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Research Paper

Defense response against postharvest pathogens in hot water treated apples

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ABSTRACT

Hot water treatment (HWT) has been demonstrated to be a safe and effective method to control postharvest diseases. In the present study, the effect of HWT (45 °C for 10 min) was investigated on the response of apple fruit against *Botrytis cinerea, Colletotrichum acutatum* and *Neofabraea vagabunda* infections. *In vivo* experiments were conducted with apples treated with HW and then inoculated with pathogen spore suspensions 0, 3, 6 and 24 h after the treatment. *In vitro* experiments were carried out in order to investigate the HWT effect on the pathogenesis enzyme activities of the three pathogens. These assays were conducted with the crude protein extracts (CPEs) derived from the hot water treated apples. A significant reduction of rot incidence was observed in fruit inoculated at 6 h (*B. cinerea* 22.7% and *C. acutatum* 11.0%) and 3 h (*N. vagabunda* 68.8%) after HWT with respect to the controls (22.0, 18.5 and 19.67 mm, respectively). CPEs extracted from hot water treated fruit inoculated at 6 h and 3 h showed the highest conidia germination inhibition (*B. cinerea* 85.6%; *C. acutatum* 52.7% and *N. vagabunda* 83.1%) with respect to the controls (74.0, 71.0 and 90.3% of conidia germination, respectively). A significant reduction of pathogenesis enzyme activities of the three pathogens was detected when pathogens were exposed to CPEs derived from hot water treated apples. The results showed that HWT can stimulate a defense response on apple against some postharvest pathogens.

1. Introduction

The postharvest life of many fruits is limited by postharvest decay due to biotic stresses (Schirra et al., 2000). During prolonged storage, the physiological changes that occur in fruit enable pathogens to develop, causing serious diseases (Barkai-Golan, 2001). The main postharvest diseases are caused by fungi that infect fruit in the field or during harvest and subsequent handling, up to marketing (Sivakumar and Bautista-Banos, 2014).

The control of postharvest diseases, necessary to obtain marketable fruit, is achieved by fungicide treatments, although, starting from the eighties, their use in the postharvest phase has become more difficult to justify (How, 1991). In fact, their application has been restricted by regulatory agencies in Europe as well as in other countries (Willer and Youssefi, 2004), while the consumer demand for organic fruit or fruit without fungicide residues has increased (El Hage Scialabba, 2007). Among alternative methods to control fruit postharvest diseases, heat applied as forced hot air or by fruit dipping in hot water, appears to be one of the most promising methods (Lurie et al., 1998). Heat can inhibit the pathogen by slowing germ tube elongation, inactivating or killing germinating spores outright, and reducing disease symptoms (Liu et al., 2012). Heat treatment can also prevent storage decay, stimulating the host-defense responses in fruit tissue (Nunes et al., 2007; Romanazzi et al., 2016 Romanazzi et al., 2016). In fact, plants have evolved several mechanisms to withstand the stress of high temperatures (Wahid et al., 2007) like the production of antifungal substances, the enhancement of wound healing, the induction of PR proteins such as chitinase and β-1,3 glucanase, or the synthesis of cell wall hydrolytic enzyme inhibitors as polygalacturonase (Schirra et al., 2000).

Notably, hot water treatment (HWT) has been successfully applied on several fruits to control postharvest pathogens such as *Monilinia* spp. of stone fruit (Casals et al., 2010), *Colletotrichum gloeosporioides* of mango (Alvindia and Acda, 2015), *C. musae* of banana (Alvindia, 2012), *B. cinerea* of tomato (Fallik et al., 2002), *Penicillium* spp. of citrus fruit and apple (Nunes et al., 2001; Nunes et al.,2007).

