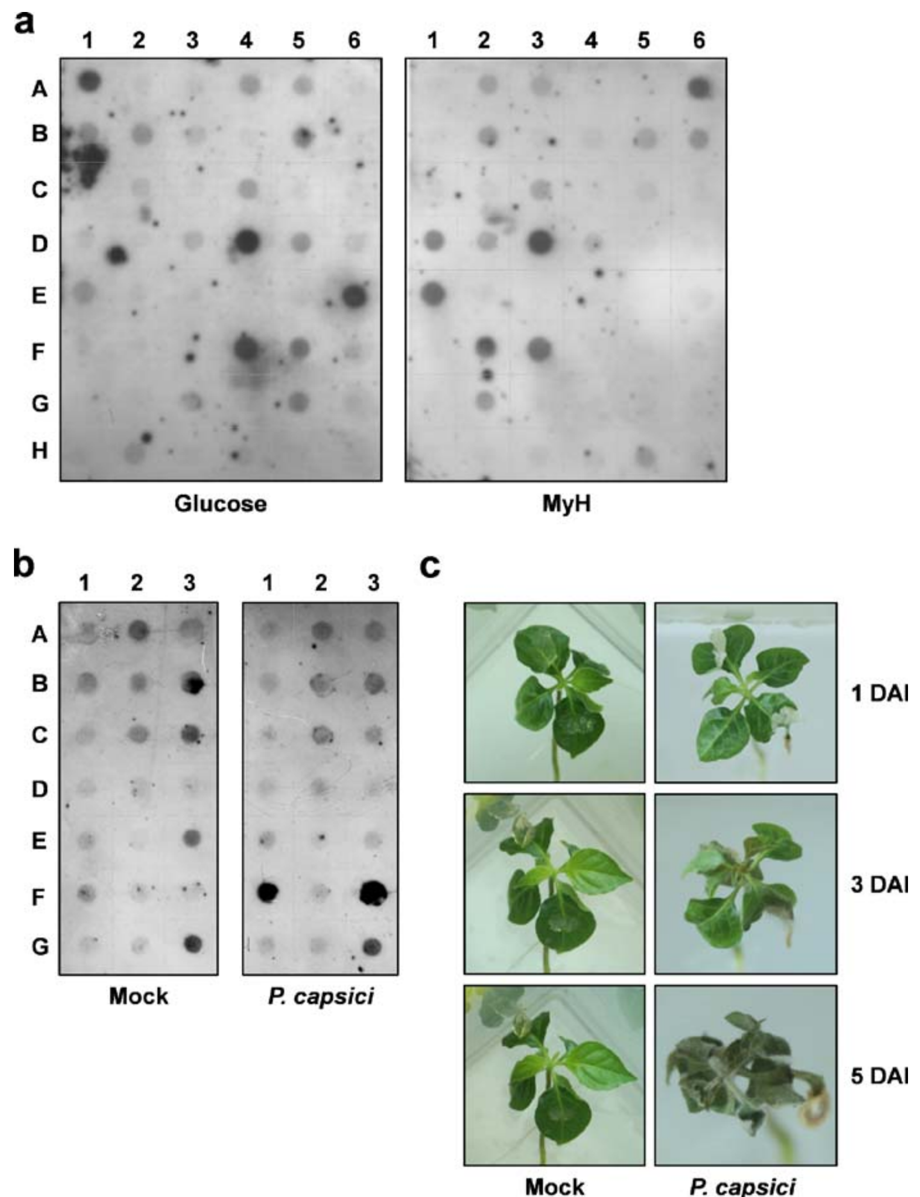

Fig. 5 *Phytophthora capsici* differentially modified transcript populations in *Capsicum chinense*. Elicitation of *C. chinense* cell suspensions with MyH modifies gene expression differentially. Different target cDNAs cloned from either a cDNA library or by RT-PCR were dot-blotted onto two identical nylon membranes. The blots were then hybridised with the corresponding control or induced probes, generated from cell suspensions (**a**) or plantlets (**b**) as described in the "Materials and methods" section. Hybridisation was carried out as described in the "Materials and methods" section and positive hybridisations were detected by chemiluminescence. Progression of symptoms in inoculated plantlets was evaluated at 24-h intervals (**c**). Each experiment was carried out at least three independent times, representative images are shown

