International Journal of Food Microbiology 126 (2008) 24–29

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Influence of additives on adhesion of *Penicillium frequentans* conidia to peach fruit surfaces and relationship to the biocontrol of brown rot caused by *Monilinia laxa*

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ARTICLE INFO

Article history:
Received 16 October 2007
Received in revised form 3 April 2008
Accepted 24 April 2008

Keywords:
Biofungicide
Biological agent
Biological control
Formulation
Stone fruit

ABSTRACT

Additives, such as sucrose, D-sorbitol, glycerol, sodium alginate, carboxymethyl cellulose, silica gel, gelatine, non-fat skimmed milk and a commercial adhesive were added to conidia of *Penicillium frequentans* at two different points in the production process of the formulation of this fungus to improve conidial adhesion. Conidial adhesion was estimated as the number of *P. frequentans* conidia (no. conidia cm^{-2}) and colony-forming units of *P. frequentans* per unit area (cfu cm^{-2}) that adhered to glass slides or to peach surfaces. The *P. frequentans* conidial concentration had a significant effect on conidial adhesion, while the shelf life of conidia did not have any effect. The highest adhesion of *P. frequentans* conidia to glass slides was observed when conidial concentrations were greater than 10^6 conidia ml^{-1}. *P. frequentans* conidial adhesion was improved when 1.5% sodium alginate or 1.5% carboxymethyl cellulose were added to the conidial mass obtained after production and before drying by the fluid bed drying process. Conidial adhesion was also enhanced when 1.5% sodium alginate, 1.5% carboxymethyl, or 1.5% gelatine were added to conidia after fluid bed drying. *P. frequentans* formulations with 1.5% sodium alginate or 1.5% carboxymethyl cellulose were more effective in reducing brown rot caused by *Monilinia laxa* than dried *P. frequentans* conidia alone. Our results show that additives can improve adhesion of *P. frequentans* conidia to fruit surfaces, resulting in more effective control of brown rot in peaches.

1. Introduction

Monilinia laxa (Aderh. and Ruhl.) Honey is an important pathogen that causes considerable pre- and post-harvest losses in stone fruit (Pascal et al., 1994). Fruit become increasingly susceptible to *M. laxa* as they ripen (Pascal et al., 1994). The use of chemicals and cold storage are considered as the primary methods to the control of post-harvest diseases (Eckert and Sommer, 1967). The development of resistance in *M. laxa* to certain fungicides (Canez and Ogawa, 1985; Gilpatrick, 1981; Katan and Shabi, 1981), new restrictions on the application of these fungicides, and environmental considerations have led to an increased interest in the use of biocontrol agents to control brown rot (Ooijkaas et al., 1998, Wilson and Pusey, 1985).

Penicillium frequentans Westling, a component of the resident mycoflora of peach twigs and flowers (Melgarejo et al., 1985), reduces peach twig blight caused by *M. laxa* (De Cal et al., 1990; Melgarejo et al., 1986). Recently, De Cal et al. (2002) developed a method for mass producing *P. frequentans* conidia, which involves a solid fermentation process. These conidia reduced brown rot fruit in *in vitro* assays (De Cal et al., 2002). *P. frequentans* produced 10^8 to 10^9 conidia g^{-1} of dry weight substrate with viability higher than 80%. However, viability was significantly reduced after 30 days at room temperature (20 to 25 °C). However, when *P. frequentans* conidia are dried by the fluid bed drying process, they maintain 100% viability after drying and 28% are still viable after 180 days at room temperature (18 to 30 °C) (Guijarro et al., 2006). Conidia of *P. frequentans* dried by the fluid bed drying process were as effective in reducing the incidence of brown rot on peach as the non-dried conidia (Guijarro et al., 2006).

