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Sodium carbonate and bicarbonate treatments induce resistance to postharvest green mould on citrus fruit

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ABSTRACT

The aim of this study was to investigate the ability of two salts, sodium carbonate and bicarbonate, to activate defence mechanisms in citrus fruit against postharvest green mould caused by *Penicillium digitatum*. In particular, once there was confirmed salt antifungal activity in the absence of direct contact with the pathogen, changes in enzyme activity and expression levels of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase (PAL), and phytoalexin (scoparone, scopoletin, umbelliferone) and sugar (glucose, fructose, sucrose) contents in treated oranges were analyzed. Overall, sodium carbonate and bicarbonate increases the activity of β-1,3-glucanase, peroxidase, and PAL enzymes in orange tissues. Gene expression analyses confirmed PAL up-regulation particularly 12 h after treatment application. HPLC analyses of peel extracts showed increased amounts of the sugars and phytoalexins, compared to control tissues, with sucrose and scoparone being the most represented. The results suggest that, although salts exert a direct antifungal effect on *P. digitatum*, they are also able to induce citrus fruit defence mechanisms to postharvest decay. The defence response seems correlated with the up-regulation of the phenylpropanoid pathway, which has a role in the adaptation to various stresses. This response could result in natural reaction to wounding and pathogen attack in citrus, enhancing its protective effect. As a consequence, the fruit might have a better chance of successful defence against the decay.

1. Introduction

The most severe postharvest fungal diseases of citrus fruit are green and blue moulds caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively. Blue mould is important on fruit kept under cold storage, whereas green mould may cause 60–80% of decay under ambient conditions (Moscoso-Ramírez et al., 2013), although numerous factors related to the fruit itself, the pathogen, and the postharvest environment can influence incidence and severity (Youssef et al., 2011). Traditionally, control is performed with synthetic fungicides. However, issues associated with their use, such as risks for human and environmental health, pathogen resistance, costs of registration and re-registration of active ingredients, *etc.*, have motivated the search for new and safer alternatives (Sanzani et al., 2012). In this context, the activity of several organic and inorganic salts has been comprehensively tested at concentrations of 2–6%, on a wide range of commodities including citrus (Smilanick et al., 1999; Palou et al., 2008; Romanazzi et al., 2012). In a previous study, several salts included in the Generally Regarded as Safe (GRAS) category were tested *in vitro* and *in vivo* against Penicillium rots of citrus fruit (Youssef et al., 2012b). Among them, sodium carbonate (SC) and sodium bicarbonate (SB) at 3% (w/v) proved to effectively reduce up to 100% disease incidence on clementines and oranges. However, little is known about their mode of action.

Much of the previous research indicated that one of the main elements underlying the efficacy of SB is the direct activity against the fungus due to its high pH (Smilanick et al., 2005; Venditti et al., 2005; Nigro et al., 2006). However, great differences in control efficacy of *P. digitatum* infections on lemons and oranges were observed among salt solutions with the same pH (Smilanick et al., 1999; Palou et al., 2001). Therefore, other possible mechanisms, such as the induction of host defence responses, might be involved.

There is a considerable interest in exploring the activation of plant defence mechanisms as alternative to traditional control methods (Sharma et al., 2002). In citrus, it has been reported that

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increased resistance against *P. digitatum* infections can be achieved by application of physical means (Kim et al., 1991; Rodov et al., 1992; Droby et al., 1993), safe chemical compounds (Venditti et al., 2005; Ippolito and Sanzani, 2011; Fallanaj et al., 2013), or antagonistic microorganisms (Fajardo et al., 1998; Droby et al., 2002). Resistance inducers are compounds that have a composition based on pathogen or plant constituents, or their analogs, so that they can react with plant receptors and activate plant defences, thus preventing pathogen infections (Terry and Joyce, 2004).

In several plant–pathogen interactions, resistance to infection is correlated with *de novo* synthesis of phytoalexins and pathogenesis-related (PR) proteins (Van Loon et al., 2006). Non-specific stress conditions, both biotic and abiotic, might activate the synthesis of these compounds, normally absent in healthy or unstressed tissues, acting on pathogens mainly by disruption of cell membranes (Smith, 1996).

Some PR proteins, such as β-1,3-glucanases (E.C. 3.2.1.39) belonging to the PR-2 family, and chitinases (E.C. 3.2.1.14) belonging to PR-3, -4, -8 and -11 families (Van Loon et al., 2006), are able to degrade fungal cell wall constituents, namely β-1,3-glucan and chitin, and thus exhibit antifungal properties. Their biosynthesis and accumulation is considered a major defence mechanism (Odjakova and Hadjiivanova, 2001). Within the plant defence strategy, also peroxidases (E.C. 1.11.1.7) play a key role, during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against multiple pathogens, and altering the ability of citrus fruit to cope with *Penicillium* infection through antioxidant activity (Ballester et al., 2006). Finally, phenylalanine ammonia lyase (E.C. 4.3.1.5; PAL) activity, which is the first enzyme in the phenylpropanoid pathway, increases in response to several kinds of stress including wounding (Ke and Saltveit, 1989). In the phenylpropanoid pathway, important compounds such as carbohydrates, either in a free state or as derivatives, are also involved. Apart from playing an important role in fruit quality properties (colour, texture, and flavour), carbohydrates are involved in citrus fruit physiology, for instance providing the substrate for the synthesis of various secondary metabolites including phenylpropanoids (Jackson, 2008).