During cold storage, apples may develop a variety of diseases caused by fungi such as green mold *(Penicillium expansum* Link), grey mold *(Botrytis cinerea,* Pers.), bitter rot *(Colletotrichum* spp.), and bull's eye rot (*Neofabraea* spp.) (Nunes et al., 2001; Borve et al., 2013; Grantina-Ievina, 2015). Several data showed the efficacy of HWT applied to apple fruits against *M. fructigena, Gloesporium* spp. (Maxin et al., 2012a), *P. expansum, Neonectria galligena* and *B. cinerea* (Fallik et al., 2002; Maxin et al., 2012b) and *N. alba* (Neri et al., 2009). Maxin et al. (2012b) demonstrated that the apples were resistant to green mold

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when the fruits were wound-inoculated after the heat treatment, indicating a potential defense induction to the pathogen infection in treated apple fruit. Studies on peach (Liu et al., 2012) and citrus (Ballester et al., 2010) reported that HWT directly inhibited fruit pathogens and elicitated the defense responses in fruit contributing to the reduction of fruit decay.

As far as we know, there are no studies on the role of fruit tissue treated with hot water on pathogenesis enzymes of postharvest pathogens. The objectives of the present research were to investigate the effect of: a) HWT on apple fruit in reducing infections by *B. cinerea*, *C. acutatum*, and *N. vagabunda*; b) crude protein extracts (CPEs) of previously hot water treated apple peel on conidia germination of the three pathogens and c) CPEs of previously hot water treated apple peel on the inhibition of the pathogenesis cell wall degrading enzymes (CWDEs) cellulase, polygalacturonase, polymethylgalacturonase and xylanase produced by the three pathogens.

2. Materials and methods

2.1. Fruit

'Golden Delicious' apple fruits (*Malus domestica* Borkh) were harvested in an orchard located in Bagnacavallo (Ravenna, Northern Italy). After harvest, fruits with no visible wounds and rots, and homogenous in size were selected, stored at 0 °C and used within 5 days.

2.2. Pathogens

The three fungal pathogens, *B. cinerea, C. acutatum* and *N. vagabunda*, belonging to the CRIOF collection, were isolated from apples showing typical rot symptoms and identified (Cameldi et al., 2017; Mari et al., 2012). *Botrytis cinerea* was grown on oat meal agar (60 g of oat meal, 10 g of sodium nitrate, 30 g of sucrose, 12 g of agar per 1000 ml of distilled water), then incubated at 25 °C for 10 days. *Colletotrichum acutatum* was grown on potato dextrose agar (PDA, Oxoid, UK) at 20 °C for 10 days. *N. vagabunda* was grown on technical agar (Oxoid, 12 g l^{-1}) amended with tomato juice (250 ml l^{-1}) at 15 °C for 15 days until use. Pathogen conidia suspensions were prepared from 10 to 15 days old colonies by scraping and suspending conidia in sterile distilled water added with 0.05% (v/v) Tween 80, and adjusted to the final concentration required with a hemocytometer.

2.3. Hot water treatment

Selected fruits were treated by dipping in water in a 10 l stainless steel tank. The water temperature was 45 °C and the duration of treatment was 10 min. The water was heated by a digital thermostat (ScanVac SHC 2000, Linge DK) with temperature stability + 0.01 °C, heater wattage 2 K W and pump with a flow rating of 15 l m^{-1}. Fruits were then divided into three groups, one for each pathogen, and artificially inoculated with the pathogen 0, 3, 6 and 24 h after HWT. For inoculation, fruits were wounded by a sterile nail (3 × 3 × 3 mm) at the equator (one wound per fruit) and then inoculated with 20 µl of the pathogen conidia suspension (10^5 conidia ml^{-1}). After inoculation, all fruits were stored at 20 °C for 6 days or 10 days depending on the inoculated pathogen. Disease severity was assessed by measuring the diameter of the lesions (mm). Untreated fruits were represented by fruit dipped in water at room temperature and inoculated with pathogens. The sample unit was represented by 4 replicates of 6 fruits each, and the experiment was performed three times.

