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Elicitation of resistance responses in grapefruit and lemon fruits treated with a pomegranate peel extract

S. Pangallo[a], M. G. Li Destri Nicosia[a], G. Raphael[b], E. Levin[b], G. Ballistreri[c], S. O. Cacciola[d], P. Rapisarda[c], S. Droby[b] and L. Schena[a]*

[a]*Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, Località Feo di Vito, Reggio Calabria 89122, Italy;* [b]*Department of Postharvest Science, ARO, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel;* [c]*Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA) – Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee, Corso Savoia 190, Acireale (CT) 95024; and* [d]*Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi, Via S. Sofia 100, 95123 Catania, Italy*

The defence responses of grapefruit and lemon to treatment with pomegranate extract (PGE) were investigated. PGE, an alcoholic extract from pomegranate peel, was recently proposed as a means of effective alternative control against postharvest rots. In *in vivo* experiments, a significant reduction of rots caused by *Penicillium digitatum* and *P. italicum* was achieved on artificially inoculated fruits without direct contact between PGE and pathogens. On lemons both pathogens were completely inhibited by PGE at 12 g dry matter L^{-1} applied 12 and 24 h before the pathogen but a significant reduction of rots was also achieved by inoculating the pathogen immediately after PGE (0 h), indicating a very quick activation of defence responses. Lower, but significant, reductions were also obtained on grapefruits. An increase in reactive oxygen species (ROS) activity, reaching its peak after 24 h, was observed, in agreement with *in vivo* efficacy trials. Similarly, the expression of five genes involved in activation of defence responses in plants (*CHI*, *CHS*, *MAPK*, *MAPKK* and *PAL*) increased following PGE application. Based on the results of the present study, the high efficacy demonstrated for PGE in previous studies can be partially attributed to the induction of resistance in host tissues.

Keywords: citrus, induction of resistance, *Penicillium* spp., pomegranate peel extract, postharvest rots

Introduction

In recent years, several plant extracts have been proposed as effective alternative control means against fungal postharvest pathogens (Gatto *et al.*, 2011; da Cruz Cabral *et al.*, 2013). In particular, an alcoholic extract (PGE) obtained from the peel of pomegranate (*Punica granatum*) proved to be effective against infection and development of postharvest rots of citrus fruit and sweet cherries (Li Destri Nicosia *et al.*, 2016). According to this study, PGE possesses several important features that enable its implementation in postharvest control strategies as a natural, safe and ecofriendly extract. PGE was characterized by a strong direct antimicrobial effect and, when applied on different fruits, provided curative and preventive activity with long persistence, a wide spectrum of activity and high efficacy under different conditions (Li Destri Nicosia *et al.*, 2016). Furthermore, the absence of signs of possible phytotoxicity and the wide availability of pomegranate peel as a waste product of processing factories suggested its possible application on a large scale (Li Destri Nicosia *et al.*, 2016).

*E-mail: lschena@unirc.it

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The persistence of the effect of PGE against postharvest rots has been demonstrated by its application 12 or 24 h before pathogen inoculation, and is related to its high phenolic content, suggesting possible induction of resistance in the host (Romeo *et al.*, 2015). Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. They have received a great deal of attention due to their diverse biological functions including a well-documented antimicrobial activity (Ahmad & Beg, 2001; Lee *et al.*, 2007; Naz *et al.*, 2007; Slusarenko *et al.*, 2008; Verástegui *et al.*, 2008). Besides the direct antimicrobial activity, phenolic compounds play a major role in plant constitutive and induced resistance to pathogens, and this property has also been exploited to achieve fruit protection during the postharvest phase (Sanzani *et al.*, 2010, 2014). The exogenous application of the flavonoid quercetin did not show a direct *in vitro* effect against *Penicillium expansum* but significantly reduced rots on apples caused by it, suggesting the induction of natural host defence responses (Sanzani *et al.*, 2009). Early studies demonstrated that chlorogenic acid is involved in resistance of potato tubers against *Phytophthora infestans*, *Streptomyces scabies* and *Verticillium albo-atrum* (Johnson & Schaal, 1952). Similarly, catechol and protocatechuic acid induced resistance of red onion to *Colletotrichum circinans* (Link *et al.*, 1929; Walker & Stahmann, 1955). Moreover, an increased level of antifungal phenols at the

infection site as a consequence of increased activity of the enzyme phenylalanine ammonia lyase (PAL) has been demonstrated for several host–pathogen combinations (Lachhab *et al.*, 2014; Fallanaj *et al.*, 2016).

