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DEVELOPMENT AND APPLICATION OF ALTERNATIVE CONTROL METHODS TO CONTROL POSTHARVEST ROTS OF FRESH FRUIT AND VEGETABLES

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Abstract

A Pomegranate Peel Extract, named PGE, has been proposed as a natural antimicrobial substance with a wide spectrum of activity against several postharvest diseases. In the present project, the evaluation of the efficiency of the extract under large scale commercial conditions was conducted, together with a deep investigation on its mechanism of action and its antimicrobial activity against major foodborne pathogen. Under large-scale commercial conditions, the efficiency of PGE was evaluated against rots of Valencia orange and clementine. The extract, proved a significant higher level of protection compared to Imazalil (IMZ), a commonly used chemical fungicide for postharvest treatments. After cold storage and shelf life period, the incidences of decay on oranges sprayed just before harvest with PGE at 12, 6, and 3 g/l, was reduced by 78.9, 76.0, and 64.6%, respectively. Similarly, postharvest dipping treatments with PGE reduced rots by 90.2, 84.3, and 77.6%, respectively. Comparable levels of protection were also achieved on clementine treated before harvest. PGE treatments proved high antimicrobial activity with long persistence resulting in high reduction in losses, longer shelf life and enhancement of the fruit quality. Furthermore, the extract showed a strong antimicrobial activity against epiphytic bacterial and fungal population suggesting its possible use as sanitizers to reduce the microbial contamination of recirculated water in packinghouses.

On the other hand, RNA-seq analyses, conducted on wounded orange fruit 0, 6, and 24 h after PGE applications, showed a significantly different transcriptome in treated oranges as compared to control samples. Our analysis showed a very quick response of gene expression (after 1h post treatment) accompanied by high up-regulation of genes (273 deferentially expressed gene). Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis showed the involvement of 1233 gene ontology (GO) terms and 35 KEGG metabolic pathways. Among these, important defense pathways were induced and antibiotic biosynthesis was the most enriched one. These findings may explain the previously documented preventive and curative activity of PGE against plant diseases.

Finally, the evaluation of the potential use of PGE as natural antimicrobial treatment to reduce the growth of foodborne pathogens using *Listeria monocytogenes* as a model pathogen *in vitro* and on fresh-cuts of melon, apple and pear, revealed high bactericidal and bacteriostatic activities of PGE. The *in vitro* results revealed that regardless of the tested concentration, PGE exerted a quick and high significant inhibitory activity against all the tested *L. monocytogenes* strains. Similarly, *in vivo* results also confirmed a strong

antibacterial activity of the extract that significantly reduced the bacterial load on fresh-cut fruit and maintained the population at low levels throughout the storage period. The findings of the present study will incorporate new knowledge on the potential use of PGE as potent alternative control mean against wide range of pathogens and will particularly contribute to the already ongoing process to register a commercial formulation of PGE.

Keywords: Pomegranate peel extract; Citrus rots; Alternative control methods transcriptomics; plant defense; *Listeria monocytogenes*, fresh-cut fruit.

Riassunto

Il progetto di ricerca riguarda lo studio di un estratto di buccia di melograno, denominato PGE (acronimo del nome inglese "PomeGranate peel Extract"), come mezzo di lotta alternativo contro svariati agenti di marciumi dei frutti nella fase post-raccolta. Nel corso dello studio vengono valutate sia le potenzialità applicative dell'estratto, saggiandolo in differenti condizioni commerciali di larga scala, sia i meccanismi di azione e l'attività antimicrobica nei confronti dei principali patogeni del post-raccolta. I risultati di questo studio mostrano l'elevata efficacia del PGE nella difesa post-raccolta di arance Valencia e clementine in condizioni commerciali di larga scala. Infatti, l'estratto ha mostrato un'azione protettiva significativamente superiore all'Imazalil, un fungicida largamente adoperato per la difesa degli agrumi in post-raccolta. L'incidenza dei marciumi sui frutti trattati immediatamente prima della raccolta e sottoposti a frigoconservazione e successiva *shelf life* è stata ridotta del 78.9, 76.0, e 64.6% , dal PGE rispettivamente alle concentrazioni di 12, 6 e 3 g/litro. Analogamente per quanto riguarda i trattamenti per immersione dei frutti in post-raccolta sono stati raggiunti livelli di riduzione dei marciumi pari al 90.2, 84.3, e 77.6%. Simili risultati sono stati ottenuti dall'applicazione dell'estratto sulle clementine prima della raccolta. La spiccata antività antimicrobica del PGE, associata alla sua lunga persistenza, sortisce oltre ad un prolungamento della *shelf life*, una forte riduzione delle perdite di prodotto ed un miglioramento della qualità dei frutti. Un'ulteriore caratteristica molto interessante dal punto di vista applicativo è l'abbattimento della carica microbica superficiale conseguente al trattamento dei frutti, che prospetta delle possibilità di utilizzo dell'estratto come sanitizzante delle acque di lavaggio e degli ambienti di stockaggio e lavorazione dei frutti. Gli effetti dei trattamenti con il PGE possono essere attribuiti all'induzione di vie metaboliche sui frutti trattati che ne influenzano la suscettibilità ai marciumi e la qualità. Infatti, dalle analisi di sequenziamento del m-RNA condotto su arance 0, 6 e 24 ore dopo il trattamento con PGE, i profili trascrittomici dei frutti trattati sono significativamente differenti rispetto al controllo non trattato. Dalle analisi si osserva l'attivazione immediata dell'espressione genica (1 ora dopo il trattamento) accompagnata da una diffusa sovrespressione genica (273 geni espressi in maniera differenziale fra trattati e non trattati). Secondo le analisi di genomica funzionale eseguite mediante *Gene Ontology* (GO) e *Kyoto encyclopedia of genes and genomes* (KEGG) per individuare i *pathways* metabolici attivati (*pathway enrichment analysis*) si rileva il coinvolgimento di 1233 termini GO e di 35 vie metaboliche KEGG. Nell'ambito di tali vie metaboliche sono incluse importanti vie deputate alla difesa, fra cui le vie preposte alla sintesi di antibiotici sono le più rappresentate. I risultati di cui sopra spiegano l'azione preventiva, oltre che curativa, dei trattamenti dei frutti con PGE nella lotta contro i patogeni. Inoltre l'azione antimicrobica del PGE è stata

valutata nei confronti di *Listeria monocytogenes*, patogeno dell'uomo di origine alimentare. Il PGE applicato in prove *in vitro* e per il trattamento di frutta porzionata (meloni, mele e pere) ha mostrato un forte effetto battericida e batteriostatico su questo patogeno-modello. Nelle prove *in vitro* è stata esercitata una forte azione battericida del PGE significativa per tutte le concentrazioni saggiate. Analogamente i risultati delle prove *in vivo* confermano la capacità dell'estratto di ridurre significativamente la carica batterica sulla frutta fresca porzionata, mantenendo la popolazione batterica a livelli contenuti durante la frigoconservazione. Le conoscenze acquisite dalla ricerca esposta nella presente tesi costituiscono un importante contributo per la valorizzazione del PGE come efficace metodo di lotta alternativo contro un'ampia gamma di patogeni ed in particolare per favorire il processo di registrazione di un formulato commerciale basato sull'estratto.

Parole chiave: estratto di melograno; marciumi dei frutti di agrumi; metodi di lotta alternativi; trascrittomica; difesa delle piante; *Listeria monocytogenes*; frutta pronta al consumo (IV gamma)

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Chapter 1. General Introduction

Over the past 50 years, the world population has increased to reach more than 7 billion people in 2017. To meet the food demand of such rapid growing population, the food production is estimated to increase by 70% by 2050 (Crist *et al.*, 2017). This increased demand for food production has in turn increased the disease pressure on crop plants (Gill and Garg, 2014). Thus, food losses are more and more a matter of serious concern. In particular, fruit and vegetables have the highest wastage rates of any other food product; almost half of all the produced fruit and vegetables are being lost mainly due to pre and post-harvest diseases, poor management techniques, and bad conservation methods (Kitinoja *et al.*, 2018; Rosegrant *et al.*, 2018). Therefore, a major effort is conducted to control these losses.

For decades, chemical fungicides have been the main post harvest disease control mean because of the their high level of efficiency, easy application and relative low cost (Barzman *et al.*, 2015). However, there is an increasing concern about their use because of the potential risks for human health, the negative impact on the environment and non-target microorganisms, and the increasing selection of pathogen resistance strains (Sanzani *et al.*, 2010; Gill and Garg, 2014). This, together with the rise of consumer awareness in food safety and healthy living, is promoting an increasing interest to safe and environmentally friendly alternative control means. Currently, the development of effective and sustainable control means for postharvest diseases is one of the main focus of modern agriculture (Wisniewski *et al.*, 2016). Therefore, the development of new biocontrol strategies is a well investigated line of research that aims to reduce or eliminate the use of chemical pesticides. These strategies are usually applied alone or as a part of integrated pest management program replacing, thereby, chemical pest control (Spadaro and Gullino, 2004; van Lenteren *et al.*, 2018). To date, several alternative methods have been applied, mainly, biocontrol products e.g. *Bacillus spp* and *Candida oleophila* (van Lenteren *et al.*, 2018; Mari *et al.*, 2016); disinfecting agents e.g. chlorine, ethanol, and organic acids (Feliziani *et al.*, 2016); physical treatments e.g. UV radiation, cold, heat, radiofrequency, and modified atmosphere (Usall *et al.*, 2016; Yao *et al.*, 2018); and more importantly Generally Regarded As Safe (GRAS) plant derivates including natural components, plant volatiles and, in particular, plant extracts (Palou *et al.*, 2016; Mari *et al.*, 2016).

1. Plant extracts

Plant extracts have recently emerged as very promising alternatives to chemicals. The high demand for environmentally sound and biodegradable products grabbed a special attention for more profound research on these substances. In fact, the use of plant extracts as antimicrobial agents has been known for centuries. They were used as remedies in folk medicine (Petereit *et al.*, 1991; Ehrhardt *et al.*, 2007). Therefore, the activity of several plant extracts has been intensively investigated. They showed different mechanism of action including direct antimicrobial activity against pathogens and/or induction of resistance in the plant host.

1.1. Plant extracts antimicrobial activity

Many plant extracts have been investigated as natural compounds to control plant diseases or to prevent fruit spoilage proving effectiveness against a wide range of plant and food borne pathogens such as *Penicillium digitatum*, *Penicillium italicum*, *Rhizopus sp.*, *Alternaria solani*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas spp.*, *Salmonella spp.*, etc. (Betoni *et al.*, 2006; Mostafa *et al.*, 2018; Latha *et al.*, 2009). The richness of these natural extracts in secondary metabolites, such as phenols, quinones, flavonoids, saponins and tannins, are reported to be the major active components of their antimicrobial activity (Gurjar *et al.*, 2012). For instance, allicin (diallyl thio sulphinate) is a volatile antimicrobial component synthetized by garlic when the tissues are damaged. Extracts of garlic showed high antimicrobial efficiency against a range of plant and food pathogens such as *Alternaria spp. Phytophthora spp., Escherichia coli*, and *Salmonella, spp*., (Ekwenye and Elegalam, 2005). Other active components including curcumin and gingerol, highly present in turmeric (*Curcuma longa Linn.*) and ginger (*Zingiber officinale Rosc.)*, respectively, were reported to also possess high antibacterial and antifungal properties against *Phytopthora infestans, Fusarium solani, Pyricularia oryzae, E. coli* and *Staphylococcus aureus,* etc. (Gurjar *et al.*, 2012; Jakribettu *et al.*, 2016). Notably, other extensively investigated and commercialized plant extracts are neem extracts (*Azadirachta indica*). They demonstrated strong and rapid antifungal, antibacterial and insecticidal activity due to the presence of important active components presented by Azadirachtin terpenoides (Lokanadhan *et al.*, 2012; Girish and Shankara, 2008). Although the main antimicrobial active components of plant extracts are generally identified, reports showed that some more components might also be responsible for the antimicrobial effect of nature substances and plant extracts (Rauha *et al.*, 2000). Furthermore, although the antimicrobial activity of these extracts is variable and relative to the extract composition, various extraction solvents such as acetone, methanol, ethanol, diethyl ether, chloroform, and water, have been investigated for better yield and quality of the extract (Jones and Kinghorn, 2006; Azmir *et al.*, 2013). In

particular, the choice of the solvent depends on the target active components to be extracted, in order to guarantee a consistent antimicrobial activity (Parekh *et al.,* 2006). A good solvent should have a high efficiency in solubilizing antimicrobial components, elevated extraction rate, fast ability to evaporate at low temperature, and low toxicity (Gurjar *et al.,* 2012). The most commonly used solvents for plant material extraction are methanol, ethanol and water (Turkmen *et al.,* 2006). Among these, water is generally less effective in solubilizing compound and its evaporation is not as fast as alcohols. On the other hand, methanol may pose toxicological issues. This latter aspect is very important since most of the antimicrobial plant extracts are used to control food and post-harvest pathogens and, therefore, the choice of the solvent is critical in order to respect the human health and the environment.