2.4. Protein extraction

Samples of apple exocarp (skin) were collected from treated fruit 0, 3, 6, and 24 h after HWT and from control fruit. All samples were ground to fine powder in a mortar prechilled in liquid nitrogen. Chilled sodium acetate buffer 20 mM, pH 5.5, containing 1% (w/v) polyvinylpolypyrrolidone (Sigma Aldrich Co., St. Louis, MO, USA) was added to the powdered samples (1 ml g^{-1} fresh weight). Extraction was made at 0 °C for 1 h under continuous stirring. The buffer extracts were then centrifuged twice at 12,000 rpm for 20 min at 4 °C and the supernatant, representing the crude protein extract (CPE), was filtered with GV Millex1 Syringe Filter Unit (Millipore Corporation, USA) to remove particles and recovered for enzymatic assays. Protein concentration in the CPE was determined by Bradford method, using BSA as standard (Bradford, 1976). Each biological replicate consisted of apple skin samples collected from 5 fruits for each treatment time.

2.5. Effect of crude protein extract on pathogen conidia germination

An aliquot (50 µl) of conidia suspension (10^5 spore ml^{-1}) of each pathogen (*B. cinerea, C. acutatum* and *N. vagabunda*) was deposited on a sterile microscope slide, and the same aliquot of CPE (50 µl), derived from fruits 0, 3, 6 and 24 h after HWT or from untreated fruit, was then added. Slides were laid on humid filter paper in a sterile Petri dish, immediately sealed with Parafilm to maintain high humidity. After 14 h of incubation at 20 °C, conidia germination was evaluated observing 30 conidia for replicate from treated and untreated samples through a microscope (Nikon Eclipse TE2000-E). For each sample, 3 microscope slides (replicates) were prepared. The experiment was repeated twice.

2.6. Effect of crude protein extract on pathogenesis enzyme activity

Cellulase (*endo*-1,4-β-glucanase), pectic enzyme (polygalacturonase and polymethylgalacturonase) and xylanase enzyme activities of *B. cinerea, C. acutatum* and *N. vagabunda* were evaluated after exposure of conidia to CPE derived from heat treated (0, 3, 6 and 24 h) and untreated fruit. Assays were performed in Petri dishes containing modified agar medium (Roberti et al., 2017). Forty microliters of pathogen suspension (10^6 conidia ml^{-1}) and the same aliquot of each CPE were placed in holes (three equidistant holes per dish) punched in the medium with a 5 mm- sterile cork borer. Control plates for each enzyme-pathogen combination were inoculated with the same volumes of conidia suspension and distilled water. After incubation at 20 °C for 48 h, enzyme activities were determined with specific colorimetric methods visualizing a clear zone of substrate degradation around the holes. The diameter of degradation halos (mm) was measured with a ruler. For each combination time of CPE, pathogen and control, three plates (replicates) were considered.

For cellulose activity, the agar medium consisted in PYE (peptone 0.5 g; yeast extract 0.1 g; agar16 g l^{-1}) supplemented with 0.5% Na-carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA) (Mugnai et al., 1997). The halo produced by *endo*-1,4-β-glucanase activity of each fungus was visualized by staining plates with a 0.2% Congo red water solution for 15 min, then discolored with NaCl 1 M. Polygalacturonase activity was determined through the Eriksson & Pettersson culture medium (Eriksson and Pettersson, 1975) enriched with sodium salt of polygalacturonic acid (Sigma-Aldrich) from citrus fruit (0.5%) and agarized with low calcium Oxoid No. 1 agar (2%, Oxoid, Basingstoke, UK). The medium was sterilized following the procedure of Ayers et al. (1966), then the pH was adjusted to 5 and 8 respectively by adding NaOH 1 M. Polymethylgalacturonase activity was determined through the above cited culture medium (Eriksson and Pettersson, 1975) enriched with Sigma citrus pectin (0.5%) and agarized with calcium-rich Oxoid No. 3 agar (2%). The growth media was sterilized following the procedure of (Durrands and Cooper, 1988) and the pH was adjusted to 8 by adding HCl 1N. The halo produced by pectic enzyme activities was visualized by 1% cetylmethyl ammonium bromide (CTAB) dissolved in distilled water, heated to 30 °C. For the xylanase assay, the medium contained 0.5% beech wood xylan (Sigma-Aldrich) in a minimal medium consisting of $NaNO_3$ 0.3%; KH_2PO_4 0.1%; $MgSO_4$ 0.05%; yeast extract 0.1%; agar 1.2% (St Leger et al.,

1997). The clearing halo was visualized by staining with Congo red, then destaining with NaCl and measured as described above. Each assay was repeated twice.

