Evaluation of a Pomegranate Peel Extract (PGE) as Alternative Mean to Control Olive Anthracnose

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Abstract

Olive anthracnose is caused by different species of *Colletotrichum* spp. and may be regarded as the most damaging disease of olive fruits worldwide, greatly affecting quality and quantity of the productions. A pomegranate peel extract (PGE) proved very effective in controlling the disease. The extract had a strong *in vitro* fungicidal activity against *Colletotrichum acutatum sensu stricto*, was very effective in both preventive and curative trials with artificially inoculated fruit, and induced resistance in treated olive tissues. In field trials, PGE was significantly more effective than copper, which is traditionally used to control the disease. The highest level of protection was achieved by applying the extract in the early ascending phase of the disease outbreaks since natural rots were completely inhibited with PGE at 12 g/l and were reduced by 98.6 and by 93.0% on plants treated with PGE at 6 and 3 g/l, respectively. Two treatments carried out 30 and 15 days before the expected epidemic outbreak reduced the incidence of the disease by 77.6, 57.0, and 51.8%, depending on the PGE concentration. The analysis of epiphytic populations showed a strong antimicrobial activity of PGE, which sharply reduced both fungal and bacterial populations. Since PGE was obtained from a natural matrix using safe chemicals and did not have any apparent phytotoxic effect on treated olives it may

be regarded as a safe and effective natural antifungal preparation to control olive anthracnose and improve olive productions.

Olive anthracnose (OA) caused by different species of *Colletotrichum* is the most important disease of olive fruit worldwide, resulting in heavy economic losses (Cacciola et al., 2012; Moral et al. 2014). Affected olive drupes show dark necrosis followed by fruit rot and mummification. Under moist conditions, abundant orange conidial masses are produced on the surface of infected drupes, while, under dry conditions, the fruits mummify and lose weight due to dehydration. Infected fruits fall prematurely to the ground and only a few mummies remain attached to the tree. The disease is commonly severe on mature fruits but green drupes of susceptible cultivars can be also infected under favorable conditions. The quality of oil from infected drupes is highly compromised due to a reddish color, high acidity, and a major reduction of polyphenols, α-tocopherol, and β-sitosterol (Moral et al. 2014). *Colletotrichum* species can also infect other olive organs including leaves, fruit pedicels, and flowers, but the economic relevance of these infections is considered of secondary importance (Cacciola et al., 2012).

According to recent taxonomic rearrangements, olive anthracnose is caused by a fungal complex, consisting of *C. acutatum sensu lato* (*s.l.*) and *C. gloeosporioides s.l.* (Damm et al., 2012; Weir et al., 2012). Furthermore, *C. karstii*, a species belonging to the *C. boninense* complex has also been associated with the disease (Schena et al., 2014). *Colletotrichum gloeosporioides s.l.* species have been rarely associated with epidemic outbreaks, though some species of this complex have shown a high level of virulence on artificially inoculated fruits (Schena et al., 2014). In any case, the most economically relevant species belong to the *C. acutatum s.l.* complex. Among these, *C. godetiae* syn. *C. clavatum* has been traditionally considered the most prevalent olive anthracnose pathogen in Italy and other central Mediterranean countries (Faedda et al., 2011; Cacciola et al., 2012; Talhinhas et al., 2015). In Spain, *C. simmondsii* is the dominant species in Catalonia while *C. godetiae*, seems to be prevalent in Andalusia (Moral et al., 2014). In Portugal, the prevalent species is *C. nymphaeae* followed by *C. simmondsii* and *C. godetiae* (Talhinhas et al. 2011). Another important species, *C. acutatum s.s.* until a few years ago had been reported only in the region of the Algarve in Portugal, and seemed to be the prevalent species on olives in Australia and South Africa (Talhinhas et al. 2011). However, recent investigations have revealed a wide diffusion of this species also in southern Italy

and in northern Tunisia (Chattaoui et al., 2016; Mosca et al., 2014). In Calabria, southern Italy, there is evidence to indicate an ongoing process of population shift from *C. godetiae* to *C. acutatum s.s.* which is now the most important causal agent of olive anthracnose (Abdelfattah et al., 2015; Mosca et al., 2014; Schena et al., 2017).

The management of olive anthracnose and other fruit rot is generally based on Integrated Pest Management (IPM) approaches, which include chemical treatments, selection of resistant cultivars and early harvesting in order to escape secondary infections (Cacciola et al., 2012). Control of the olive fruit fly attacks may also be useful to reduce the disease since the fly may act as vector and facilitate infection through oviposition wounds (Malacrinò et al., 2015; 2017). In olive-growing areas where anthracnose is endemic, frequent rains in the fall and high humidity may reduce the persistence of fungicide treatments. Therefore, several fungicide treatments are needed to control the disease. Furthermore, the need to avoid fungicide residues in olive oil and to comply with the regulation on preharvest fungicide intervals can leave the drupes unprotected in a developmental stage of very high susceptibility and in a season when environmental conditions are conducive to the disease. In Calabria, two to three preventive treatments from late September to the end of December with copper formulates are considered quite effective against the disease, whereas, high humidity conditions can significantly reduce the efficacy of treatments (Agosteo et al., 2007; Graniti et al., 1993; Pennisi et al., 1993). In Australia, copper-based fungicides and strobilurins applied under field conditions failed to control anthracnose disease irrespective of the timing of application (Sergeeva, 2011). Spring treatments commonly carried out to control peacock spot caused by *Spilocea oleagina* may also help reduce blossom blight and reduce latent infections which represent an important inoculum source for fall infections (Moral et al., 2014). Recently, two commercial formulates containing trifloxystrobin+ tebuconazol and pyraclostrobin have been registered in Italy on olive only for application during spring and early summer, respectively.