1.2. Plant induced resistance

Several natural alternative control substances and, particularly, plant extracts haven't only showed a direct antimicrobial activity against major plant pathogens but, interestingly, they induce resistance in the host plant similar to the one induced by pathogen infection (Oostendorp *et al.*, 2001; Burketova *et al.*, 2015). This is an important feature that is very valuable as pest control strategy especially in organic agriculture and also in integrated pest management program that aims to reduce the use of chemical pesticides. These natural treatments are considered plant resistance inducers or so-called plant resistance activators. They induce the plant's own defense mechanisms through the activation of battery of defense genes protecting, therefore, the plant against biotic and abiotic stresses (Burketova *et al.*, 2015; Conrath, 2011). The protection level depends mainly on the type of the elicitor, the way and timing of its application as well as the plant genotype and developmental stage (Alexandersson *et al.,* 2016). There are two forms of induced resistance: systemic acquired resistance (SAR), and induced systemic resistance (ISR). Both forms are triggered by prior infection or artificial treatment, and they are efficient against broad spectrum of pathogens, and they can be differentiated by the nature of the elicitor and the metabolic pathways involved (Burketova *et al.,* 2015). SAR is triggered by local pathogen infection or artificial treatments such as BTH, probenazole. It relies on pathways regulated by salicylic acid (SA) and it induces the accumulation of pathogenesis-related proteins (Bernsdorff *et al.,* 2016). Whereas ISR is initiated by the colonization of the roots by rhizobacteria or fungi and it is triggered by mobile signals consisting of jasmonic acid (JA) and ethylene (ET) and, unlike SAR, it doesn't involve the accumulation of pathogenesis-related proteins, but instead it activates the production of antimicrobial peptides (defensins) (Oostendorp *et al.*, 2001). Therefore, the level of efficiency of plant resistance elicitors usually depends on their ability to enhance plant resistance mechanism. The plant's response to resistance inducers is generally associated with the activation of signal

transduction pathways leading to the alterations in cell wall composition, production of phytoalexins and anti-microbial protein, deposition of callose, production of reactive oxygen and nitric oxide, accumulation of pathogenesis-related (PR) proteins, etc (Alexandersson *et al.*, 2016; Oliveira *et al.*, 2016). The efficiency of resistance elicitors in higher plants is well documented and the advances in research is accompanied by the commercialization of products that respect the human health and the environment and, in the same time, prove high efficiency in controlling plant pathogens. For instance, one of the widely known and effective plant extracts is the ethanolic extract of knotweed (*Reynoutria sacchalinensis (F. Schmidt) Nakai*) commercialized under the name of Milsana®. It is categorized as resistance inducer against powdery mildew of cucumber, wheat and roses (Vechet *et al.*, 2009; Burketova *et al.*, 2015; Pasini *et al.*, 1997; Fofana *et al.*, 2002). It induces the expression of genes responsible of the biosynthesis of flavonoids and the accumulation of phytoalexins and hydrogen peroxide. Many other plant extracts were also tested as possible resistance inducers such as the aqueous extracts of neem leaves (*Azadirachta indica Juss.*) which triggered the activation of defense related genes such as phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) with rapid accumulation of phenolic compounds (Paul and Sharma, 2002). Similarly, extracts of *Hedera helix* also proved implication in inducing defense responses to control *Erwinia amylovora* in apple rootstock (Baysal and Zeller, 2004). Another interesting example demonstrating that plant wastes could also serve as plant inducers, is extracts of sugar beet waste. These extracts showed, under greenhouse conditions, high efficiency in controlling *Phytophthora infestans.* The treated potatoes showed induction of the pathogenesis-related protein (PR-1 and PR-2) (Moushib *et al.*, 2013).

2. Pomegranate peel extracts

Although the global trend has shifted towards alternative control means and particularly plant extracts, finding good extract that provides at once several features such as high antimicrobial efficiency, long persistence, and ability to induce resistance in the host plant is essential to build up a potent integrated pest management strategy. In this regard, extracts from pomegranate peel (*Punica granatum* L.) have emerged as very promising antimicrobial substances. Their medical applications in ancient times have pushed researchers into more profound studies about the application of these extracts not only in the medical sector but also in other biology fields including plant protection. Serval studies started with phytochemical screening of different parts of the pomegranate fruit revealing high predominance of bioactive components in the peel part (Singh *et al.*, 2002; Orak *et al.*, 2012). These bioactive constituents are represented by phenolic components, mainly punicalagin, and ellagic and gallic acids. These molecules are reported to be very potent antioxidants components (Tehranifar *et al.*, 2011;

Zahin *et al.*, 2010). However, the concentration of these active components varies from an extract to another depending on many factors such as extraction method, fruit maturity stage, variety, etc (Shwartz et al., 2009; Romeo et al., 2015). Therefore, many studies have investigated the antimicrobial activity of different pomegranate peel extracts (PPEs) and evaluated their potential use as biocontrol method against major plant diseases. Different PPEs showed, in *in vitro* trials, high significant inhibitory activity against various major plant and food pathogens including *Botrytis cinerea*, *Penicillium digitatum*, *Alternaria alternata, Stemphylium botryosum, Fusarium oxysporum, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella spp.*, etc (Rongai *et al.*, 2015; Glazer *et al.*, 2012; Rongai *et al.*, 2018; Tayel *et al.*, 2009; Nuamsetti *et al.*, 2012; Oraki *et al.*, 2011). Although, in the agronomic sector, most of the investigations have yet been *in vitro* trials, numerous studies were carried out under *in vivo* conditions. For instance, (Elsherbiny et al., 2016) demonstrated that a methanolic extract of pomegranate peel exerts both preventive and curative antimicrobial activity against dry rot of potato tubers caused by *Fusarium sambucinum.* Similarly, another PPE showed high efficiency in reducing rots caused by *P. digitatum* on citrus fruit (Tayel *et al.*, 2009). While, other reports showed the effect of these extracts in preserving the quality and extending the shelf life of fruit when the extract is incorporated in the coating formulations (Rongai *et al.*, 2018; Nair *et al.*, 2018). This high antimicrobial activity of pomegranate peel extracts pushed for more pronounced investigations on its possible toxicological effects on the human health. Studies proved its safe use and suggested its potential application as food additive or as treatment against several human diseases (El-Desouky et al., 2015; Jahromi et al., 2015).

Recently a particularly promising extract from pomegranate peel has been identified as PGE (Romeo *et al.*, 2015). It is a concentrated aqueous extract obtained from 80% ethanol/water mixture after evaporation of ethanol under vacuum at 40 °C. PGE extract solution is supplemented with 1% citric acid as antioxidant or acidifying agent, regarded as safe and widely accepted by the public opinion and authorities (Romeo *et al.*, 2015). A chemical characterization of PGE showed that the extract is rich in polyphenols with 66.97g gallic acid equivalents/kg of total Phenolics presented mainly by gallic acid and punicalagin, and 21.64mg cyanidin 3-glucoside equivalents/kg of total anthocyanins. PGE proved highly effective against a wide range of major fungal diseases under both experimental and commercial conditions. It showed a wide spectrum of activity being effective against grey mold caused by *Botrytis cinerea* on table grapes and sweet cherries, anthracnose caused by *Colletotrichum acutatum sensu stricto* and *Colletotrichum godetiae* on olives, brown rot caused by *Monilinia spp.* on sweet cherries, green and blue molds caused by *P. digitatum* and *P. italicum* on citrus and blue mold caused by *Penicillium expansum* on apples (Li Destri Nicosia *et al.*, 2016; Pangallo *et al.*, 2017a; Pangallo *et al.*, 2017b). PGE showed a strong

antimicrobial effect with preventive and curative activity, and a long persistence even when applied under commercial conditions (Pangallo *et al.,* 2017b). Furthermore, recent investigations have demonstrated the induction of resistance in citrus and olive fruit treated with PGE, although specific studies to identify the involved genes in the induced resistance have not been yet conducted (Pangallo *et al.,* 2017a).

Scope of the thesis

Considering the potential use of PGE as natural antimicrobial and plant protection preparation, the aim of the present study was to advance the current knowledge of this extract. In this context, specific investigations were conducted to: i) evaluate its efficacy, under large scale commercial conditions, against postharvest blue and green mold of citrus as pre and postharvest treatment. ii) investigate its mechanism of action using a transcriptomic approach to determine the genes and pathways activated in citrus tissues after the extract treatment; and iii) evaluate the potential use of PGE as natural antimicrobial sanitizer to control a major foodborne pathogen such as *Listeria monocytogenes*. Obtained results will significantly contribute to the already ongoing process to register a commercial formulation of PGE.

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Chapter 2. Pre- and postharvest applications of a pomegranate peel extract to control decay of citrus fruit during storage and shelf life

Abstract: Blue and green mold rots caused by *Penicillium italicum* and *Penicillium digitatum* are the major postharvest citrus diseases. These diseases are commonly controlled with conventional chemical compounds but legislative restrictions, consumer concerns and the developments of resistant strains of the pathogens have increasingly led to the search for alternative methods of control. A pomegranate peel extract (PGE) was very effective in controlling postharvest rots of valencia orange and clementine, under large scale commercial conditions. The extract, proved a significant higher level of protection compared to Imazalil (IMZ), a commonly used fungicide for postharvest treatments. After cold storage and shelf life period, the incidence of decay on oranges sprayed before harvest with PGE at 12, 6 and 3 g/l was reduced by 78.9, 76.0, and 64.6%, respectively. Similarly, postharvest dipping treatments with PGE reduced rots by 90.2, 84.3, and 77.6%, respectively. Comparable levels of protection were also achieved on clementine treated before harvest. The high level of efficacy and the consistence of results on different fruit species (clementine and orange) and with different application methods (pre- and postharvest) was evidence of reliability and flexibility, making PGE a potent antimicrobial treatment against postharvest diseases of citrus. PGE also showed a strong antimicrobial activity against epiphytic fungal and bacterial populations suggesting its possible use as sanitizers to reduce the microbial contamination of recirculated water in packinghouses. The results of the present study encourage the replacement of chemical fungicides and sanitizers with PGE to control citrus postharvest rots. This may lead to significant reductions of losses, lower environmental impact and improved quality and safety of products without chemical residues.

Keywords: Citrus rots; Alternative control methods; *Penicillium digitatum; Penicillium italicum*; Pomegranate peel extract; Valencia orange; Clementine

1. Introduction

Citrus is one of the most significant agricultural crops worldwide and, particularly, in Italy which ranked as the second largest European citrus producer after Spain (Eurostat, 2017). Oranges (*Citrus sinensis* (L.) Osbeck) and clementine (*Citrus clementina* hort. ex Tanaka) are considered the leading grown citrus species in the world with a production reaching 73 and 33 Million tonnes in 2017, respectively (FAO, 2017). These citrus species are characterized by a longer shelf life compared to other tropical and sub-tropical fruits. However, post-harvest losses are of serious concern. Each year, 30 to 50% of the total citrus production is wasted due to postharvest diseases (Porat *et al.*, 2000). These diseases include Sour rot, Brown rot, Alternaria rot, Stem-end rots, and Penicillium rot caused by *Geotrichum candidum*, *Phytophthora spp*., *Alternaria spp*., *Diplodia natalensis* and *Phomopsis*, and *Penicillium. ulaiense*, respectively. The incidence of these fungal pathogens on the fruit is generally low, but when the environmental conditions are convenient for the pathogen growth, serious damages are observed. However, blue and green mold rots caused by *P. italicum* and *P. digitatum* are the major postharvest citrus diseases (Youssef *et al.*, 2012). Both pathogens require wounds to infect the fruit, therefore, fruit injuries caused during harvest, packaging house manipulation, transport or storage, help to initiate the pathogen infection (Palou, 2014). Accordingly, good harvest and post-harvest handling is critical to prevent fungal diseases. Although in most citrus industries, these preventions are already delicately considered, blue and green molds are still inducing high economic losses to the citrus sector. Accordingly, a major effort is dedicated to search for efficient control means. For decades, the application of chemical fungicides in packaging houses, before fruit storage, has been the main management technique to prevent postharvest diseases. For instance, Imazalil (IMZ), widely used chemical fungicide, proved high efficiency in controlling postharvest pathogens (Altieri *et al.*, 2013). However, with the increase of consumer awareness about chemical residues together with the latest European legislative restrictions, a special attention has recently been given to the alternative control means (Erasmus *et al.*, 2015; Palou, 2014; Wisniewski *et al.*, 2016). Up to date, several alternative control methods have been used including the application of antagonists and the use of resistance inducers and natural fungicides, however, their application in the citrus sector is still limited (Janisiewicz and Korsten, 2002; Talibi *et al.*, 2014). Their inconsistent activity under practical commercial conditions, low persistence as well as the risk of fruit injury are the main limitations of these alternative methods (Palou *et al.*, 2008; Youssef *et al.*, 2012). Therefore, a continuous effort is dedicated to this line of research in order to find effective alternatives to control plant diseases. Accordingly, some of plant extracts proved high efficiency and persistence as alternative fungicides to control postharvest rots. In particular, pomegranate peel extract, called PGE, has recently proved to efficiently control wide range of

postharvest fungal pathogens including *P. digitatum* and *P. italicum* (Romeo *et al.*, 2015; Li Destri Nicosia *et al.*, 2016). Its composition is proved to be rich in phenolics including gallic acid and particularly punicalagins which are phenolic components only found in pomegranate (Romeo et al., 2015). This extract is characterized by preventive and curative effect, wide spectrum of activity, high efficiency and long persistence (Pangallo et al., 2017b). Studies not only proved its direct antimicrobial activity but also its ability to induce resistance in treated fruit tissues (Pangallo *et al.*, 2017a; Pangallo *et al.*, 2017b; Belgacem *et al.*, 2019).

The aim of the present study was to evaluate, under practical commercial conditions, the effect of PGE treatments against postharvest rots of clementine and orange fruit through pre and postharvest application. The effect of PGE was further compared to a chemical control product (IMZ), and two salts (potassium bicarbonate (KHCO3) and sodium bicarbonate (NaHCO3)) which have already been proposed as effective and safe alternative means.

2. Material and Methods

2.1. Treatments

A concentrated extract of pomegranate peel (PGE) (120 g/l of dry matter) was obtained according to Romeo *et al.* (2015) from ripe pomegranate (*Punica granatum* L.) fruit cv. "*Mollar De Elche*". The extract was stored at 4°C and diluted before use with tap water to obtain three concentrations of 12, 6 and 3 g/l. KHCO3 and NaHCO3 (Sigma Aldrich S.r.l., Milan, Italy) were dissolved in tap water to get 2% (W/V) solutions. A commercial formulation of IMZ (Deccozil 50, Decco, Italy) was used with the recommended concentration of 0.1%. All solutions were prepared just before use.

2.2. Preharvest treatments

Experiments were conducted in commercial orchards of Valencia oranges (GPS coordinates: 38°13'06.24"N105 – 16°14'11.54"E) and clementines (GPS coordinates: 38°13'06.24"N – 16°14'11.54"E) in April 2016 and November 2017, respectively. Uniform, symptomless, and not damaged plants were selected for the experiments. In both years, treatments were conducted according to a completely randomized block design of 3 replicates, each consisting of 5 plants. Plants were sprayed with approximately 10 litters of solution containing PGE at three different concentrations, salts (NaHCO3 and KHCO3) or tap water (control), using a backpack atomizer. The following day, the treated fruit were harvested, put in plastic boxes and stored for 1 week at 4±1°C and 95-98% RH.

Fruit from untreated plants were stored without any additional treatment (field control), or dipped in IMZ solution (chemical control) as described above for postharvest treatments. All fruit were cold stored for 7 days and then subjected to other 7 days of shelf life at 20±2°C. The fruit incidence was controlled at the end of the cold storage and shelf life. For each treatment, 2400 oranges or 6000 clementines placed in 12 plastic boxes (three replicates of 4 boxes) were visually inspected to determine the incidence of rots.

For both Valencia oranges and clementine fruit, the epiphytic bacterial and fungal population was evaluated before and at the end of cold storage. From 5 fruit, five disks of flavedo and albedo were cut using a sterile cork borer of 10 mm diameter.
Disks were put into sterile cups containing 100 ml of sterile water and shacked with rotary shaker at 200 rpm for 30 min. A volume of 100 µl of the obtained solutions were serially diluted and plated on PDA media to determine the fungal and bacterial population as described above respectively. the number of colony forming units was recorded and converted to CFU/ fruit.