2.7. Statistical analysis

All data were analyzed using one-way ANOVA and the least significant differences test (LSD) was used to separate differences among the means; statistical significance was considered as $P < 0.05$. All analyses were performed with Statgraphics software (version centurion 15.0). The experiments were carried out in a completely randomized block design.

3. Results

3.1. Effect of hot water treatment on apples inoculated with postharvest pathogens

The effect of a HWT was investigated on apples inoculated, after treatment, with three postharvest pathogens. Data showed that the efficacy was dependent on the pathogen and on the delay of inoculation after HWT. Lesions produced by *B. cinerea* were significantly reduced by HWT, compared to untreated fruit, at all inoculation times, particularly at 6 h after treatment (22.7%). Similarly, fruit hot water treated and inoculated with *C. acutatum* showed significantly reduced lesions only when inoculation was performed 6 and 24 h after treatment, although the reduction was slight: 11.0 and 10.7% respectively (Fig. 1). The best disease control was observed in fruit inoculated with *N. vagabunda* 3 h after HWT; in fact, the lesion diameters were reduced by 68.8% with respect to the control.

3.2. Influence of crude protein extract from hot water treated fruit on pathogen germination

Crude proteins, extracted from fruits 0, 3, 6 and 24 h after HWT, significantly inhibited conidia germination of all pathogens with respect to the control (Fig. 2). A high inhibition of *B. cinerea* was obtained with crude proteins extracted from fruits 6 and 24 h after HWT (85.6% and 68.5% respectively, towards control fruits); at the same times, 6 and 24 h after HWT, a lower inhibition of conidia germination was obtained in the case of *C. acutatum* (52.7% and 28.6% respectively). On the other hand, for *N. vagabunda* the best control was observed with CPEs derived from fruits 3 h after HWT (83.1%).

3.3. Pathogenesis enzyme activity assays

The effect of CPEs derived from fruits 0, 3, 6, 24 h after HWT was assayed on pathogenesis enzyme activity of *B. cinerea*, *C. acutatum* and *N. vagabunda.*

Endo-1,4-β-glucanase activity of all three pathogens was almost always reduced by CPEs derived from fruit at all four times after HWT compared to water and controls (Table 1). Moreover, for all pathogens the halos produced by CPEs of untreated fruits were always lower than those produced by water control. The *endo*-1,4-β-glucanase activity of *B. cinerea* was totally inhibited by CPEs from fruits 3, 6, 24 h after HWT, while no difference was found between the activity of CPEs from fruits 0 h after HWT and untreated fruits. Endo-1,4-β-glucanase activity of *C. acutatum* was significantly inhibited by CPEs from fruits at all times after HWT (average of 28.9% inhibition with respect to the control). The enzyme activity of *N. vagabunda* was significantly inhibited by CPEs from fruits 0, 3, 6 and 24 h after HWT (average of 48.2% inhibition compared to untreated fruits).

The polygalacturonase activity (Table 1) of both *B. cinerea* and *N. vagabunda* was statistically similar after exposure to water or CPEs from untreated fruits, while the enzyme activity of *C. acutatum* in the presence of crude proteins extracted from untreated fruit was lower than