Table 1 *Capsicum chinense* cDNAs differentially modified by *Phytophthora capsici*. The relative abundance of transcripts is represented with arrows

Array position	Identity	Accession[a]	Functional category[b]	Cells[c]	Plants[d]
A1	ACC oxidase	AJ879117	01.08.02	↓	–
A2	Polyubiquitin	AJ879118	14.07.05	↑	–
A3	Esterase	AJ879119	01.06	↑	↑
A4	Putative receptor-associated prot.	AJ879936	18.02.07	↓	–
A5	Glutathione S-transferase	AJ879121	01.20.37.01	↓	–
A6	Pathogenesis-related prot. (PR10)	AJ879115	32.05.01	↑↑	–
B1	Metallothionein-1	AJ879116	32.05.01.03.03	↓	–
B2	Arachidonic acid-induced protein	AJ879120	30.01.09.09	–	–
B3	Ubiquitin conjugated enzyme	AJ879070	14.07.05	–	–
B4	Metallothionein	AJ879063	32.05.01.03.03	–	–
B5	Cell wall protein-1	AJ879062	42.27.03	↑	–
B6	Ω-6 desaturase	AJ879071	01.06.05	↑	–
C1	ATPase like	–	02.45.15	↓	–
C2	ABC Transporter protein	AJ879065	20.03.25	↑	–
C3	Ω-6 fatty acid desaturase	AJ879069	01.06.05	↑	–
C4	Extensin-like protein	AJ879072	70.01	–	–
C5	Anionic peroxidase	AJ879066	32.01.01	–	–
C6	GST/peroxidase	AJ879067	32.01.01	–	–
D1	Pathogenesis-related protein	AJ878871	32.05.01	↑↑↑	–
D2	Ribosomal protein L37a	AJ879068	12.01.01	↑	–
D3	Pathogenesis-related protein	–	32.05.01	↑↑↑	–
D4	Glutathione peroxidase	AJ973135	32.01.01	↓↓↓	–
D5	14-3-3 protein	AM040266	30.07	–	–
D6	Ethylene response protein	AM040273	36.20.18.02	–	–
E1	Pathogenesis-related protein	–	32.05.01	↑↑↑	–
E2	Cytosol. malate dehydrogenase	AM040268	02.10	–	–
E3	Alcohol dehydrogenase	AM040269	02.16.01	–	–
E4	Glutathione S-transferase -12	AM040271	01.20.37.01	–	–
E5	Putative zinc finger protein	AM040274	16.17.09	–	–
E6	Cell wall protein-3	AM040272	42.27.03	↓↓↓	–
F1	Phosphate-induced protein	AM040267	30.07	–	–
F2	Hypothetical protein-1	AM040275	99	↑↑↑	–
F3	Pathogenesis-related protein	–	32.05.01	↑↑↑	–
F4	FAD-linked oxidase	AM040276	16.21.05	↓↓↓	–
F5	Replication factor A	AM040277	10.01.03.05	↓↓	–
F6	Auxin-induced protein	AM040278	36.20.18.01	–	–
G1	Actin	AM168448	70.04.03	–	–
G2	MAP kinase 1	AJ608158	30.01.05.01.03	↑	↑↑↑
G3	MAP kinase 2	AJ608159	30.01.05.01.03	↑↑↑	–
G4	MAP kinase 3	AJ608160	30.01.05.01.03	–	↑↑↑
G5	Ornithine decarboxylase	–	01.01.05.01.01	↓↓	–
G6	Type-I WRKY transcript. Factor	AM900560	11.02.03.01.01	–	–
H1	Non-expresser of PR prot. (NPR1)	AM900559	36.20.16.05	–	–
H2	V-ATPase	AJ879064	02.45.15	–	–