2.3. Postharvest treatments

Oranges (cv Valencia) were collected in April 2016 from a commercial orchard located in Calabria, Southern Italy (GPS coordinates: 38°13'06.24"N – 16°14'11.54"E). Experiments were conducted by fruit dipping for 30 s in an IMZ solution, used as chemical control, or for 5 min in PGE at 12, 6 and 3 g/l, $KHCO_3$ or $NaHCO_3$. Oranges dipped in tap water and untreated oranges were used as controls. Fruit were dried and waxed on the commercial packing line. Treated fruit were cold-stored for 7 days at 4°±1°C and -98% RH, and then subjected to 7 days of shelf life at 20± 2°C. The disease incidence was determined at the end of the cold storage and shelf life. For each treatment, 2400 fruit arranged in 12 plastic boxes (three replicates of 4 boxes) were visually inspected to determine the disease incidence. To evaluate the sanitizing activity of the tested treatments, three subsamples of 50 ml were collected from each dipping solution and maintained for 3h at 5°C. After shaking, a volume of 100 µl of water suspensions were serially diluted and plated on triplicate plates of Potato Dextrose Agar (PDA) and PDA amended with ampicillin and streptomycin sulphate (250 mg/l each) to detect the bacterial and fungal load, respectively. After incubation at 22°C for 3–4 days, the number of colony forming units was recorded and converted to CFU/ml.

2.4. Statistical analyses

After analysis of variance (ANOVA), significant differences between treatments were determined according to Tukey's test at a significance level of

$P < 0.05$. Percentages were converted into Bliss angular values (arcsine √%) before analysis.

3. Results

3.1. Evaluation of natural decays

Clementine and orange fruit were evaluated once the rot softening symptoms appeared on the peel. The identification of the fungal mycelium and spores, on the fruit rots, showed that 80% of rots were caused by *P. digitatum*, followed by *P. italicum* (around 20%), and lastly by both fungal pathogens (around 10%). However, grey mold infections caused by *Botrytis cinerea* were detected only on clementine and represented around 5% of the detected rots.

3.2. Preharvest treatments

At the end of cold storage, low rot incidence was detected on all treated orange, before harvest, where the controls, orange sprayed with tap water and untreated, showed only 6.5% and 5.9% of rot symptoms, respectively (Fig.1, A). Among all treatments, only PGE (12g/l) showed significant rot reduction of 92.3 %. While, at the end of the shelf life, the disease incidence highly increased to reach 81.4% with control fruit. This was greatly reduced by PGE treatments to reach 90.2, 84.3, and 77.6%, when treated with PGE 12, 6, 3 g/l, respectively. Comparing to other treatments, PGE was significantly more effective than tested salts and IMZ.

A higher incidence of decay was observed with clementine fruit where the control treated fruit reached 32% of decay at the end of cold storage (Fig.1, B). PGE effectively reduced the rot incidence by 98.1, 94.1, and 91.3% at the three tested concentrations, respectively. While, lower efficiency was detected with IMZ, KHCO3 and NaHCO3 with rot incidence reduction of 89.1, 84.7, and 87.2%, respectively.

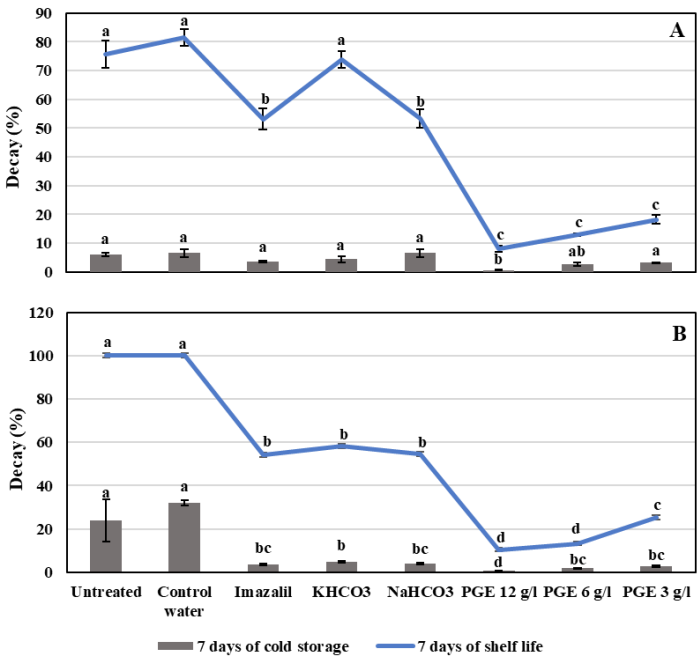

Fig 1. Incidence of decays after 7 days of cold storage and 7 days of shelf life of untreated and treated Valencia orange **(A)** and clementine fruit **(B)**, before harvest. Treatments include PGE (12, 6, and 3 g/l), 0.1% Imazalil, 2% NaHCO3 and KHCO3. Untreated fruit and fruit sprayed with tap water were used as controls. Imazalil was applied after harvest by dipping. Bars indicate standard errors of the means. For each time, different letters indicate significant differences ($P < 0.05$) among treatments.

The microbiological analysis of the epiphytic fungal and bacterial population on Valencia orange (Fig 2) and clementine (fig 3) fruit revealed high microbial reductions, before and after cold storage, on fruit treated with PGE comparing to the controls (untreated and water dipped fruit). Overall, the antimicrobial activity of PGE was higher on bacteria than fungi, regardless of the fruit species or time of sampling. While, no significant differences were detected between orange control fruits, PGE treatments and in particular PGE 12g/l was the most effective treatment with a bacterial and fungal reductions varying between 94.3-97.7% and 83.1-89.5%, respectively. Significant but lower microbial reductions were also achieved by PGE at 6 and 3 g/l and, to a lesser extent, with IMZ. Salts, NaHCO3 and KHCO3 induced significant but much lower reductions only on the bacterial populations of clementine fruit.

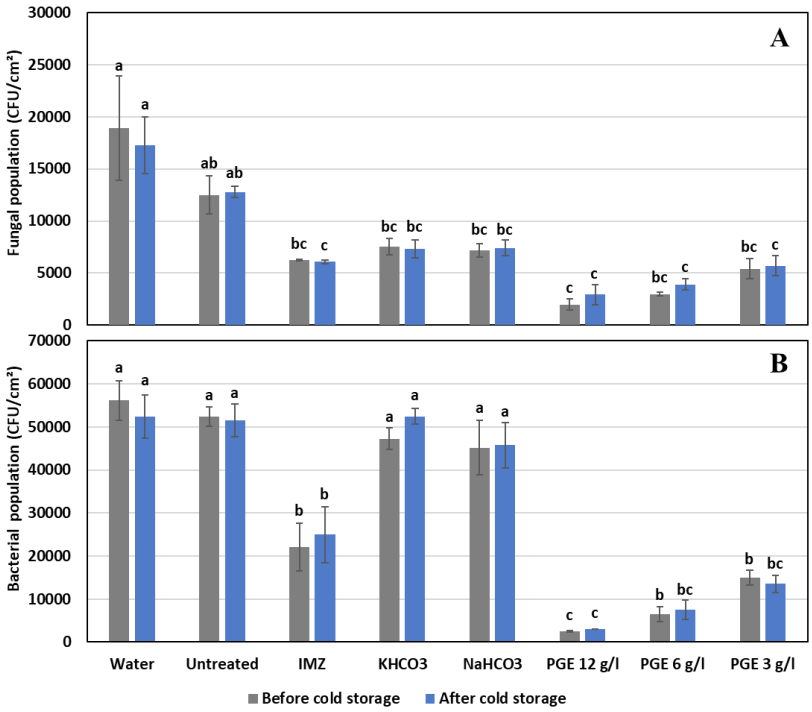

Fig 2. Epiphytic population of Fungi **(A)** and bacteria **(B)** on Valencia oranges before and after cold storage. Different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

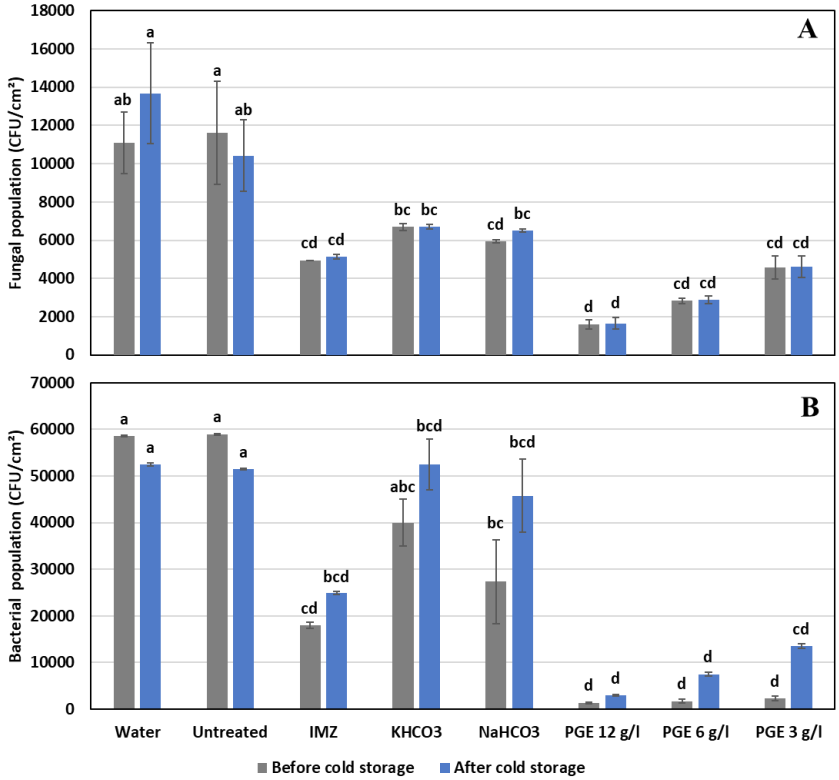

Fig 3. Epiphytic population of fungi **(A)** and bacteria **(B)** on clementine before and after cold storage. Different letters indicate significantly different values according to Tukey's test ($P \leq 0.05$).

3.3. Postharvest treatments

After 7 days of cold storage, untreated orange and oranges dipped in tap water (controls) showed low rot incidence of 12,29 and 13.3%, respectively (Fig. 4). This incidence was further reduced with PGE treatments to reach a reduction of 97.5, 71.3 and 58.2% when treated with PGE concentrations of 12, 6 and 3 g/l, respectively. However, no significant difference was detected between control fruit and IMZ treatment. After 7 days of shelf life, the disease incidence highly increased to reach 75.8% with fruit dipped in tap water. This was significantly reduced with fruit treated with PGE 12, 6 and 3 g/l, where the reductions reached 78.9, 76.0, and 64.6%, respectively. Lower but still significant reductions of rots were obtained with IMZ (49.6%) and NaHCO3 (48.8%).

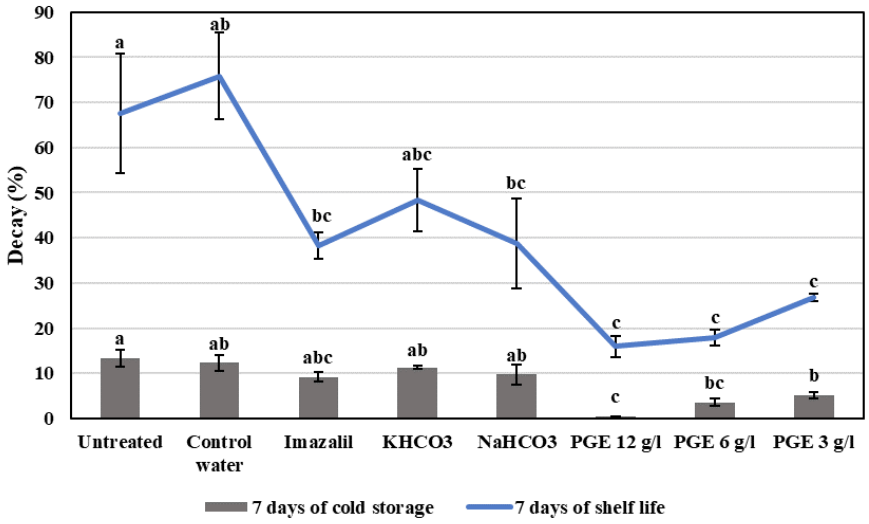

Fig 4. Incidence of decays (%) after 7 days of cold storage and 7 days of shelf life of untreated and treated Valencia oranges after harvest. Treatments include PGE (12, 6, and 3 g/l), 0.1% Imazalil, 2% NaHCO3 and KHCO3. Untreated oranges and oranges dipped in tap water were used as controls. Bars indicate standard errors of the means. For each time, different letters indicate significant differences ($P < 0.05$) among treatments.

A microbiological analysis of the aqueous dipping treatment solutions revealed a strong antimicrobial activity of the extract against bacteria and fungi (Fig. 5). While, the bacterial population in tap water dipping solution was $4x10^9$ CFU/ml, PGE significantly reduced it by 98.5 (PGE 12g/l), 98.4 (PGE 6g/l), and 78.0% (PGE 3g/l). A much lower but significant reduction was revealed in the dipping solutions containing KHCO3 and IMZ. The total fungal counts in tap water dipping solution reached $2.3x10^7$ CFU/ml. This contamination was reduced by 44.1% in water containing IMZ and by 86.7, 85.3, and 58.8% in dipping water containing PGE at 12, 6 and 3 g/l, respectively. Overall, PGE showed high significant bactericidal and fungicidal activity comparing to all tested treatments (IMZ and salts), except with the lowest concentration of PGE (3g/l) where no significant fungicidal effect was observed comparing to IMZ.

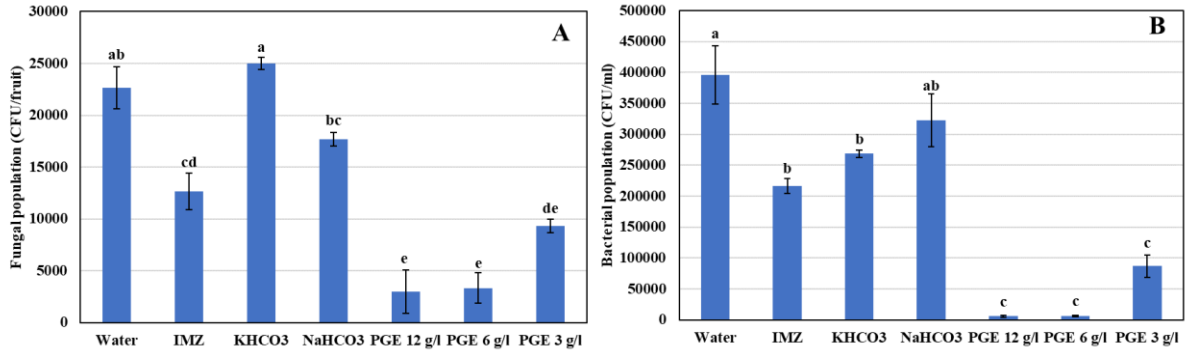

Fig 5. Fungal **(A)** and bacterial **(B)** populations of postharvest dipping solutions on Valencia oranges. bars are standard errors of the mean. For each group of microorganisms, different letters indicate significant differences ($P < 0.05$) among treatments.