Despite taking precautions to enhance the survival of a biocontrol strain, severe environmental conditions may drastically limit the establishment of a biocontrol agent on a host target site (Burges, 1998). Therefore, a formulation of biocontrol agents could be used to protect them when applied on aerial plant parts. Sticker additives improve spore adhesion to the target leaf and prevent wash-off by rain (Schisler et al., 2004). Many of them also have humectant properties (Burges, 1998). Degrees of acquisition and retention of microorganisms are likely to be related to differences of the biological agents themselves (species, formulation), the plant to be treated (plant species, type of leaf surface), and environmental conditions (position of leaves, precipitation, wind, etc.) (Burges, 1998).

Adhesion of microorganisms on plant surfaces is usually a two-step process. Initial adhesion results from a pre-existing glycoprotein layer or from a component of the conidial cell wall. Subsequently, stronger adhesion results from metabolic activation and protein synthesis (Osherov and May, 2000, 2001). The first stage, which occurs as soon as conidia are hydrated, is characterized by relatively weak adhesive forces and appears to involve hydrophobic interactions (Doss et al.,

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doi:10.1016/j.ijfoodmicro.2008.04.018

1993). The second stage involves a step associated with respiration and growth and conducts to stronger binding; it occurs when viable conidia have been incubated for several hours under conditions that promote germination (Filonow, 2001a; Robert et al., 1994).

The objectives of our study were to find additives that improve the adhesion of *P. frequentans* conidia to fruit surfaces maintaining a high biomass, and that increased its biocontrol against brown rot. Additives were chosen by their sticker described properties (Burges, 1998).

2. Materials and methods

2.1. Cultures

The isolate of *P. frequentans* (ATCC number 66108) used in this study was part of the mycoflora of twigs and flowers of peach grown in Spain (Melgarejo et al., 1985). A monosporic isolate of *M. laxa* (ATCC number 66106) was obtained from an apricot (Zaragoza, Spain). Both fungi were stored on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) slants at 4 °C and slants were used to start cultures on PDA that were grown in darkness at 25 °C for 7 days to produce conidia. Each isolate was grown in 90-mm diameter plastic Petri dishes containing 20 ml of PDA. Conidia of *M. laxa* were produced in PDA that had been amended with 1% acetone v/v, as described previously (Pascual et al., 1990).

Conidia of *P. frequentans* were produced in a solid fermentation system (De Cal et al., 2002). The fungus was grown on a mixture of peat (Gebr. BRILL substrate GmbH&Co. KG, Germany); vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain); and lentil meal (1:1:0.5; w: w: w). Five hundred grams of the above substrate (40% moisture content) were placed in a plastic 600 cm^3 volume bag that is designed specifically for solid fermentation (Valmic^R). The bag was then sealed and sterilized by autoclaving at 1.2 kg cm^{-2} and 120 °C for 1 h, on 3 consecutive days. Bags were then inoculated with a conidial suspension of *P. frequentans* in sterile distilled water, to provide 10^5 conidia g^{-1} dry substrate. This was again sealed and incubated in darkness at 20 to 25 °C for 5 days. Conidia were harvested from solid fermentation bags by suspending the substrate in sterile water. Suspensions of conidia from solid fermentation were then shaken in a rotary shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 160 rpm for 30 min, filtered through several layers of glass wool, washed with sterile distilled water, and concentrated by centrifugation at 10,000 rpm (Sorvall RC5C Plus, GMI, Minnesota, USA) for 10 min. Pellets of fresh conidia (F) were obtained. F conidia were dried in a fluid bed dryer to reduce moisture content below 15%. F conidia were resuspended in sterile distilled water and filtered through 1 μm filter paper in a Büchner funnel (Guijarro et al., 2006). This conidial paste was introduced in a fluid bed dryer (FBD model 350s, Burkard Manufacturing Co Ltd., Hertfordshire, UK) at the highest air flow rate at 40 °C (Guijarro et al., 2006). The moisture content of each final product of conidia was measured using a humidity analyzer (BOECKEL, GmbH +Co, Hamburg, Germany). Germination of dried conidia was tested by the bioassay described in Guijarro et al. (2006), always resulting above 80%. These F conidia resuspended in water and further dried were named "dried" conidia (D).