The activation of resistance responses, associated with the use of alternative control means, is considered to be the preferred approach in integrated control strategies against postharvest diseases (Ippolito *et al.*, 2000; Droby *et al.*, 2002). During ripening and senescence of fruit and other vegetative plant parts after harvest, disease resistance generally declines leading to increased susceptibility to pathogen attack. Hence, the use of resistance inducers may be useful to restore an acceptable level of resistance. In this regard, a variety of physical and biological elicitors are capable of inducing resistance responses, but the nature of responses to different elicitors is largely unknown (Wilson & Wisniewski, 1994; Anwer, 2015). The use of plant extracts as inducers of resistance is a developing and very promising field of research in plant protection (Song *et al.*, 2013; Benouaret *et al.*, 2014).

Aspects of induced natural defence responses that are important to its possible use in postharvest plant protection include the speed, magnitude and timing of the response in relation to pathogen inoculum load and the maturity of the produce. In this regard, one of the first cellular responses to early infection processes is the production of reactive oxygen species (ROS), via consumption of oxygen in a so-called 'oxidative burst' (Torres *et al.*, 2006). ROS have been reported to mediate defence gene activation by interaction with other signalling components such as mitogen-activated protein kinase (MAPK) cascades (Zhang & Klessig, 2001). Moreover, ROS can mediate the generation of phytoalexins and other secondary metabolites involved in arresting the growth of pathogens (Torres, 2010). The plethora of secondary metabolism products related to the defence response includes pathogenesis-related proteins (PR proteins), which are host proteins produced in response to attack by pathogens or by a related event and which constitute a marker of systemic acquired resistance (SAR) (Ryals *et al.*, 1994; van Loon *et al.*, 2006).

The aim of the present study was to investigate defence responses elicited by PGE applied to citrus fruit after harvest. *In vivo* assays, providing spatial separation of pathogens and PGE, were performed on artificially inoculated fruit to determine the magnitude and practical relevance of the induced resistance in the citrus fruit–*Penicillium* sp. pathosystem. Furthermore, the ROS response and the expression of genes known to be involved in plant defence reactions were examined in PGE-treated tissues with and without pathogens.

Materials and methods

Fruits, pathogens and pomegranate extract

All experiments were conducted with freshly harvested organic lemons (cv. Monachello) and grapefruits (cv. Sunrise). Fruit of uniform size and maturity were surface sterilized by immersion in a 2% sodium hypochlorite solution for 1 min and washed twice with tap water. After a couple of hours of air-drying, fruits were fixed on polypropylene honeycomb panels using double-sided sticky tape. Fruits were maintained at room temperature (22–24°C) in closed plastic boxes containing wet paper to ensure high relative humidity (RH).

Fungal isolates of *Penicillium digitatum* and *Penicillium italicum* were obtained from decayed lemons and used to prepare suspensions of conidia as previously described (Li Destri Nicosia *et al.*, 2016).

The extract of pomegranate peel (PGE) was obtained from ripe pomegranate fruits as previously reported (Romeo *et al.*, 2015). The stock solution contained 120 g dry matter L^{-1}; 1% citric acid was added as antioxidant. The PGE was stored at 5°C until use.

In vivo experiments to evaluate the induction of resistance

Surface-sterilized fruit were wounded with a sterile needle (2 mm diameter) to have three equidistant 2 mm deep wounds in the equatorial zone. Each wound was treated with 10 μL of diluted pomegranate extract (PGE) containing 12, 1.2 or 0.12 g dry matter L^{-1}. Wounds treated with 1% citric acid served as mock controls. After 0, 12 or 24 h (lemons) and 6 or 24 h (grapefruits), another identical series of wounds was made approximately 5 mm from the previous ones and inoculated with 10 μL of a spore suspension containing 5×10^4 conidia mL^{-1}. Lemons were inoculated with *P. digitatum* or *P. italicum*, while grapefruits were only inoculated with *P. digitatum*.

