Eur J Plant Pathol (2011) 131:669–683
DOI 10.1007/s10658-011-9841-z

Ethylene, but not salicylic acid or methyl jasmonate, induces a resistance response against *Phytophthora capsici* in Habanero pepper

Rosalía Núñez-Pastrana · Guadalupe Fabiola Arcos-Ortega · Ramón Armando Souza-Perera · Carlos Alberto Sánchez-Borges · Yumi Elena Nakazawa-Ueji · Francisco Javier García-Villalobos · Adolfo Alberto Guzmán-Antonio · José Juan Zúñiga-Aguilar

Accepted: 11 July 2011 / Published online: 27 July 2011

Abstract We sprayed defence-related plant growth regulators (salicylic acid, methyl jasmonate and ethephon) on one-month-old Habanero pepper seedlings cultivated in vitro. Twenty-four hours later, we inoculated the seedlings with a virulent strain of *Phytophthora capsici* and periodically evaluated the disease symptoms. At the concentrations used, neither salicylic acid nor methyl jasmonate generated a protective effect in the seedlings, which died less than 10 days post inoculation. However, the treatment with 5 mM ethephon delayed or prevented disease symptoms in 30% of the seedlings. Interestingly, blocking the ethylene receptor with a previous application of 300 μM silver nitrate impeded the protective effects of ethephon. This result demonstrated that the plant resistance response required the perception of ethylene. Analysis of transcript populations in ethephon-treated seedlings revealed a direct correlation between survival and the accumulation of PR1, a gene marker of the systemic acquired resistance (SAR). Although the ethephon treatment also modified transcript levels of the plant defensin PDF1.2, a marker of the induced systemic resistance (ISR), in this case the accumulation also occurred when the ethylene receptor was blocked, suggesting a non-specific effect. The ethephon treatment did not modify the expression of NPR1 (a key transcriptional regulator of plant defence). Interestingly, transgenic pepper seedlings overexpressing endogenous PR10 or esterase genes, which are induced by the ET treatment, completely resisted the infection, which corroborated the importance of these genes in the defence response. Our results suggest that ethylene induced a systemic defence response in susceptible seedlings, possibly in an NPR1-independent pathway.

Keywords Pathogenesis-related proteins · Phytohormones · Phytophthora blight

Abbreviations

dpi	Days post inoculation
ET	Ethylene
JA	Jasmonic acid
MeJA	Methyl jasmonate

Electronic supplementary material The online version of this article (doi:10.1007/s10658-011-9841-z) contains supplementary material, which is available to authorized users.

R. Núñez-Pastrana · R. A. Souza-Perera · C. A. Sánchez-Borges · Y. E. Nakazawa-Ueji · F. J. García-Villalobos · A. A. Guzmán-Antonio · J. J. Zúñiga-Aguilar (✉)
Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Chuburná de Hidalgo, Mérida 97200 Yucatán, México
e-mail: zuniga@cicy.mx

G. F. Arcos-Ortega
Centro de Investigaciones Biológicas del Noroeste, Mar Bermejo No. 195, Playa Palo de Santa Rita, La Paz, B. C. S., México

PB Phytophthora blight
SA Salicylic acid

Introduction

Phytophthora blight (PB) is a deadly disease that affects all species of the genus Capsicum and many species within the families Solanaceae and Cucurbitaceae (Kreutzer et al. 1940). The disease is characterised by root and crown rot; leaf, fruit and stem blight; and the rapid wilting and death of infected plants (Ristaino et al. 1993). Even though the causal agent of PB (the oomycete *Phytophthora capsici)* was first described on bell pepper in New Mexico almost 90 years ago (Leonian 1922), no effective management programs have been developed to stop its spread in pepper crops.

There are virtually no PB-resistant species in the genus Capsicum; the commercially valueless *Capsicum annuum* cultivars "AC2258" (Palloix et al. 1990) and "Serrano Criollo de Morelos" (CM334) (Gil-Ortega et al. 1991) are among the few exceptions. Research efforts to find the genetic basis of resistance have determined that it can be polygenic, with quantitative trait loci (QTL) mapping to several chromosomes (Sugita et al. 2006). However, efforts to generate resistant cultivars through breeding programs have not resulted in cultivars with consistent levels of resistance.

Manipulation of the host response has been proposed as one mechanism by which oomycete pathogens block the plant defence response (Kamoun 2006). If this mechanism is in effect, a previous triggering of the correct defence response should render the susceptible pepper species resistant to later infection by *P. capsici*. Indeed, the application of chemicals has been used to study the susceptibility of peppers to PB. Baysal et al. (2005) proposed that the activation of defence-related enzymes such as L-phenylalanine ammonia-lyase (PAL), chitinase, and β-1,3-glucanase may contribute to the induction of resistance against *P. capsici* in *C. annuum* seedlings that were sprayed with acibenzolar-S-methylbenzo [*1,2,3*]thiadiazole-7-carbothioic acid-S-methyl ester (ASM). In another report, the exogenous application of DL-β-amino-n-butyric acid (BABA) induced a resistance response to PB in *C. annuum* (Sunwoo et al. 1996). In these works, the exact mechanisms by which the chemical treatments induced a systemic response were not determined.

The establishment of plant systemic defence responses requires the orchestrated action of plant growth regulators (PGRs). Salicylic acid (SA) mediates the establishment of systemic acquired resistance (SAR), a robust response triggered by the specific recognition of pathogenic microorganisms and characterised by the induction of genes encoding pathogenesis-related (PR) proteins. Ethylene (ET) and jasmonic acid (JA) play key roles in the induced systemic resistance (ISR), a response activated by non-pathogenic rhizobacteria (Van Loon et al. 1998) that includes the expression of non-"SAR" PR proteins, principally plant defensin 1.2 (PDF1.2) and thionin 2.1 (THI2.1). Despite the fact that different signals activate SAR and ISR, their signal transduction networks are complex and may share some components. In some responses, the protein encoded by the non-expressor of PR proteins gene 1 (NPR1) can activate either SAR or ISR, according to the nature of the pathogenic signal and the defence hormone involved (Pieterse and Van Loon 1999). For example, SA can induce a gene encoding the protein PR1 (a molecular marker of SAR) through NPR1-dependent and NPR1-independent mechanisms (Clarke et al. 1998); however, ET induces PR1 in an NPR1-independent pathway (Shah et al. 2001). In any case, once activated, PR1 displays antimicrobial activity against different pathogens.

Because the role of phytohormones in the establishment of plant systemic responses is well established, we tested the hypothesis that a previous treatment with SA, MeJA (methyl jasmonate) or ET could promote a resistant response against PB in a susceptible cultivar of Habanero pepper. We also evaluated the expression of SAR and ISR gene markers to obtain further evidences about the possible mechanisms of defence. Our results revealed that a previous spraying with ET reduced or impeded infection in seedlings inoculated with *P. capsici*. The resistance phenotype was correlated with the expression of the PR1 gene. Since it seemed that expression of PR proteins correlated with the resistance phenotype, we overexpressed PR10 and esterase homologue genes to evaluate their contribution to defence. In both cases the overexpression conferred resistance

to *C. chinense* seedlings against *P. capsici*. These results demonstrated that the external application of ET induced a defence response against PB in Habanero pepper, probably by the promotion of a NPR1-independent SAR-like response.

Materials and methods

In vitro culture of Habanero pepper

Habanero pepper seeds from Seminis® were surface sterilised with 80% ethanol for 5 min, rinsed three times with sterile distilled water, washed with sodium hypochlorite (1.6%) for 15 min and finally rinsed three times with sterile distilled water. Surface-sterilised seeds were germinated in Petri dishes with cotton and water. Once the radicle emerged, the seeds were transferred to Magenta boxes containing synthetic media. The medium contained Murashige and Skoog salts (Sigma), thiamine (40 mg l^{-1}, Sigma), myo-inositol (100 mg l^{-1}, Sigma), L-cysteine (25 mg l^{-1}, Fluka), sucrose (30 g l^{-1}) and gelrite (2.2 g l^{-1}, Sigma); the pH of the medium was adjusted to 5.6, and it was autoclaved at 1.1 kg cm^{-2} (121°C) for 15 min. The Magenta boxes were covered with a lid that allowed gaseous interchange through a sponge. Habanero pepper seedlings were grown for 30–35 days (when they had four leaves) at 25°C under an 18-h photoperiod at a light intensity of 19.64 μmol m^{-2} s^{-1}.