Table 1 (continued)

Array position	Identity	Accession[a]	Functional category[b]	Cells[c]	Plants[d]
H3	TMV-induced protein	–	32.05.03	↑↑	↑
H4	Proteinase inhibitor	–	18.02.01.02.03	↑↑↑	–

[a] GenBank Accesion number

[b] The functional category was assigned by comparison of the deduced aminoacid sequence with the *Arabidopsis thaliana* sequences in the MIPS FunCat catalogue. 01 Metabolism; 02, energy; 04, storage protein; 10, cell cycle and DNA processing; 11, transcription; 12, protein synthesis; 14, protein fate (folding, modification, destination); 16, protein with binding function or cofactor requirement (structural or catalytic); 18, regulation of metabolism and protein function; 20, cellular transport, transport facilitation and transport routes; 30, cellular communication/signal transduction mechanism; 32, cell rescue, defence and virulence; 34, interaction with the environment; 36, systemic interaction with the environment; 38, transposable elements, viral and plasmid proteins; 40, cell fate ; 41, development (systemic); 42, biogenesis of cellular components; 43, cell type differentiation; 45, tissue differentiation; 47, organ differentiation; 70, subcellular localisation; 73, cell type localisation; 75, tissue localisation; 77, organ localisation; 98, classification not yet clear-cut; 99, unclassified proteins

[c] Changes in gene expression in pepper cell suspensions elicited with *P. capsici* mycelium homogenates

[d] Changes in gene expression in six-leaf-stage pepper plantlets infected with *P. capsici* mycelium

transferase 12, AM040271), proteins involved in signal transduction pathways (14-3-3 protein, EMBL AM040266; MAPK3, EMBL AJ698160), and one ethylene response element homologue (ethylene response protein, EMBL AM040273). Interestingly, transcript levels for NPR1 (EMBL AM900559) and WRKY cDNAs (EMBL AM900560), whose homologues are central regulators of the plant defence responses, were not modified under the elicitation conditions.

Modification of transcript population during infection of *Capsicum chinense* plantlets with *Phytophthora capsici*

Under laboratory inoculation conditions, first signs of necrosis were observed in the inoculated leaves around 1 day after inoculation (DAI). Necrosis reached the apical meristem by three DAI, and the whole plantlet was covered after five DAI (Fig. 5c). No single plantlet survived beyond 5-6 DAI. According to this, systemic changes in the transcript population were evaluated in non-inoculated leaves at different periods between 6 and 24 HAI. We found fewer modifications in the transcript patterns compared to those observed in vitro (Fig. 5b and Table 1). Like in cell suspensions, neither NPR1 nor WRKY genes were induced during the *in planta* interaction. Noteworthy, the most evident modification was the remarkably strong induction of MAPK1 (EMBL AJ608158) and MAPK3 (EMBL 698159) genes.

Discussion

Even though the role of the soilborne oomycete *P. capsici* as the causal agent of Phytophthora blight was first described in pepper almost a century ago (Leonian 1922), only few highly resistant cultivars have been developed, and no effective management programmes to control its spread in pepper crops have been developed (Hausbeck and Lamour 2004). Since most species in the genus Capsicum are susceptible to different races of *P. capsici* (Oelke et al. 2003) their relationship could be defined as a compatible interaction.

Most common plant defence responses include alkalinisation of the extracellular medium (Felix et al. 1993), explosive production of reactive oxygen species (Davies et al. 2006), activation of signal transduction pathways such as the MAPK pathway (Eckardt 2002), and induction of defence-related genes (Eckardt 2002). Elicitation with MyH produced these responses in *C. chinense*.