Incidence of decay (percentage of infected wounds) and disease severity (diameter of the lesions) were evaluated 4 days after pathogen inoculation. All experiments contained three replicates of five fruits, with each replicate kept in a separate plastic box. Each experiment was repeated at least twice. The data were submitted to analysis of variance (ANOVA) and means were compared using Duncan's test ($P < 0.05$) to determine if treatments were significantly different. Percentages were converted into Bliss angular values (arcsin $\sqrt{\%}$) before analysis.

Quantification of ROS

Surface-sterilized grapefruits were wounded and treated as described before with 10 μL of PGE at 12 g dry matter L^{-1}. Fruits treated with citric acid (1%) or sterile water were used as controls. After 6, 24 and 48 h, small disks of peel (10×10 mm) were removed from around the wounds with a scalpel and stained with dichlorofluorescein (DCF) for 10 min in the dark to avoid light-induced oxidation of the dye. The slices were then removed, floated on fresh phosphate-buffered saline (PBS) to wash off excess dye, affixed to a glass slide and examined with an inverted laser-scanning confocal microscope (Fluoview 500; Olympus Ix 81) equipped with a 488 nm argon-ion laser. The intensity of the fluorescence signal was estimated by calculating the average pixel intensity, using the confocal microscope's image analysis system. For each treatment and assessment time, the intensity of fluorescence was evaluated on three peel discs from four different fruits.

Expression of resistance genes in PGE-treated grapefruit

The relative expression of key genes involved in resistance was evaluated in grapefruit peel tissue treated with PGE, i.e.

chitinase (*CHI*), chalcone synthase (*CHS*), mitogen-activated protein kinase and mitogen-activated protein kinase kinase (*MAPK* and *MAPKK*, respectively) and phenylalanine ammonia lyase (*PAL*).

Surface-sterilized fruit were wounded at five equidistant sites in the equatorial zone and 10 μL of PGE at 12 g dry matter L^{-1} was pipetted into each wound. Wounds treated with water or 1% citric acid served as controls. The same experiment was repeated in the presence of the pathogen by adding 10 μL of a suspension containing 5×10^4 conidia of *P. digitatum* to wounds already treated with PGE, water or citric acid (controls). After 0, 6, 24 and 48 h wound sites (3 × 3 mm) were removed with a scalpel under sterile conditions, immediately frozen in liquid nitrogen and stored at −80 °C. All experiments were performed using three biological replications and each replicate consisted of five wounds, collected from a single fruit.

RNA extraction

Frozen tissue was ground in liquid nitrogen with a mortar and pestle. RNA was extracted from 30 mg of ground tissue using the SV Total RNA Isolation System kit (Promega) according to the manufacturer's instructions. Nucleic acids were treated with the DNA-free DNA Removal kit (Invitrogen) to remove DNA according to the manufacturer's instructions. The quality and concentration of RNA was evaluated by gel electrophoresis and with a ND1000 spectrophotometer (NanoDrop). Purified RNA samples were stored at −80 °C.

Relative expression of target genes

Total RNA (1 μg) was used to synthesize cDNA using the Verso cDNA Synthesis kit (Thermo Scientific) and subjected to quantitative PCR (qPCR) to determine the relative expression of the selected genes. Reactions were carried out in a total volume of 20 μL containing 1 μL cDNA, 5 μL ABsolute qPCR SYBR Green ROX mix (ABgene) and 0.3 μM forward and reverse primers as described by Hershkovitz *et al.* (2012). Amplifications were conducted in MicroAmp 96-well plates (Applied Biosystems) and consisted of 15 min at 95 °C; then 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. The relative expression of genes was calculated according to the comparative C_t method (Livak & Schmittgen, 2001), with actin used as an internal standard (Hershkovitz *et al.*, 2012).

Results

In vivo assays on artificially inoculated fruits

On lemons, PGE at 12 g L^{-1} completely inhibited the development of rot when applied 12 and 24 h before inoculation with *P. italicum* and *P. digitatum* (Fig. 1b,c). However, when inoculation with *P. italicum* and *P. digitatum* was performed immediately after the application of PGE (0 h), inhibition percentage of decay was 23% and 77%, respectively (Fig. 1a). A lower, but significant, reduction of decay was also achieved with PGE at 1.2 g L^{-1} (against both pathogens) and at 0.12 g L^{-1} (against *P. digitatum* only). The highest efficacy was observed when the pathogens were inoculated 24 h after treatment with PGE as compared to 0 and 12 h. In general, higher efficacy was demonstrated against *P. digitatum* as compared to *P. italicum* (Fig. 1).