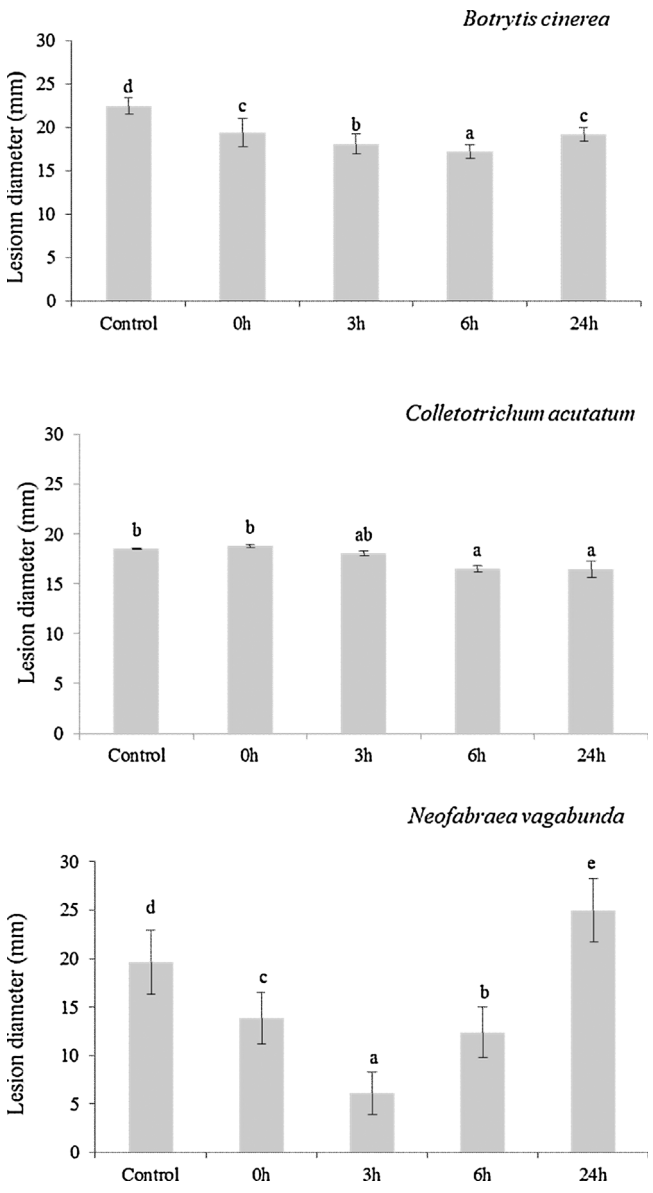

Fig. 1. Effect of hot water on apple fruits inoculated with *Botrytis cinerea, Colletotrichum acutatum and Neofabraea vagabunda* 0, 3, 6 and 24 h after the treatment. Disease severity (diameter-mm) was measured at the end of incubation time. The experiment includes 4 replicates of 6 fruits at each time point. Data were analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) followed by LSD test, $P < 0.05$. Different letters indicate significant differences between untreated control and hot water treatment at each time point.

water control (25.8%). In the case of *B. cinerea,* the enzyme activity was significantly reduced by CPEs derived from fruits 3, 6 and 24 h after HWT; the percentages of reduction were 53.1%, 42.9% and 53.1% respectively, compared to the untreated control. The polygalacturonase activity of *C. acutatum* was reduced by crude proteins extracted from fruits at all times after HWT, ranging from 37.1% (0 h) to 62.9% (24 h) compared to the untreated control. Concerning *N. vagabunda*, the enzyme activity was significantly reduced only by CPEs from fruits 0, 3, 6 h after HWT. On the contrary, an increase in polygalacturonase activity was observed in the presence of CPEs from fruits 24 h after HWT.

The best reduction of *B. cinerea* polymethylgalacturonase activity (Table 2) was achieved with CPEs from fruits 0 and 3 h after HWT (41.0% on average compared to untreated fruits). A slight reduction (17.7%) was observed in CPEs from fruits 24 h after HWT for *C. acutatum* and from fruits 0, 3, 6 h after HWT for *N. vagabunda* (10.5%).

