



Postharvest control of brown rot and Rhizopus rot in plums and nectarines using carnauba wax

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ABSTRACT

The effects of *Copernicia cerifera* wax (carnauba wax) on the development of *Monilinia fructicola* and *Rhizopus stolonifer* *in vitro* and on infection in nectarines and plums were investigated. Inhibition of mycelial growth and spore germination were assessed on potato dextrose agar amended with carnauba wax at concentrations of 1%, 2%, 3% and 4.5%. The spore germination of both fungi on nectarines covered with carnauba wax was evaluated by scanning electron microscopy. The post-infection and protective effects of carnauba wax (4.5% and 9%) toward brown rot and *Rhizopus* rot in plums and nectarines were investigated. In the protective tests, fruit were wounded, covered with carnauba wax and then inoculated. For the post-infection tests, fruit were wounded, inoculated and then covered with carnauba wax. There was no mycelial growth of *M. fructicola* at any of the wax concentrations. *R. stolonifer* was completely inhibited by carnauba wax at all concentrations except at 1%. There was no germination of spores *in vitro* for both *M. fructicola* and *R. stolonifer* at any concentrations of carnauba wax. There was 50% inhibition of spore germination for *M. fructicola* and 90% for *R. stolonifer* on the surface of nectarines covered with 9% carnauba wax. Protective application of 4.5% and 9% carnauba wax significantly reduced incidences of both diseases in nectarines and plums. Post-infection control of both diseases by the application of wax was inefficient.

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1. Introduction

The main postharvest diseases of plums and nectarines in Brazil are brown rot, caused by *Monilinia fructicola* (G. Wint.) Honey, and *Rhizopus* rot, caused by *Rhizopus stolonifer* (Erhenb.:Fr.) Vuill. (Martins et al., 2005). The fungus *M. fructicola* can infect immature fruit, remaining quiescent until maturation, and mature fruit (Wade and Cruickshank, 1992). However, *R. stolonifer* only infects mature fruit via wounds (Ogawa, 1995). Both diseases are very aggressive, with fast colonization and dissemination in mature fruit. Until recently, control of postharvest diseases in plum and nectarine from Brazil was performed using fungicides (Chitarra, 1997). Currently, the demands of consumers and environmental organizations for residue-free products, together with the rules of the Integrated Fruit Production (IFP) certification program for fruit in Brazil, have initiated a more extensive search for products that can substitute for fungicides. With this need in mind, evaluations of the efficiency of alternative control methods and of methods that reduce the use of fungicides are becoming more numerous (Mari et al., 2003; Tripathi and Dubey, 2004).

Several products and techniques for postharvest control have been tested for use as fungicide alternatives, including controlled and/or modified atmosphere (Smith et al., 1987; Nava and Brackmann, 2002), treatment with UV-C irradiation (González-Aguilar et al., 2004; Shama, 2007), use of ozone gas (Palou et al., 2002) and coating the fruit with commercial waxes (Waks et al., 1985; Jeong et al., 2003). These methods have shown some promising results as postharvest treatments.

For several fruits, the purpose of waxing is to impart a shiny appearance to the fruit and to reduce weight loss by slowing down senescence during storage. Waxes act as protective films for fruits, maintaining the pulp's firmness for a longer period of time (Banks, 1985). They typically modify the atmosphere around the fruit, raising carbon dioxide levels and reducing oxygen levels (Petracek et al., 1998). In this environment, fruits possess lower metabolic activities, especially respiration (Oliveira et al., 2000) and transpiration (Chitarra and Chitarra, 2005). These attributes can induce greater fruit resistance to pathogens, increasing their shelf life.

Carnauba wax is extracted from leaves and leaf buds of the “Carnauba” tree (*Copernicia cerifera* Mart.), which belongs to the Palmae family. This species is native to Brazil and is abundant in the North and Northeast regions of the country (Lorenzi, 2002). Carnauba wax is commercially used as a glazing agent in cosmetics, pharmaceuticals, candy coatings, dental wax, etc.

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According to Jacomino et al. (2003), in commercial concentrations, carnauba wax prevents rot in guavas, besides improving the physico-chemical properties of fruit. Joyce et al. (1995) reported that waxing extended the storage lifetime of avocado both through reduction in water loss and modification of the internal atmosphere. Besides these attributes, carnauba wax is a potential antifungal. Evidence that this wax acts as a potential antifungal agent was reported by Bompeix and Morgat (1977) on apple, where the use of carnauba wax reduced postharvest decay by 70%. Additionally, Cruz et al. (2002) reported that *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Colletotrichum lindemuthianum* and *Colletotrichum musae* were inhibited *in vitro* by different wax fractions and purified enzymes (like chitinase and glucanases) from carnauba wax.

The objective of the present work was to evaluate the protective and post-infection effects of carnauba wax on brown rot and Rhizopus rot in plums and nectarines.

2. Materials and methods

2.1. *In vitro* experiments

2.1.1. Inoculum origin

The fungi *M. fructicola* and *R. stolonifer* were isolated from infected plum and nectarine fruit from a wholesale market in São Paulo, Brazil. With the assistance of a histological needle, *M. fructicola* and *R. stolonifer* spores were collected aseptically and transferred to Petri dishes containing potato dextrose agar (PDA) culture medium. The growth period for pathogens at room temperature was three days for *R. stolonifer* and seven days for *M. fructicola*.