A lower efficacy of PGE was found in trials with grapefruits compared to lemons, although a significant reduction of the incidence of decay was demonstrated at PGE concentrations of 12 and 1.2 g L^{-1} (Fig. 2). In particular, the incidence of *P. digitatum* decay was reduced by 68% and 31% (Fig. 2a) and by 60% and 32% (Fig. 2b) when PGE was applied 6 and 24 h before the pathogen, respectively. No significant reductions were found using PGE at the lowest concentration (0.12 g L^{-1}).

Quantification of ROS

Reactive oxygen species activity was quantified by measuring fluorescence after staining grapefruit peel with DCF (Fig. 3). At 6 h after PGE application, the fluorescence signal around wounds was significantly higher in PGE-treated fruits compared to the water control but not significantly different to that found in the citric acid control. After 24 h, the fluorescence signal was about 5-fold greater in treated fruits compared to the controls (water and citric acid) at 24 h and remained significantly higher at 48 h, although at a lower level.

Analysis of gene expression in grapefruits treated with PGE

On grapefruits treated with PGE but not inoculated with the pathogen, the expression of most investigated genes (*CHI*, *CHS*, *MAPKK* and *PAL*) was up-regulated at 6 h following the treatment (Fig. 4, left column); *MAPK* was the single exception. At 6 h the up-regulation was particularly evident for *MAPKK* with an expression level about 40 times higher than the water control. Significant levels of overexpression were also observed for *MAPKK* at 0 h, *PAL* at 48 h and *CHS* at 24 and 48 h.

A different response was observed in fruits treated with PGE and inoculated with *P. digitatum* (Fig. 4, right column). The presence of the pathogen generally delayed the fruit response: only two genes (*MAPK* and *MAPKK*) were slightly up-regulated at 6 h, while all genes showed a significantly higher expression after 24 h. On the other hand, the presence of the pathogen determined a generally much higher gene expression for *PAL* and *MAPK*. This up-regulation was particularly evident at 24 and 48 h but a significant increase of expression was also revealed at 0 h (*PAL*) and 6 h (*MAPK*). *CHS* and *CHI* were also overexpressed at 0 and 48 h, respectively.

Discussion

The results of the present study demonstrated that PGE applied to citrus fruit activated the defence response in peel tissues. In *in vivo* experiments, a significant reduction of rots caused by *P. digitatum* and *P. italicum* was achieved when PGE was applied to wounds near the inoculation point, without direct contact with the pathogens. This represents indirect but clear evidence that induction of resistance mechanisms is taking place in

