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Investigating the effects of crab shell chitosan on fungal mycelial growth and postharvest quality attributes of pomegranate whole fruit and arils

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ABSTRACT

Postharvest disease management for pomegranate fruit remains a critical challenge and the need for effective alternative treatments is essential in order to minimise losses. This study investigated the *in-vitro* and *in-vivo* antifungal activities of crab shell chitosan and fludioxonil (a registered postharvest fungicide) as a control against *Botrytis* sp., *Penicillium* sp. and *Pilidiella granati* isolated from pomegranate fruit. Mycelial growth inhibition was evaluated using potato dextrose agar amended with varying concentrations of 0–1 g/L for fludioxonil, and 0–10 g/L of chitosan. Complete mycelial growth inhibition was observed at 0.10 g/L and 10 g/L for fludioxonil and chitosan, respectively. Chitosan concentrations causing a 50% reduction in mycelial growth (EC_{50}) were 0.47, 1.19, and 2.21 g/L for *P. granati, Botrytis* sp., and *Penicillium* sp., respectively. The EC_{50} concentrations for fludioxonil were 0.02, 0.48, and 0.90 mg/L for *Penicillium* sp., *P. granati,* and *Botrytis* sp., respectively. For *in-vivo* investigation of artificially inoculated pomegranate fruit; chitosan effectively reduced rot incidence by 18–66%, and was most efficient when applied as a preventative treatment regardless of cultivar ('Herskawitz' and 'Wonderful'). Additionally, chitosan treatments (0, 2.5, 7.5 and 15 g/L) were applied on minimally processed pomegranate arils as edible coating prior to packaging and storage at 4 °C for 14 days. The chitosan treated arils best maintained physico-chemical quality attributes and significantly lower microbial counts for mesophilic aerobic bacteria, yeast and moulds. This study showed that crab shell chitosan has a potential as a green fungicide for postharvest disease management of pomegranate.

1. Introduction

Pomegranate (*Punica granatum* L.) has become one of the most lucrative super fruit globally, due to the health benefiting properties such as vitamins, minerals, dietary fibre and polyphenols (tannins, anthocyanins and ellargic acid) (Gil et al., 2000; Bhowmik et al., 2013; Mditshwa et al., 2013). The antioxidant activities of pomegranates can neutralise nearly twice as many free radicals as red wine and seven times as many as green tea (Gil et al., 2000; Seeram et al., 2008). Clinical studies have shown that polyphenols from pomegranate can lower risk of heart disease and retard cancer progression (Stover and Mercure, 2007; Aviram and Rosenblat, 2012; Bhowmik et al., 2013).

Pomegranate disease management is predominantly achieved by the application of synthetic fungicides pre- and postharvest. One of the commonly applied fungicides during commercial handling of pomegranates and other pome fruit is fludioxonil (Palou

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and del Río, 2009; Karaoglanidis et al., 2011). However, intensive fungicide usage is limited due to public health concerns, and the emergence of fungicide-resistant strains of the various fungal genera. This has necessitated the need to explore alternatives for synthetic fungicides (Liu et al., 2007). One of such alternative fungicide is chitosan, a non-toxic biodegradable compound derived from the outer shell of crustaceans. It has shown potential as a new class of plant protectant against pre- and postharvest diseases for various horticultural commodities (Bautista-Baños et al., 2006; Liu et al., 2007; Ali et al., 2010). These studies showed the potential of chitosan to control fruit decay, delay the ripening process and maintain quality attributes of a fresh food product. However, there is limited information in the literature on the effective concentrations of crab shell chitosan, which could inhibit fungal mycelial growth on pomegranate 'Herskawitz' and 'Wonderful' cultivars (cvs.) fruit. Also, the role of chitosan as a preventative or curative alternative to synthetic fungicide for pomegranates has not been investigated.

Pomegranates are colonised by various naturally occurring and/or pathogenic fungi and bacteria (Petersen et al., 2010). The colonisation of the fruit can be superficial, or may cause extensive internal postharvest spoilage and decay (Palou and del Río, 2009; Munhuweyi et al., 2016). The most common postharvest diseases of pomegranates are heart rot (*Alternaria alternata* or *Aspergillus niger*), grey mould rot (*Botrytis cinerea*) and, green/blue mould rot (*Penicillium* spp.) (Thomidis, 2014). Other disease causing pathogens include *Rhizopus* spp., *Nematospora* spp., *Pilidiella granati*, *Collectotrichum gloesporiodes* and *Pestalopsis vercolour*. These pathogens are often site-specific and vary from region to region depending on the geographical conditions and location (Palou and del Río, 2009). Based on our preliminary study on the fungal pathogens associated pomegranate fruit in South Africa; *Botrytis* sp., *Penicillium* sp. and *Pilidiella granati* were identified as the most important postharvest disease causing pathogens. Thus, these were fungal pathogens investigated in this study.