Non-specific elicitors derived from the fungal cell wall are potent inducers of apoplastic alkalinisation in many plant species (Felix et al. 1993). Indeed, alkalinisation of the culture medium has been proposed as a rapid and convenient assay to assess the perception of chitin in *Solanum lycopersicum* (Felix et al. 1993). In our work, the alkalinisation of the medium was maintained until at least 50 h after MyH addition. These results are similar to those observed in *C. annuum* cell suspensions treated with *P. capsici* filtrate and homogenates (García-Pérez et

al. 1998), in which a constant pH increment lasted at least 24 h.

Addition of MyH also produced intra- and extracellular production of H_2O_2 and activation of PR proteins. Davies et al. (2006) observed a high production of H_2O_2, by 50–70 min after *A. thaliana* cell suspensions were treated with 100 μg ml^{-1} glucose equivalents of a *Fusarium oxysporum* elicitor. Activation of Capsicum β-1,3-glucanases was previously found during the interaction between *C. annuum* and compatible or incompatible isolates of *P. capsici* (Kim and Hwang 1994). Furthermore, García-Pérez et al. (1998) proposed that *C. annuum* β-1,3-glucanases were associated with resistance to *P. capsici*, because the activity of an intracellular enzyme increased 24 h after cell suspensions of a resistant cultivar were inoculated with mycelium homogenates and culture filtrates. Thus, the activation of β-1,3-glucanases with Rf values of 0.05 and 0.11 might represent a *C. chinense* defence-related response. Although relative migration (Rf) has been used almost exclusively in chromatography to identify analytes and determine their molecular mass, there are many examples of its use to identify PR proteins by native gel electrophoresis (García-Pérez et al. 1998).

The above evidence supports our proposal that the H_2O_2 production, medium alkalinisation and activation of β-1,3-glucanases observed in cell suspensions from *C. chinense* represent pepper-specific responses induced by *P. capsici* elicitors, yet they can be also produced by other biotic and abiotic stresses.

In our elicitation system, two MAPK-like enzymes were activated fast and transiently. Even though we did not demonstrate the identity of the enzymes, different confirmatory experiments support our proposal that their activities corresponded to *C. chinense* MAPK, especially their recognition by a specific antibody directed against bisphosphorylated (activated) MAPK. This antibody was generated against a eukaryotic-conserved amino acid sequence surrounding the MAPK signature domain (pTEpY); since the deduced amino acid sequence of at least three MAPK cDNAs cloned from *C. chinense* cell suspensions revealed they possess the conserved domain (data not shown), it is reasonably possible that the corresponding coded proteins were expressed in the cell suspensions and cross-reacted with the antibody. On the other hand, as the role of many plant MAPKs in the establishment of the defence response against pathogens has been solidly demonstrated (Zhang and Klessig 2001), the finding that they were activated after elicitation of pepper cells should not be surprising. However, it should be pointed out that *C. chinense* is highly susceptible to *P. capsici*; thus, the physiological significance of MAPK activation during the in vitro elicitation with oomycete isolates must be further clarified.

Elicitation of the cell suspensions also produced the differential accumulation of defence-related transcripts. From a small array of 46 homologous genes representing different features of the metabolic pathways, five PR-protein clones displayed maximum up-regulation, and two MAPK genes were also induced, particularly MAPK-2. This is especially interesting because transcriptional activation is not the main regulation mechanism of most plant MAPKs (Zhang and Klessig 2001). On the other hand, down-regulation of different genes also correlated with the establishment of a defence response. Specifically, inactivation of ROS-scavenging enzymes is needed for the establishment of the defence-related oxidative burst; we found repression of genes coding ROS-scavenging enzymes, in coincidence with the establishment of an oxidative burst in cell suspensions. Ethylene-related genes were not modified by the treatment, which suggests that the defence response did not involve the ethylene-mediated signalling pathway; alternatively, the elicitation with MyH could inhibit ethylene synthesis and probably ethylene-mediated gene expression.