A significant reduction in xylanase activity (Table 2) of *B. cinerea*

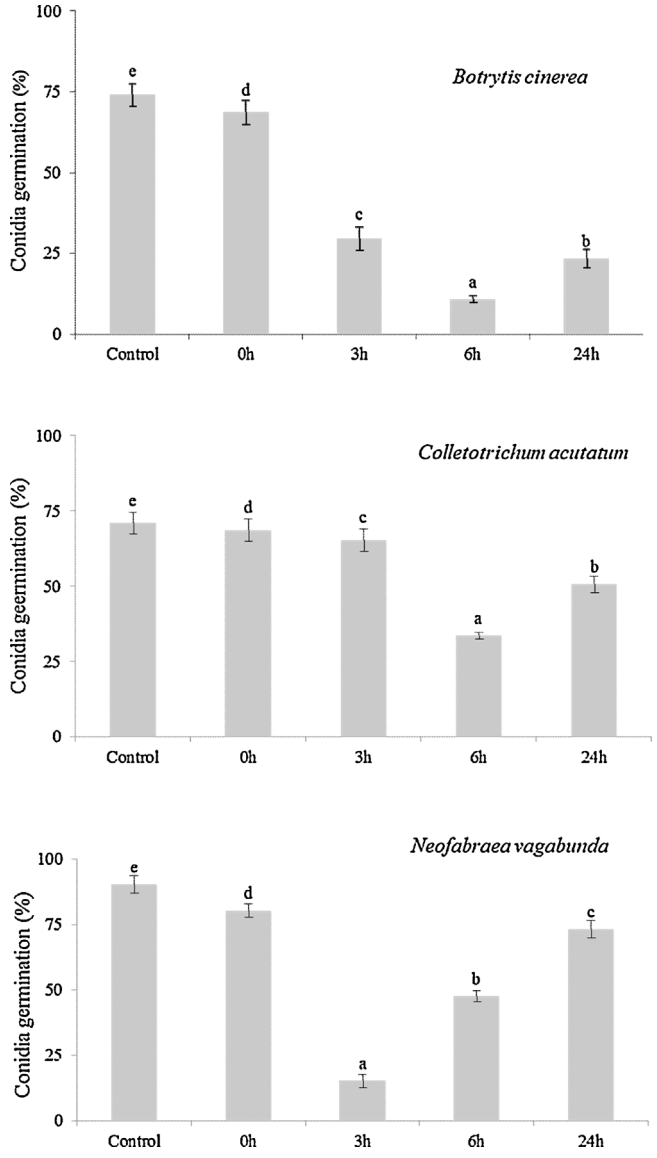

Fig. 2. Effect of crude protein extracts from untreated apple fruits (control) and fruits treated with hot water and collected 0, 3, 6, 24 h after treatment on spore germination of *Botrytis cinerea, Colletotrichum acutatum* and *Neofabraea vagabunda.* Data are the means of germinated conidia of 30 conidia observed for each time point. Data were analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) followed by LSD test. Different letters indicate significant differences according to LSD test, $P < 0.05$.

was detected in CPEs from fruits 0, 3, 6 h after HWT. The highest reduction of *C. acutatum* xylanase activity was exerted by CPEs from fruits 6 h after HWT (36.9% with respect to untreated fruits). In addition, the crude proteins extracted from fruits 0, 3, 6, 24 h after HWT were effective in reducing the xylanase activity of *N. vagabunda*, in particular CPEs from fruits 3 and 6 h after HWT (52.9% on average).

4. Discussion

This work demonstrated a reduction of symptoms caused by apple postharvest pathogens, mainly for *N. vagabunda* when fruits were first dipped in water at 45 °C for 10 min and then challenged with the single pathogens at different times Maxin et al. (2012b) tested the efficacy of HWT against apple fruit rot caused by *B. cinerea, N. galligena* and *P. expansum,* concluding that the greatest effect of HWT against these pathogens was mediated by heat-induced acquired resistance of fruit rather than a direct mortality effect on conidia. Our results obtained using a single temperature (45 °C) partially confirmed this thesis. The effect of treatment on reduction of disease symptoms was evident with the exception of *C. acutatum*, immediately after HWT (0 h). Moreover, the efficacy of treatment was more pronounced against *N. vagabunda,* especially in fruits inoculated 3 h after HWT, partially in agreement with Spadoni et al. (2014) and Maxin et al. (2012b). In contrast, on peach fruit, a HWT before *M. fructicola* inoculation led to a stimulation of conidia germination and no control of brown rot (Spadoni et al., 2015); otherwise, when fruits were inoculated and then treated with hot water the reduction of disease was over 50% (Spadoni et al., 2013). As asserted by Maxin et al. (2012a) there are at least two components which may contribute to the mode of action of hot water: [1] a direct and lethal effect of heat on fungal inoculum within or outside the fruit, and [2] an indirect effect mediated by a stress-induced physiological response of the fruit. In the case of peach, there is probably only a direct effect on the pathogen conidia, while our results on apple support the hypothesis of the induced resistance against the tested postharvest pathogens, even if a direct effect cannot also be excluded as already reported by Spadoni et al. (2015).