Restrictions in the use of copper fungicides due to the toxicity of Cu^{2+} ions and their adverse effects in soil and water have prompted research into substances that may substitute its use. In this context, the role of olive oil as a major component of the Mediterranean diet is urging effective alternative control means to ensure high quality productions and the absence of chemical residues. In Calabria, field trials on the cultivars ‘Cassanese’ (moderately susceptible) and ‘Ottobratica’ (highly susceptible) with sodium bicarbonate ($NaHCO_3$) significantly reduced the proportion of drupes

affected by anthracnose, even though the efficacy of treatments depended on the susceptibility of the cultivars (Agosteo et al., 2007). Recently, several fungi isolated from olive proved to inhibit the growth of *C. acutatum,* but only *in vitro* tests were performed (Landum et al., 2016). In another study, nutrients and microorganisms from the olive carposphere were found to play an important role in the infection processes of *C. godetiae* (Agosteo et al., 2015).

In recent years, particular interest has been shown in plant extracts i.e. biodegradable substances with low toxicity to the environment that exhibit a direct fungicidal or fungistatic activity that can also induce resistance in treated plant hosts (Chen and Dai 2012; Palou et al., 2016). Typically, bioactive compounds contained in plant extracts are products of secondary metabolism produced by the plant for its own protection against pests and pathogens. They have been used in a number of host/pathogen combinations and some have proved very effective, suggesting the possible development of natural antifungal compounds that would be as effective as synthetic fungicides (Schena et al., 2007). Among others, an alcoholic extract obtained from the peel of pomegranate (PGE) seems to be promising for the development of alternative control strategies against fungal plant pathogens. It has shown a high efficacy against several postharvest diseases caused by major necrotrophic fungi including *Botrytis cinerea* on table grapes and sweet cherries, *Monilinia laxa* on sweet cherries, *Penicillium digitatum* and *Penicillium italicum* on lemons and grapefruits and *Penicillium expansum* on apples (Li Destri Nicosia et al., 2016; Romeo et al., 2015). Currently, the available data indicate the possible implementation of PGE to control plant diseases in different host pathogen combinations since it showed a broad spectrum of activity, strong direct antimicrobial effects and both curative and preventive activities with long persistence even when applied under semi-commercial conditions (Li Destri Nicosia et al., 2016). Furthermore, recent investigations have demonstrated the elicitation of resistance responses in grapefruit and lemon fruits treated with PGE (Pangallo et al., 2016).

The aim of the present work was to evaluate the efficacy of PGE as an alternative control method against olive anthracnose. Experiments were conducted *in vitro*, on artificially inoculated fruits and under field conditions to determine the efficacy, the mechanisms of action and the reliability of PGE as a safe and effective control mean.

MATERIAL AND METHODS

Pomegranate extract and inoculum preparation. A concentrated pomegranate peel extract (PGE) was obtained according to Romeo et al. (2015) from organic ripe pomegranate (*Punica granatum* L.) fruit cv. "Wonderful", harvested in Acireale (Italy). The concentrated solution containing 120 g/l of dry matter and 1% citric acid as antioxidant, was stored at 5°C until use.

A fungal isolate of *C. acutatum s.s.* was obtained from a rotted olive fruit collected in Calabria, southern Italy. A conidial suspension of the pathogen was serially diluted with sterile water and plated on potato dextrose agar (PDA, Sigma- Aldrich) in order to obtain cultures from single conidia. The identity of *C. acutatum s.s.* was confirmed according to morphological features and by sequencing the ITS1-5.8S-ITS2 region and the β-tubulin 2 gene as described by Schena et al. (2014).

To produce the inoculum, *C. acutatum s.s.* was grown on PDA for 10 days and conidia were collected with a spatula, suspended in sterile distilled water, filtered through a double layer of sterile muslin cloth (Artsana, Rome, Italy) and uniformly mixed. The concentration of the conidia in the suspension was determined using a haemocytometer chamber (Brand Gmbh, Wertheim, Germany) and diluted to have stock solutions containing 10^5 conidia/ml.

***In vitro* antifungal activity of PGE.** The effect of PGE on spore germination and germ tube elongation of *C. acutatum s.s.* was determined using the procedure recently described by Li Destri Nicosia et al. (2016). Briefly, a conidia suspension of the pathogen was transferred in Eppendorf tubes containing potato dextrose broth (PDB) and PGE at three different concentrations (0.12, 1.2, and 12 g/liter) and incubated at 22°C for 20 hours. Water replaced the PGE solution in control samples. Three groups of 50 spores each were randomly selected and examined under a microscope to determine the percentage of germinated conidia and the average length of the germ tubes. Conidia were considered germinated when the length of the germ tube was at least equal to the diameter of the non-germinated conidia.