By the use of a laboratory-made array, analysis of gene expression in systemic tissues during the Habanero pepper *in planta* infection with *P. capsici* revealed a small number of modifications. Interestingly, as with elicitation of cell suspensions, the expression of ACC oxidase and ethylene-response genes was not modified during *in planta* infection. Also, neither NPR1 nor WRKY genes were apparently induced by the elicitation of cell suspensions or the infection of plantlets (Table 1). Thus, the defence response in cell suspensions, if actually promoted, may be independent of the processes involving these genes.

In *Arabidopsis thaliana*, *Nicotiana attenuata*, *N. tabacum* and other species, NPR1 functions in defence and is present as a single-copy gene (Rayapuram and Baldwin 2007), but the WRKY family of transcription factors contains numerous members (Eulgem et al.

1999). Thus, we must consider that the WRKY gene homologue analysed in this work could have a role other than a defence response to pathogens; however, a comparison of its deduced amino acid sequence in databases revealed that the best four matches correspond to Solanaceae homologues with specific functions in the plant defence response (Hofmann et al. 2008; Maeo et al. 2001; Park et al. 2005; Skibbe et al. 2008).

Apart from a couple of PR transcripts, outstanding accumulation of two MAPK transcripts (MAPK1 and MAPK3) dominated among all genes tested *in planta*. This is extraordinary because MAPK activation was supposed to be related to the defence response; however, no plantlets survived the infection. Accepting that gene induction may not represent protein synthesis or kinase activation, a probable explanation is that gene induction of MAPK would not be sufficient to sustain an efficient defence response. Alternatively, since it has been proposed that infection success by the oomycete in compatible interactions relies on its ability to suppress the host defence response either by blocking the correct perception of effectors, the proper activation of signalling intermediates, the effective triggering of the defence-related transcription, or the inactivation of secreted hydrolytic enzymes (Reviewed by Göhre and Robatzek 2008), it might be possible that molecular manipulation of the *C. chinense* defence response resides some point downstream of the MAPK signalling cascade. Remarkably, during the writing of this paper, ethylene treatments that induced resistance against Phytophthora blight in *C. chinense* plantlets completely abolished the *P. capsici*-mediated *in planta* induction of the MAPK1 and MAPK3 genes (Núñez-Pastrana, unpubl.). The inverse relationship between transcriptional activation of these two MAPK and the resistance phenotype suggests these MAPK may play a negative role during the *P. capsici* colonisation of *C. chinense* plantlets. Even though a direct connection between these two phenomena must be demonstrated, the negative role of the MAPK pathway during plant defence responses has been demonstrated in several plant species (Frye et al. 2001).

In contrast with previous reports employing lyophilised mycelium homogenates (García-Pérez r. 1998), we used whole mycelium homogenates that were autoclaved. This treatment must denature the protein fraction; thus, it is possible that the elicitation principle resides most likely in the non-protein components of the homogenate. Since whole homogenates are comprised of a mixture of both intra and intercellular components, the elucidation of the elicitor's chemical nature is important to obtain further evidence about the infection strategy of the oomycetes.

As predicted, results from this work demonstrate that whole mycelium homogenates from *P. capsici* are able to activate a defence response when added to pepper cell suspensions, including the activation and induction of MAP kinases, but they displayed a negative effect over the induction of NPR1 and WRKY1 genes. These data would support the role of the molecular manipulation of the host defence response through Phytophthora effectors as a key event that guarantees the oomycete success in colonisation, resulting in the absence of highly-resistant species in the genus Capsicum against the Phytophthora blight disease. Use of cell suspensions to study plant-pathogen interactions has many advantages, because external factors that influence the plant metabolism can be better controlled. Thus, addition of oomycete effectors to cell suspensions may be used to understand the mechanisms of infection or the cellular responses modified by the pathogen.

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