4. Discussion

In the present study, different control substances were applied under large-scale commercial conditions as pre- and post-harvest treatments to control major citrus pathogens. Regardless of the concentration, PGE showed the highest efficiency, comparing to all tested substances, in controlling orange and clementine rots both before and after harvest. In particular, PGE showed higher efficiency comparing to IMZ which is a chemical treatment currently considered as the most effective fungicide to control postharvest citrus rots despite the identification of several resistant strains (Kinay *et al.*, 2007; Pérez *et al.*, 2011; Erasmus *et al.*, 2015). This together with the consistency of PGE effect in controlling the epithetic microbial population on clementine and orange fruit, and in both pre and postharvest, makes the extract very potent reliable antibacterial substance. This is an important feature comparing to other alternative strategies particularly biocontrol agents which are considered as one of the most viable alternatives. However, their inconsistence efficiency mainly due to their sensibility to the external environmental conditions and host species, makes their application very limited (Sui *et al.*, 2015; Ippolito *et al.*, 2005). Furthermore, results of this study showed low but significant effect of salts in controlling citrus green and blue molds. This confirms previous studies reporting that the antimicrobial activity of salts in controlling citrus diseases is limited due to several restrictions such as limited persistence, inconsistent activity, risk of fruit injury, lack of preventive effect, etc (Youssef *et al.*, 2012; Smilanick, 2010; Palou *et al.*, 2002). Therefore, the application of salts could be useful as an integrated approach or in combination with other control means in integrated pest management systems, while PGE could be used as an alternative to chemicals. This together with the continuous growing public concerns about health and environmental risks concerning chemical residues, PGE could be considered an important control strategy particularly for cash crops such as citrus.

Noteworthy, the high level of protection achieved by the extract in both pre and post-harvest treatments makes the integration of PGE in control strategies more practical, flexible and particularly effective, regardless of the timing of disease appearance. In this case, the extract might be important as field treatment for citrus productions that don't undergo, during packaging and commercialization, specific postharvest treatments e.g. washing and waxing. This is often the case in Italy where early varieties of clementine are quickly introduced into the distribution chain without any treatments or mechanical operations leaving the fruit with few leaves as an indication of product freshness. Similar case is found in Europe but with different fruit species that cannot be subjected to dip treatments such as strawberries and table grapes (Nigro *et al.*, 2006). Furthermore, the field application of PGE could be considered easy, particularly for less technologically advanced countries where simple equipment including conventional fungicides sprayers could also be used for PGE field treatments. Moreover, postharvest dipping treatments can be easily and cheaply integrated into citrus packinghouse operations by adding the active ingredient to the washing water usually used to wash the fruit and/or reduce temperature (hydro-cooling).

PGE preharvest treatments showed strong antimicrobial activity, before and after cold storage, against epiphytic fungal and bacterial populations associated with oranges and clementine. This broad range of antimicrobial activity of the extract could negatively affect the microbiome of the carposphere which plays an important role in protecting the fruit (Chalutz and Wilson, 1990). This together with the persistent activity of PGE after 7 days of cold storage and 7 days of shelf life suggest the possible reduction of the fruit natural antagonists which could represent a relevant issue from a practical point of view. However, the capability of PGE in inducing resistance in fruit tissues could explain the antimicrobial persistence and efficiency of the extract overtime (Belgacem *et al.*, 2019). This feature could be further exploit to protect washed citrus fruit where washing water may flash the fruit microorganisms and, therefore, affect the consistency of the carposphere microbiota. Furthermore, the application of PGE, as a postharvest dipping treatment, might be also strategic against water-borne microorganisms. The extract could be useful to reduce the microbial contamination of the recirculated water and substitute the use of chlorine and other sanitizers commonly used in citrus packinghouses.

In conclusion, results of this study demonstrated the potential use of PGE as an effective alternative control treatment to control pre and postharvest citrus rots. It is worth mentioning that the trials of this study were carried out under large scale commercial conditions where most of other alternative control means usually proved an antimicrobial activity under laboratory conditions but not under commercial conditions. Furthermore, the substitution of IMZ chemical treatments

with PGE could result in significant reduction in losses, enhancement of the fruit quality, and reduced environmental impact from the lack of chemical residues (Romeo *et al.*, 2015). Therefore, the potential use of PGE is not restricted to organic production but also it could be incorporated in conventional and/or integrated farming systems. This together with the wide availability of PGE, as a by-product of processing industries, and the absence of phytotoxicity effect, might speed up the costly process for PGE registration as a natural fungicide (Li Destri Nicosia *et al.*, 2016; Pangallo *et al.*, 2017b).

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Chapter 3. Transcriptomic Analysis of Orange Fruit Treated with Pomegranate Peel Extract (PGE)

Abstract: A Pomegranate Peel Extract (PGE) has been proposed as a natural antifungal substance with a wide range of activity against plant diseases. Previous studies showed that the extract has a direct antimicrobial activity and can elicit resistance responses in orange tissues. In the present study, the transcriptomic response of orange fruit toward PGE treatments was evaluated. RNA-seq analyses, conducted on wounded fruits 0, 6, and 24 h after PGE applications, showed a significantly different transcriptome in treated oranges as compared to control samples. The majority (273) of the deferentially expressed genes (DEGs) were highly up-regulated compared to only 8 genes that were down-regulated. Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis showed the involvement of 1233 gene ontology (GO) terms and 35 KEGG metabolic pathways. Among these, important defense pathways were induced and antibiotic biosynthesis was the most enriched one. These findings may explain the underlying preventive and curative activity of PGE against plant diseases.

Keywords: orange; pomegranate peel extract; PGE; RNA-seq; transcriptomics; plant defense.

1. Introduction

The peel of pomegranate, accounting for approximately 50% of the total fruit weight, is a rich source of phenolic components, including phenolic acids and flavonoids such as anthocyanins and hydrolyzable tannins. The latter compounds are mainly represented by punicalagins, ellagic acid and its derivatives (Venkataramanamma *et al.*, 2016; Fischer *et al.*, 2011). Ellagitannins are the most important and abundant phenolic compounds in pomegranate peel and are responsible for strong antioxidative and antimicrobial activities (Tehranifar *et al.*, 2011; Zahin *et al.*, 2010). Therefore, pomegranate peel extracts have recently received great attention as valuable natural compounds for a number of applications. For instance, they were proposed as effective alternative means to inhibit the germination and growth of several mammalian pathogenic bacteria including *Listeria monocytogenes*, *L. innocua*, *Staphylococcus aureus*, *Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Salmonella spp.* (Al-Zoreky, 2009; Gullon *et al.*, 2016).

Of particular relevance are their potential agricultural applications. An alcoholic extract from pomegranate peel, named PGE, proved high efficacy in controlling several plant diseases when applied both before and after harvest. A high level of protection was achieved against *Botrytis cinerea* on table grapes and sweet cherries, *Monilinia spp.* on sweet cherries, *Penicillium digitatum* and *Penicillium italicum* on citrus species, *Penicillium expansum* on apples and *Colletotrichum spp.* on olives (Romeo *et al.*, 2015; Li Destri Nicosia *et al.*, 2016; Pangallo *et al.*, 2017a). In addition to its wide spectrum of activity, several other important features were reported on PGE, such as the high level of efficacy, in both preventive and curative applications, a complex mechanism of action, which includes direct fungicidal and bactericidal activities, and the capability of inducing resistance in the host tissues (Pangallo *et al.*, 2017a; Pangallo *et al.*, 2017b). The induction of resistance in host tissues treated with PGE was indirectly demonstrated on citrus and olive fruit inoculated with *P. digitatum* and *P. italicum* (Pangallo *et al.*, 2017a), and *Colletotrichum acutatum* (Pangallo *et al.*, 2017b). In fact, rots were also significantly reduced when no direct contact was made between the PGE and the pathogens. Furthermore, the application of PGE on grapefruits caused a significant increase of reactive oxygen species (ROS) which reached a peak after 24 h post treatment (Pangallo *et al.*, 2017a). Analyses revealed the activation of several genes involved in plant defense responses such as CHI, CHS, MAPK, MAPKK, and PAL. These PGE features seem to be a direct consequence of its rich content in phenols (Romeo *et al.*, 2015). In fact, phenolic components are potent antimicrobial agents that exert a direct effect on fungal pathogens and can also induce resistance in the plants (Alsaggaf *et al.*, 2017). For instance, quercetin, a common polyphenol in plant tissues induced resistance in plants and fruits by acting on the transcription level of defense genes (Sanzani *et al.*, 2010; Jia *et al.*, 2010). The knowledge of genes

and pathways involved in the induced resistance contributes to the understanding of the mechanisms of action of PGE and may have important practical implications facilitating the development of appropriate formulation and methods of application to better control postharvest diseases (Spadaro and Droby, 2016).

Therefore, the aim of the present study was to investigate the impact of PGE on the transcriptome of treated orange fruits in order to investigate the molecular basis of the induced resistance after PGE applications.

2. Materials and Methods

2.1. Experimental Design and Sampling

A stock solution of PGE containing 120 g/l of dry matter and 1% citric acid used as antioxidant was obtained according to Romeo *et al.* (2015). The solution was stored at 5 °C and diluted just before use.

Freshly harvested oranges (Citrus sinensis cv. Valencia) from organic agriculture were wounded with a sterile needle around the pedicel to produce three equidistant wounds (2 mm deep and wide) and were treated with 20 µL of PGE (12 g/l), 1% citric acid or sterile water (control). Citric acid was included in the trials since it is commonly used to stabilize PGE (Romeo *et al.*, 2015). Samples were taken at three time intervals after treatments: 1) soon after treatment "1h", 2) 6 h after the treatment "6 h" 3) and 24 h after the treatment "24 h". At each time point, albedo and flavedo were excised around the wounding sites using an 8 mm diameter cork-borer. For each treatment, three replicates, each consisting of 9 wounds from three different oranges were collected (n = 27). Samples were immediately frozen in liquid nitrogen, ground using a mortar and pestle, and stored at −20 °C. Total RNA was extracted from 30 mg of ground fruit tissue using the SV Total RNA Isolation System kit (Promega). The extracted RNA was purified using the DNA-free kit (Invitrogen). Each RNA sample was adjusted to have a total volume of 50 µl of total RNA. Library construction and sequencing were conducted at Macrogen Inc. (Seoul, Korea) using an Illumina Hi-Seq 2500 System to obtain 100 bp paired-end reads. Reads were deposited in the Sequence Read Archive with the accession number (PRJNA428949).

2.2. Data Analysis

The quality of the obtained raw reads was evaluated using the FastQC tool, version 0.11.3 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and was trimmed with Trimmomatic V 0.36 (Bolger *et al.*, 2014) using a 4-base wide sliding window trimming approach with an average quality of 15 and minimum read length of 36. Reads were mapped to the genome draft of sweet orange (C. sinensis) version 2 (Xu *et al.*, 2013) using TopHat 2.1.1 (Trapnell *et al.*, 2009). The mapped reads were assembled into transcripts using the default setting of

Cufflinks except that the library normalization method was set to geometric which employs the DESeq normalization method (Trapnell *et al.*, 2010; Anders and Huber, 2010). Significant changes in transcript expression were determined using Cuffdiff as implemented in Cufflinks with the default 0.05 q-value cut-off. Gene expression values (FPKM) were used to conduct Principal Coordinates Analysis (PCoA) and to construct heatmaps, using Qlucore v3.3 (Qlucore, Lund, Sweden) bioinformatic software. A list of significantly differentiated genes (q-value ≤ 0.049 corresponding to a p-value of 0.02187 $R2 \geq 0.2728$) was selected and the corresponding sequences were extrapolated from the Citrus sinensis genome reference. These genes were mapped and annotated, and their Gene Ontology (GO) terms (Level 2), and pathways were analyzed using Blast2GO version 2.6.6 using default parameters (Conesa *et al.*, 2005).

3. Results

After quality filtering and adaptor trimming, the High-Throughput Sequencing resulted in a total of 767,487,068 sequences for read 1 and read 2 combined, and an average of 14,212,723 paired-end reads per sample (Table 1). Reads mapping on the genome draft of sweet orange (C. sinensis) resulted in the identification of 30,142 mapped genes.

Table 1. Summary of the results of transcriptomic analysis on oranges treated with PGE, citric acid or water (control) and analyzed 1, 6 and 24 h post treatment (hpt).

Treatment	Replicates	Sampling Time (hpt)	Read 1	Read 2
Citric acid	R1	1	16509545	16509545
	R2	1	10819465	10819465
	R3	1	12792832	12792832
	R1	6	11027909	11027909
	R2	6	14228323	14228323
	R3	6	8719836	8719836
	R1	24	15550388	15550388
	R2	24	24863143	24863143
	R3	24	11287417	11287417
H_2O	R1	1	13733858	13733858
	R2	1	16898374	16898374
	R3	1	9761332	9761332
	R1	6	22501980	22501980
	R2	6	20726432	20726432
	R3	6	20954767	20954767
	R1	24	15517302	15517302
	R2	24	18455542	18455542
	R3	24	20239137	20239137
PGE	R1	1	9514582	9514582
	R2	1	8451256	8451256
	R3	1	12624217	12624217

R1	6	11596104	11596104
R2	6	14399695	14399695
R3	6	10650424	10650424
R1	24	13176031	13176031
R2	24	9556111	9556111
R3	24	9187532	9187532

While 30,142 genes were included in the analysis, 585 genes remained after filtering the variance according to the Qlucore software's recommendation. Among those, 281 genes were differentially expressed (DEG) and significantly differentiated fruit treated with PGE from those treated with water and citric acid, regardless of the sampling time point.

At all sampling time points (1, 6, and 24 h), the great majority of the DEGs (273) were upregulated in the PGE-treated fruit as compared to water and citric acid, since only a small fraction of genes (8) was down-regulated (Fig. 1). Overall, differences between control and PGE-treated fruits increased over the time since the expression level of the upregulated DEGs tended to increase while the downregulated genes showed an opposite expression pattern.

Furthermore, multivariate Principal Component Analysis (PCA) revealed a clustering of the transcriptomes into two groups where the PGE-treated samples were distinctly separated from the control ones (water and citric acid). The clusters representing samples receiving different treatments were further divided into three sub clusters, corresponding to the sampling time i.e., '1h', '6h' and '24 h' (Fig. 2).