Antifungal activity of PGE on detached fruit. All the trials were performed on healthy detached olives (cv. Leccino) collected in the Monopoli area, southern Italy, where local climatic conditions are generally not conducive for olive anthracnose. Drupes, selected for uniformity in size and color and for the absence of olive fruit fly damage were surface disinfected by immersion in a 2% sodium

hypochlorite solution for 1 min, rinsed with tap water, air-dried and fixed on polypropylene honeycomb panels as previously described (Li Destri Nicosia et al., 2016). All experiments were performed using three replicates of ten fruits each, with each replicate kept in separate plastic boxes. In all the experiments, fruits were kept 0.5-1 cm apart to avoid nesting, and wounded with a nail to make 1x1 mm injuries. Wounds were treated with 10 µl of PGE at the concentration of 12, 1.2 or 0.12 g/l and inoculated with 10 µl of a spore suspension of *C. acutatum s.s.* Mock wounds, treated with 1% citric acid or sterile water and inoculated with the pathogen served as controls.

To evaluate the preventive effect, wounds were treated with the PGE and incubated for 1, 12 or 24 h before inoculation with the pathogen. Whereas, to determine the curative effect, drupes were first inoculated with the pathogen and then treated with PGE after 6 h, 12 h or 24 h. Finally, to evaluate the induction of resistance in the host tissues, PGE and pathogen were applied at two different sites approximately 3 mm apart on each olive fruit. Drupes were first treated with PGE and then inoculated with the pathogen, after 6 h, 12 h or 24 h.

All experiments were repeated twice and olives were maintained at 20°C in lidded plastic boxes containing wet paper to ensure high relative humidity (RH). The percentage of infected wounds was evaluated daily starting 4 days post inoculation.

Field trials on naturally infected olives. Field trials were carried out in 2015 and 2016 in intensive olive orchards located in the Gioia Tauro and Oppido Momertina areas, of Calabria, southern Italy, which are very close together. In 2015, treatments were applied to olives of cv Arbosana and the experiment was planned soon after the appearance of the first rotted olives (November 10). The incidence of the disease at the time that treatments were applied was approximately 5% of the pending olives. Before treatments, plants were shaken vigorously to bring down all the rotted olives. In 2016, two different treatments were applied to olives of cv Ottobratica, approximately 30 (October 13) and 15 (October 28) days before the expected epidemic outbreak of the disease.

In both years, plants (three per treatment) were selected within a randomized block design to be uniform in terms of tree size of and quantity of pending production. Treatments included PGE at three different concentrations (12, 6 and 3 g/L), copper (Bordeaux mixture 20 GD Caffaro, 20% Cu^{++}) and $NaHCO_3$ (2% w/v) (Sigma-Aldrich). Three olive trees treated with tap water and three untreated trees were used as controls. All the treatments were applied using a commercial electric driven back

sprayer (Mod. Serena, Italdifra, Castelfidardo, AN, Italy) and each tree received approximately 3 liters of solutions/water.

The incidence of the disease expressed as percentage of infected olives was determined 3 and 7 days after the treatment in 2015, and 30 days after the first treatment in 2016. In both years, 100 olives were randomly collected from each tree (300 olives per treatment) and accurately observed for the presence of anthracnose symptoms. Leaves and fruits were also examined to exclude the presence of any symptoms of phytotoxicity.

Effects of treatments on epiphytic fungal and bacterial populations. The effects of treatments on fungal and bacterial populations on olive drupes was evaluated in 2015 on treated and untreated olives two days after field treatments. Three replications of 10 apparently healthy fruit were randomly picked from each olive tree to comprise 90 olives per thesis. Collected drupes were shaken in 100 ml of sterile distilled water on a rotatory shaker at 150 rpm for 30 min. One hundred microliters of the rinse water were serially diluted and plated on PDA containing ampicillin and streptomycin sulfate (250 mg/l) to determine fungal colonies and PDA without antibiotics to detect bacteria. The plates (three per replication) were incubated at 22°C for 3–4 days. The number of fungal and bacterial colonies (CFU) was recorded and converted into CFU fruit^{-1}.

Statistical analyses. In all the trials, data were submitted to the analysis of variance (ANOVA) and means were compared using Tukey's test ($P<0.05$) to determine the significance of the treatments. In the curative essay on detached olive a two factor essay (General Linear Model) was performed, in order to determine the effect of treatment and timing and their interactions on the rot development. Percentages were converted into Bliss angular values (arcsine √%) before analysis.

RESULTS

***In vitro* antifungal activity of PGE.** The germination of conidia of *C. acutatum s.s.* was completely inhibited in PDB containing PGE at 12 and 1.2 g/l (Fig. 1). Furthermore, a significant reduction of the germination (46.3%) was also achieved with PGE at 0.12 g/l, although the length of germ tubes with this concentration of extract was not significantly different compared to the controls (water and citric acid).

The citric acid had an inconsistent inhibiting effect on the germination of the conidia and did not affect the length of the germ tubes (Fig. 1).