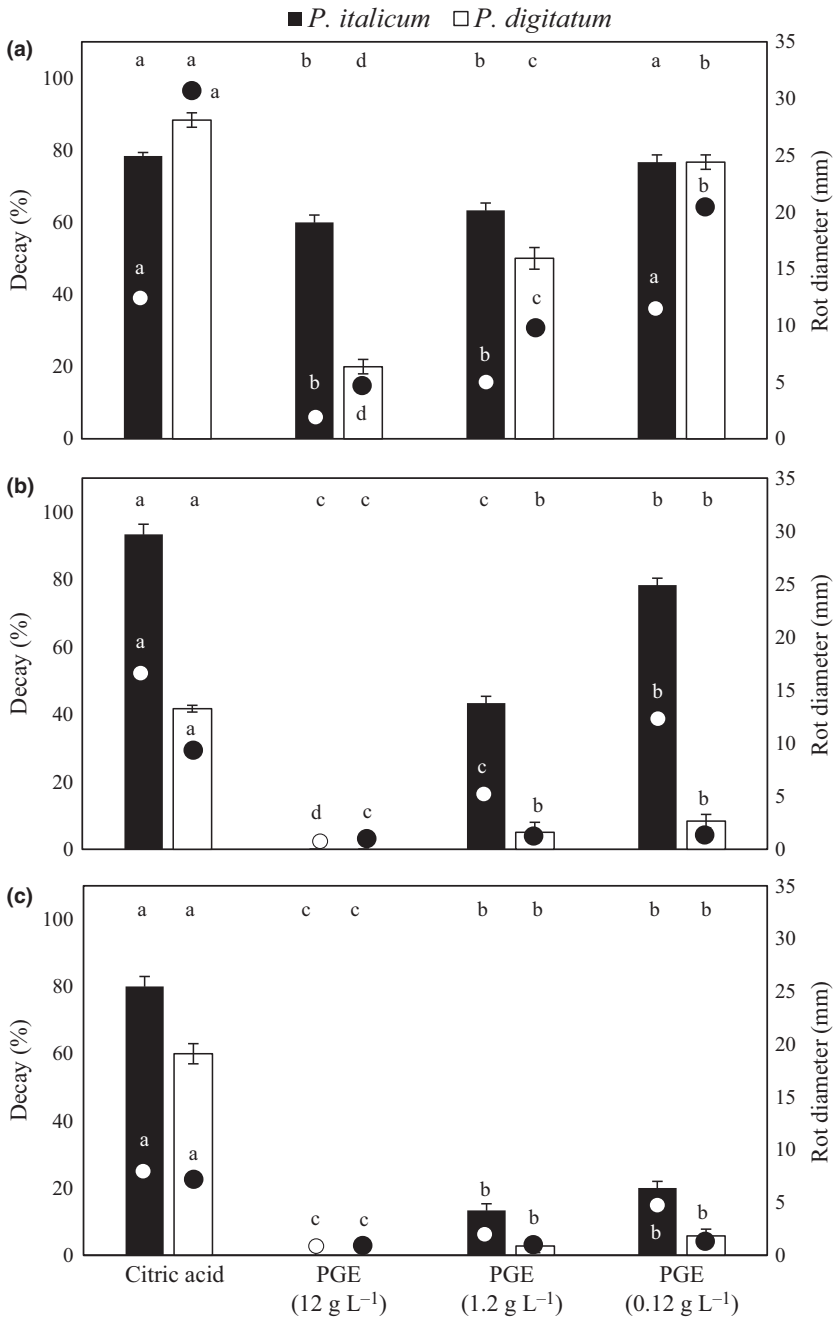

Figure 1 Incidence of decay (columns) and rot diameter (circles) in lemons treated with pomegranate peel extract (PGE) and inoculated with *Penicillium italicum* or *P. digitatum*, in spatially separated wounds. PGE was applied at different concentrations (12, 1.2 or 0.12 g dry matter L^{-1}) immediately (a), or 12 h (b) and 24 h (c) before the pathogens. Fruit treated with a 1% solution of citric acid and inoculated with the pathogens served as controls. Bars indicate standard errors of the means. Different letters indicate significantly different values according to Duncan's test ($P \leq 0.05$). Letters at the top of the figure or by the circles refer to incidence of decay or rot diameter, respectively.

PGE-treated tissue and is responsible for the inhibition of pathogen development and infection of the fruit (Zhu *et al.*, 2010). This effect was demonstrated on both lemons and grapefruits, with a higher level of protection achieved on lemons than on grapefruits. On lemons, *P. italicum* and *P. digitatum* were completely inhibited by the application of 12 g dry matter L^{-1} PGE at 12 or 24 h before inoculation of the pathogen adjacent to the point of PGE application, but a significant reduction of decay was also achieved by inoculating the pathogen immediately after PGE treatment, indicating a quick activation of defence responses in fruit peel tissue. Furthermore, significant reductions of decay were also obtained using PGE diluted to 1.2 g L^{-1} or 0.12 g L^{-1}. On grapefruits, a significant reduction of decay was achieved when 12 g L^{-1} or 1.2 g L^{-1} PGE was applied 6 or 24 h prior to inoculation. However, none of the investigated concentrations and times of application permitted complete inhibition of decay, while the lowest PGE concentration (0.12 g L^{-1}) did not have a significant impact on pathogen infection and development. Overall, these results highlight the complexity of mechanisms involved in the induced resistance, which was modulated by PGE concentration, time of application