Chemical treatments

One-month-old in vitro seedlings were sprayed with 1 ml of 5 mM ethephon (Sigma) or different concentrations of SA (Productos Químicos Monterrey) (1, 5 and 10 mM) or MeJA (Sigma) (10, 100 and 150 μM). Sets of seedlings were sprayed with the corresponding solvents (SA and ethephon were dissolved in H_2O; MeJA was dissolved in 0.1% Tween 20) as controls. To inhibit the perception of ET, the seedlings were sprayed with 300 μM $AgNO_3$ (Sigma) 24 h before the application of ethephon (1 ml of a 5 mM solution). After each chemical treatment, the Magenta boxes were hermetically closed for 24 h, and then the seedlings were inoculated with *P. capsici*.

In vitro culture of *Phytophthora capsici* and inoculation of Habanero pepper

A virulent strain of *P. capsici* was grown for six days on potato dextrose agar (PDA, Difco) at 28°C before being used to inoculate pepper seedlings. One-month-old Habanero pepper seedlings were inoculated in vitro by placing a mycelium plug from *P. capsici* onto the surface of two opposing leaves (the third and fourth true leaves). Disease symptoms were then recorded periodically [at 3, 10 and 20 days post inoculation (dpi)]. In mock-inoculated seedlings, two PDA plugs were used.

Transient genetic transformation of Habanero pepper

Transient genetic transformation of Habanero pepper seedlings was performed according to Arcos-Ortega et al. (2010). One-month-old seedlings were infiltrated with *A. tumefaciens* (strain LBA4404) suspensions harbouring the plasmids pCAMex (empty binary vector), pCAMex::PR10 (*C. chinense* PR10 cDNA inserted into the pCAMex binary vector) or pCAMex:: Esterase (*C. chinense* esterase cDNA inserted into the pCAMex binary vector). The infiltrated seedlings were rinsed with sterile Murashige and Skoog (MS) medium, and the co-cultivation was left in the dark for 48 h at 25°C. After co-cultivation, the seedlings were washed thoroughly with sterile MS medium, and they were then rinsed four times with 250 mg l^{-1} cefotaxime (claforan). The efficiency of transformation was monitored in parallel seedlings by a GUS histochemical assay. Finally, transgenic seedlings were inoculated with a plug of *P. capsici*, and the disease symptoms were recorded periodically (at 5 and 10 dpi).

ET measurements

To measure the production of ET, a 1-ml sample of gas was removed with a gas-tight syringe (SGE Analytical Science) from the atmosphere of hermetically closed Magenta boxes containing the mock- or *P. capsici*-inoculated seedlings. Gas samples were collected every 24 h over a period of six days. The concentration of ET was determined by gas chromatography (Hewlett Packard gas chromatograph, 8690 Series II) with a GS-Q FSOT column (Alltech, 30 m L×0.53 mm ID) and an FID detector. Nitrogen was

used as the carrier gas at a flow rate of 10 ml min^{-1}. The temperature of the oven was 70°C, and injector and detector temperature was 200°C. ET was used as an external standard. Three seedlings per treatment were analysed, and a statistical analysis was performed to determine the standard error in the mock- and *P. capsici*-inoculated seedlings.

Scanning electron microscopy

Electron micrographs from inoculated Habanero pepper seedlings that were previously treated with 5 mM ethephon and/or 300 μM $AgNO_3$ were obtained as follows. Three dpi, the inoculated leaves were cut and prepared for scanning electron microscopy. The leaves were fixed with 5% (v/v) formaldehyde (Sigma), and they were then submerged sequentially for 1 h into different solutions containing increasing concentrations of ethanol (30, 50, 70 and 96% v/v). The dehydrated leaves were submerged twice into absolute ethanol for 1 h each, and they were dried using a model Sandri-795 Tousimis® drier, substituting ethanol with liquid CO_2, and subsequently with gaseous CO_2. Finally, the dried samples were treated with a 21-nm thick gold cover using a Denton® Vacuum Desk II metaliser. The metalised samples were observed under a model JSM-6360LV JEOL® Scanning Electron Microscope.

RNA extraction and gene-expression analysis

RNA was extracted from the leaves of mock-inoculated pepper seedlings and from seedlings inoculated with a plug of *P. capsici* mycelium. Both types of seedlings were previously treated in vitro with 5 mM ethephon and/or 300 μM $AgNO_3$. The treated leaves were collected at 0, 4, 8, 12, 24, 48 and 72 h post inoculation, and total RNA was isolated using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using oligo-dT and SuperScript™ III reverse transcriptase (Invitrogen). The transcript levels of specific genes in inoculated seedlings were quantified by means of reverse transcription coupled to quantitative real-time PCR (qRT-PCR). Each cDNA used as a qRT-PCR substrate represented different RNA pools, as follows: i) soon after inoculation (0 and 4 h); ii) a middle time after inoculation (8, 12 and 24 h); and long after inoculation (48 and 72 h). The RNA and cDNA concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific). The qRT-PCR analysis was performed using combinations of gene-specific primers for each cDNA (Table 1). Each amplification assay was performed in triplicate in a 48-well plate (StepOne model, Applied Biosystems). The reaction mix (20 μl final volume) consisted of 10 μl of SYBR Green PCR Master Mix 2X (Applied Biosystems), 1 μl of each primer (250 nM final concentration) and 3 μg of cDNA. A blank (no-template control) was also incorporated into each assay for each primer pair. The cycling program was as follows: one initial denaturation step at 95°C for 10 min, then 30 cycles of 15 s at 95°C, 30 s at 54°C and 30 s at 72°C. In the case of PDF1.2 transcription analysis, the number of cycles was 40. After completion of the program, melting-curve data were collected to verify PCR specificity, contamination and the absence of primer dimers. Variations in the concentration of every cDNA sample were normalised using actin as an internal standard. Fold changes in gene expression were based on the ΔCt calculation ($2^{-\Delta\Delta Ct}$ method). ΔCt corresponded to the Ct of one selected gene subtracted from the Ct of actin for each treatment. Fold changes in expression were based on the calculation of the ΔΔCt that corresponds to the ΔCt in treated (water, ethephon or $AgNO_3$-ethephon) and inoculated leaves subtracted from that of mock-inoculated leaves. An analysis of variance (ANOVA) followed by Tukey's multiple range test ($P \leq 0.05$) was performed to determine the significance of the differences in fold expression between the different treatments.

Results

Exogenous application of ET prevented *Phytophthora capsici* infection of susceptible Habanero pepper seedlings

In non-treated seedlings and in those sprayed with control solutions, *P. capsici* rapidly infected the plant tissue (Figs. 1 and 2). The inoculated leaves showed necrosis and withering at nearly 3 dpi, and the necrosis reached the leaf peduncle and hypocotyls by 4 dpi. Between 4 and 5 dpi, the necrosis covered the upper part of the plant, and between 6 and 10 dpi

Table 1 Oligonucleotide sequence of the primers used in the real-time RT-PCR assays

Gene	Sequence primer	Amplicon size (pb)
ACC oxidase	[a]F: 5′-AAGTGCAACCATGGGACTTC-3′ [b]R: 5′-TGCTTTCCCAGTCTGTGTTG-3′	212
Actin	F: 5′-TTCCCTCTATGCCAGTGGAC-3′ R: 5′-GGCTGTGGTGGTGAAAGAGT-3′	183
NPR1	F: 5′-GCACAGAGGACAACAGTGGA-3′ R: 5′-TCAGTGAACGCTTTGGTCAG-3′	260
PDF1.2	F: 5′-CAAGGGGTTGTGCCTTAGTA-3′ R: 5′-TTCCTGCAGAAGCATTTAAGA-3′	105
PR1	F: 5′-CTTGTTAGTCTCATGATACTAGCC-3′ R: 5′-TCATTTTAGTAAGGGACTTTGTCCGG-3′	459

[a] Forward primer
[b] Reverse primer

the mycelium grew profusely throughout the entire seedling (Fig. 2). We obtained similar results with seedlings sprayed with different concentrations of SA or MeJA (Fig. 1); priming the plant defences with SA or MeJA did not alter the normal progress of the disease. In contrast, previous spraying of seedlings with a concentration of ethephon that did not induce the abscission of leaves produced a significant modification of the disease symptoms (Fig. 2). Nonetheless, the ethephon treatment had a heterogeneous effect on the population. At 3 dpi (when 100% of control seedlings were infected), 60% of the treated seedlings showed no symptoms of infection. Interestingly, though the percentage of asymptomatic seedlings was reduced to 30% by 10 dpi, these seedlings resisted infection for more than 20 dpi (Table 2).