Efficacy of PGE on artificially inoculated olives. *Preventive effect.* PGE significantly reduced the incidence of rot caused by *C. acutatum s.s.* at all tested concentrations when applied 1, 12 and 24 h before inoculation with the pathogen (Fig. 2A). In particular, PGE at 12, 1.2 and 0.12 g/l applied 1 hour before the pathogen reduced percentage of rotting by 76.7, 56.7, and 50%, respectively. The same extract concentrations applied 12 and 24 h before the pathogen reduced the incidence of rot by 91.7, 83.3, and 62.5% and by 85.7, 60.7, and 53.5%, respectively (Fig. 2). Citric acid did not significantly affect the incidence of rot.

Curative effect. PGE at 12 and 1.2 g/l significantly reduced the incidence of decay when applied 6, 12 and 24 h after the pathogen (Fig. 2B). At the lowest tested concentration (0.12 g/l), significant reductions were achieved at 6 and 24h, but not at 12 h. Rots were reduced by 62.2, 48.2, and 24.1% at 6 h, by 63.0, 44.4 and 18.5% at 12 h and by 50.0, 40.0, and 23.5% at 24 h, using PGE at 12, 1.2 and 0.12 g/l, respectively. Overall, the highest level of protection was achieved with PGE at 12 g/l, followed by PGE at 1.2 and 0.12 g/l, although differences were not statistically significant in most of the cases (Fig. 2B). Citric acid did not significantly affect the incidence of rot.

The ANOVA two factor essay confirmed that the development of rot was significantly affected by the PGE treatment ($F= 63,41$; $df = 4$; $P<0.001$) and by the timing of application ($F= 10,16$; $df = 2$; $P<0.001$) but excluded any significant interaction time X treatment ($F= 1,59$; $df = 8$; $P= 0.168$).

Induction of resistance. Using PGE at 12 and 24 g/l a significant reduction of rot was achieved without direct contact between the pathogen and PGE, suggesting the induction of resistance in olives treated with the extract (Fig. 3). In particular, PGE at 12 g/l reduced rots by 53.5, 59.3 and 40.7% in olives inoculated with the pathogen 6, 12 and 24 h after the extract, respectively. In the

same inoculation times, PGE at 1.2 g/l reduced rots by 46.4, 37.0, and 33.3%, respectively. Furthermore, a significant reduction was achieved with PGE at the lowest concentration (0.12g/l) at 24 h ($P \leq 0.05$).

Field trials on naturally infected olives. In 2015, the incidence of natural rot on olive trees sharply increased after 10th November (treatment date) and reached 100% of the drupes after just three days on untreated and water-sprayed plants (Fig. 4). On the contrary, the development of natural rot was completely inhibited on olives trees treated with PGE at 12 g/l and was reduced by 98.6 and 93.0% on plants treated with PGE at 6 and 3 g/l, respectively (Fig. 4). PGE was significantly more effective than copper and $NaHCO_3$ at all tested concentrations ($P \leq 0.05$). In particular, three days after treatments, the incidence of infected drupes on plants treated with copper and $NaHCO_3$ was significantly reduced by 58.8 and 55.0%, respectively. No significant differences were revealed between $NaHCO_3$ and copper (Fig. 4). Seven days after the treatment, PGE at 12, 6, and 3 g/l still reduced the incidence of rotted olives compared to the controls by 84.4%, 73.0%, and 72.2%, respectively. A significant reduction was also revealed for $NaHCO_3$ and copper, but the entity of the reduction (around 27%) was much lower compared to PGE treatments (Fig. 4).

Trials conducted in 2016 confirmed a high efficacy of PGE, which significantly reduced rot at all concentrations. However, a lower level of protection was generally observed compared to 2015. In particular, rot was reduced by 77.6, 57.0, and 51.8% using PGE at 12, 6 and 3 g/l, and by 46.5 and 36% with copper and $NaHCO_3$, respectively (Fig. 5). PGE at 12 g/l was significantly more effective than copper and $NaHCO_3$. Notably, no signs of phytotoxicity were detected on the plants in either of the trials.

Effects of treatments on fungal and bacterial populations. PGE treatments significantly reduced fungal and bacterial populations at all tested concentrations (Fig. 6). In particular, fungal populations were reduced by 99.5, 83.9, and 75.9% using PGE at 12, 6, and 3 g/l, and by 68.3 and 52.5% using copper and $NaHCO_3$, respectively (Fig. 6A). A slightly higher level of population was revealed on olives sprayed with water when compared to untreated olives, but differences were not significant ($P \leq 0.05$).

A similar trend was determined for bacteria (Fig. 6B). Their population was reduced by 98.2, 91.4, 85.2, on olives treated with PGE at 12, 6, and 3 g/l and by 78,7 and 55% on those treated with copper

and $NaHCO_3$, respectively. Significant differences were observed between $NaHCO_3$ and PGE-treated drupes. As for fungi, a slightly higher level of population was revealed on olives sprayed with water compared to untreated olives, but the differences were not significant ($P \leq 0.05$).

