Tropical Plant Pathology, vol. 35, 5, 271-276 (2010)

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RESEARCH ARTICLE / *ARTIGO*

Control of grey rot of apple fruits by biologically active natural products

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ABSTRACT

Biorend SC (chitosan), BC-1000 EC (grapefruit extract plus bioflavonoids) and ECO-100 SC (bioflavonoids plus organic acids, citric phytoalexins, fatty acids, glycerides and sugars), respectively, suppressed grey rot of apple caused by *B. cinerea* by 80.1%, 79.0% and 76.5% when used as post-harvest treatments under controlled conditions. When applied as combined pre- and post-harvest treatments Biorend SC inhibited fruit rot by 49.9 %, while BC-1000 EC and ECO-100 SC were ineffective. None of the products inhibited fruit rot when applied as pre-harvest treatments under controlled conditions or as post-harvest treatments under commercial conditions. The algal polysaccharide ulvan used in post-harvest treatments suppressed grey rot by 56.0% under controlled conditions, but had no inhibitory effect on combined pre- and post-harvest treatments. The inability of products to activate defense mechanisms (chitinase and peroxidase) of fruits was consistent with the unsuccessful control of rot by pre-harvest treatment. The results suggest that the natural products used have potential for use in integrated management of *Botrytis* rot when applied after harvest.
Key words: *Malus* sp., *Botrytis cinerea*, alternative control, post-harvest disease.

RESUMO

Controle da podridão cinzenta da maçã por produtos naturais biologicamente ativos

Biorend SC (quitosana), BC-1000 EC (extrato de toranja mais flavonóides), e ECO-100 SC (bioflavonóides mais ácidos orgânicos, fitoalexinas cítricas, ácidos graxos glicerídeos e açúcares) inibiram em 80,1%, 79,0% e 76,5%, respectivamente, a podridão causada por *Botrytis cinerea* quando utilizados no tratamento pós-colheita de frutos de maçã sob condições controladas.Tratamento combinado de Biorend SC, com aplicação tanto em pré como no pós-colheita, proporcionou 49,9% de inibição da podridão, enquanto BC-1000 e ECO-100 EC não foram efetivos. Nenhum desses produtos inibiu a podridão cinzenta, quando utilizados em tratamento de pré-colheita em condições controladas ou em tratamento de pós-colheita em condições comerciais. O polissacarídeo algal ulvana, utilizado nos tratamentos de pós-colheita, reduziu em 56% a podridão cinzenta das maçãs em condições controladas, mas não teve efeito inibitório nos tratamentos combinados de pré e pós-colheita. A incapacidade dos produtos em ativar mecanismos de defesa (quitinases e peroxidases) nos frutos, após o tratamento em pré-colheita, foi consistente com a falta de controle da podridão nesse tipo de ensaio. Pelos resultados, sugere-se que os produtos naturais utilizados apresentam potencial para a utilização no manejo integrado da podridão de Botrytis quando aplicados em pós-colheita.
Palavras-chave: *Malus* sp. *Botrytis cinerea*, controle alternativo, doença pos-colheita.

INTRODUCTION

Post-harvest losses of apple fruits in Chile are mainly caused by fungal pathogens, and typically reach 5% (Alvarez et al., 2004). Grey rot of apple, caused by *Botrytis cinerea* Pers., is a severe disease worldwide on pome fruits where advanced storage technologies are available. Synthetic fungicides continue to be widely used against grey rot. However, alternative control methods are needed because of growing concerns regarding risks of fungicides to human health and the environment, progressive reduction in registered fungicides available for apple protection, development of fungicide-resistant strains of *B. cinerea,* and increased consumer demand for organic foods (Spadaro et al., 2004).