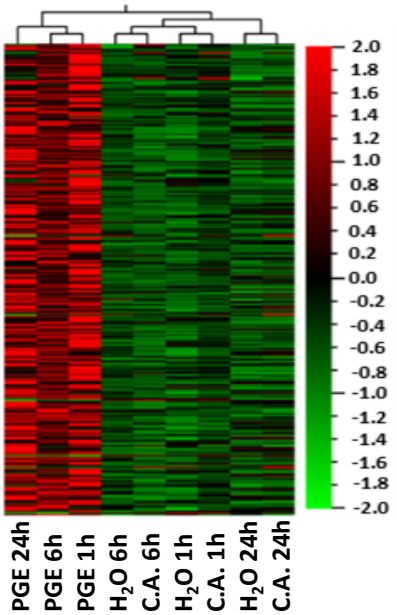

Fig 1. Hierarchical clustering heatmap of differentially expressed genes (DEGs) in orange fruit treated with PGE, citric acid (C.A.) or water (H_2O) 1, 6 and 24 h post treatment (hpt). Colors indicate the level of expression as indicated in the scale on the right side of the figure.

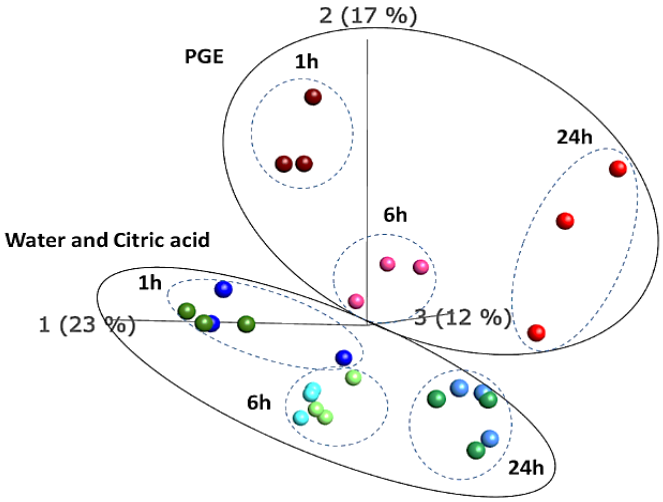

Fig 2. Principal Component Analysis (PCA) of all transcripts from oranges treated with PGE, citric acid or water (control) and analyzed 1, 6 and 24 h post treatment (hpt).

3.1. Gene Ontology Enrichment

The GO terms and metabolic pathways of the DEGs were identified by performing functional enrichment analyses. In total, 253 genes were annotated with 1233 GO terms and were assigned to biological process, cellular component, or molecular function (Fig. 3). Among the "Biological process" category, the prominent functional groups for both induced and repressed genes were related to the cellular process (126 DEGs), metabolic process (121), single organism process (74), cellular component organization (31), and localization (31). While for the 'Molecular function' category, most of the terms belonged to the catalytic activity (95) and binding groups (87). Cell (131), Cell part (131) and Organelle (109) were the most enriched groups in the 'Cellular component' category.

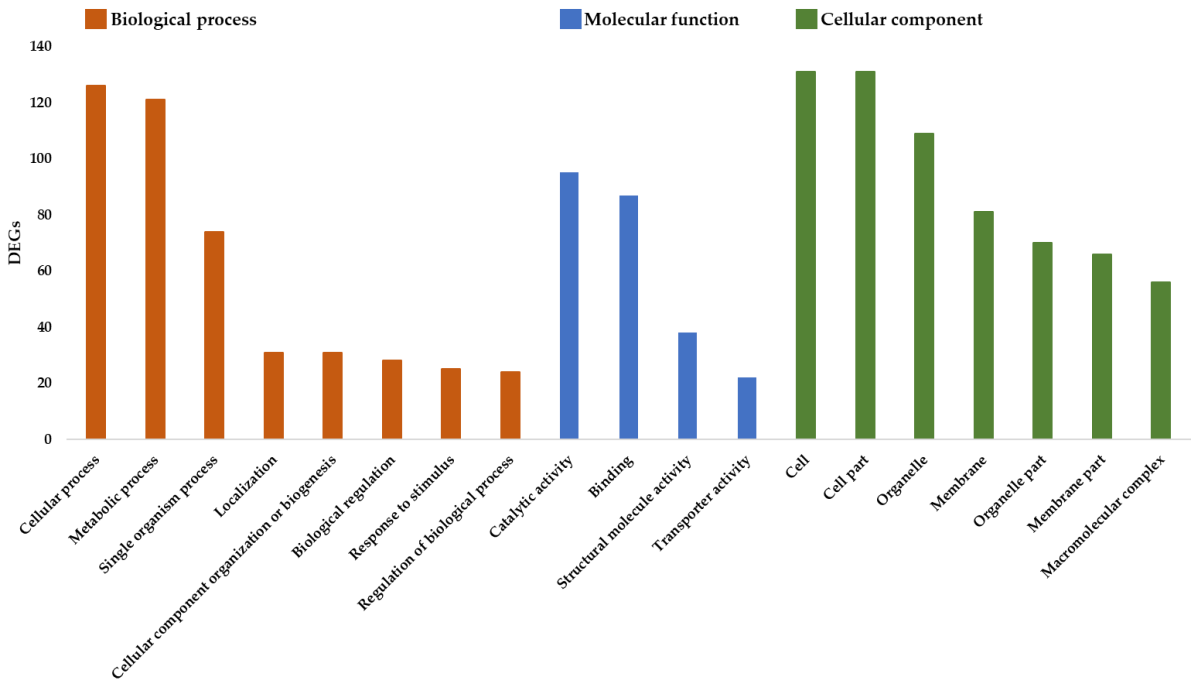

Fig 3. Functional annotation of the differentially expressed genes using Gene Ontology terms.

3.2. KEGG Pathways

KEGG pathway enrichment analysis of the up-regulated and down-regulated genes after PGE treatment showed the involvement of 35 metabolic pathways (Table 2). Among these pathways, 34 were up-regulated while only 1 pathway involved in monoterpenoid biosynthesis was down-regulated. Overall, a large pool of transcripts fell within the area of primary metabolism, i.e., carbohydrate and energy metabolism (16 pathways), amino acid metabolism (4 pathways), and nucleotide metabolism (2 pathways), while other transcripts were mapped to the area of secondary metabolites biosynthesis (6 pathways), and xenobiotics biodegradation and metabolism (6 pathways). Several identified genes translated to enzymes that were involved in multiple pathways. In other cases, multiple enzymes were found to be activated within the same pathways after PGE treatment.

Table 2. List of KEGG pathways of orange fruits treated with PGE and their corresponding genes.

Category	Pathway	Number of genes	Enzymes in pathway
Carbohydrate and Energy metabolism	Glycolysis/Gluconeogenesis	3	ec:5.3.1.1, ec:4.1.2.13, ec:2.7.2.3
	Pyruvate metabolism	3	ec:1.1.1.37, ec:4.4.1.5, ec:3.1.2.6
	Pentose phosphate pathway	3	ec:2.7.1.15, ec:2.2.1.2, ec:4.1.2.13
	Glyoxylate and dicarboxylate metabolism	2	ec:1.1.1.37, ec:1.1.3.15
	Fructose and mannose metabolism	2	ec:5.3.1.1, ec:4.1.2.13
	Pentose and glucuronate interconversions	2	ec:1.1.1.22, ec:4.2.2.2
	Amino sugar and nucleotide sugar metabolism	1	ec:1.1.1.22
	Inositol phosphate metabolism	1	ec:5.3.1.1
	Ascorbate and aldarate metabolism	1	ec:1.1.1.22
	Citrate cycle (TCA cycle)	1	ec:1.1.1.37
	Oxidative phosphorylation	6	ec:1.10.2.2, ec:1.9.3.1, ec:1.6.5.3
	Carbon fixation pathways in prokaryotes	1	ec:1.1.1.37
	Carbon fixation in photosynthetic organisms	4	ec:1.1.1.37, ec:5.3.1.1, ec:4.1.2.13, ec:2.7.2.3
	Methane metabolism	2	ec:1.1.1.37, ec:4.1.2.13
	Nitrogen metabolism	1	ec:1.7.1.1
	Sulfur metabolism	5	ec:3.6.2.1, ec:2.5.1.48, ec:2.7.7.4, ec:2.7.1.25
Lipid metabolism	Glycerolipid metabolism	1	ec:3.1.1.3
Nucleotide metabolism	Purine metabolism	5	ec:3.6.1.3, ec:2.7.7.4, ec:2.7.4.6, ec:2.4.2.7, ec:2.7.1.25
	Pyrimidine metabolism	1	ec:2.7.4.6
Amino acid metabolism	Cysteine and methionine metabolism	6	ec:1.1.1.37, ec:2.5.1.6, ec:2.1.1.14, ec:1.13.11.54, ec:3.3.1.1, ec:2.5.1.48
	Phenylalanine metabolism	1	ec:2.1.1.104

	Selenocompound metabolism	4	ec:2.1.1.14, ec:2.5.1.48, ec:2.7.7.4
	Glutathione metabolism	4	ec:2.5.1.18, ec:1.11.1.15
Biosynthesis of secondary metabolites	Antibiotic biosynthesis	10	ec:1.1.1.37, ec:1.1.3.15 ec:2.5.1.48, ec:2.2.1.2, ec:5.3.1.1, ec:2.7.7.4, ec:4.1.2.13, ec:2.7.4.6, ec:2.7.2.3
	Monoterpenoid biosynthesis	2	ec:4.2.3.20
	Phenylpropanoid biosynthesis	4	ec:1.11.1.7, ec:2.1.1.104
	Flavonoid biosynthesis	1	ec:2.1.1.104
	Monobactam biosynthesis	2	ec:2.7.7.4
	Stilbenoid, diarylheptanoid and gingerol biosynthesis	1	ec:2.1.1.104
Xenobiotics biodegradation and metabolism	Fluorobenzoate degradation	1	ec:3.1.1.45
	Toluene degradation	1	ec:3.1.1.45
	Metabolism of xenobiotics by cytochrome P450	3	ec:2.5.1.18
	Drug metabolism - cytochrome P450	3	ec:2.5.1.18
	Drug metabolism - other enzymes	2	ec:3.1.1.1
	Chlorocyclohexane and chlorobenzene degradation	1	ec:3.1.1.45

4. Discussion

In the present study, a transcriptomic analysis was conducted to evaluate the impact of PGE on the expression of genes in oranges treated at different intervals after treatment (1, 6 and 24 hpt). PGE treatment significantly influenced the gene expression (253 DEGs) compared to the control, while citric acid, commonly utilized to stabilize the extract, did not have any impact. Importantly, a significant impact was revealed at all investigated time points, including 1 hpt, indicating a very quick response of the host tissue. The enrichment analysis showed the involvement of genes mainly in the catalytic and metabolic processes. These results were in accordance with the KEGG analysis where pathways identification revealed that PGE acts entirely on the metabolic pathways of the orange fruit. Among the 35 metabolic pathways, 34 were activated while only 1 pathway, involved in monoterpenoid biosynthesis, was down-regulated. Most of the enriched pathways were involved in primary metabolism, followed by secondary metabolite biosynthesis and xenobiotic metabolism.

The upregulation of primary metabolism indicates an increased demand for energy and biosynthesis, which in turn may modulate signal transduction cascades that lead to plant defense responses (Rojas *et al.*, 2014). For instance, cysteine and methionine metabolism contained the most up-regulated genes comparing to other pathways, with 6 DEGs coding for 6 different enzymes. Cysteine and methionine are known to be very sensitive amino acids to almost all forms of reactive oxygen, and their metabolism has a crucial role in oxidation resistance in plants (Bin *et al.*, 2017). Similarly, 6 DEGs coding for 3 enzymes were up regulated in the oxidative phosphorylation step. This pathway is important for producing cellular energy, which results in the activation of the host defense mechanisms and suppression of the pathogen colonization of the host tissue. Similarly, we found an upregulation of 5 enzymes, involved in the carbon fixation pathways, which are important for the synthesis of new molecules (metabolites) (Bolton, 2009). In addition, among the activated genes, 3 DEGs coded for a very important enzyme, Glutathione S-transferases (GST, ec:2.5.1.18). This enzyme has variety of functions in plant metabolism, but is usually over-expressed after a pathogenic infection (Dean *et al.*, 2005). Particularly, it plays major role in plant susceptibility to fungal infection where it is involved in the detoxification step of lipid hydroperoxides produced by peroxidation of membranes. Studies showed its involvement in plant defense signaling, the NPR1-independent SA-mediated pathway (Ghanta *et al.*, 2011), hypersensitive reaction and the increase of secondary metabolite production (Guerriero *et al.*, 2018). Other important carbohydrate and energy pathways were also highly upregulated such as 'Pentose phosphate', 'Pyruvate metabolism', 'Glycolysis/Gluconeogenesis', etc. These pathways were reported to be involved in the oxidase activity responsible for production of ROS (Couée *et al.*, 2006; Bolton, 2009). Therefore, the high transcription level of genes involved in primary metabolism providing energy and intermediate components explains the induction and overexpression of other metabolisms including xenobiotic metabolism and secondary metabolite biosynthesis.

The high upregulation of a battery of genes involved in primary metabolism was accompanied by a high expression of a subset of genes implicated in key pathways of secondary metabolism biosynthesis. The activation of this metabolism reconfirms the assumption of potential involvement of plant defense mechanism triggered by PGE treatment. Particularly, phenylpropanoid biosynthesis, one of the most important components of the plant defense system, was significantly up-regulated after PGE treatment (Qi *et al.*, 2018). Phenylpropanoids exhibit a broad spectrum of antimicrobial activity, play a major role as chemical or physical barriers against plant infections, and as signal molecules involved in local and systemic plant defense mechanisms. They participate in the formation of secondary resistance metabolites and are precursors to flavonoids, isoflavonoids, and stilbenes which were also activated following PGE treatment (Dixon *et al.*, 2002). Many of these metabolites have an

antifungal effect, and their overproduction by the plant is considered to be part of a specific antimicrobial defense system (Wink and Schimmer, 2010).

Interestingly, a pathway producing terpenoid volatiles (monoterpenoid biosynthesis pathway) was the only downregulated pathway in this study. Terpenoid volatiles are emitted by plants to communicate with the environment. In sweet orange, these terpenoids are most importantly D-Limonene, a monocyclic monoterpene, which accounts for approximately 97% of the total terpenes in oil glands of orange flavedo (Dugo and Di Giacomo, 2002). The D-limonene down-regulation was reported to be tightly associated with the activation of the defense responses in the fruit. Rodríguez and co-workers (2014) (Rodríguez *et al.,* 2014) showed that the downregulation of D-limonene is followed by the up-regulation of genes involved in disease resistance genes.