The above results suggested a role for ET in the establishment of a defence response against PB; however, there are reports in the literature of non-specific effects induced by ethephon (Lawton et al. 1994). To determine if this defence-like response was induced specifically by ET, we repeated the ethephon treatment in seedlings in which the ET perception was blocked by the application of silver nitrate (300 μM $AgNO_3$), a chemical compound that impairs the capacity of the receptor to transduce the signal after ET is bound (Beyer 1976). The optimal concentration of $AgNO_3$ to block the ET perception was determined experimentally by a leaf abscission assay (Supplementary Material 1). We found that the addition of $AgNO_3$ to pepper seedlings not only impeded the protective effects of ethephon but also allowed a faster proliferation of mycelium *in planta* (Fig. 2), which suggested that the resistance phenotype depended on the perception of ET. A closer observation of the infected leaves using scanning electron microscopy revealed that by three dpi the mycelium had dispersed profusely through the tissue (Fig. 3a.b), and hyphae began to emerge from the leaf surface (Fig. 3c). However, in the ET-treated leaves, most of the mycelium remained within the agar plug and only occasionally disseminated in its vicinity, neither penetrating significantly nor disturbing the leaf surface, which appeared healthy and turgid (Fig. 3d–f). Interestingly, the previous addition of $AgNO_3$ to block the ET perception produced a more aggressive dispersal of the mycelium than that observed in control seedlings (Fig. 3g–i). These results reinforce the association of the defence response against *P. capsici* with the perception of ET.

Endogenous ET did not accumulate during the progress of Phytophthora blight in vitro

Because the Habanero pepper seedlings used in this study were susceptible to PB but ET treatment prevented the establishment of the disease, it is possible that the production of endogenous ET was impaired during the infection of untreated seedlings. To assess this possibility, we measured the concentration of ET in the Magenta boxes during the progress of PB infection in vitro (Fig. 4). In the pepper seedlings inoculated with *P. capsici*, ET did not accumulate within the jars during the first 3 dpi, and only a slight accumulation occurred between 4 and 5 dpi (Fig. 4, black boxes). On the contrary, ET accumulated rapidly beginning at 1 dpi in seedlings that were treated with 5 mM ethephon (Fig. 4, open circles), regardless of whether they were previously sprayed with $AgNO_3$ (Fig. 4, black diamonds) and subsequently inoculated with *P. capsici* (Fig. 4, black circles). Under the ethephon treatments, the accumu-

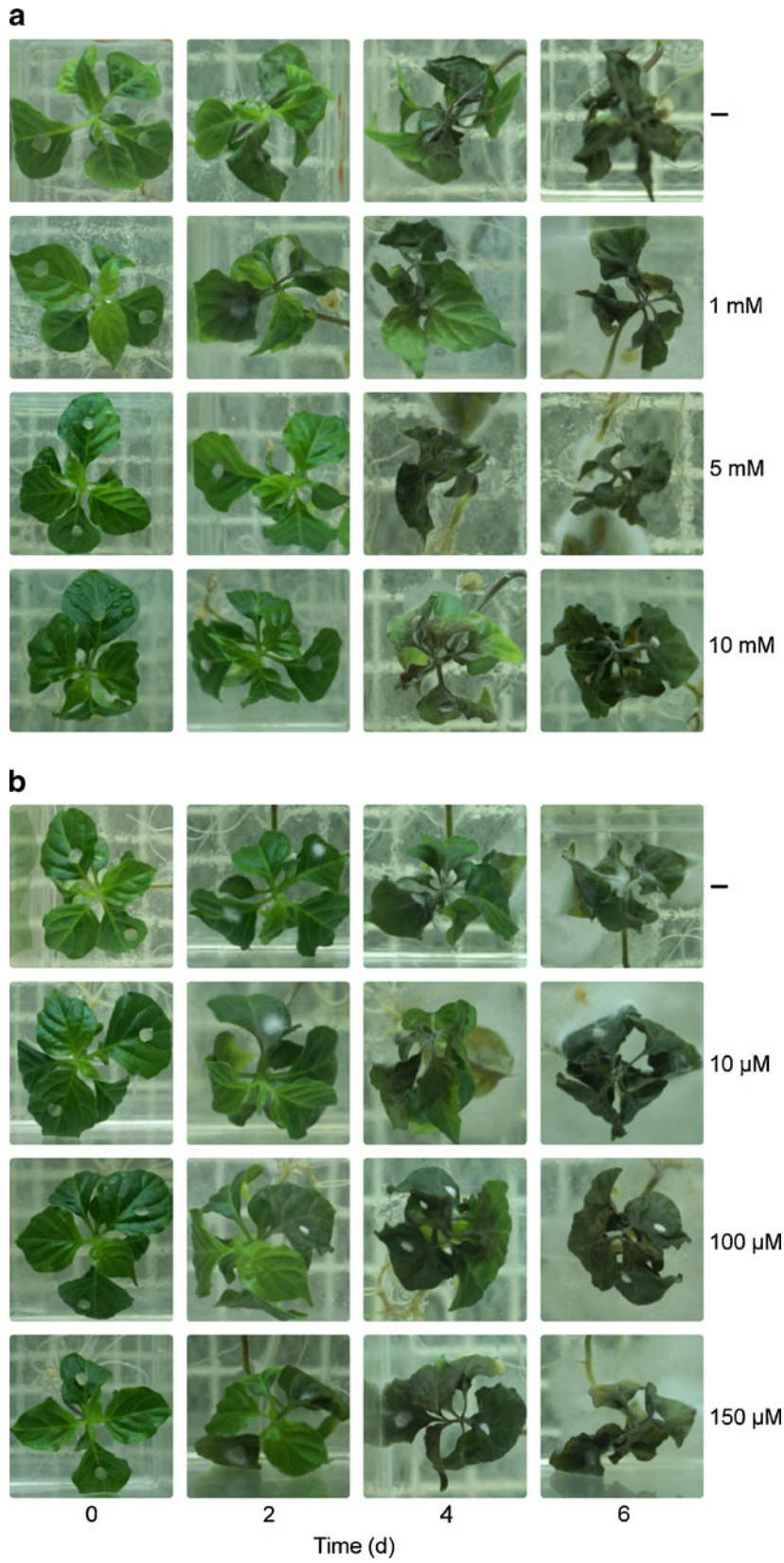

Fig. 1 Effect of salicylic acid and methyl jasmonate in the pepper resistance against *Phytophthora capsici*. One-month old Habanero pepper seedlings were sprayed with different concentrations of salicylic acid (**a**) and methyl jasmonate (**b**). 24 h later they were inoculated in vitro with *Phytophthora capsici* and the disease symptoms were periodically evaluated. Control seedlings (−) were sprayed with water (salicylic acid treatment) or 0.1% Tween 20 (v/v) (methyl jasmonate treatment)