DISCUSSION

In the present study, we demonstrated the feasibility of PGE as a very effective natural antifungal preparation to control olive anthracnose caused by *Colletotrichum* species. Preliminary *in vitro* tests and experiments conducted on artificially inoculated drupes highlighted a strong antimicrobial activity and a high efficacy of PGE in preventive and curative applications. Similar results were obtained in previous investigations focused on important necrotrophic pathogens such as *B. cinerea, P. digitatum, P. italicum* and *P. expansum* (Romeo et al., 1015; Li Destri Nicosia et al., 2016). The high efficacy of PGE also against typical hemi-biotrophic fungi like *C. acutatum* spp. (Guidarelli et al., 2011) further proof its broad spectrum of activity that may contribute towards making economically sustainable the costly process needed for the registration and commercialization of natural products.

On drupes artificially inoculated with *C. acutatum s.s.*, a significant reduction of rot was also achieved without direct contact between PGE and the pathogen, suggesting a strong induction of resistance, enough to inhibit the pathogen development and the infection of the drupes. Previous investigations demonstrated that PGE can increase the activity of reactive oxygen species (ROS) and activate genes involved in defense responses such as chitinase, chalcone synthase, mitogen-activated protein kinase and mitogen-activated protein kinase kinase and phenylalanine ammonia lyase in grapefruits (Pangallo et al., 2016). Although the molecular nature of the induced resistance in olives was not investigated, our results support a non-host specific induction, confirming previous speculations on the broad range of application of PGE.

The induction of resistance may have also played a role in the high efficacy observed when PGE was applied 12 or 24 hours before the pathogen. The ability of PGE to protect olives for several hours after its application is a very important feature and may prove synergic with the observed curative activity. Indeed, PGE significantly reduced the incidence of rot also on fruit treated 6, 12 and 24 h after the pathogen inoculation, i.e. sufficient time for conidia to germinate and start the infection process. It has been reported that latent infections established during early fruit development phases plays an important role in the disease cycle of olive anthracnose by favoring the survival of the pathogen during the summer period and represents an important source of inoculum for epidemics on ripening fruit (Moral et al. 2014; Talhinhas et al. 2011). In this context, a natural compound able to reduce latent infections and protect fruits after its application may be extremely useful.

The results obtained in field trials by simulating practical commercial conditions were particularly remarkable. Indeed, while many alternative control methods have proved effective under laboratory conditions, most of them failed when tested in large-scale trials. The highest level of protection was achieved in 2015 when PGE was applied soon after the appearance of the first rotted olives i.e. at the early ascending phase of the disease outbreaks. After three days, the incidence of the disease on control plants (untreated or sprayed with water) reached 100%, while natural rot was completely inhibited on olive trees sprayed with PGE at 12 g/l and reduced by 98.6 and by 93.0% on plants treated with PGE at 6 and 3 g/l, respectively. The high incidence of the disease on control plants after only three days, suggests that most olives may have already been infected when treated with the extract, although still asymptomatic. These results, in agreement with above discussed tests on artificially inoculated drupes, indicate a strong curative action of PGE. On the other hand, the existence of ongoing infections at the treatment time may explain the low efficacy of copper, which is a typical protective chemical. Interestingly, a very high level of protection was achieved on PGE-treated olives, even 7 days after treatments, confirming a long-term protection of the treated olives with very important practical implications.

A high level of efficacy against olive anthracnose was also achieved in 2016 using a different application strategy since two anticipated treatments were carried out approximately 30 and 15 days before the expected epidemic outbreak of the disease. Compared to 2015, PGE provided a lower level of protection (ranging from 77.6 and 51.8%, according to the concentration), yet it was significantly more effective than copper and $NaHCO_3$. These results may suggest that the most successful strategy is the application of PGE at the early ascending phase of the disease outbreaks. However, more investigations are needed to define dosage, time, and method of application in consideration of the many factors that can affect the efficacy of treatments under field conditions. For instance, in 2016 recurrent heavy rains created very conducive conditions for the disease and prevented the access to the field during the early phases of the epidemic outbreak when differences between treated and untreated trees were likely to be higher. Nevertheless, it is important to highlight that both field trials were conducted under very conducive conditions due to the high sensitivity of the investigated olive cvs (Arbosana and Ottobratica) and the prolonged wet periods typical of the investigated area (Cacciola et al., 2012).

Overall, our results demonstrate a very high potentiality of PGE in controlling olive anthracnose since it provided very high levels of protection and it was significantly more effective than copper, which is traditionally used to control the disease (Cacciola et al., 2012). Furthermore, copper has serious ecological drawbacks since its use can lead to pollution of aquifers and to an accumulation in the soil, with toxic effects for both the microbial population and the fauna of the land and crops. It is also of concern that it may have harmful effects on human health (Cacciola et al., 2012). In field trials, we also used $NaHCO_3$, which has been reported as an alternative control mean for olive anthracnose (Agosteo et al., 2007). Although $NaHCO_3$ was the least effective treatment, it still provided a significant reduction of rot. This salt may be useful in integrated control strategies in consideration of its safety and low costs as well as the lack of legislative restrictions that allows its distribution before harvesting (Nigro et al., 2006).