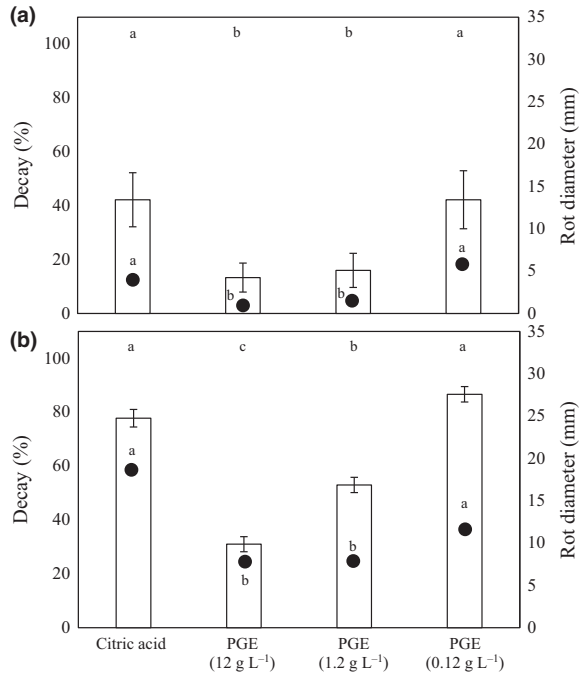

Figure 2 Incidence of decay (columns) and rot diameter (circles) in grapefruits treated with pomegranate peel extract (PGE) and inoculated with *Penicillium digitatum*, in spatially separated wounds. PGE was applied at different concentrations (12, 1.2 or 0.12 g dry matter L^{-1}) 6 h (a) or 24 h (b) before the pathogen. Fruit treated with a 1% solution of citric acid and inoculated with *P. digitatum* served as controls. Bars indicate standard errors of the means. Different letters indicate significantly different values according to Duncan's test ($P \leq 0.05$). Letters at the top of the figure or by the circles refer to incidence of decay or rot diameter, respectively.

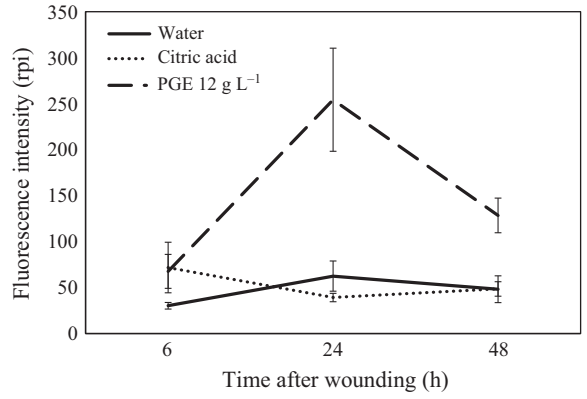

Figure 3 Quantification of reactive oxygen species (ROS) expressed as the relative pixel intensity (rpi) in grapefruit peel 6, 24 and 48 h after treatment with pomegranate peel extract (PGE). Fruits treated with water or citric acid were used as controls. Values represent the mean ± standard error (n = 12).

and host species. It is difficult to explain the higher induced resistance effect in lemons compared to grapefruits; it is assumed that the physiological state of the fruit played a major role in this effect because lemons used in this study were at the light-green stage while grapefruits were harvested in the middle of the harvesting season with full colour development. This highlights the fact that fruits at different physiological stages react differently to chemical elicitors such as PGE and possibly other biotic and abiotic elicitors. Many metabolic activities in defence responses are related to the developmental stage of the plant and the capability of citrus fruit to activate defence responses progressively decreases during ripening or progressing of the harvesting season (McCollum *et al.*, 1997; Alkan & Fortes, 2015). In agreement, Droby *et al.* (1993) found that exposure of harvested grapefruit to ultraviolet (UV) light induces resistance against *P. digitatum* but the UV dose required for development of maximum resistance increases as the season progress.