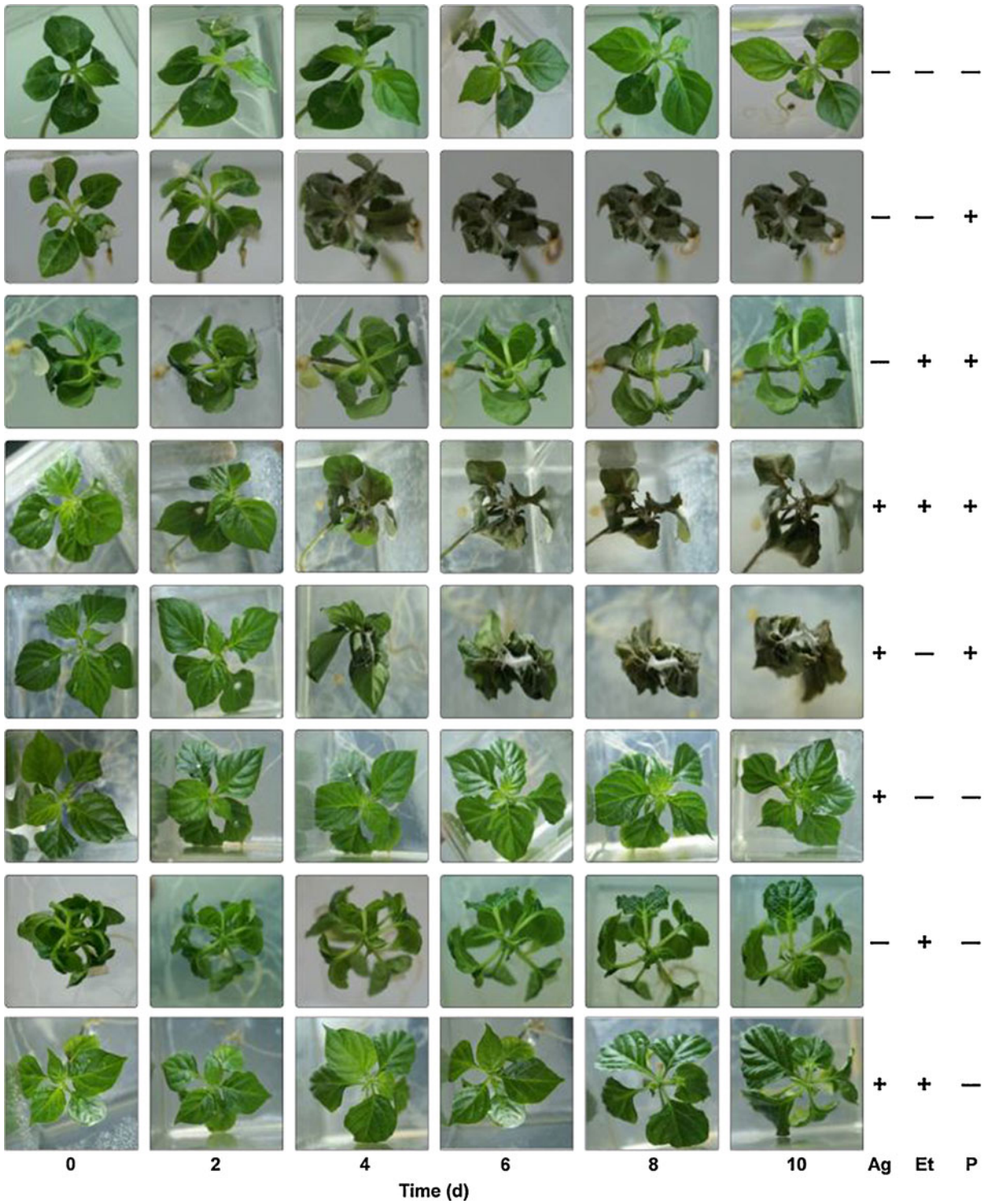

Fig. 2 Effect of ethylene in the pepper resistance against *Phytophthora capsici*. One-month old Habanero pepper seedlings were sprayed with 300 μM silver nitrate (Ag) or 5 mM ethephon (Et). 24 h later they were inoculated in vitro with *P. capsici* and the disease symptoms were periodically evaluated. Control seedlings were sprayed with water

Table 2 Effect of exogenous application of ethylene on the severity of Phytophthora blight in Habanero pepper

Dpi[a]	Treatment[b]	Disease severity[c] (% seedlings)			
		–	+	++	++++
3	W	0	10	90	0
	Eth	60	40	0	0
	Ag+Eth	0	20	80	0
10	W	0	0	0	100
	E	30	10	30	30
	A+E	0	0	0	100
20	W	0	0	0	100
	E	20	0	10	70
	A+E	0	0	0	100

[a] Days post inoculation

[b] Treatments in pepper seedlings consisted of spraying with water (W), ethephon (Eth) or $AgNO_3$ and ethephon (Ag+Eth) prior to the inoculation with *P. capsici*

[c] Severity level of the infection was denoted as: (–), no lesion was observed; (+), only one of the two inoculated leaves was necrotized; (++), the two inoculated leaves were necrotized; (++++), the whole plant was necrotized and mycelium was profusely growing

lation of ET reached a maximum between 3 and 4 dpi and then decreased gradually. In seedlings that were mock inoculated (Fig. 4, open boxes) or treated with 300 μM $AgNO_3$ (Fig. 4, open diamonds), there was no accumulation of ET during the whole observation period (0 to 5 d).

Toxic effects of ethephon or silver nitrate were not responsible for the inhibition of the mycelium growth *in planta*

To test whether toxic effects of the treatments with ethephon or $AgNO_3$ halted the mycelium spread *in planta*, the oomycete was cultivated on PDA plates directly in the presence of 300 μM $AgNO_3$ and/or 5 mM ethephon. The addition of both chemicals to the culture medium, alone or in combination, delayed the growth of *P. capsici* dramatically, especially when added together, yet the growth was not completely inhibited (Fig. 5). Although the in vitro effect of ethephon might correlate with its capacity to block the establishment of the disease, the effects of $AgNO_3$ and the combination of $AgNO_3$ plus ethephon are indeed contrary to the results obtained *in planta*, as the mycelium spreading was optimal in seedlings sprayed with $AgNO_3$ and ethephon (compare Figs. 2 and 3 with Fig. 5). Thus, we could discard the possibility that a toxic effect of the chemical treatment was responsible for inhibiting the growth of the pathogen.

The ethephon treatment modified the expression of defence-related genes in Habanero pepper seedlings

In previous studies, the inoculation of pepper seedlings with *P. capsici* induced the expression of two MAPK genes (CcMPK1 and CcMPK2), whereas the expression of several defence-related genes was either not modified, like NPR1 and WRKY, or even slightly reduced, like PR10 and esterase homologues (Nakazawa et al. 2010). To gain insight into the regulation of the defence pathway induced by ET, we then performed qRT-PCR to quantify the transcript levels of the PR1 and PDF1.2 genes, which are molecular markers for SAR and ISR, respectively. Both genes were induced by ethephon, but the expression of PDF1.2 was even higher when the ET perception was blocked, which suggests a non-specific effect (Fig. 6). Induction of PR1 occurred long after the ethephon spraying (48 to 72 h). ET specifically induced the ACC oxidase gene (used as a marker for ET perception), but it did not modify the expression of NPR1 (Fig. 6).