Biological control and induced resistance, employed alone or as part of an integrated pest management approach, have emerged as promising tools for fruit rot control while exerting low environmental impact and reducing the need for synthetic fungicides. Particularly promising are biologically active natural products that function by means of endogenous elicitors (arising from the plant itself) and exogenous elicitors (coming from the pathogen or other natural sources) which induce biochemical defense responses in plant tissues against pathogens (Ebel & Mithöfer, 1997; Tripathi & Dubey, 2004). Elicitation may occur through

increased activity of peroxidase and/or lipoxigenase, two important enzymes involved in the plant defense system that respectively disrupt cell membrane permeability and oxidize phenols into compounds that are toxic to pathogens. Plant pathogens elicit the activity of chitinases that degrade chitin components of the pathogen's cell wall (Ebel & Mithöfer, 1997).

Products that elicit plant defense responses are available for post-harvest fruit protection in some countries and are under development in others (Spadaro et al., 2004). Ulvan, a water-soluble polysaccharide derived from the green alga *Ulva* spp., is the principal biopolymeric fraction of the algal cell wall (Paulert et al., 2009). Foliar applications of ulvans were reported to induce resistance in apple against *Colletotrichum gloeosporioides* Penz, and to reduce severity of local and systemic disease by 65% (Araújo et al., 2008). Biorend SC, a commercial product based on poly-D-glucosamine and chitin acetate with fungistatic activity against pathogens, elicits plant defenses because of chitosan effects (Hadwiger, 1999). Also, and based on results from the company, the manufacturer recommends it as appropriate for organic production of grapevine, citrus, berries, avocado, stone fruits, etc (http://www.biorend.cl/).

Because of their potential to elicit plant defense responses against pathogens, several natural products with known biological activity were evaluated for the control of grey rot of apple fruits caused by *B. cinerea*, with the objective of testing whether the products Biorend SC, ulvan, BC-1000 EC and ECO-100 SC used in the field and under commercial conditions were able to protect apple fruits against grey rot.

MATERIALS AND METHODS

Fruits and pathogens

Fruits of apple cv. Fuji for use in experiments were selected for uniform size, color, and maturity, and for freedom from visible wounds, defects and decay. An isolate of *Botrytis cinerea* (strain 256) from a fruit of cv. Fuji grown in San Fernando (6th Region of Chile) that causes grey rot in apple fruits and is sensitive to thiabendazole fungicide was used for inoculations. The pathogen was grown on potato dextrose agar (PDA) at 22^{o}C for 15 days after which spores (conidia) were recovered in sterile distilled water. For use in inoculations the spore density was adjusted to 10^5 spores/mL water by means of a Neubauer chamber.

Reagents and agro-products

All reagents for preparation of crude extracts and for enzymatic analysis were of analytical grade (Sigma Chemical Co., St. Louis, Mo, USA and Merck, Darmstadt, Germany). The following natural products were evaluated: Biorend SC, which contains 2.5% chitosan derived from crab chitin, was provided by BIOAGRO S.A., Chile; BC-1000 EC and ECO-100 SC, products containing seed and pulp extracts of grapefruit, were provided by Chemie Chile S.A.; and ulvan, which was prepared according to Paulert et al. (2009). Briefly, dried tissues of *Ulva* spp. were autoclaved in distilled water and residues of the alga were removed by repeated filtration through filter paper (80 g.m^{-2}). Water-soluble polysaccharide in the filtrate was precipitated with 2.5 volumes of absolute ethanol for 48 h at -20ºC. The precipitate (ulvan, minimum 80%) was dried in a forced-air oven at 40-45ºC for 48 h. The fungicide Mertec® 40 SC (active ingredient thiabendazole; 2-thiazol-4-yl-1H-benzimidazol), was provided by Syngenta S.A.

Identification of grey rot in apple fruits

Grey rot was identified in apple fruits based on the presence of rot symptoms and mycelium and sporulation of *B. cinerea* as signs of the disease. Rot severity was estimated by measuring the diameter of rotted areas of tissues.