Some inhibitors of pathogenesis enzyme activity are present in the skin of healthy apples. Polygalacturonase inhibitors of *C. acutatum* were extracted from 'Cripps Pink' apple (Gregori et al., 2008) such as from grapefruit against *P. italicum* and *B. cinerea* (D'hallewin et al., 2004) and from bean against *Aspergillus niger* (Berger et al., 2000). Our experiments partially confirmed these results, showing a reduction of *endo*-1-4-β-glucanase activity of *B. cinerea*, *C. acutatum* and *N. vagabunda* and a reduction in the activity of *C. acutatum* polygalacturonase after exposure to crude proteins extracted from untreated 'Golden Delicious' apples.

In addition, our results show that the target pathogens produce an enzyme pool able to degrade different cell-wall polysaccharides, such as pectin, hemicelluloses and celluloses allowing the fungus to attack the cell (Annis and Goodwin, 1997). Recent studies focused the attention on CWDEs families that are specifically present in plant pathogens (Kubicek et al., 2014), also showing the activities of hydrolytic enzymes from different fungi in relation to different types of substrate (Couturier et al., 2012; King et al., 2011). Typically, pectinase enzymes are

Table 1
Endo-1,4-β-glucanase and polygalacturonase activities (halo-mm^2) of *Botrytis cinerea*, *Colletotrichum acutatum* and *Neofabraea vagabunda* after exposure to distilled water (water), crude protein extracts from untreated apple fruits (control) and fruits treated with hot water and collected 0, 3, 6, 24 h after the treatments (HWT–0 h, HWT–3 h, HWT–6 h, HWT–24 h). Each value is the mean of three replicates ± standard errors. Data were analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) followed by LSD test. Within the same species the same letters represent no significant differences according to LSD test ($P < 0.05$).

Treatment	*Endo-1,4-β-glucanase*			*Polygalacturonase*		
	Botrytis cinerea	*Colletotrichum acutatum*	*Neofabraea vagabunda*	*Botrytis cinerea*	*Colletotrichum acutatum*	*Neofabraea vagabunda*
Water	254 ± 0.00c	177 ± 1.36c	220 ± 3.23c	170 ± 2.00d	229 ± 3.08e	51 ± 0.72b
Control	141 ± 1.88b	145 ± 1.04b	127 ± 1.36b	147 ± 7.07cd	170 ± 1.57d	47 ± 0.90b
HWT-0h	122 ± 0.60b	105 ± 1.32a	69 ± 0.94a	126 ± 0.65c	107 ± 0.60c	31 ± 1.17a
HWT-3h	0 ± 0.00a	127 ± 1.72a	69 ± 0.00a	69 ± 0.49a	89 ± 0.55b	59 ± 0.44c
HWT-6h	0 ± 0.00a	95 ± 0.00a	62 ± 1.67a	84 ± 0.55b	89 ± 0.55b	55 ± 0.44c
HWT-24h	0 ± 0.00a	85 ± 1.48a	63 ± 3.00a	69 ± 0.94a	63 ± 0.00a	79 ± 0.90d

Table 2
Polymethylgalacturonase and xylanase activities (halo-mm^2) of *Botrytis cinerea*, *Colletotrichum acutatum*, *Neofabraea vagabunda* after exposure to distilled water (water), crude protein extracts from untreated apple fruits (control) and fruits treated with hot water and collected 0, 3, 6, 24 h after the treatments (HWT–0 h, HWT–3 h, HWT–6 h, HWT–24 h). Each value is the mean of three replicates ± standard errors. Data were analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) followed by LSD test. Within the same species the same letters represent no significant differences according to LSD test ($P < 0.05$).