In agreement with *in vivo* trials, an increased ROS activity that reached its peak at 24 h after treatment was observed in grapefruits treated with PGE. The production of ROS in plants in response to microorganisms has been proposed to prime the establishment of different defensive reactions against pathogens (Torres, 2010). Furthermore, ROS may serve as a signal to trigger an oxidative burst in host tissue (Hershkovitz *et al.*, 2012). Similarly, gene expression analyses highlighted the overexpression of all investigated genes (*CHI*, *CHS*, *MAPK*, *MAPKK* and *PAL*) in the peel of grapefruit treated with PGE as compared to control fruits. Interestingly, one of the investigated genes (*MAPK*) was highly activated by the PGE treatment only in the presence of *P. digitatum*. A possible explanation of this result is that PGE might induce resistance in fruit tissue by a priming effect. Priming is a mechanism that leads to a physiological state that enables plants to respond more rapidly and/or more robustly after exposure to biotic or abiotic stress. The primed state has been related to increased, more efficient, activation of the defence response and enhanced resistance to a challenging stress (Aranega-Bou *et al.*, 2014). In previous studies, the up-regulation of both *MAPKK* and *MAPK* was observed in grapefruit, tomato fruit and grapevines treated with *Metschnikowia fructicola*, *Cryptococcus laurentii* and protein hydrolysates, respectively (Jiang *et al.*, 2009; Hershkovitz *et al.*, 2012; Lachhab *et al.*, 2014). In general, MAPK cascades transfer signals from upstream receptors to downstream cellular effectors with the consequent modification of downstream signalling proteins (Zhang & Klessig, 2001). These cascades have been associated with typical plant defence responses including the production of PR proteins, the production of ROS and cell death (Pedley & Martin, 2005). PAL and CHS are key enzymes of the phenolic biosynthesis pathway and are involved in the synthesis of phytoalexins. Their involvement in the resistance response of grapefruit to biocontrol agents and abiotic stress has been reported previously (Droby *et al.*, 1991, 2002; Arras, 1996; González-Candelas *et al.*, 2010; Hershkovitz *et al.*, 2012). Among defence molecules, chitinase (CHI) is a well-characterized antifungal protein that

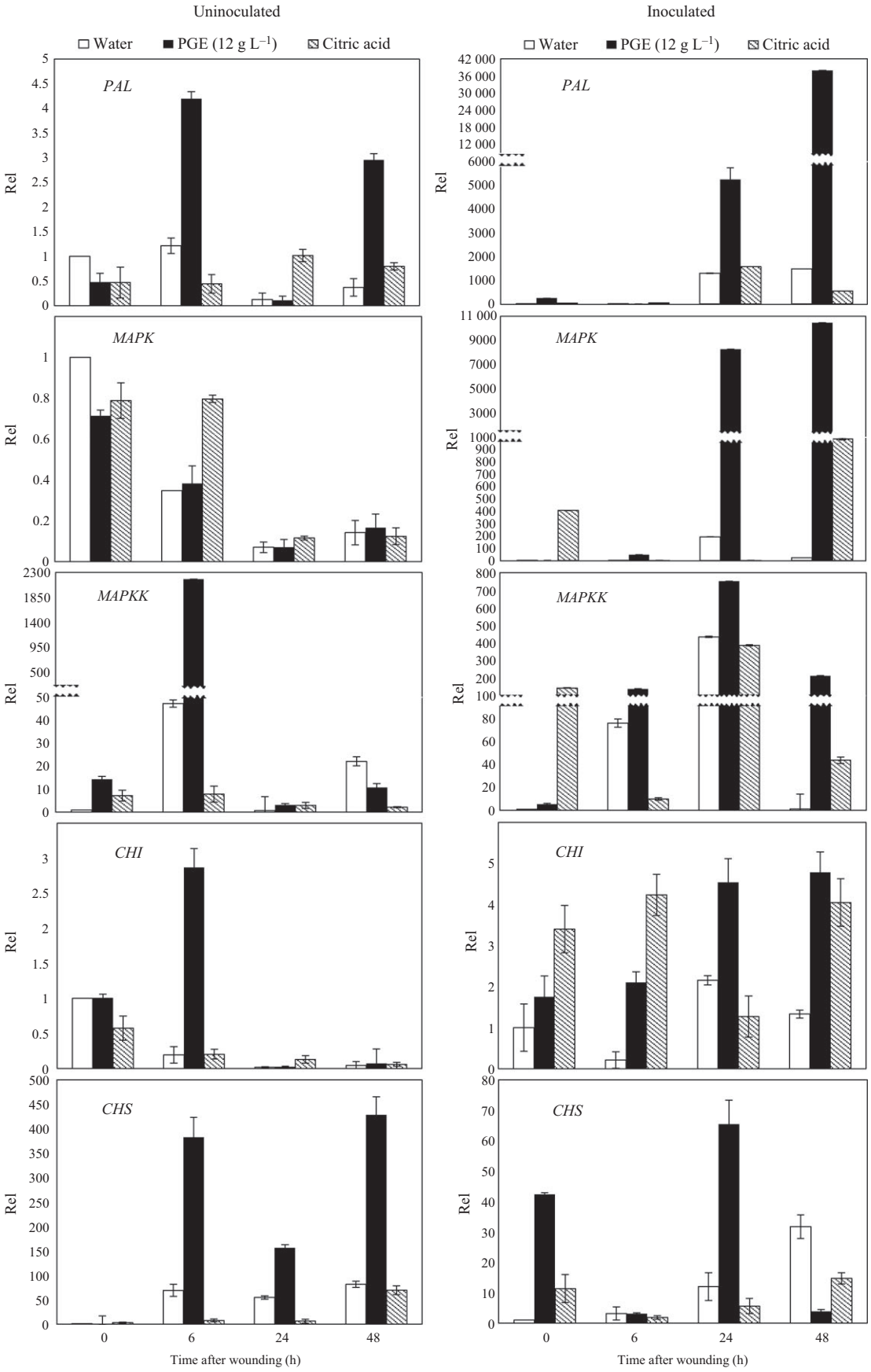
Uninoculated
Inoculated
Water
PGE (12 g L−1)
Citric acid
PAL
MAPK
MAPKK
CHI
CHS
Rel
Time after wounding (h)
0
6
24
48