Effectiveness of pre-harvest treatments

Apple trees cv. Fuji in a 15-year-old organic orchard located in San Fernando, Chile, were sprayed with Biorend SC (5.6 mL/L), ECO-100 SC (2 mL/L) or ulvan (1 g/L) at 50, 30 and 15 days before harvest. The treatments were applied in 2,000 L water/ha by means of a gun sprayer operated at 2.07 x 10^6 Pa (Table 1). Control trees were sprayed with water only. Harvested fruits were immersed in a 5% sodium hypochlorite solution for 3 minutes, rinsed with sterile distilled water, and air dried. Two wounds, each 2 mm deep and 2 mm in diameter, were made at the equator of each fruit by means of a nail which was sterilized after each wounding by immersion in ethanol and flaming. Each wound was inoculated with 20 µL of a conidial suspension of *B. cinerea* or with water only for controls, by means of a micropipette with sterile tips. The inoculated apples of each replicate were placed on a cardboard tray, which was enclosed in a perforated polyethylene bag, packed in a separate apple box and kept in commercial cold storage for 33 days at 0±1ºC. There were four replicates each with 10 fruits per treatment.

Effectiveness of postharvest treatments

Post-harvest fruit treatments were evaluated under controlled conditions and in commercial storage. In a controlled storage assay, fruits of cv. Fuji from the organic orchard were surface-disinfested, air dried, and wounded as described for the pre-harvest assay. For treatments the wounded fruits were dipped in solutions of Biorend SC (250 mL/L), BC-1000 EC (2.5 mL/L), ECO-100 SC (1.5 mL/L), ulvan (0.1 g/L), or Mertec 40 SC (2.5 mL/L) in distilled water, or in distilled water only for controls (Table 1). After treatment, the fruits were air dried at room temperature (15-18°C) for 48 h. The wounds were then inoculated with *B. cinerea* and stored as described for the pre-harvest

TABLE 1 - Commercial products with the corresponding active ingredients and doses used in pre- and post-harvest treatments

Commercial name	Active ingredient	Dose	
		Pre-harvest	Post-harvest
Biorend SC	Chitosan [2.5 % of acetate of poly-D-glucosamine plus acetate of chitosan]	5.6 mL/L	250 mL/L
ECO-100 SC	Bioflavonoids, ascorbic acid, citric fitoalexins, citric and lactic acids, sugars, fatty acids and glycerids, and organic acids	2.0 mL/L	1.5 mL/L
BC-1000 EC	Grapefruit extract plus bioflavonoids	-	2.5 mL/L
Ulvan (crystals)	Algal polysacharide	1.0 g/L	0.1 g/L
Mertec 40 SC	Thiabendazole	-	2.5 mL/L

experiment. There were four replicates each with 20 fruits per treatment. In an assay conducted in commercial storage, fruits of cv. Fuji from the organic orchard were placed in bins in a hydro-cooling system, sprayed with aqueous solutions of Biorend SC, BC-1000 EC or ECO-100 SC at the doses indicated in Table 1, and kept in a commercial cold storage at 0±1ºC. A total of four bins, each containing 550 kg fruit, were used per treatment. After four months of storage, fruits with rot symptoms and signs of *B. cinerea* were weighed and percentage of grey rot inhibition was calculated.

Effectiveness of combined pre- and post-harvest treatments

The pre-harvest treatments of combined assays were run in parallel with pre-harvest treatments already described. Trees in the old organic orchard were sprayed once with water solutions of Biorend SC, ECO-100 EC or ulvan at 30 days before harvest. Control trees were sprayed with tap water. Immediately before treatments were applied, half of the fruits on the tree were tagged in each treatment and covered with polyethylene bags. The treatments were applied in 2,000 L water / ha by means of a gun sprayer operated at 2.07 x 10^6 Pa (Table 1). The plastic bags were removed after fruits that were not covered with the bags had dried. After harvest, the fruits were surface disinfested, rinsed and air dried as before. Half of the fruits of each treatment were immediately stored at -80°C for use in testing chitinase and peroxidase activities. The remaining fruits were wounded and post-harvest treatments were applied by dipping as described above. Treated fruits were air dried at room temperature (15-18ºC) for 48 h, inoculated with *B. cinerea,* packed in plastic bags and stored for 33 days at 0±1ºC as before. There were four replicates each with 20 fruits per treatment.