The aims of this study were; i) to investigate the antifungal efficacy of crab shell chitosan on fungal pathogens isolated from pomegranate cvs. 'Herskawitz' and 'Wonderful' fruit, and ii) to elucidate on the role of crab shell chitosan as an effective edible coating for minimally processed pomegranate arils. To achieve these aims three set objectives were investigated; a) the *in-vitro* sensitivity of mycelial growth of *Botrytis* sp., *Penicillium* sp., and *P. granati* isolated from pomegranate fruit to varying concentrations of chitosan and fludioxonil (positive control); b) antifungal effect of crab shell chitosan as an edible coating on whole pomegranate fruit before infection (as preventative) and post infection (as curative); and c) the effects of chitosan as an edible coating on the physico-chemical and microbial quality of pomegranate arils during cold storage at 4 °C for 14 days.

2. Materials and methods

2.1. Plant material

Pomegranate fruit cvs. 'Herskawitz' (an early season variety) and 'Wonderful' (a late season variety) were aseptically harvested at commercial maturity from an orchard located in Wellington, Western Cape region, South Africa (GPS S33° 39.276 E18° 59.399). Fruit of uniform size, shape, and free of any external defects were selected and washed with tap water (1 min). Thereafter, samples were coated with 70% (v/v) ethanol for 30 s, 0.35% (v/v) sodium hypochlorite (NaOCl) for 2 min, and 70% (v/v) ethanol for 30 s as described by Fourie et al. (2002), then dried overnight under laminar airflow. This was done to remove dirt and any unwanted surface flora. A separate batch of fruit (100 per cultivar) was set aside for aril processing.

2.1.1. Aril preparation and packaging

Processing and packaging of arils was performed at a commercial pack-house facility, in accordance with good manufacturing practice guidelines recommended for food product. Pomegranate fruit husks were manually processed for aril extraction by trained personnel. Arils were uniformly mixed and divided into four equal lots for the following coating treatments: (a) control (untreated with chitosan), but arils were washed in 1% acetic acid v/v, (pH 5.6) in order to expose the samples to similar acetic acid concentration used in chitosan preparation; (b) 2.5 g/L chitosan; (c) 7.5 g/L chitosan; and (d) 15 g/L chitosan. Treatments were performed by dipping each batch of arils in respective solution for 60 s, thereafter drained with a colander, and collected on a tray before air drying. Aril portions of 125 g were weighed into $15.5 \times 11.5 \times 3.5\ cm^3$ polypropylene trays (Blue Dot Packaging, Cape Town, South Africa) that had been previously sterilised with ethylene oxide. The trays were heat sealed using a semi-automated machine (Food Processing Equipment, South, Africa) with a polymeric film POLYID® 107 polyethylene (thickness 55 μm; carbon dioxide transmission rate, 600–700 mL/m^2.day at 38 °C, 90% relative humidity and 1 bar) provided by Barkai Ployon Ltd., (Kibbutz Barkai, Israel). A label of $7.0 \times 3.8\ cm^2$ area was placed onto each package film to simulate the labels found in the retails market packages.

The packaged products were immediately cooled down to 2 °C whilst at the pack-house, before transportation in ice-packed cooler boxes fitted with data loggers (Gemini Data Loggers, United Kingdom) to the plant pathology research laboratory. The temperature inside the cooler boxes ranged between 3.5-4.5 °C, on arrival. Packaged samples were stored at 4 °C and 95% for 14 d. Microbial and physico-chemical properties of pomegranate fruit samples were taken on fresh arils prior to packaging and storage as a baseline on day 0. Four packages from each experimental condition were taken for analyses on each sampling day 3, 7, 10 and 14.

2.2. Pathogen sampling

Fungal pathogens, *Botrytis* sp. (STE-U 7866), *Pilidiella granati* (STE-U7864) and *Penicillium* sp. (STE-U 7865) were previously isolated and characterised from naturally infected pomegranates (cv. 'Herskawitz') harvested from a commercial orchard located in the Wellington area, Western Cape, South Africa (GPS S33° 39.276 E18° 59.399). Virulence and pathogenicity of the isolates was verified by fulfilling the Koch's postulates. The pathogen isolates are stored in the Stellenbosch University, Department of Plant Pathology (STE-U) culture collection under the given accession numbers. Cultures were grown on Potato Dextrose Agar (PDA) (Merck Pty. Ltd., Modderfontein, South Africa) for 7–14 days at 25 °C before each trial.

2.3. Media preparation

Fludioxonil (Sigma-Aldrich, Steinheim, Germany) was dissolved in acetone to make a 1 g/L stock solution. Crab shell chitosan (Sigma-Aldrich, Steinheim, Germany) was purified as described by Laflamme et al. (1999) and dissolved in 1% glacial acetic acid and pH adjusted to 5.6 to make a 10 g/L stock solution. The purification was achieved by extensive grinding of chitosan to a powder, washing repeatedly in distilled water, pelleting at low-speed centrifugation (Eppendorf AG, Hamburg, Germany), and then air drying. The chitosan sheets were subsequently solubilized by stirring in 0.25 M HCl, centrifuged at 13000*g* for 10 min at 4 °C to remove insoluble material, and precipitated by neutralisation with 2.5 M NaOH. The chitosan pellets, recovered by centrifugation at 25000*g* for 15 min, were thoroughly washed with deionised water to remove salts and lyophilised. Mycelial growth inhibition was tested on PDA amended with 0 (control), 0.05, 0.1, 0.5, 0.75 and 1.0 g/L fludioxonil, or with 0.15, 0.3, 0.45, 0.6, 0.75, 1.5, 3.0, 4.5, 6.0 and 10 g/L chitosan. In all