The analysis also revealed that 9 enzymes, detected in the induced pathways, are also implicated in the biosynthesis of antibiotics. This means that the produced secondary metabolites are explicitly antibiotic substances. Considering that antibiotics are phytochemicals that are known to have antimicrobial and antiviral properties in plants (Lattanzio *et al.,* 2006; Wink and Schimmer, 2010), the induction of the production of these components might be one of the main mechanisms of action of PGE, especially that its activation continued to increase until 24h post treatment. This induction could be explained mainly by the high concentration in PGE of phenolic compounds (Romeo *et al.*, 2015). Phenolic components are considered very potent antimicrobial agents that exert a direct effect on the pathogen by the suppression of microbial enzyme systems, increasing the permeability of the cell, etc. (Alsaggaf *et al.*, 2017). However, phenolics were also reported to induce resistance in the plant. A study on quercetin, a polyphenol that is found in fruits, vegetables, herbs, etc. (de Oliveira *et al.,* 2016), showed that quercetin application induces resistance in plants and fruits by acting on the transcription level of defense genes (Sanzani *et al.,* 2010; Jia *et al.*, 2010). Therefore, this suggests that the PGE has a dual mode of action by directly affecting microbial growth, as a phenolic substance, as well as inducing several defense-related gene responses in plants. The co-existence of more than one mechanism of action is considered an important feature to increase efficacy and ensure high levels of protection under different conditions and in different phases of the disease cycle (Spadaro and Droby, 2016). In particular, the activation of resistance responses may protect commodities from future wound infections, avoid the establishment of latent infections and restrict fungal growth and sporulation. In this context, the results of the present study support previous speculations of the primary role of induced resistance in the persistent efficacy of PGE after its application (Li Destri Nicosia *et al.*, 2016). Furthermore, its curative effect may be related to the rapid activation of responses that reduce or block the ongoing fungal colonization (Pangallo *et al.*, 2017b).

The results also showed an overall high expression of defense genes involved in xenobiotic metabolism in oranges treated with PGE. Xenobiotics are foreign

chemical contaminants that can be absorbed and accumulated in plant cells (Sandermann Jr, 1992). To detoxify these components, the plant induces the expression of several genes involved in the xenobiotic metabolism. The activation of 5 pathways responsible for plant detoxification, and more specifically involving cytochrome P450 and glutathione S-transferases (GSTs), suggests a fruit response to the PGE treatment. In other words, the plant seems to be able to detoxify the extract, and this process should be very effective since PGE did not cause any symptoms of phytotoxicity in treated organs (Li Destri Nicosia *et al.*, 2016; Pangallo *et al.*, 2017b).

5. Conclusions

In conclusion, the results of the present study provide a comprehensive picture of the impact of PGE on the gene expression of treated oranges, highlighting the induction of multiple metabolic responses. These responses are likely to collectively implement a defense system capable of counteracting fungal infections. In particular, GO analysis and pathway mapping of the DEGs showed the induction of important defense pathways, including the phenylpropanoid pathway. However, the massive up-regulation of genes suggest that the induction of defense mechanisms by PGE might be energetically costly for the fruit, which could lead to massive redistribution of energy resources. This would not be an issue in fruits or other mature organs but may be for young growing organs or plantlets. Future investigations will be needed to evaluate these aspects and to experimentally determine the role and function of specific differentially regulated genes in order to dissect their participation in the resistance to pathogens.

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Chapter 4. Effectiveness of a pomegranate peel extract (PGE) in reducing *Listeria monocytogenes* in vitro and on fresh-cut pear, apple and melon.

Abstract: Pomegranate peel extract (PGE) is a new promising natural alternative control substance with large spectrum of activity against wide range of pathogenic microorganisms. In the present study, PGE was firstly investigated as natural antimicrobial against *Listeria monocytogenes* both *in vitro* and on fresh-cut fruit. The *in vitro* results showed quick and strong bactericidal and bacteriostatic activity against 5 different strains which were almost completely inhibited by the extract. Furthermore, it significantly decreased growth rate and maximum growth of all tested strains. *In vivo* trials, confirmed a strong antibacterial activity of the extract that significantly reduced the bacterial load on fresh-cut apple, pear and melon and maintained the population at low levels throughout the storage period (7 days). PGE at 12 g/l reduced *L. monocytogenes* by 1.89, 1.24, and 0.9 log units soon after treatment and by 1.53, 3.89, and 2.99 log units, after 7 days of storage on pear, apple, and melon, respectively. This high antibacterial activity could be mainly explained by the high content in polyphenols and hydrolysable tannins mainly represented by punicalagins and ellagic acid. Overall, results of this study suggest a potential industrial application of PGE to reduce the growth of the pathogenic microorganisms in fresh-cut fruit and ensure a microbial safety in case of contamination.

Keywords: PGE, *Listeria monocytogenes*, antimicrobial activity, fresh-cut fruit.

1. Introduction

In recent years, the demand for healthy and ready-to-eat fresh-cut products has highly increased and, therefore, the industry is in continuous search for new and improved methods to maintain the quality and extend the shelf-life of products. Fresh-cut fruit and vegetables are minimally processed products (trimmed, peeled and/or cut) that offer to consumers high nutritional value, freshness, convenience and flavour similar to the original raw intact product (Gómez-López *et al.*, 2009; Del Nobile *et al.*, 2009). However, these products deteriorate faster than the unprocessed raw materials, mainly due to the damages caused by peeling operation as well as the other minimally processing operations (Prakash *et al.*, 2018; Rolle and CHISM III, 1987). This alters the processed product and makes it more vulnerable to microbial contamination and colonization with the consequent reduction of quality and shelf life (Prakash *et al.*, 2018).

Microbial contamination may represent a direct critical risk for human health because of the proliferation of important pathogens such as *Listeria monocytogenes* (Leverentz *et al.*, 2006). This bacteria is an important human pathogens that can contaminate fresh-cut produces in any step of the processing chain (Chaves *et al.*, 2016). Therefore, several methods and strategies have been developed and used by the fresh-cut industries in attempt to reduce the occurrence and the risk associated to foodborne diseases. Sanitizers, including chlorine (Wu and Kim, 2007), organic acids (Mani-Lopez *et al.*, 2012), heat treatments (Bermúdez-Aguirre and Corradini, 2012), ultraviolet (UV) light (Yaun *et al.*, 2004), and ozone (Wysok *et al.*, 2006) have been widely applied to disinfect and reduce the initial bacterial load on fruit and vegetables. However, these methods have shown several drawbacks such as the formation of potential carcinogenic by-products from using chlorine, low efficiency in reducing the bacterial population, chemical residues, destruction of nutrients and the alteration of sensory characteristics (Chaves *et al.*, 2016; Gil *et al.*, 2009). This, together with the increase of the consumer awareness in food safety and healthy living, has increased the interest to safe and environmentally friendly alternative control means and mainly plant substances such as essential oil and plant extracts.

Recently, a pomegranate peel extract (PGE) proved to be very effective in controlling fungal postharvest rots on different fruit species (Li Destri Nicosia *et al.*, 2016). Experiments demonstrated a complex mechanism of action which include the induction of resistance in treated host tissues and a strong antimicrobial activity against both fungi and bacteria (Pangallo *et al.*,2017; Pangallo *et al.*, 2017b; Belgacem *et al.*, 2019). The high antimicrobial activity was associated to the high content of phenolic and flavonoid compounds in PGE (Romeo *et al.*, 2015) Although PGE has never been tested against potential human bacterial pathogens, other extracts from pomegranate peel were able to reduce the germination and growth of several pathogenic bacteria including *Listeria monocytogenes, L. innocua, Staphylococcus aureus, Escherichia coli,*

Yersinia enterocolitica, *Pseudomonas aeruginosa*, and *Salmonella* spp. (Al-Zoreky, 2009; Gullon *et al.*, 2016; Pangallo *et al.*, 2017c). Furthermore, edible coatings formulated with a pomegranate peel extract and other anti-browning agents were used to extend the shelf life of fresh-cut persimmon fruit (Taberner *et al.*, 2016).

The aim of the present study was to evaluate the potential use of PGE as natural antimicrobial to reduce the growth of foodborne pathogens using *Listeria monocytogenes* as a model pathogen *in vitro* and on fresh cuts of melon, apple and pear.

2. Material and Methods

2.1. Pomegranate peel extract (PGE) and Bacterial strains

All experiments were conducted using a stock solution of an aqueous pomegranate peel extract (PGE) prepared according to Romeo *et al.* (2015). The solution was stored, before use, at 5±1 °C and diluted to have 3 concentrations of PGE containing 12 (**PGE-12**), 2.4 (**PGE-2.4**), and 1.2 (**PGE-1.2**) g/l of dry matter. Since the pH of these solutions was very low (2.7, 2.8, 3.1, respectively), PGE-12 was adjusted with phosphate buffer to increase the pH to 4.4 (**aPGE-12**) and included in experiments with fresh-cut fruit plugs in order to evaluate the potential impact of solution acidity on the antimicrobial activity.

Four strains of *L. monocytogenes* belonging to the Spanish Type Culture Collection (CECT 4031, serovar 1/2, CECT 933, serovar 3a, CECT 940, serovar 4d, CECT 4032, serovar 4b) and one strain that was previously isolated from fresh-cut lettuce (Lm 230, serovar 1/2 a, Abadias *et al.*, 2014) were used in the present study. Strains were grown individually in tryptone soy broth supplemented with 6 g/l of yeast extract (TSYEB). After 24 h of incubation at 37±1 ºC, bacterial cells were harvested by centrifugation (9800 × *g* for 10 min at 10 ºC) and resuspended in a saline solution (8.5 g/l NaCl) to obtain single-strain stock suspensions. The concentration of each strain suspension was determined by plating duplicate 10-fold serial dilutions on TSA media (TSA, Biokar Diagnostics, Beauvois, France) enriched with 6 g/l of yeast extract, 2.5 g/l glucose and 2.5 g/l K_2HPO_4, TSAYE) and incubated at 37 ºC for 24 h.

2.2. *In vitro* assays

To evaluate the bactericidal activity of PGE, 50 µl of *L. monocytogenes* suspensions (approximately 10^8 UFC/ml) were added to 5 ml of PGE at three different concentrations (12, 2.4 and 1.2 g/l). Sterile water was used as control. For each strain and concentration three replicates were used. After 2, 5, 10 and 30 min of contact time at 20°C, bacterial suspensions of *L. monocytogenes* were 10-fold serially diluted in saline peptone (8.5 g L^{-1} NaCl and 1 g L^{-1} peptone)

and plated on TSA (TSA, Biokar Diagnostics, Beauvois, France) enriched with 6 g/l of yeast extract, 2.5 g/l glucose and 2.5 g/l K_2HPO_4, TSAYE). After 24 h of incubation at 37 ºC, the number of colony forming units was recorded and converted to CFU/ml.

To evaluate the impact of PGE on the growth parameters of *L. monocytogenes*, 20 µl of bacterial suspensions containing approximately 10^5 CFU/ml were added to 180 µl of TSBYE to obtain a final PGE concentration of 2.4 or 1.2 g/l in a round-bottomed 96-well microplate (Greiner, Frickenhausen, Germany). TSBYE without PGE served as a control and each treatment was replicated four times. The microplate was incubated for 36 h at 37±1ºC and the absorbance of suspensions was recorded every 30 min using a spectrophotometer (Epoch Microplate Spectrophotometer, Biotek-Instruments, Winooski, USA) set at λ = 700 nm. Plates were automatically agitated before measurements.

2.3. *In vivo* assays

Experiments were performed on apples (cv. *Golden Delicious*) and pears (cv. *Conference*) obtained from local packinghouses in Lleida (Catalonia, Spain) and on melons (cv. *Cantaloupe*), purchased from a local supermarket. For all fruit species, two independent trials were conducted using different fruit batches. In both trials, fruits were preliminary washed with tap water, surface disinfected with ethanol 70 % and dried at room temperature. Fruits were peeled and cut with a sterilized cork-borer to have cylindrical plugs of 1.2 cm diameter × 1.0 cm long (weighting approximately 1 g).

Fruit plugs were inoculated with *L. monocytogenes* by dipping in a bacterial suspension (10^6 CFU/mL) containing the five strains of the pathogen, for 2 min. The bacterial suspension was obtained by mixing equal volumes of the single-strain stock solutions. Inoculated fruit plugs were air dried at room temperature for 30 min and incubated overnight at 5 ºC. Plugs of each fruit were then divided into 6 uniform groups and subjected to different treatments including PGE-12, PGE-2.4, PGE-1.2, aPGE-12, and distilled water. Other plugs did not receive any treatment. Treatments were performed by dipping the inoculated plugs for 10 min at 150 rpm. After drying for 30 min at room temperature, plugs from each treatment were further divided into two sub-groups, each consisting of 6 replicates. Sub-groups were used to determine the concentration of bacterial cells soon after the treatment or after 7 days of storage at 10±1 ºC. To determine bacterial population, plugs were put in a sterile bag containing 9 ml of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England) and blended in a homogenizer (Minimix® 100, Interscience, France) for 120 s at 12 strokes/s. The homogenized mixtures were then serially diluted in saline peptone, plated on duplicate plates of selective Palcam agar (Biokar Diagnostics, Beauvois, France) and incubated at 37±1 ºC for 48 h. The bacterial concentration was expressed as log CFU/g.

2.4. Statistical analysis

Prior analyses, all CFU mL^{-1} data were transformed to $\log_{10}$ CFU mL^{-1} or $\log_{10}$ CFU g^{-1}. For the bacterial growth experiment, curves were fitted using the DMFit 3.5 Excel add-in provided by ComBase (https://www.combase.cc) and growth parameters (lag time, growth rate, and maximum population density) were determined using the Gompertz model.
Data were analysed using general linear model analysis with JMP®8, 2004 software (JMP®8, SAS Institute, Cary, NC, USA). After analysis of variance (ANOVA), significant differences between treatments were determined according to Tukey's test at a significance level of $P < 0.05$.

3. Results

3.1. *In vitro* assays

In vitro experiment showed a strong bactericidal activity of PGE. The number of viable cells (log CFU/mL) of *L. monocytogenes* was always significantly reduced by the extract (Table 1). No significant differences were observed among the 3 tested concentrations of PGE. In addition, the incubation time did not have a relevant influence on the bactericidal activity as similar results were achieved after 2, 5, 10 and 30 min of contact. On the contrary, important differences were observed among *L. monocytogenes* strains. Strains CECT 4031 and CECT 933 were the most sensitive since their population was always below the detection limit for almost all tested concentrations and incubation times. A slightly higher tolerance was revealed for the strain CECT 4032. Strains CECT 940 and Lm230 showed the highest rates of survival, but still their population was reduced at least by 3.3 log units after 2 minutes of incubation with all the PGE doses.