Statistical analysis

All assays were performed according to a completely randomized design and rot inhibition in percentage was calculated. The data were subjected to ANOVA and Tukey`s test.

Determination of chitinase- and peroxidase activity

Apple fruits stored for 2 months at -80°C after pre-harvest treatments were defrosted and peeled. To prepare crude extracts for testing enzyme activities, 1 g of peel of each fruit was macerated in 1 mL of 0.1 M Tris-HCl pH 7.5 (containing 10 mM β-mercaptoethanol, 10% glycerol and 10 mM PMSF (p-methyl sulfonyl fluoride), filtered through cheesecloth, stored at -28°C. The crude extracts were thawed, centrifuged at 5,000 x *g* and assayed for chitinase and peroxidase activities. Quantification of proteins was done as described in Bradford (1976) using Bovine Serum Albumin (BSA) as a standard. Chitinase activity was visualized in agarose gels according to Ríos (2007), using 1% agarose gel containing 0.04% glycolchitin (Sigma) as substrate, prepared in 0.1 M sodium acetate pH 5.0. β-1,4-chitinase activity was visualized as dark spots on a blue fluorescent background (Figure 1). Images were recorded with a digital camera, processed using the Adobe Photoshop 6.0 image program and analyzed using the Gel-Proanalyzer 3.1. Program (Media Cybernetics). Dark spot diameters produced by *Serratia marcescens* chitinase were used as standards to calculate enzyme activity. Chitinase activity was expressed as Units/mg protein. Controls were run with untreated fruits. Peroxidase activity was determined spectrophotometrically following changes in absorbance at 470 nm, after mixing 100 uL of crude extract with 2.9 mL substrate (25% v/v guaiacol, 100 mM H_2O_2, 50 mM phosphate pH 6.0). Peroxidase activity was expressed as the increase in absorbance at 470 nm/min/g fresh weight (Stadnik & Buchenauer, 2000).

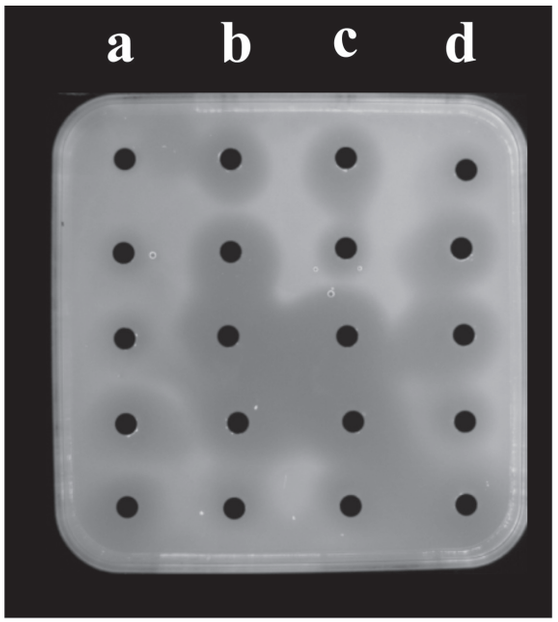

FIGURE 1 - A plate used for estimation of chitinase activity. The plate contained gelled 1% agarose containing 0.04% glycolchitin (chitinase substrate) prepared in 0.1 M sodium acetate pH 5.0. The holes in columns were loaded with 40 mL of crude extracts of apple peelings containing the same amount of proteins from: a, the control; b, pre-harvest; c, post-harvest and d, combined pre- and post-harvest treatments. Diameters of the dark halos around the holes indicate chitinase activity and should be compared within each column. Mean chitinase activity in columns b, c and d was 84.6 $\pm$ 8.7 U/mg of protein and showed no significant differences from the control (71.9 U/mg protein).