2.1.2. Effect of carnauba wax in the mycelial growth of *R. stolonifer* and *M. fructicola*

Mycelial plugs from the edges of *M. fructicola* and *R. stolonifer* colonies were transferred to the centre of Petri dishes containing PDA and carnauba wax at concentrations of 1%, 2%, 3% and 4.5%; PDA without carnauba served as a control. Carnauba wax (Megh-wax ECF 124, produced by MEGH Indústria e Comércio Ltda) was added to the PDA after media autoclaving. Megh-wax ECF 124 has pH of 8.5–10.5 with a translucent liquid aspect, brown colour, anionic charge and active ingredient content of 17–19%. Plates were incubated at 25 °C with a 12 h photoperiod. The colony diameters of *M. fructicola* were measured daily using the average of two perpendicular measurements for six days. *R. stolonifer* colony diameters were assessed only on the third day of incubation. The evaluations were performed until the fungal colonies in the control treatments reached 90 mm in diameter. The experimental design was completely randomised with seven replications and five treatments, and the experiment was performed twice.

2.1.3. Effect of carnauba wax on the germination of *R. stolonifer* and *M. fructicola*

Pure colonies of *M. fructicola* and *R. stolonifer* grown on PDA were used to prepare suspensions of 10^5 spores mL^{-1} with 20 mL sterile distilled water per Petri dish. One millilitre of each suspension was transferred to a test tube containing 9 mL of carnauba wax at concentrations of 1%, 2%, 3% and 4.5%. Tubes containing 9 mL of distilled water served as the controls. Three 30 μL aliquots from each tube were placed in plastic Petri dishes, which were put inside a plastic box containing filter paper and 30 mL of distilled water. The boxes were covered and incubated in growth chambers at 25 °C under light for 12 h. After this period, the germination process was stopped by addition of one drop of lactoglycerol to every drop of each fungal suspension (Silva et al., 2008). Germination percentage was calculated based on observation of 100 conidia per aliquot,

which were visualised in an optic microscope at 400 $\times$ magnification. Conidia were considered to have germinated when the germ tube length was equal to or greater than the conidial length. The experimental design was completely randomised with three replicates. The experiment was performed twice.

2.2. *In vivo* experiments

2.2.1. Scanning electron microscopy analysis

R. stolonifer and *M. fructicola* cultures were grown on PDA for three and seven days, respectively, at 25 °C prior to spore collection. Spore suspensions were prepared with sterile distilled water and adjusted to 10^5 spores mL^{-1} . Eight mature nectarines were wounded (0.5 mm in diameter by 5.0 mm in depth) with a sterile needle at two points in the fruit equatorial region. Four nectarines were immersed in 9% carnauba wax for 3 min and four fruit served as control treatments. Suspensions (30 μL) of *M. fructicola* or *R. stolonifer* spores were placed over each wound. The fruit remained in a moist chamber at 25 °C for 12 h. After this period, fragments of 1 cm $\times$ 1 cm from the inoculated regions were excised for scanning electron microscopy analysis. The fragments were placed on glass slides inside Petri dishes with moistened filter paper and exposed to 2% (w/v) osmium tetroxide (OsO_4) vapour for 12 h in the dark and inside a well-ventilated fume hood (Kim, 2008). The samples were then transferred to plastic boxes with silica gel and kept at 25 °C for 72 h. The fragments were sputter coated with gold (approximately 30 nm thick) and examined with scanning electron microscopes (Carl Zeiss DSM 940-A and LEO 435 VP operating at 5 kV and 20 kV). The experiment was performed twice.

2.2.2. Effects of carnauba wax on brown rot and Rhizopus rot

The post-infection and protective effects of commercial carnauba wax were evaluated. The plum variety used in the experiments was Reubennel, and the nectarines were of the Sun-ripe, Sungold and Caldesi varieties, depending on the availability of fruit in the market. Mature fruit were superficially disinfected through immersion in a sodium hypochlorite solution (0.2% of active chlorine) for 3 min. The fruit were then placed on paper towels to dry for 20 h and were later inoculated.

The spore suspension was prepared by addition of 20 mL of distilled water to the fungal culture. The inoculum concentration in the suspension was calibrated at 10^5 spores mL^{-1} for *M. fructicola* and *R. stolonifer*. The fruit were wounded (0.5 mm in diameter by 5 mm in depth) with a hypodermic needle in the equatorial region, and 30 μL of the *M. fructicola* or *R. stolonifer* spore suspension was placed over the wound. After inoculation, fruit were maintained in a humidified chamber for 4 h on plastic trays. After the treatments, each fruit was individually kept in a 500 mL plastic pot.

The wax was applied preventively before inoculation or post-infection after inoculation of the wounded fruit. The fruit remained immersed in 0%, 4.5% and 9% wax concentrations for 3 min using a 4 L glass beaker. The assay was comprised of 24 treatments of 20 fruit each, including two wax concentrations and the control treatment, two pathogens, two hosts and two application strategies, for a total 240 plums and 240 nectarines (480 fruit in total). This experiment was repeated using 30 additional fruit per treatment. After the wax treatment, each fruit was kept in an individual plastic pot and stored at room temperature (25 ± 2 °C).

2.2.3. Experimental evaluation and statistical analyses

Brown rot incidence (percentage of infected fruit) and severity (lesion diameter on infected wounds) were assessed five days after inoculation. Rhizopus rot incidence was assessed three days after inoculation. The assays were based on a randomised experimental design.

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