The over-expression of PR10 and esterase genes in Habanero pepper seedlings prevented their infection by *P. capsici*

To determine if the overexpression of defence-related genes could have an effect on the resistance against PB, we transformed one-month-old Habanero pepper seedlings with PR10 and esterase genes and evaluated the response at different times after their inoculation in vitro with *P. capsici*. In pepper seedlings transiently transformed with the empty binary vector (pCAMex), the mycelium dispersed throughout the seedling between 3 and 4 dpi (data not shown). At five days, the mycelium not only had grown massively but was also dispersed throughout the culture medium (Fig. 7). Conversely, transformation with either the esterase or the PR10 gene rendered the transgenic seedlings completely resistant to the oomycete

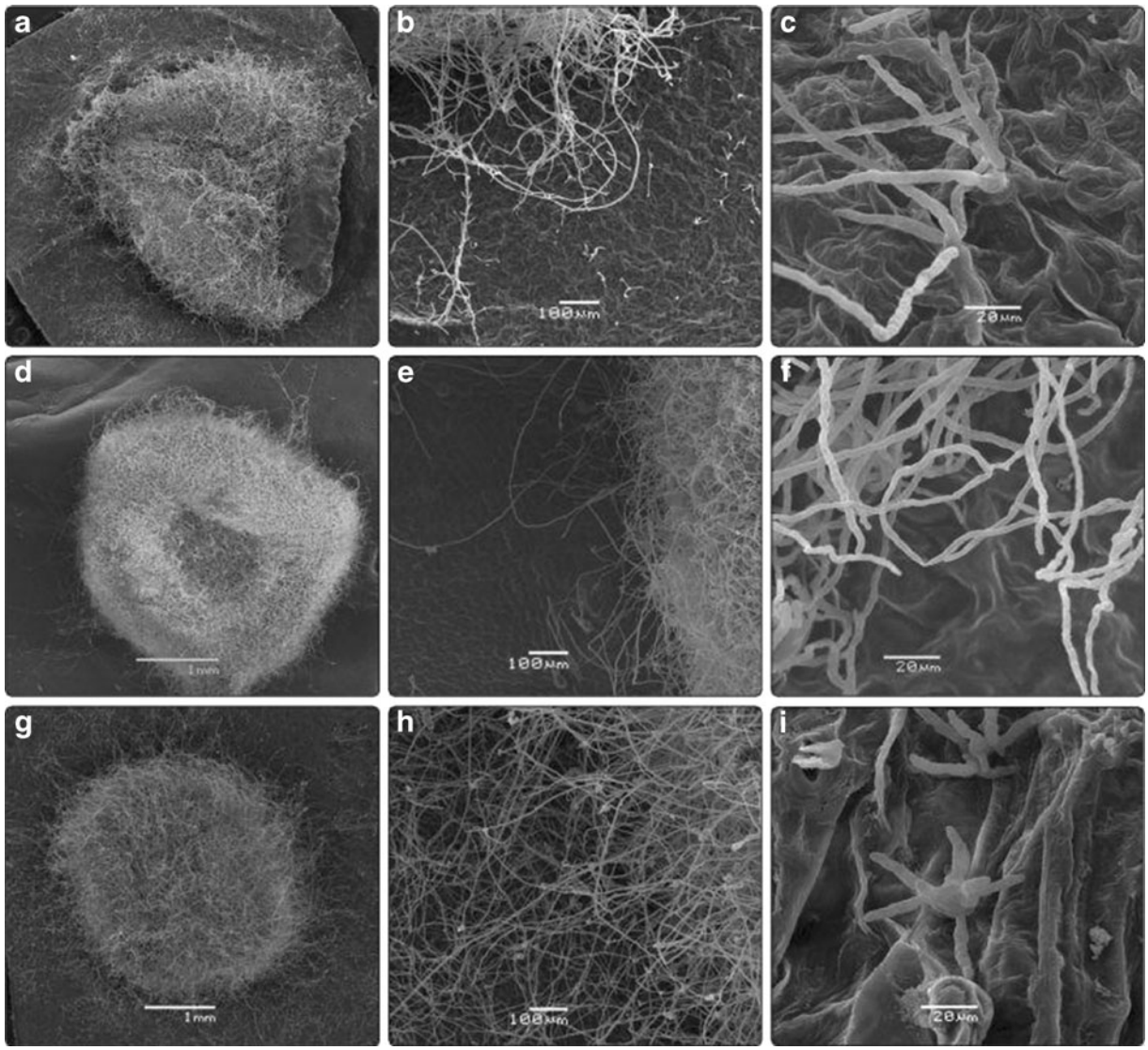

Fig. 3 Effect of ethylene on the growth of *Phytophthora capsici* on the Habanero peppers leaves. Inoculated leaves of seedlings from the different treatments were detached and observed by scanning electron microscopy. Pepper leaves inoculated with *P. capsici* (**a–c**), pepper leaves sprayed with 5 mM ethephon, and then inoculated with *P. capsici* (**d–f**), pepper leaves sprayed with 300 μM $AgNO_3$ and 5 mM ethephon, and then inoculated with *P. capsici* (**g–i**). Bars= 1 mm (**a, d, g**), 100 μm (**b, e, h**) or 20 μm (**c, f, i**), respectively

(Fig. 7), even 10 dpi. This behaviour was consistent in three independent experiments.

Discussion

With few exceptions (Gil-Ortega et al. 1991), most of the species within the genus Capsicum are susceptible to the lethal disease Phytophthora Blight. Different strategies have been used in attempts to dissect the genetic nature of the resistance, to investigate its biochemical and molecular regulation, and to identify the hormonal signalling network involved in the resistance response. A different approach, which has been used to study the regulation of defence against PB, involves the use of biotic and abiotic elicitors to induce systemic resistance in susceptible pepper cultivars (Ahmed et al. 2000; Baysal et al. 2005). However, despite the efforts to understand the basis of the compatibility between pepper species and *P.*

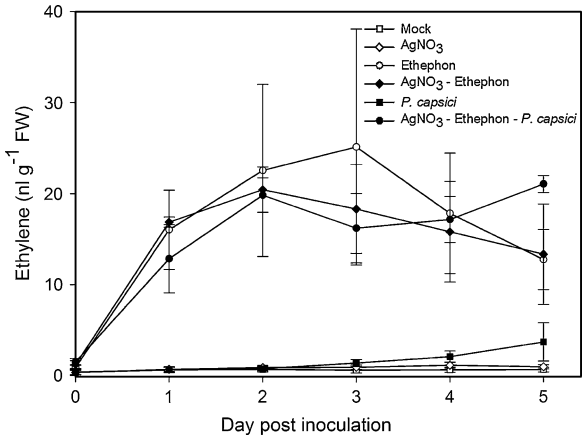

Fig. 4 Accumulation of ethylene in Habanero pepper seedlings inoculated in vitro with *Phytophthora capsici*. One-month-old seedlings were sprayed with water, 300 μM $AgNO_3$, 5 mM ethephon, and then mock inoculated or inoculated with *P. capsici*. All treatments were sequentially applied in 24-h intervals. After each treatment, 1 ml of the air in the jar was periodically sampled and analised by gas chromatography. Seedlings were mock inoculated (*white square*) (two PDA plugs were put on the leaves), sprayed with $AgNO_3$ (*white diamond*), sprayed with ethephon (*white circle*), sprayed with $AgNO_3$ and ethephon (*black diamond*), sprayed with water and inoculated with *P. capsici* (*black square*), or sprayed with $AgNO_3$ and ethephon and inoculated with *P. capsici* (*black circle*). Each point is the mean of three independent experiments with a single seedling. Bars represent standard errors of the means

capsici, the mechanisms by which this pathogen blocks the defence response are not clear.

In the present work, we carried out PB challenge experiments with pepper seedlings in vitro based on two general considerations. First, we wanted to avoid the interference of biotic or abiotic environmental stresses, as a casual induction of systemic responses could mask the results. Second, because there is a direct correlation between the age of the pepper plants and their response to PB (Kim et al. 1989), it is possible that physical barriers and the history of the plant's interaction with its environment contribute to the resistant phenotype; thus, we believed that the inoculation of young seedlings could offer a clear cause-and-effect response.