Figure 4 Relative expression levels (Rel) determined by reverse transcription-quantitative PCR (RT-qPCR) of phenylalanine ammonia lyase (*PAL*), mitogen-activated protein kinase (*MAPK*), mitogen-activated protein kinase kinase (*MAPKK*), chitinase (*CHI*) and chalcone synthase (*CHS*) in grapefruit peel wounded with pomegranate peel extract (PGE), then left uninoculated (left side graphs) or then inoculated with *Penicillium digitatum* (right side graphs). Gene expression levels were determined immediately after the treatment and after 6, 24 and 48 h. Fruits treated with water or citric acid instead of PGE were used as controls. Vertical lines represent the standard error for an average of three biological replicates.

hydrolyses the chitin present in various fungal cell walls. It is present in healthy tissues at low basal levels and it accumulates during compatible and incompatible host–pathogen interactions (van Loon *et al.*, 2006). There is compelling evidence that chitinase can act directly by degrading the pathogen cell wall or indirectly by releasing oligosaccharide elicitors of the defence reaction, both of which are potential defence mechanisms against fungal infection (Cheong *et al.*, 2000). Several reports have demonstrated the induction of CHI in fruits treated with biotic and abiotic elicitors after harvest (Kamal Abo-Elyousr *et al.*, 2010; Hershkovitz *et al.*, 2012; Benouaret *et al.*, 2014).

The levels and timing of expression of the five investigated genes in the peel of grapefruits treated with PGE changed significantly in the presence of a pathogen. *Penicillium digitatum* delayed the activation of most genes: the highest levels of activation were generally achieved at 6 or 24 h in uninoculated or inoculated fruits, respectively. The data seem to suggest the existence of complex mechanisms by which the pathogen modulates the response of fruits to PGE treatment and actively delays the activation of resistance genes. Fungal effectors can modulate plant responses, although the molecular basis of this phenomenon is far from being fully elucidated (Kloppholz *et al.*, 2011; Rafiqi *et al.*, 2012; de Sain & Rep, 2015).

The present study reports information about the induction of resistance in citrus fruit treated with PGE, a plant extract proposed as effective alternative control means against postharvest rots. Although several studies have focused on fruit gene expression in response to cold stress (Maul *et al.*, 2008), pathogen infection (González-Candelas *et al.*, 2010), biocontrol agents (Jiang *et al.*, 2009; Hershkovitz *et al.*, 2012) and organic and inorganic salts (Youssef *et al.*, 2014), limited information is currently available for plant natural extracts (Mekbib *et al.*, 2007; Song *et al.*, 2013; Talibi *et al.*, 2014). From the practical standpoint, the activation of resistance responses is important to protect commodities from any eventual wound infections and to restrict fungal growth and sporulation. The results in the present study suggest that the high efficacy demonstrated for PGE under different conditions, on different hosts and against different pathogens, is at least partially determined by the induction of resistance in host tissues (Li Destri Nicosia *et al.*, 2016). In particular, induced resistance is likely to play a major role in two important PGE features: the persistent efficacy after its application and the wide spectrum of its activity. Furthermore, induced resistance may also play a role in the curative effect of PGE that has been reported previously, due to the rapid activation of resistance responses that may reduce or block the colonization of host tissues (Li Destri Nicosia *et al.*, 2016).

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