Treatment	*Polymethylgalacturonase*			*Xylanase*		
	Botrytis cinerea	*Colletotrichum acutatum*	*Neofabraea vagabunda*	*Botrytis cinerea*	*Colletotrichum acutatum*	*Neofabraea vagabunda*
Water	102 ± 1.60c	161 ± 0.76d	113 ± 0.00c	0.0 ± 0.00a	436 ± 0.29c	434 ± 2.63c
Control.	94 ± 0.55c	96 ± 1.00b	85 ± 1.48b	304 ± 1.29d	452 ± 0.97c	498 ± 2.97c
HWT-0h	64 ± 0.00a	120 ± 0.65c	76 ± 1.00a	166 ± 1.23b	311 ± 2.54a	299 ± 1.44b
HWT-3h	47 ± 1.31a	120 ± 0.65c	76 ± 1.00a	227 ± 0.97c	343 ± 2.35ab	232 ± 1.16a
HWT-6h	84 ± 0.55b	113 ± 0.00c	76 ± 1.20a	205 ± 0.43c	285 ± 1.89a	237 ± 1.64a
HWT-24h	85 ± 1.46b	79 ± 0.90a	107 ± 0.60c	289 ± 1.39d	395 ± 3.50b	325 ± 1.92b

produced first in the largest amounts (Miedes and Lorences, 2004), in agreement with our results, reporting a good polymethylgalacturonase and polygalacturonase activity for three pathogens, except for *N. vagabunda* in the case of polygalacturonase. Polygalacturonase produced by fungal pathogens plays a critical role in pectic degradation; necrotrophic fungi such as gray mold have an expanded family of polygalacturonases (PGAs), suggesting a high capacity of pectin degradation (Zhao et al., 2013)while other fungi contain only a small number of PGAs, which play an auxiliary role in breaking pectin down.

It is also interesting to note *xylanase* activity amounts, particularly expressed by *C. acutatum* and *N. vagabunda* and it was not showed by *B. cinerea* in water condition. In several plant pathogens, CDWEs like pectinases and xylanases were demonstrated to be related to pathogenicity or virulence (Douaiher et al., 2007; Kikot et al., 2009) suggesting that the enzymatic activity is specifically attributed to the fungus species. As expected, our results confirmed that the degradation of pectic polymers and hemicellulosic polysaccharides by the target pathogens is a very important process in the colonization of fruit tissue as confirmed in *in vivo* experiments. We focused our analysis on pectinases, cellulases, and hemicellulases because they represent the major plant CWDEs in fungal pathogens. Nevertheless, many studies revealed that fungi exhibit considerable differences in the number and variety of CWDEs (Zhao et al., 2013).

The HWT increased the inhibition of enzyme activity, starting at 0 h after the treatment, with the exception of *endo*-1-4-β-glucanase and polygalacturonase activities of *B. cinerea*, and polymethylgalacturonase activity of *C. acutatum*. It is also demonstrated that the effect of HWT can be related to an inhibition of pathogen spore germination by the activity of proteins, which are enhanced by the temperatures in apple fruit skin. In fact, the crude protein extract of hot water treated fruit peel reduced pathogen spore germination. This effect was time dependent and the highest spore inhibition was detected in crude extract starting 3 h after the treatment. The activity of proteins contained in the crude extract is confirmed by our results on pathogenesis enzyme inhibition. Crude extracts presumably contain inhibitors of pathogenesis enzymes, which pathogens produce to degrade the fruit cell wall, mainly peel tissue. Inhibitors of *endo*-1-4-β-glucanase, pectic enzyme (polygalacturonase and polymethylgalacturonase) and xylanase were differently produced as a result of heat treatment.

The efficacy *in vivo* is not completely explained by our experiments, probably the effect of hot water treatment on the increase of pathogenesis enzyme inhibitors may be due not to an individual or a few enzymes but to an enzyme pool as stated by Amnuaysin et al. (2012) and Spadoni et al. (2015).

5. Conclusions

Our results give further characterization of the mechanisms of HWT in reducing fruit rot symptoms. This study shows for the first time the role of HWT on pathogenesis enzyme activity exerted by some postharvest pathogens. More investigation are necessary to understand the exact contribution of HWT to pathogenesis enzyme inhibitors to better control tested fungal pathogens, in order to improve its efficacy.

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