The results revealed that in Habanero pepper seedlings cultivated and inoculated in vitro, neither SA nor MeJA substantially modified the normal progress of the disease. On the contrary, spraying with ET clearly induced a resistance response against PB, delaying the appearance of symptoms by more than 10 days and halting the infection in 30% of the seedlings. Even though more experiments are needed to determine the basis of this heterogeneous induction of defence against *P. capsici*, the irregular kinetics of

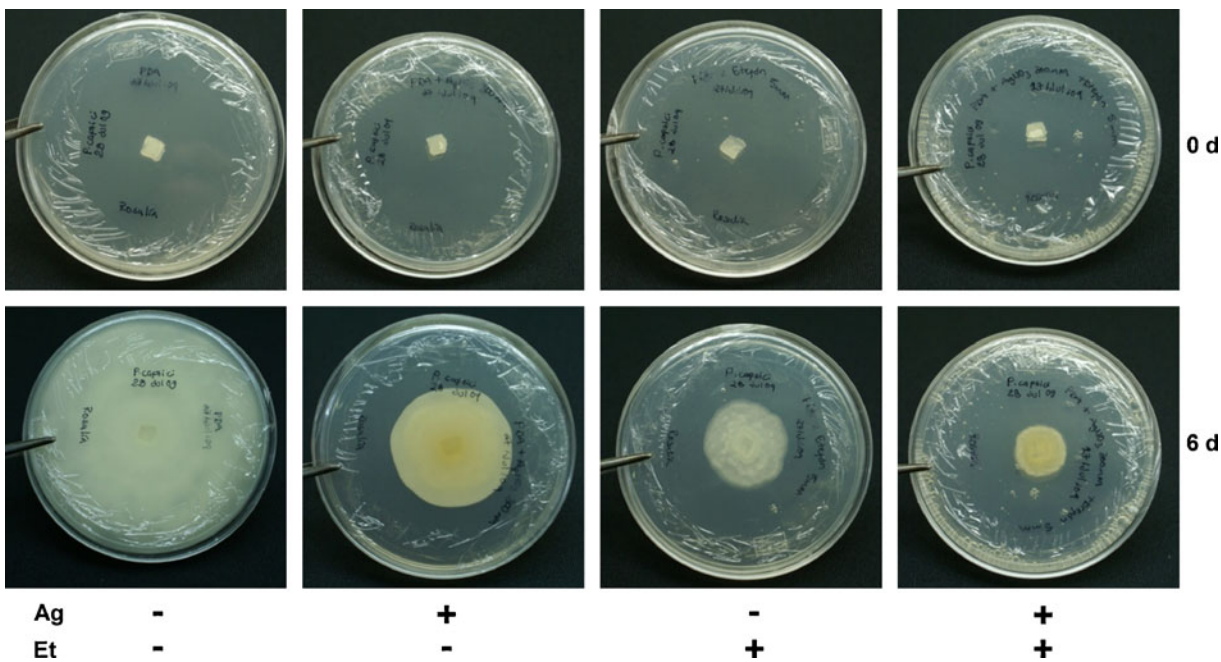

Fig. 5 Effect of $AgNO_3$ and ethephon on the *Phytophthora capsici* growth in vitro. Mycelium pieces (0.5 cm^2) were seeded onto PDA plates containing $AgNO_3$ (Ag) and/or ethephon (Et) and the growth was periodically monitored (0 and 6 days)

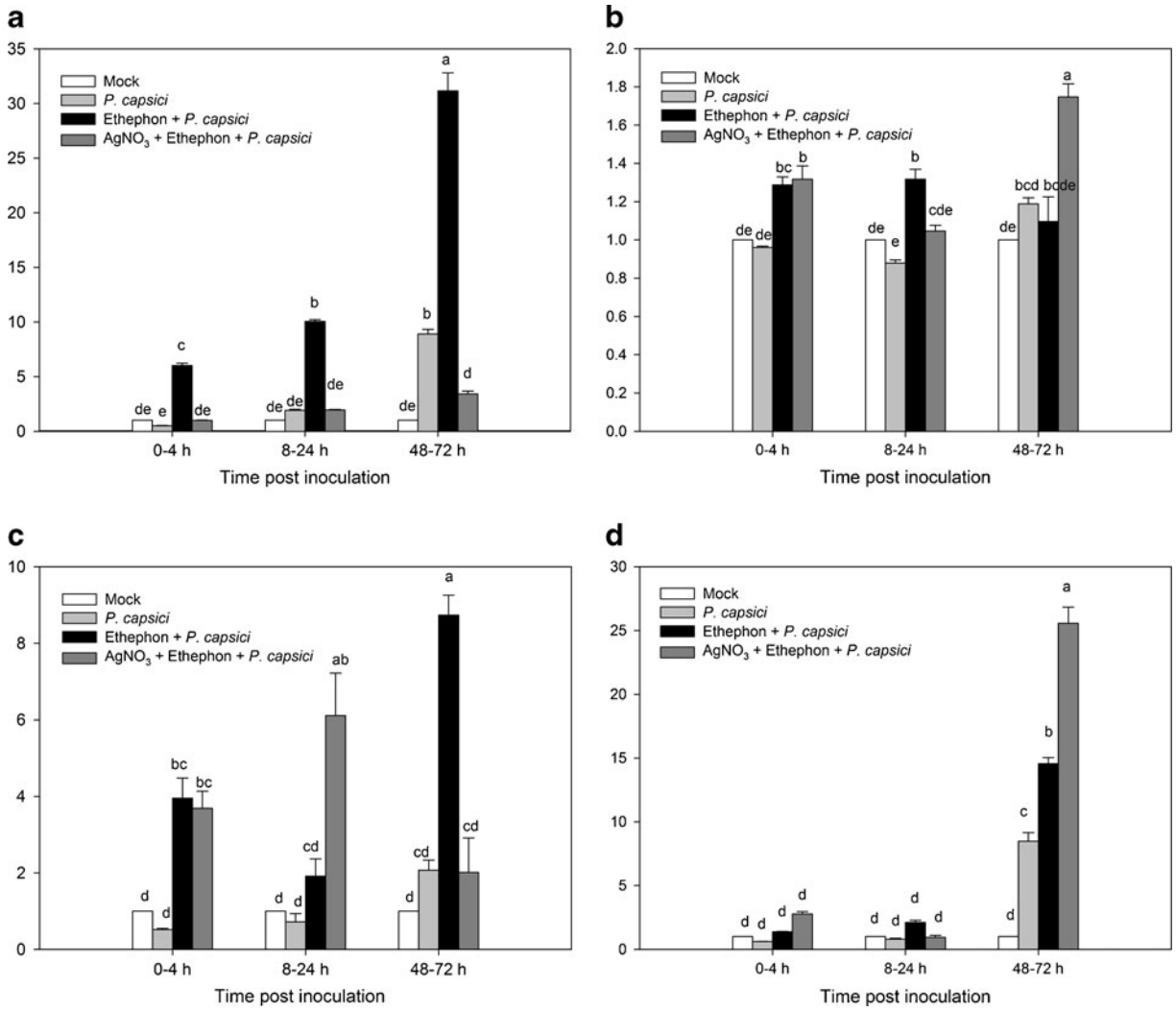

Fig. 6 Effect of ethylene on the gene expression of defence molecular markers. Three-microgram pools of cDNA from three seedlings under the different treatments were amplified by qPCR with gene-specific primers for ACC oxidase (A), NPR1 (B), PR1 (C) and PDF1.2 (D) genes. As control, seedlings were inoculated with an agar plug (mock). Fold changes in gene expression were based on the $2^{-\Delta\Delta Ct}$ method. Actin was used for normalization of each transcript. Vertical bars indicate standard errors from three independent amplifications. Columns with different letters differ significantly according to Tukey's multiple range test ($P \leq 0.05$)

the ET release after the spraying with ethephon (note the standard errors in Fig. 4) might have an important contribution to the resistance phenotype. In this respect, the existence of ET mutants in peppers would help to conduct clearer experiments. Nonetheless, the electron micrographs showed that whenever the ethephon treatment induced a resistance phenotype, a clear inhibition of both the mycelium penetration and dispersion was produced, yet the mycelium viability was not affected noticeably (Fig. 3d–f). The use of $AgNO_3$ to block the ethylene receptor confirmed an ethylene-specific effect in the host. These results positively suggest that an active plant response induced by ET inhibited the oomycete growth and its ability to penetrate the leaf surface, and it is possible that both responses were decisive to prevent tissue infection by *P. capsici*.