Table 1. Concentration of *L. monocytogenes* cells ($\log_{10}$ CFU/ml) after 2, 5, 10 or 30 min of incubation in PGE solution at three different concentrations (1.2, 2.4 or 12.0 g/l) or water (control). Separate statistical analyses were conducted for each strain and incubation period. Different letters indicate significantly different values according to Tukey's test ($P < 0.05$).

Strain	Treatment	Incubation period (min)			
		2	5	10	30
CECT 933	Water	7.11 a	7.04 a	7.08 a	7.11 a
	PGE 1.2 g/l	<dl b	<dl b	<dl b	<dl b
	PGE 2.4 g/l	<dl b	<dl b	<dl b	<dl b
	PGE 12 g/l	<dl b	<dl b	<dl b	<dl b
CECT 940	Water	7.62 a	7.08 a	6.97 a	6.63 a
	PGE 1.2 g/l	3.59 b	3.49 b	3.32 b	3.23 b
	PGE 2.4 g/l	3.67 b	3.48 b	3.11 b	3.00 b
	PGE 12 g/l	3.61 b	3.54 b	3.43 b	3.30 b

Lm230	Water	6.93	a	6.90	a	6.93	a	6.90	a
	PGE 1.2 g/l	3.52	b	3.49	b	3.38	b	3.34	b
	PGE 2.4 g/l	3.48	b	3.41	b	3.30	b	3.28	b
	PGE 12 g/l	3.61	b	3.45	b	3.40	b	3.23	b
CECT 4032	Water	7.28	a	7.18	a	7.08	a	7.28	a
	PGE 1.2 g/l	2.28	b	1.89	b	1.37	b	1.30	b
	PGE 2.4 g/l	2.56	b	0.92	b	1.74	b	0.23	b
	PGE 12 g/l	1.56	b	<dl	b	<dl	b	<dl	b
CECT 4031	Water	6.70	a	6.85	a	6.74	a	6.65	a
	PGE 1.2 g/l	0.20	b	0.52	b	<dl	b	<dl	b
	PGE 2.4 g/l	<dl	b	<dl	b	<dl	b	<dl	b
	PGE 12 g/l	<dl	b	<dl	b	<dl	b	<dl	b

< dl: below detection limit

The analysis of the growth parameters of *L. monocytogenes* in TSBYE showed a significant impact of PGE on the maximum cell growth of all investigated strains (Table 2). Interestingly, the effect of PGE was directly correlated to its concentration since significant differences were always revealed between the two tested concentrations. In particular, PGE-2.4 reduced the maximum cell growth between 46.9% (CECT 9333) and 62.9% (CECT 4031) as compared to the control (TSBYE without PGE). With PGE-1.2 reductions ranged between 18.4% (strain CECT 4032) and 35.3% (CECT 933).

PGE-2.4 significantly reduced also the growth rate of all strains with reductions ranging between 41.6% (CECT 4032) and 63.9% (CECT 933) as compared to the control. Lower, but still significant reductions were also achieved with PGE-1.2 for 4 out of 5 strains. Similarly, the duration of the lag phase was increased by PGE for 4 out of 5 strains.

Table 2. Growth kinetic parameters (lag time, growth rate, and max absorbance) of the five tested strains of *L. monocytogenes* cultured in standard TSBYE (control) or in TSBYE amended with PGE at 1.2 and 2.4 g/l. For each parameter and strain, different letters indicate statistically different values according to Tukey's test ($P<0.05$).

Strains	**Medium**	**Lag time, λ (h)**		**Growth rate, μ (Absorbance at λ = 700 nm)**		**Max absorbance**	
Lm230	TSBYE	7.5	c	0.002229	a	0.564225	a
	TSBYE+PGE 1.2 g/l	7.9	b	0.001435	b	0.370295	b
	TSBYE+PGE 2.4 g/l	8.4	a	0.001005	c	0.242917	c
CECT 933	TSBYE	9.3	c	0.001122	a	0.491362	a
	TSBYE+PGE 1.2 g/l	11.3	b	0.000610	b	0.317862	b
	TSBYE+PGE 2.4 g/l	13.0	a	0.000405	c	0.261084	c
CECT 940	TSBYE	7.9	c	0.002180	a	0.492187	a
	TSBYE+PGE 1.2 g/l	8.3	b	0.001366	b	0.359077	b

	TSBYE+PGE 2.4 g/l	8.9	a	0.000972	c	0.234525	c
CECT 4031	TSBYE	9.0	b	0.001738	a	0.402777	a
	TSBYE+PGE 1.2 g/l	9.3	ba	0.001000	b	0.313139	b
	TSBYE+PGE 2.4 g/l	9.9	a	0.000685	c	0.149649	c
CECT 4032	TSBYE	6.7	c	0.002335	a	0.495242	a
	TSBYE+PGE 1.2 g/l	7.4	b	0.002658	a	0.403998	b
	TSBYE+PGE 2.4 g/l	8.2	a	0.001364	b	0.216037	c

3.2. *In vivo* trials

3.2.1. Effect PGE treatments on *L. monocytogenes* population on fresh-cut pear

In both trials, a similar population of *L. monocytogenes* ranging from 6.2 and 6.5 log CFU/g was detected after inoculation (untreated) and on plugs dipped in water (control) (Fig. 1). On these samples, the bacterium population greatly increased after seven days of storage reaching approximately 8.0 log CFU/g in the first trial and more than 8.7 log CFU/g in the second trial.

In both trials, PGE-12 and PGE-2.4, significantly reduced the population of *L. monocytogenes* soon after the treatment of fresh-cut pear plugs (Fig. 1). In particular, PGE-12 and PGE-2.4 reduced the bacterium by 1.61 and 1 log units (first trial), and by 2.17 and 1.13 log units (second trial), respectively. In the second trial, a significant reduction was also achieved with the aPGE-12 by 1.22 log units, which indicated that the effect was due to the PGE extract by itself and not due to low pH of PGE solution. Overall, a higher concentration of PGE was needed to significantly reduce the growth of the bacterium after 7 days of storage. In particular, PGE-12 proved very effective reducing the bacterium by 1.46 and 1. 6 log units in the first and second trial, respectively.

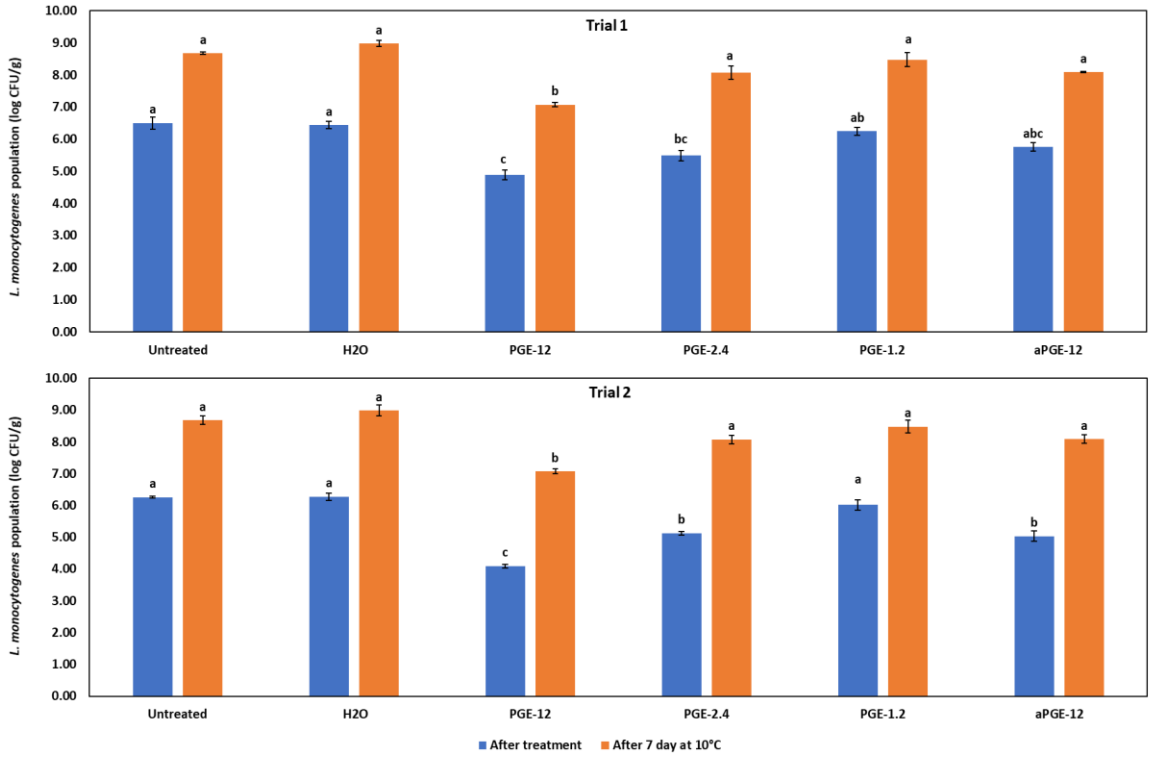

Fig 1. Population of *L. monocytogenes* (log CFU/g) determined in trials 1 and 2 on fresh-cut pear plugs after treatments (dark grey column) and after 7 days of storage at 10°C (grey column). Bars indicate standard errors of the means. For each assessment time and for each trial, different columns with different letters indicate significant differences between treatments according to Tukey's test ($P < 0.05$).

3.2.2. Effect of PGE on *L. monocytogenes* population on fresh-cut apple

Soon after treatments, on both untreated and water treated samples, a higher population of *L. monocytogenes* was revealed in the first trial (6.0 and 5.7 log CFU/g) as compared to the second trial (around 5.2 log CFU/g) (Fig. 2). An opposite situation was observed after 7 days of storage in the second trial where the bacterial population increase was more pronounced in the second trial than the first with a bacterial population of 7.5 and 7.9 log CFU/g on untreated and water treated samples, respectively.

PGE proved very effective in reducing the population of *L. monocytogenes* on fresh-cut apple. After treatments, significant reductions were recorded with PGE-12 and PGE-2.4 in both trials and with PGE-1.2 in the second trial. A significant reduction was also achieved with aPGE-12 that was only evaluated in the first trial. Overall, PGE-12 and PGE-2.4 reduced the population of the bacterium between 0.69 and 1.8 log units.

Moreover, after 7 days of storage, a high efficacy of PGE was also revealed where the bacterium population was reduced in both trials by more than 3 log units with PGE-12 and PGE-2.4. However, PGE at the lowest concentration (PGE-1.2) did not show any significant effect.

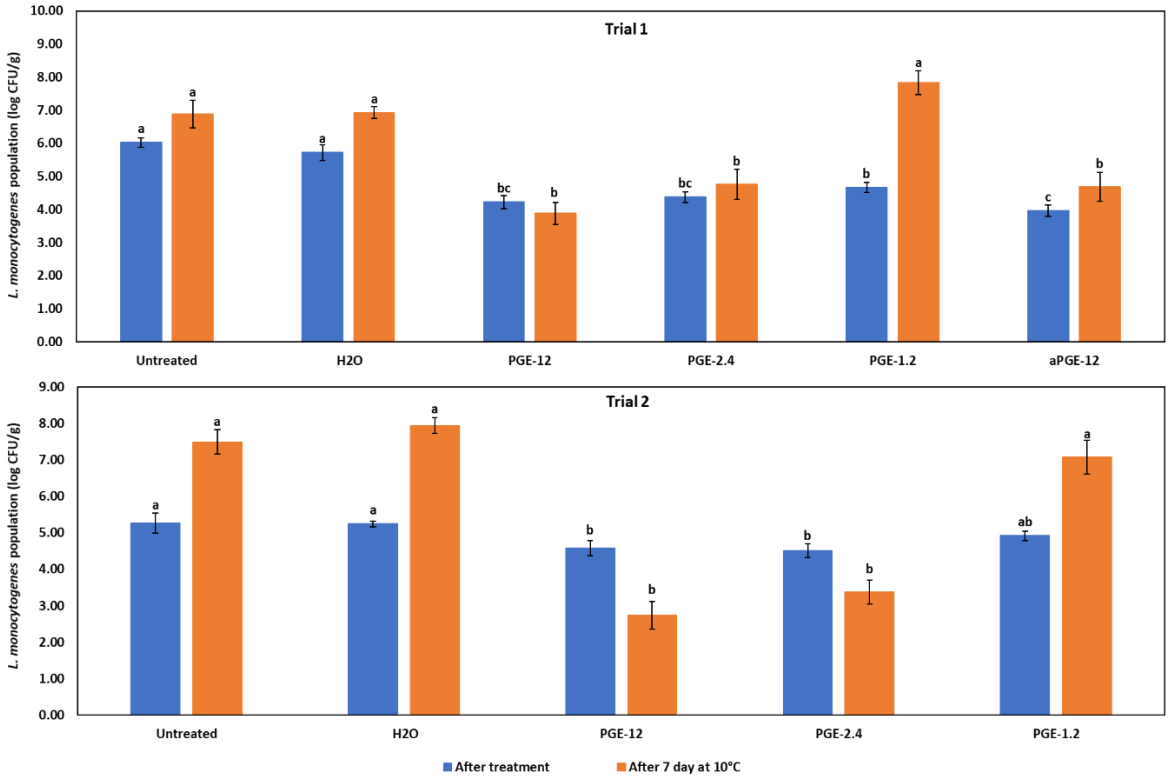

Fig 2. Population of *L. monocytogenes* (log CFU/g) determined in trials 1 and 2 on fresh-cut apple plugs after treatments (dark grey column) and after 7 days of storage at 10°C (grey column). Bars indicate standard errors of the means. For each assessment time and for each trial, different columns with different letters indicate significant differences between treatments according to Tukey's test (P< 0.05).

3.2.3. Effect of PGE on *L. monocytogenes* population on fresh-cut melon

Initial population of *L. monocytogenes* was 7.1 log CFU/ml in the first trial and 6.7 log CFU/ml in the second one (Fig. 3).

Soon after treatments, a significant reduction of *Listeria* population was achieved with all PGE treatments. In particular, compared to untreated fresh-cut melons, PGE-12, PGE-2.4 and PGE-1.2 reduced the bacterial population by 0.88, 0.57, and 0.46 log units (first trial) and by 0.94, 0.59, and 0.26 log units (second trial), respectively. aPGE-12 was only tested in the second trial and reduced the bacterium population by 0.85 log units.

After 7 days of storage at 10 ºC, the population of the bacterium increased, reaching a count > 9.0 log CFU/g with untreated and water samples in both trials. On the contrary, on melon plugs treated with PGE, the bacterium population was reduced by more than around 3 log units with PGE-12 as compared to the untreated samples. However, PGE at the lowest concentration (PGE-1.2) did not show any significant effect.