RESULTS AND DISCUSSION

Pre-harvest treatments

Pre-harvest treatments with Biorend SC, ECO-100 EC or ulvan did not significantly reduce severity of apple grey rot during post-harvest storage (data not shown). Any product remaining on the apple surface following the final treatment was probably removed during surface disinfection with sodium hypochlorite.

Post-harvest treatments

In the controlled storage tests, Biorend SC, BC 1000 EC, ECO-100 SC and ulvan applied after harvest each significantly inhibited grey rot by about 80%, but were less effective than Mertec 40 SC (Figure 2). Biorend SC contains chitosan [(β-1,4-linked glucosamine) which has antifungal activity (Hadwiger, 1999) and strongly inhibits mycelial growth and spore germination of *Botrytis cinerea* and other post-harvest fungal pathogens in culture, including *Alternaria alternata*, *Rhizopus stolonifer*, and *Colletotrichum gloeosporioides* (Bautista-Baños et al., 2003; Liu et al., 2007; Amborabé et al., 2008). Our findings in apple are consistent with reports of *in situ* inhibition by chitosan of post-harvest decay caused by *B. cinerea* on strawberries (El Ghaouth et al., 1992), grapes (Romanazzi et al., 2002) and tomatoes (Liu et al., 2007).

BC-1000 EC and ECO-100 SC, although containing active ingredients different from chitosan (Table 1), showed inhibitory effects similar to Biorend SC in commercial conditions (data not shown). The former contains grapefruit extracts (GSE) which have fungicidal effects (Xu et al., 2007). These authors showed antifungal activity of GSE on *B. cinerea* and the control of rot in table grapes stored at 0–1 °C in both *in vitro* and *in vivo* experiments. The 79% and 77% inhibition of post-harvest grey rot by BC-1000 and ECO-100 SC, respectively (Figure 2), agrees with effects reported by Xu et al. (2007) for grapes and by Bensch & Guerrero (2001) for blueberries.

Grey rot inhibition by ulvan (56%) was weaker than that reached by the other treatments. Ulvan is able to reduce infection by apple pathogens (Araújo et al., 2008) but may require longer than the 48 h between treatment and plant inoculation used in the present research to adequately elicit plant responses (Araújo et al., 2008; Cluzet et al., 2004). Ulvan is a complex branched polysaccharide mainly composed of disaccharide (i.e. ulvanobiuronic acid 3-sulfate) repeating units which induce resistance in several plant species (Araújo et al., 2008; Cluzet et al., 2004; Paulert et al., 2009). Its structure differs from that of chitosan, a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). These differences may be related to the 80% and 56% rot inhibition produced by Biorend SC and ulvan, respectively. It is known that the elicitation of plant defense responses requires

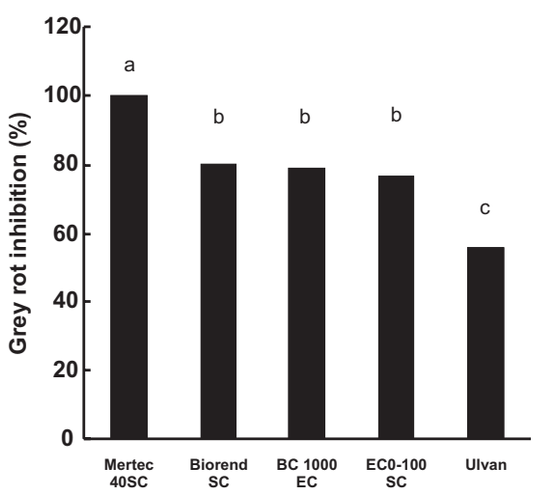

FIGURE 2 - Effect of post-harvest treatments on the percentage of inhibition of the development of grey rot in apple fruits cv. Fuji under controlled conditions. Different letters indicate significant differences by Tukey's test ($P < 0.05$). All treatments were significantly different from the control (water).

polysaccharides with specific chain length and structure, such as pectic fragments (Roco et al., 1993), linear β-1,3-glucans (Klarzynski et al., 2000), chitin fragments (Kaku et al., 2006) or chitosan (Hadwiger, 1999).