The Habanero pepper response to ET is consistent with other reports in which a positive effect of ET on the resistance of *Solanum lycopersicum* to *P. capsici* was reported (Francia et al. 2007). Interestingly, in this report, the authors found that ET had a negative

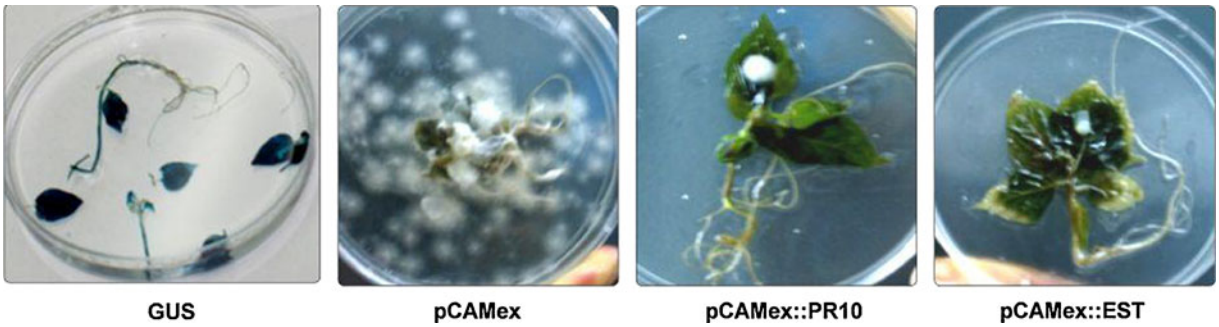

Fig. 7 Overexpression of resistance genes induced during systemic resistance in Habanero pepper seedlings. One-month-old seedlings transformed with defence genes PR10 (pCAMex::PR10) or esterase genes (pCAMex::EST) were inoculated with *Phytophthora capsici*, and the disease symptoms were observed after 5 days. As control, seedlings were transformed with the empty binary vector (pCAMex). Efficiency of transformation was evaluated in additional seedlings by the GUS histochemical assay (GUS)

effect against *Fusarium oxysporum*, which is in agreement with recent findings by our group indicating that spraying with ethephon reversed the natural resistance of Habanero peppers to a local strain of *F. oxysporum* (Núñez-Pastrana, unpubl.). These coincident results support our proposal that ET induced a systemic response against *P. capsici* and also suggest that the signalling networks of defence are conserved within the Solanaceae family.

In plants, a network of PGR coordinates the establishment of the main systemic responses. SA mediates the establishment of SAR during the plant interaction with pathogens, and ET and MeJA are involved in the development of ISR during the plant interaction with non-pathogenic microorganisms (Van Loon et al. 1998). Thus, the fact that ET induced a systemic response in Habanero pepper might imply the involvement of an ISR rather than a SAR response. This assumption is reinforced by the fact that the ET levels showed a modest increase within 4 dpi with *P. capsici*, when all seedlings were completely infected. Conversely, in the ethephon-treated seedlings, there was an almost ten-fold increase within the first 24 h after inoculation that rose to almost 20 fold and remained at that level for at least five days.

A qRT-PCR evaluation of defence gene markers was performed to analise the nature of the observed systemic response. The results revealed that the application of ET to Habanero pepper seedlings induced the expression of PDF1.2, a molecular marker for ET and MeJA. However, because the expression of this gene was higher in the $AgNO_3$-treated seedlings (that ultimately died), it is likely that PDF1.2 did not contribute to the resistance induced by ET. On the contrary, it induced the expression of PR1, a broadly accepted molecular marker for SAR. In addition, the ET treatment barely induced the expression of the NPR1 homologue; thus, the expression of PR1 seemed to be independent of NPR1. The pivotal role of NPR1 in switching between the ET, JA and SA signalling pathways relies on its cytosolic activation (Spoel et al. 2003); however, gene expression of NPR1 is also positively regulated during the activation of plant defence responses (Yu et al. 2001). In fact, PR1 has both NPR1-dependent and NPR1-independent expression (Shah et al. 2001). Furthermore, there are examples of the NPR1-independent induction of PR1 by ET (Kim and Hwang 2000). Additionally, the regulation of basic PR1 genes by ET has been demonstrated in different species, including *Nicotiana tabacum* (Eyal et al. 1993), and especially in *Arabidopsis thaliana*, where the promoters of PR1 genes have MeJA-responsive elements (Santamaria et al. 2001). In our study, the highest expression of PR1 occurred at relatively late times after inoculation (48 and 72 h). Thus, even though the ET treatment induced a systemic response, the NPR1-independent induction of PR1 would suggest the involvement of a SAR-like response, rather than a classical ISR. These results are in agreement with other reports in the Solanaceae family, where resistance against oomycetes depended on the late expression of PR1 (van't Klooster et al. 1999). The deduced amino acid sequence of the *Capsicum chinense* PR1 cDNA analysed in this study shares 97% identity with a

basic PR1 protein from *Solanum tuberosum* (Supplementary Material 2) (Hoegen et al. 2002).

In some cases, the expression of PR genes has been associated with the chemical induction of systemic resistance (Baysal et al. 2005). However, in others, such as *A. thaliana*, the use of PR genes as molecular markers has revealed that the resistance induced by BABA is independent of PRs (Jakab et al. 2001). We found that PR1 is induced by ET in coincidence with the establishment of a defence response. In addition, the overexpression of homologous PR and defence genes induced by ET (PR10 and esterase, Núñez-Pastrana, unpubl.) conferred to the Habanero peppers a resistance phenotype, which strongly supports the hypothesis that PR genes are important players in the ET-induced response. The PR10 gene is a member of a multigenic family consisting of relatively diverse members that are usually induced upon pathogen attack and environmental stresses, and these genes participate in developmental processes (Liu and Ekramoddoullah 2006). The deduced amino acid sequence of the Habanero pepper PR10 cDNA analysed in this study shares a 77% identity with that of a PR10 gene from *C. annuum* (Supplementary Material 2), whose transcripts were induced in its incompatible interactions with tobacco mosaic virus and *Xanthomonas campestris* pv. *vesicatoria*. This PR10 gene had ribonucleolytic enzyme activity (Park et al. 2004). In *C. annuum*, an esterase gene (PepEST) that is highly expressed during an incompatible interaction between ripe pepper fruit and the fungus *Colletotrichum gloesporioides* has been described. The over-expression of PepEST in transgenic Arabidopsis resulted in enhanced resistance to *Alternaria brassicicola* (Ko et al. 2005).

In recent years, evidence from Phytophthora species has indicated that pathogen effectors manipulate plant defence responses after their translocation into the host cytoplasm (Whisson et al. 2007). The members of the genus Phytophthora possess apoplastic and cytoplasmic effectors that are sufficient to trigger systemic resistance in their hosts (Kamoun et al. 1997). For instance, *P. capsici* possesses capsicein, a member of the α-class elicitins that has been shown to induce protection of tobacco against *P. nicotianae* (Ricci et al. 1989). *P. capsici* also possesses phospholipase-like proteins with significant similarity in their amino termini to capsicein (Nespoulous et al. 1999). Thus, although it is possible that peppers are able to recognise different effectors during their interaction with *P. capsici*, the oomycete must effectively block the establishment of a defence response. This hypothesis implies that if the correct signalling pathway is activated at the right time, the host systemic response should make the cell metabolism refractory to further manipulation by the pathogen. Our results demonstrated that in the *C. chinense-P. capsici* pathosystem, the previous application of ET activated a systemic response that prevented infection by the pathogen, probably through an NPR1-independent mechanism. Thus, it is possible that components of the signalling pathway activated by ET are targets of the oomycete effectors; this is supported by the fact that in Habanero pepper ET levels did not rise significantly during the normal course of the infection. In addition, because treatment with ET reversed the natural resistance of Habanero pepper to *F. oxysporum*, the response activated by ET could be specific to some pathogens, but it could also be antagonistic to the defence responses devoted to other pathogens.