The analysis of epiphytic populations on olive drupes highlighted a strong and broad antimicrobial activity of PGE, which significantly reduced both fungal and bacterial populations. The high content of punicalagins in PGE (Romeo et al., 2015) is likely to play a role in this activity since these phenolic compounds, characteristic of pomegranate, are well known for their antimicrobial activity (Glazer et al., 2012; Taguri et al., 2004). These results suggests the need for specific studies to evaluate the degradation rate of PGE and its long-term impact on natural microbial populations. Furthermore, its toxicity for humans and other mammals needs to be evaluated. However, the fact that pomegranate peel extracts are commonly used as food additives, functional food ingredients or biologically active components in nutraceutical preparations is more than encouraging (Akhtar et al., 2015). Furthermore, the medicinal properties of pomegranate peel tannins have been traditionally used to treat several common ailments and prevent bone loss (Spilmont et al., 2015). In this context, it is also important to highlight that PGE is obtained using safe and food grade chemicals such as ethanol and citric acid (Romeo et al., 2015) and in the present study, as well as in previous investigations, did not show any phytotoxic effects (Li Destri Nicosia et al., 2016).

ACKNOWLEDGEMENTS

This research was funded by The Italian Ministry of Education, University and Research (MIUR) with grant "Modelli sostenibili e nuove tecnologie per la valorizzazione delle olive e dell'olio extravergine di oliva prodotto in Calabria - PON Ricerca e competitività 2007–2013 (PON03PE_00090_02). The authors wish to thank Mrs. Ann Davies for the revision of the English text.

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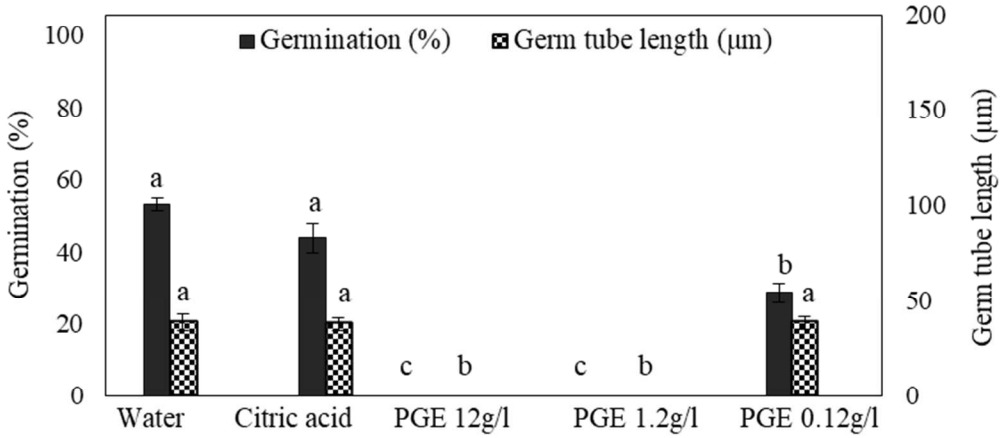

Fig. 1 - Effect of pomegranate peel extract (PGE) on germination and germ tube elongation of conidia of *Colletotrichum acutatum s.s.* incubated for 20 h at 22°C in potato dextrose broth (PDB) containing PGE at different concentrations (12, 1.2 or 0.12 g/l). Conidia incubated in PDB containing water and 1% citric acid served as a control. Bars indicate standard errors of the means. For both investigated parameters, germination and germ tube length, different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