A lack of effectiveness of post-harvest treatments of apple fruits with Biorend SC, BC 1000 EC and ECO-100 SC when applied under commercial conditions after harvest (data not shown) agreed with results reported for chitosan by Hernández-Muñoz et al. (2008). The treatments may also have been ineffective against any latent or unnoticed fruit infections caused by *B. cinerea* prior to harvest. Because the fruit in the commercial test were not air dried before cold storage it was possible that surface moisture on the fruit following treatment may have persisted sufficiently to favor infection by *B. cinerea,* such as through tiny wounds in the fruits.

Biorend SC applied before and after harvest suppressed grey rot by about 50% but ECO-100 SC and ulvan were ineffective (Table 2). The finding that Biorend SC applied as both pre- and post-harvest treatments was less effective than when applied only after harvest (Figure 2) suggests that the latter treatment should be preferred.

In the study of combined pre-and post harvest treatments, chitinase activity was similar in peelings of treated and untreated fruits that were covered or not covered during treatment, which suggested that pre-harvest applications of Biorend SC, ECO-100 SC or ulvan did not elicit the defense of the apple fruit cv Fuji. Results obtained for chitinase activity in fruits treated with Biorend SC differ from those of chitinase elicitation in melon plants in response to chitin oligosaccharides (Roby et al., 1987). In the latter system, chitinase induction occurred six hours after treatment and was maximal at 12-24 hours. Therefore, the similar chitinase activities observed between control and treatment with Biorend SC after 33 days storage at 0±1°C cannot be considered as a lack of elicitation of chitinases, but due to the basal chitinase activity of apple peel (Figure 1).

Mean peroxidase activities in peel of treated apple fruits were numerically several times higher than the mean activity of the controls (data not shown) but the standard deviations were high (in some instances as high as the peroxidase activity) and the differences were not significant. Whether elicitation occurred remains unclear and the data may represent basal levels of the enzyme. Results obtained with ECO-100 SC and with BC-1000, for which peroxidase elicitation was not observed after 33 days of cold storage, appear to differ from those reported for table grapes (*Vitis vinifera* cv. Red Globe) treated with grapefruit seed extract (GSE) and stored at 2±1°C (Xu et al., 2009). Peroxidase activity in the grapes increased to a maximum of 1.6 times the basal activity after 21 days of storage and subsequently decreased below the basal level (Xu et al., 2009).

In summary, under controlled conditions, Biorend SC reduced the incidence and severity of grey rot of apple caused by *B. cinerea* both in combined and single treatments (post-harvest). Post-harvest treatments with BC-1000, ECO-100 or ulvan inhibited the progress of rot but their effectiveness was weaker than for Biorend SC. Given that post-harvest treatments did not inhibit fruit rot under commercial conditions, but substantially suppressed the disease under controlled conditions, it is considered important that variables that differed in the two groups of tests be investigated in future tests. These variables should include the timing, volume and method of treatment application, water quality or any other factor that could influence results, and the use of single compared to combined treatments using different products. In addition, pre-harvest treatments applied at earlier growth stages than used in the present work should be tested for control of grey rot of apple fruits, considering that infection of calyces could occur during blooming.

TABLE 2 - Effect of combined pre- and post-harvest treatments on the inhibition of development of apple grey rot under controlled conditions

Treatments	Rot inhibition (%)
Biorend SC	49.9 a
ECO-100 EC	0.9 b
Ulvan	6.4 b
Control (Water)	0.0 b

ACKNOWLEDGEMENTS

This research was supported by a grant from the Cyted Project "Desarrollo de tecnologías para manejo integrado de enfermedades del manzano" (Grant number: 106PI0302). M.J.S is a research fellow of CNPq.

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TPP 47 - Received 16 November 2009 - Accepted 19 October 2010
Section Editor: John C. Sutton