Finally, although the role of JA has been suggested to be crucial in the establishment of the defence of *C. annuum* against *P. capsici* (Ueeda et al. 2006), the results from our model suggest that ET, rather than SA or JA, is the key phytohormone involved in restricting PB in Habanero pepper.

Acknowledgements The authors would like to thank Q. Angela Kú Gonzalez for technical assistance in obtaining the scanning electron micrographs, and Dr. Felipe Barahona Pérez for technical assistance in the ethylene measurements. This project had funding from CONACYT grant P48831. RNP and GFAO gratefully acknowledge CONACYT for the scholarships 208245 and 56153, respectively.

References

Ahmed, S. A., Pérez, C., & Candela, M. E. (2000). Evaluation of induction of systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. *European Journal of Plant Pathology, 106*, 817–824.

Arcos-Ortega, G. F., Chan-Kuuk, R. A., González-Kantún, W. A., Souza-Perera, R., Nakazawa-Ueji, Y. E., Avilés-Berzunza, E., et al. (2010). *Agrobacterium tumefaciens*-transient genetic transformation of Habanero pepper (*Capsicum chinense* Jacq.) leaf explants. *Electronic Journal of Biotechnology, 13*, 7–8.

Baysal, Ö., Turgut, C., & Mao, G. (2005). Acibenzolar-S-methyl induced resistance to *Phytophthora capsici* in pepper leaves. *Biologia Plantarum, 49*, 599–604.

Beyer, E. M. (1976). A potent inhibitor of ethylene action in plants. *Plant Physiology, 58*, 268–271.

Clarke, J. D., Liu, Y., Klessig, D. F., & Dong, X. (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. *The Plant Cell, 10*, 557–569.

Eyal, Y., Meller, Y., Lev-Yadun, S., & Fluhr, R. (1993). A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. *The Plant Journal, 4*, 225–234.

Francia, D., Demaria, D., Calderini, O., Ferraris, L., Valentino, D., Arcioni, S., et al. (2007). Wounding induces resistance to pathogens with different lifestyles in tomato: role of ethylene in cross-protection. *Plant, Cell & Environment, 30*, 1357–1365.

Gil-Ortega, R., Palazón-Español, C., & Cuartero-Zueco, J. (1991). Genetics of resistance to *Phytophthora capsici* in the Mexican pepper SCM-334. *Plant Breeding, 107*, 50–55.

Hoegen, E., Strömberg, A., Pihlgren, U., & Kombrink, E. (2002). Primary structure and tissue-specific expression of the pathogenesis-related protein PR-1b in potato. *Molecular Plant Pathology, 3*, 329–345.

Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J. P., et al. (2001). B-Aminobutiric acid-induced resistance in plants. *European Journal of Plant Pathology, 107*, 29–37.

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology, 44*, 41–60.

Kamoun, S., Lindqvist, H., & Govers, F. (1997). A novel class of elicitin-like genes from *Phytophthora infestans*. *Molecular Plant-Microbe Interactions, 10*, 1028–1030.

Kim, Y. J., & Hwang, B. K. (2000). Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiologia Plantarum, 108*, 51–60.

Kim, Y. J., Hwang, B. K., & Park, K. W. (1989). Expression of age—related resistance in Pepper plants infected with *Phytophthora capsici*. *Plant Disease, 73*, 745–747.

Ko, M. K., Jeon, W. B., Kim, K. S., Lee, H. H., Seo, H. H., Kim, Y. S., et al. (2005). A *Colletotrichum gloeosporioides*-induced esterase gene of nonclimacteric pepper (*Capsicum annuum*) fruit during ripening plays a role in resistance against fungal infection. *Plant Molecular Biology, 58*, 529–541.

Kreutzer, W. A., Bodine, E. W., & Durrell, L. W. (1940). Cucurbit diseases and rot of tomato fruit caused by *Phytophthora capsici*. *Phytopathology, 30*, 972–976.

Lawton, K. A., Potter, S. L., Uknes, S., & Ryals, J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. *The Plant Cell, 6*, 581–588.

Leonian, L. H. (1922). Stem and fruit blight of pepper caused by *Phytophthora capsici* species nov. *Phytopathology, 12*, 401–408.

Liu, J. J., & Ekramoddoullah, A. K. M. (2006). The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. *Physiological and Molecular Plant Pathology, 68*, 3–13.

Nakazawa-Ueji, Y. E., Núñez-Pastrana, R., Souza-Perera, R. A., Santana-Buzzy, N., & Zúñiga-Aguilar, J. J. (2010). Mycelium homogenates from a virulent strain of *Phytophthora capsici* promote a defence-related response in cell suspensions from *Capsicum chinense*. *European Journal of Plant Pathology, 126*, 403–415.

Nespoulous, C., Gaudemer, O., Huet, J. C., & Pernollet, J. C. (1999). Characterization of elicitin-like phospholipases isolated from *Phytophthora capsici* culture filtrate. *FEBS Letters, 452*, 400–406.

Palloix, A., Daubeze, A. M., Phaly, T., & Pochard, E. (1990). Breeding transgresive lines of pepper for resistance to *Phytophthora capsici* in a recurrent selection system. *Euphytica, 51*, 141–150.

Park, C. J., Kim, K. J., Shin, R., Park, J. M., Shin, Y. C., & Paek, K. H. (2004). Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *The Plant Journal, 37*, 186–198.

Pieterse, C. M. J., & Van Loon, L. C. (1999). Salicylic acid-independent plant defence pathways. *Trends in Plant Sciences, 4*, 52–58.

Ricci, P., Bonnet, P., Huet, J. C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., et al. (1989). Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. *European Journal of Biochemistry, 183*, 555–563.

Ristaino, J. B., Larkin, R. P., & Campbell, C. L. (1993). Spatial and temporal dynamics of *Phytophthora* epidemics in commercial bell pepper fields. *Phytopathology, 83*, 1312–1320.

Santamaria, M., Thomson, C. J., Read, N. D., & Loake, G. J. (2001). The promoter of a basic PR1-like gene, *AtPRB1*, from *Arabidopsis* establishes an organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate. *Plant Molecular Biology, 47*, 641–652.

Shah, J., Kachroo, P., Nandi, A., & Klessig, D. F. (2001). A recessive mutation in the *Arabidopsis SSI2* gene confers SA- and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. *The Plant Journal, 25*, 563–574.

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defence pathways through a novel function in the cytosol. *The Plant Cell, 15*, 760–770.

Sugita, T., Yamaguchi, K., Kinoshita, T., Yuji, K., Sugimura, Y., Nagata, R., et al. (2006). QTL analysis for resistance to Phytophthora blight (*Phytophthora capsici* Leon.) using an intraspecific doubled-haploid population of *Capsicum annuum*. *Breeding Science, 56*, 137–145.

Sunwoo, J. Y., Lee, Y. K., & Hwang, B. K. (1996). Induced resistance against *Phytophthora capsici* in pepper plants in response to DL-B-amino-n-butyric acid. *European Journal of Plant Pathology, 102*, 663–670.

Ueeda, M., Kubota, M., & Nishi, K. (2006). Contribution of jasmonic acid to resistance against Phytophthora blight in *Capsicum annuum* cv. SCM334. *Physiological and Molecular Plant Pathology, 67*, 149–154.

Van Loon, L. C., Bakker, P. A. H. M., & Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology, 36*, 453–483.

Van't Klooster, J. W., Vleeshouwers, V. G. A. A., Kamoun, S., & Govers, F. (1999). Characterization of a cDNA encoding a pathogenesis-related protein PR1 from potato (*Solanum tuberosum*) (Accession No. AJ250136) (PGR99-182). *Plant Physiology, 121*, 1384.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J., Gilroy, E. M., et al. (2007). A translocation signal for delivery of oomycete effector proteins inside host plant cells. *Nature, 450*, 115–118.

Yu, D., Chen, C. & Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *The Plant Cell, 13*, 1527–1539.