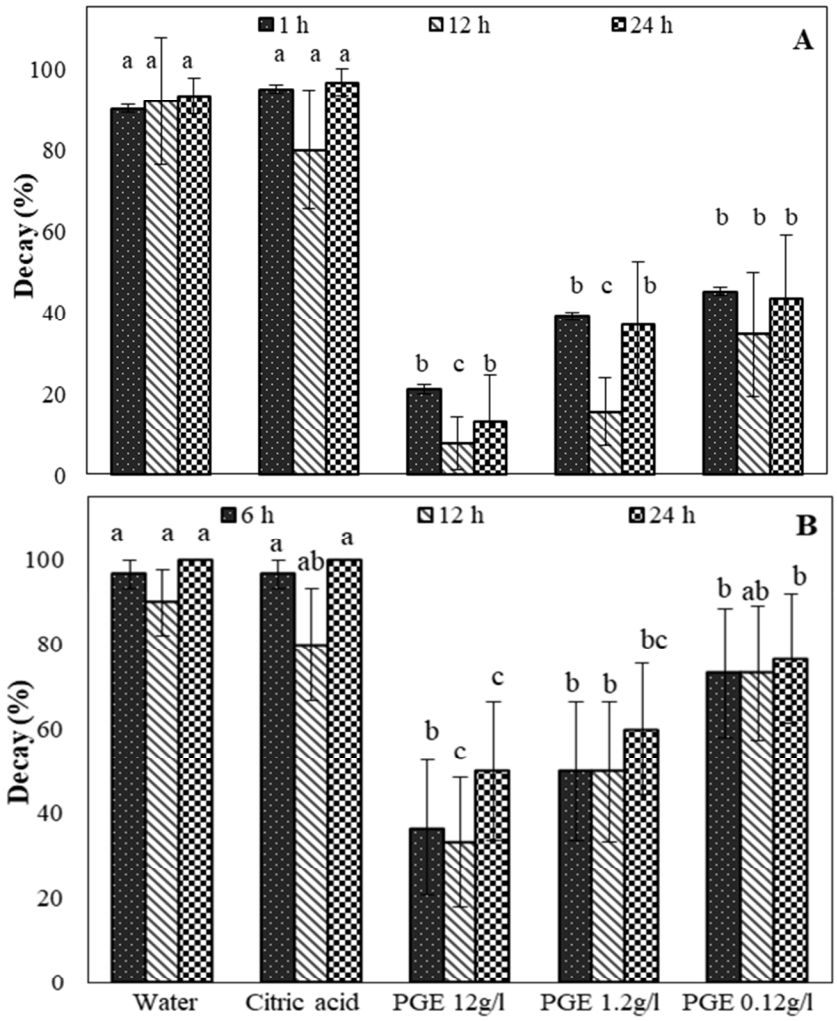

Fig. 2 - Preventive (A) and curative (B) effect of pomegranate peel extract (PGE) on artificially inoculated olives. In preventive trials fruit were first treated with PGE and then inoculated with a spore suspension of *Colletotrichum acutatum s.s.* after 1, 12 or 24 h. In curative trials, olives were first inoculated with a spore suspension of the pathogen and then treated with PGE after 6, 12 and 24 hours. In both trials PGE was tested at different concentrations (12, 1.2 or 0.12 g/l) and fruit mock treated with water or 1% citric acid and inoculated with the pathogen served as controls. Bars indicate standard errors of the means. For each assessment time, different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

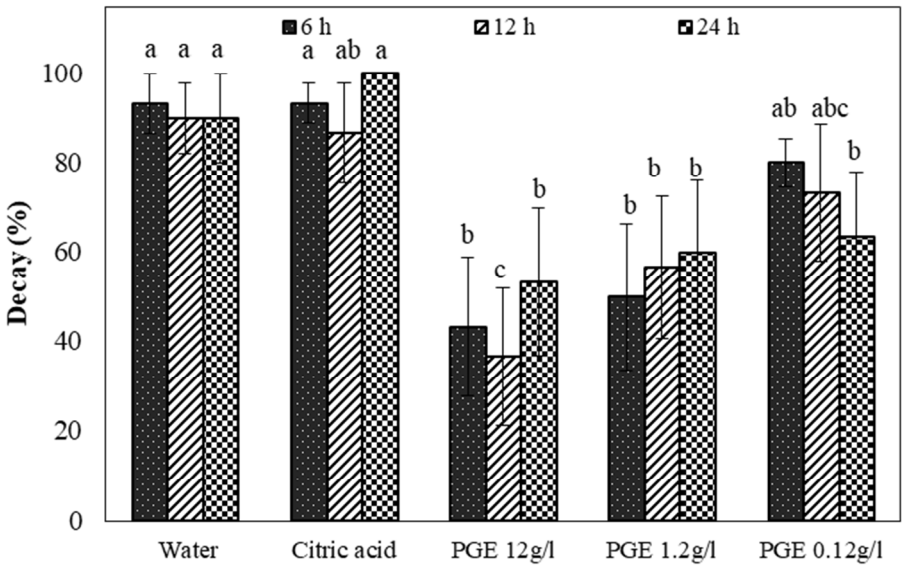

Fig. 3 - Incidence of decay in olives treated with pomegranate peel extract (PGE) and inoculated with *Colletotrichum acutatum s.s.*, in spatially separated wounds (induction of resistance). PGE was applied 6, 12 or 24 hours before the pathogen at different concentrations (12, 1.2 or 0.12 g/l). Fruit mock treated with water or 1% citric acid and inoculated with the pathogen served as controls. Bars indicate standard errors of the means. For each assessment time, different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

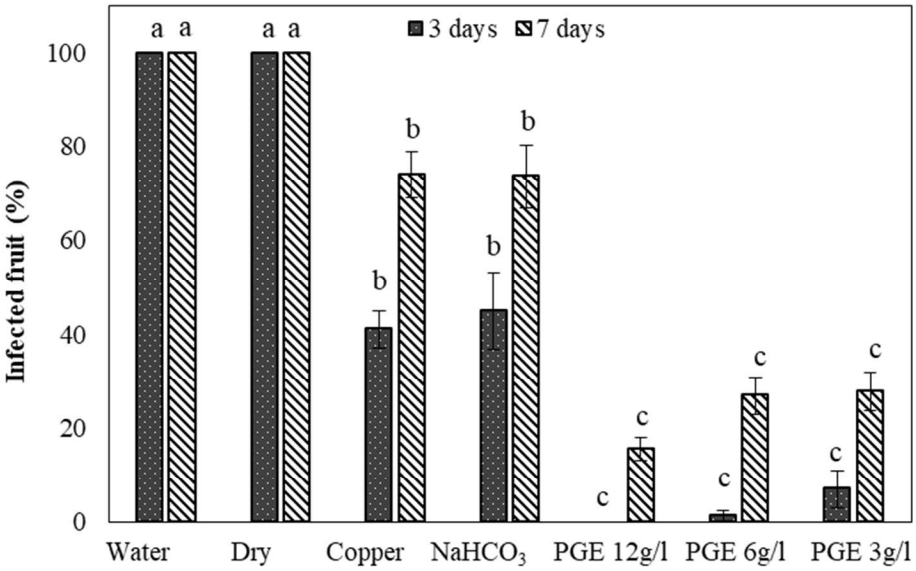

Fig. 4 - Incidence of rotted drupes on olive trees of cv Arbosana treated in 2015 with PGE at three different concentrations, copper or NaHCO3. Olive trees untreated or sprayed with tap water were used as controls. The incidence of rotted olives was evaluated 3 and 7 days after treatments. Bars indicate standard errors of the means. For each assessment time, different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