In the present study, chemical, biochemical, and molecular analyses were carried out in order to evaluate changes in enzyme activity, gene expression levels and phytoalexin and carbohydrate contents in citrus fruit following SC and SB treatments. These results would support their putative role as resistance inducers against *Penicillium* decay of citrus fruit.

2. Materials and methods

2.1. Plant material

Oranges [*Citrus sinensis* (L.) Osbeck] cv. Valencia late were harvested at veraison from a local orchard in Castellaneta (Italy), selected for uniformity of size and absence of symptoms of any disorders, and immediately processed. Fruit were surface-sterilized with a 2% commercial bleach solution for 2 min, washed with tap water and air-dried at room temperature.

2.2. Testing of salts as resistance inducers

Oranges were wounded once (5 mm depth × 3 mm wide) with a sterile nail-head along the equatorial axis. For each treatment, 30 μL of 3% (w/v) SB or SC solutions were introduced into each wound. Fruit treated with sterile distilled water were used as a control. Treated fruit were placed in a tray, which was then wrapped into a plastic bag. After 48 h of incubation at 20 °C and high relative humidity (RH, 90–95%), another wound was made approximately 5 mm away from the previous one. This wound was air-dried and inoculated with 10 μL of a 10^4 conidia mL^{-1} suspension of *P. digitatum*. Each treatment had 3 replicates made up of 4 oranges each. Replicates were again wrapped in a plastic bag (90–95% RH) and maintained at 20 °C for two weeks. The incidence of decay (percentage of infected wounds, %) and disease severity (lesion diameter, mm) were recorded. The whole experiment was performed twice.

2.3. Tissue sampling for extractions

Fruit were individually wounded with a sterile nail (3 mm wide × 5 mm deep) at eight points on the equatorial surface. Samples were designated as follows: (i) unwounded fruit; (ii) fruit wounded and treated with water; (iii) fruit wounded and treated with SB; (iv) fruit wounded and treated with SC. In each wound, 30 μL of 3% salt solutions or sterile distilled water were applied. For each treatment fruit were randomized and arranged into 5 lots for tissue excision at different time intervals (0, 12, 24, 48, 72 h). Each lot was made up of 3 replicates and each replicate consisted of 4 fruit. The whole experiment was repeated twice. Fruit were arranged in plastic boxes, which were then individually wrapped into plastic bags (90–95% RH) and 20 °C for 72 h. At the established time intervals, tissue cylinders (5 mm) from each lot were excised from the inoculation site. The excised tissues were rapidly frozen in liquid nitrogen, mixed and ground to a fine powder using a commercial blender, lyophilized using a freeze-dryer and pump (VaCo 10-D-N2, Zirbus Dry Technology GmbH, Germany) and stored at −80 °C until use for enzyme, gene expression and metabolic assays.

2.4. Enzyme assays

From each sample and sampling time, 10 g of fine tissue powder were homogenized with 50 mmol sodium acetate buffer pH 5.6 (1:1, w/v), centrifuged (15 min at 10,000 × *g* and 4 °C) and the supernatant filtered through filter paper by a Buchner funnel. Proteins were precipitated in 60% acetone ($v\,v^{-1}$) at −20 °C and the resulting pellet, following centrifugation (30 min at 10,000 × *g* and 4 °C), was washed 3 times with 60% cold acetone. The pellets were dried, resuspended in 2 mL of 50 mmol sodium acetate buffer (pH 5.6) and kept at −20 °C until use. The protein concentration was determined according to Bradford (1976) with the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., USA).

Chitinase activity was assayed using dye-labeled carboxymethylchitin-RBV (Loewe Biochemica GmbH, Germany) following the method of Wirth and Wolf (1990). The assay was carried out by mixing 100 μL of protein extracts, 100 μL of CM-chitin-RBV and 200 μL of 50 mmol phosphate buffer (pH 6.4). After 2 h of incubation at 37 °C, the reaction was stopped with 100 μL of 2 N HCl, cooled, centrifuged (10,000 × *g*) and the absorbance of the supernatant measured at 550 nm (Multiskan EX, Labsystem). Chitinase activity was calculated according to Wirth and Wolf (1990) and expressed in international units (U) μg^{-1} of total protein. One U is defined as the amount of enzyme required to catalyze the formation of 1 nmol min^{-1} of product.

β-1,3-Glucanase activity was determined following the method of Abeles and Forrence (1979), by incubating 62.5 μL of protein extracts for 2 h at 37 °C in 62.5 μL of 4% (w/v) laminarin. The reaction was stopped by adding of 375 μL of 3,5-dinitrosalicylic acid (DNS) and by heating the sample in boiling water for 10 min and then rapidly cooling it in ice. The absorbance of each sample was measured at 492 nm (Multiskan EX, Labsystem) and activity values reported as μmol glucose equivalents μg^{-1} of total protein min^{-1}.

Peroxidase activity was determined using guaiacol as substrate (Hammerschmidt et al., 1982). The reaction mixture, consisting of 100 μL of crude extract and 100 μL of 50 mmol sodium acetate