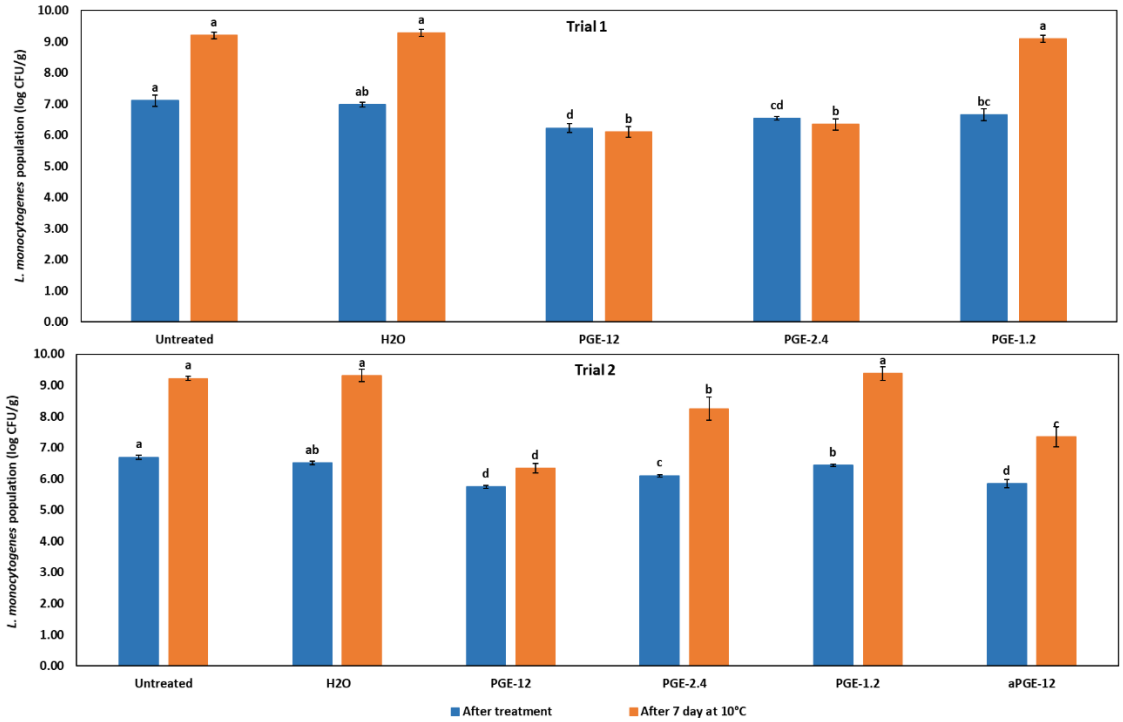

Fig 3. Population of *L. monocytogenes* (log CFU/g) determined in trials 1 and 2 on fresh-cut melon plugs after treatments (dark grey column) and after 7 days of storage at 10°C (grey column). Bars indicate standard errors of the means. For each assessment time and for each trial, different columns with different letters indicate significant differences between treatments according to Tukey's test (P< 0.05)

4. Discussion

The present study represents the first investigation of PGE as natural antimicrobial to reduce and control the growth of foodborne pathogens on ready-to-eat fresh-cut fruit. Experiments were conducted using *L. monocytogenes* as a model species in light of its primarily importance as food contaminant, and future investigations will be needed to evaluate the efficacy of PGE against other foodborne microorganisms. Overall, *in vitro* and *in vivo* results showed high bactericidal and bacteriostatic effects of PGE against *L. monocytogenes*. In particular, the *in vitro* results (Table 1) revealed that regardless of the tested concentration, PGE exerted a quick and high significant inhibitory activity against all the *L. monocytogenes* tested strains by reducing the population by at least 3.3 log units after short time of contact (2 minutes). This high antibacterial activity could be explained by the composition of the extract. In fact, PGE is rich in polyphenols and hydrolysable tannins mainly represented by punicalagins and ellagic acid that have been reported to exert a strong antimicrobial activity against Gram-negative and Gram-positive bacteria (Wu *et al.*, 2018). In any case, the absence of the outer membrane in *L. monocytogenes*, as a Gram-positive bacterium, makes it easier for the extract to alter and, therefore, causing a loss of the bacterium cellular components (Li *et al.*, 2014).

The antimicrobial activity of other extracts from pomegranate peel against a variety of food-borne pathogens including *L. monocytogenes, E. coli*, and *S. aureus* has been already reported (Wu *et al.*, 2018; Gullon *et al.*, 2016; Li *et al.*, 2014). However, PGE, seems to be more effective due to its higher content in polyphenols and, being obtained with food grade ethanol, it can be considered a safe and eco-friendly antimicrobial preparation (Al-Zoreky, 2009; Romeo *et al.*, 2015). Furthermore, PGE bactericidal activity also proved to be stronger against *Listeria*, comparing to other plant extracts such as cherry pomace extracts (Kołodziejczyk *et al.*, 2013), and similar to the activity of plum extract which also reduced the *Listeria* population to under the detection level after 5 minutes of contact (Sójka *et al.*, 2015).

PGE also revealed strong bacteriostatic effect and its activity was significantly influenced by the concentration of the extract (Table 2). In particular, the log phases of the tested bacteria strains grown in broth media containing PGE were significantly longer. This delayed response of the growth indicates that PGE can negatively modify the growth environment making it longer for the bacteria to adjust (Swinnen *et al.*, 2004). More importantly, PGE showed high efficiency in reducing the growth rate as well as the maximum growth of *L. monocytogenes*. This effect may be attributed to the richness of the extract in tannins that may combine with proteins and cause their precipitation (Wu *et al.*, 2018; Singh *et al.*, 2019). Likely tannins of the extract may combine with proteins of the bacterial membrane as well as with protein of the culture media forming complexes that lead to the lysis and death of the bacteria. Moreover, the high concentration of polyphenols of PGE causes the decrease of pH gradient around the cell membrane and the increase of its permeability, leading to cell death (Singh *et al.*, 2018).

In vivo results confirmed a strong antibacterial activity of PGE that significantly reduced the bacterial load on fresh-cut apple, pear and melon and was able to maintain the population at low levels throughout the storage period (7 days). However, the reduction of the bacterial population in the *in vivo* experiments was overall lower as compared to *in vitro* conditions. This could be mainly explained by the presence of organic matter as well as to the presence of a solid matrix, that increase the bacterial survival and decrease the contact between the treatment and the bacteria (Rodgers *et al.*, 2004; KIM *et al.*, 1999). For the same reason, a higher concentration of PGE seems to be needed to control the bacterium in practical *in vivo* conditions as confirmed by the low efficacy of the lowest tested concentration of PGE-1.2. Interestingly, the aPGE-12 (pH 4.4) showed a slight lower efficacy as compared to normal PGE (pH 2.7), both soon after treatments and after 7 days of storage at 10 ºC. This result confirms that the composition of PGE rather than its low pH was the main determinant factor for its activity. In this context, the higher concentration of polyphenols compared to other plant extracts, make pomegranate peel extracts particularly promising for future applications especially that it already proved major beneficial effects on

human health (Sorrenti *et al.*, 2019; Howell and D'Souza, 2013). However, the overall lower efficacy of the aPGE-12 indicate that pH still plays a role in determining the level of efficacy. This aspect needs to be taken into account in future applications and/or in the development of commercial formulations. On a practical point of view, the analysis of previous reports suggests that PGE enables higher levels of reductions of *L. monocytogenes* populations compared to other alternative sanitizers such as vanillin, citrox, N-acetyl-l-cysteine, hydrogen peroxide and peroxyacetic acid (Abadias *et al.*, 2011). The antimicrobial efficiency of these treatments against *Listeria* spp. on fresh-cut fruit has been widely investigated and it is generally recognized by the scientific community (Abadias *et al.*, 2011; Silveira *et al.*, 2008; Rodgers *et al.*, 2004). For instance, after storage, the microbial reductions observed with PGE treated apple plugs were almost double the reduction obtained after treatment with hydrogen peroxide, which is an environmental friendly substance against pathogenic bacteria of fresh-cut fruits (Abadias *et al.*, 2011; Ukuku and Fett, 2002). Therefore, the effectiveness of PGE in reducing the bacterial growth as well as its effect on the sensory quality of the fruit should be further evaluated under typical commercial processing conditions. Moreover, the antimicrobial activity of PGE and/or its spectrum of activity could be further enhanced by combining it with other alternative control means. For instance, the combination of PGE with a bacteriophage may be strategic since the latter showed broad spectrum of activity against *L. monocytogenes* strains on fresh-cut melons and pears but not on apples (Oliveira *et al.*, 2014).

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Chapter 5. General discussion and conclusion

Due to the growing public concern for human health and environmental pollution, the use of conventional pesticides to control postharvest diseases is needed to be minimized. Therefore, many alternative control strategies have been evaluated for their potential use as a part of integrated pest management systems but only few of them have been found to be effective in practical commercial conditions. Hence, considering the previously documented high efficacy of PGE against a wide range of postharvest diseases the aim of the present PhD thesis was to extend the current knowledge of PGE in order to lay the bases for its practical use in integrated control strategies and/or as alternative to chemical compounds.

In this context, the present study was the first investigation conducted under large-scale commercial conditions evaluating the antimicrobial activity of PGE as pre- and post-harvest treatment against citrus rots. Overall, PGE treatments proved high antimicrobial activity and long persistence resulting in high reduction in losses, longer shelf life and enhancement of the citrus fruit quality. In particular, both pre- and post-harvest PGE treatments were much more effective in reducing postharvest rots than sodium and potassium bicarbonate which are two salts commonly used as alternative means in integrated control strategies (Palou et al., 2008). This together with the widely reported inconsistent efficiency of many formulations tested under practical commercial conditions and based on biocontrol agents, make the integration of PGE in citrus postharvest control strategies very promising especially that PGE proved flexible and particularly effective regardless of the timing of application. In particular, PGE field applications might be easily implemented in practical conditions and may be strategic for fresh fruit productions where fruit are packed and marketed without undergoing specific treatments such as washing and waxing. Furthermore, the higher efficiency of PGE compared to IMZ, a chemical treatment currently considered as the most effective fungicide to control postharvest citrus rots, suggests a potential use of PGE not only strictly for organic production but also incorporated in conventional and/or integrated farming systems.

Although PGE proved very effective in its current formulation, its mechanism of action remained unclear. Therefore, the investigation of its mechanisms of action was considered necessary so it may be used to further improve its efficacy and facilitate the development of commercial formulations. To this aim, the mode of action of PGE was studied on oranges using a transcriptomic analysis to have a

holistic view on the global gene expression in fruit treated tissues. Our analysis showed a very quick response of gene expression accompanied by high up-regulation of genes. In particular, GO analysis and pathway mapping of the DEGs showed the induction of important pathways involved in defense including mainly the antibiotic biosynthesis pathways. The induction of such important defense pathways together with the reported direct antimicrobial activity exerted by PGE confirm previous preliminary data on the co-existence of more than one mechanism of action (Pangallo et al., 2017a; Pangallo et al., 2017b). This is considered an important feature to ensure high levels of protection on different hosts and environmental conditions, against different pathogens, and in different phases of the disease cycle. Future investigations will be needed to determine the role and function of specific differentially regulated genes in order to dissect their participation in the resistance to pathogens. The obtained results represent valuable information to improve future control strategies through the identification of better methods and timings of application. For instance, the massive and quick activation of resistance genes in treated tissues makes the application of PGE a good strategy to prevent latent infections as well as to control already established infections (curative action). Another possible use, worth further investigation, is the application of PGE on aerial plant parts to protect organs such roots or tubers that cannot be easily treated. Similarly, early treatments of leaves and/or other plant organs such as flowers may be used to protect the new vegetation and fruit during growing and ripening phases as well as harvesting, packaging and storage.

Overall, the optimization of PGE control strategies may be useful to further improve its efficacy, broaden its fields of application and reduce the concentration of its active ingredients while keeping a high level of efficacy. The latter might need to be investigated to reduce the potential risk of PGE on the environment as well as the human health. In fact, the reduction of concentrations may be strategic to reduce the costs of PGE treatments and make it commercially competitive on the market. Although PGE is obtained from a costless waste product such as the pomegranate peel, the extraction costs may still be an important issue.

Considering the high antibacterial and antifungal activity of PGE the third objective of the present thesis was the evaluation of its potential use as natural antimicrobial treatment to reduce the growth of foodborne pathogens. Using *Listeria monocytogenes* as a model pathogen, experiments conducted *in vitro* and on fresh cuts of melon, apple and pear, revealed high bactericidal and bacteriostatic activity of the extract. The *in vitro* results revealed that regardless of the tested concentration, PGE exerted a quick and high significant inhibitory

activity against all the tested *L. monocytogenes* strains. This high bactericidal activity exerted by PGE was stronger than other reported plant extracts such as cherry and plum extracts (Sójka et al., 2015; Kołodziejczyk et al., 2013). Similar results were found *in vivo* trials where PGE significantly reduced the bacterial load on fresh-cut fruit and maintained the population at low levels throughout the storage period. Obtained results confirmed a primary role of phenolic compounds in determining the efficacy of PGE. Consequently, the higher efficiency of PGE as compared to other alternative sanitizers such as vanillin, citrox, hydrogen peroxide and peroxyacetic acid makes PGE particularly promising for future applications. Data also indicated that pH may play a role in determining the level of efficacy and this aspect needs to be considered in future applications and/or in the development of commercial formulations.

In conclusion, the findings of the present work incorporate new knowledge on the potential use of PGE as a safe and potent alternative control mean against a wide range of pathogens and will contribute to the already ongoing process to register a commercial formulation. According to our results PGE not only protects fruit and vegetable against fungal rots, avoiding the use of dangerous chemical compounds, but also improve the quality of production by preventing the proliferation of foodborne pathogens. In this context other potential application methods such as the incorporation of PGE in industrial fruit washing system or its use as water sanitizer are worth of further investigations.

Future studies also remain to be conducted in order to exclude any kind of phytotoxicity and to determine the effect of PGE on fruit quality parameters such as color, taste, texture, etc. In fact, the massive up-regulation of genes determined in the present study by transcriptomic analysis suggests that the induction of defence mechanisms might be energetically costly for the fruit, which could lead to massive redistribution of energy resources. This would not be an issue in fruits or other mature organs but may be for young growing organs or plantlets. Furthermore, although other pomegranate peel extracts have been frequently used in traditional human medicine and are widely recognized as safe for human health (Al-Zoreky, 2009; Jahromi et al., 2015), specific toxicological analyses will need to be performed during the registration process. Overall, our findings on PGE contributed to highlight the potential of plant extracts as effective natural preparations to control plant diseases and to prevent foodborne pathogens and will surely spur new researches in this fascinating field.

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