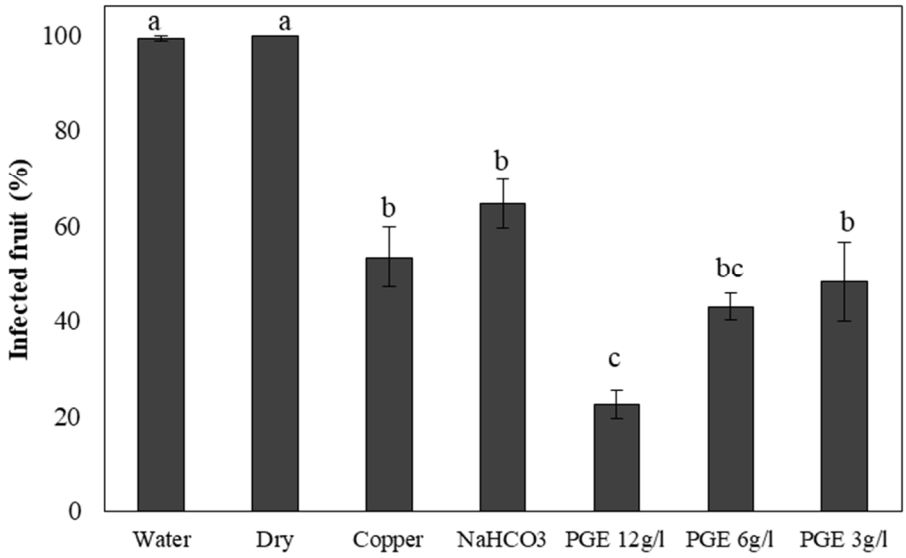

Fig. 5 - Incidence of rotted drupes on olive trees on cv Ottobratica treated in 2016 with PGE at three different concentrations, copper or NaHCO3. Olive trees untreated or sprayed with tap water were used as controls. Treatments were made 15 and 30 days before the evaluation of the incidence of the disease. Bars indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

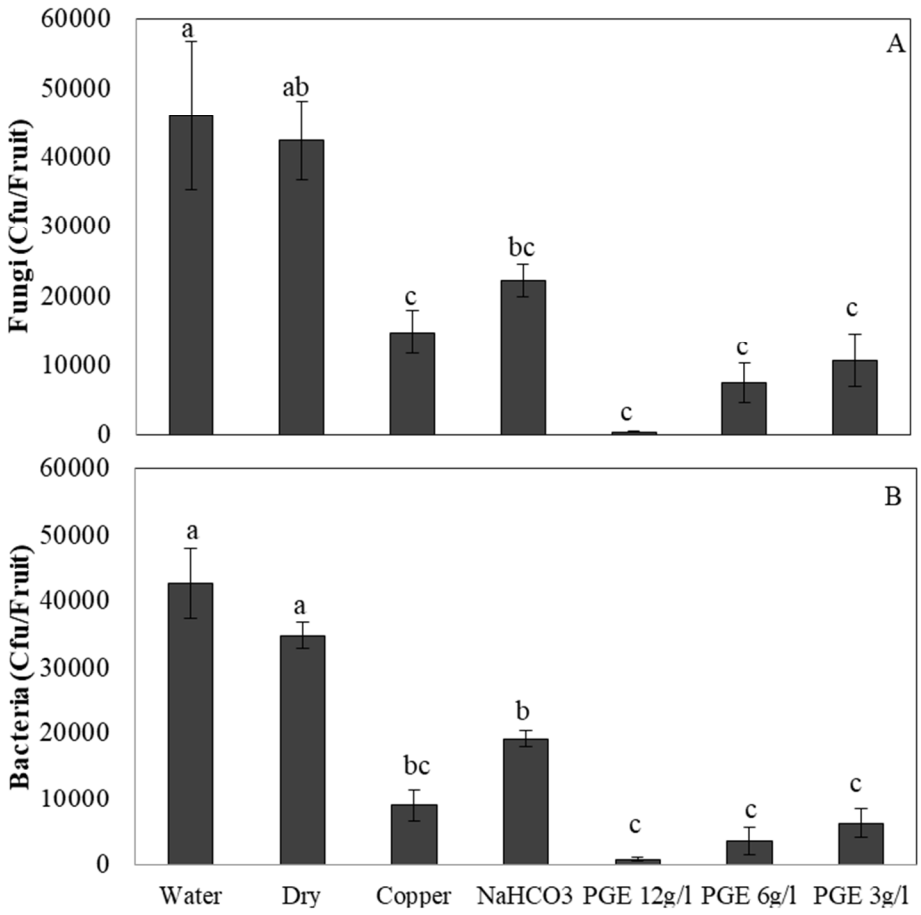

Fig. 6 - Consistence of fungal (A) and bacterial (B) populations on olive drupes of cv Arbosana treated in 2015 with PGE at three different concentrations, copper or NaHCO3. Drupes untreated or sprayed with tap water were used as controls. Bars indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).