Two kinds of conidial formulations were produced: i) formulations in which additives ($_x$) were added to conidia Before the Drying process (BD_x conidia), and ii) After the Drying process (AD_x). To obtain BD_x conidia pellets, F conidia were resuspended in 5 ml of the different additive solutions ($_x$) in a 250 ml centrifuge tube, shaken on a vortex mixer (Reax Top, Heildoph, Rose Scientific Ltd. Alberta, Canada) for 10 s and then kept for 10 min at room temperature (20–25 °C). Conidial suspensions were then filtered through 1 μm filter paper in a Büchner funnel and dried as previously described (Guijarro et al., 2006). To obtain AD_x conidia pellets, D conidia were resuspended in 5 ml of the different additive solutions ($_x$) in a 250 ml centrifuge tube, shaken on a vortex mixer (Reax Top, Heildoph, Rose Scientific Ltd. Alberta, Canada) for 10 s and then kept for 10 min at room temperature (20–25 °C) before use.

2.2. Additives

Sticker compounds, such as sugars (sucrose and D-sorbitol), polyalcohols (glycerol), hydrophobic compounds (sodium alginate and carboxymethyl cellulose), desiccant compounds (silica gel), a commercial adhesive (96% di-menteno, Nu-film-17 (Miller Chemical and Fertilizer Co., Hanover, Pennsylvania, USA) (as a positive control), gelatine, and non-fat skimmed milk were used as additives at doses shown in Table 1. Additives and concentrations were selected based on previous research (Burges, 1998; Guijarro et al., 2007; Sabuquillo et al., 2005). Additives were dissolved in distilled water w:v (solid compounds) or v:v (liquid compounds) and then autoclaved at 1.0 kg cm^{-2}, 120 °C for 20 min.

2.3. The effect of additives on germination of conidia P. frequentans and M. laxa

The effect of additives at different concentrations (0.375, 0.75, 1.3, 1.5, 1.875, 3.75, 7.5%) on the germination of fresh conidia of *P. frequentans* in sterile Czapek broth (Difco, Detroit, MI) was evaluated using a bioassay described in De Cal et al. (1988). Suspensions of 1×10^6 conidia ml^{-1} of *P. frequentans* F conidia, *P. frequentans* D conidia, or *M. laxa* conidia were prepared in sterile Czapek broth. Additive solutions were prepared as described above at 1.3× for each concentration and additive. Thirty μl droplets of conidial suspensions were mixed on a slide with 15 μl of each additive solution to give a concentration of 10^6 conidia ml^{-1}. Aliquots of 45 μl of each mixture were placed on sterile glass slides. All slides were placed in 15-cm-diameter glass Petri dishes lined with moist paper and incubated for 16 h at 20 to 25 °C in darkness. Germination of 50 conidia was recorded per each drop and three drops were made for each sample. A spore was considered germinated when the germ tube was longer than the length of the spore (usually more than 7 μm). The complete experiment was repeated twice.

2.4. Effect of conidial concentration on adhesion

The effect of conidial concentration on adhesion was determined for *P. frequentans* D conidia on glass slide surfaces. Glass slides were previously cleaned with 95% ethanol and sterilized at 80 °C for 24 h. Then they were placed in polycarbonate vessels each with 100 ml of five different concentrations of *P. frequentans* D conidia suspensions

Table 1
Effect of additives ($_x$) on the germination of *Penicillium frequentans* (F and D conidia) and *Monilinia laxa* conidia[z]

Additives ($_x$)	Rate (%)	Germination of conidia (%)		
		Penicillium frequentans		*Monilina laxa*
		F conidia	D conidia	
Sodium alginate	1.5	91 a	83 ab	64 b
Carboxymethyl cellulose	1.5	90 a	78 a	27 a
Gelatine	1.5	94 a	83 ab	66 bc
Glycerol	7.5	93 a	94 c	33 a
Silica gel	7.5	92 a	87 ab	28 a
Non-fat skimmed milk	7.5	89 a	91 bc	73 c
D-sorbitol	1.875	87 a	NT	82 d
Sucrose	1.875	92 a	75 a	59 b
Nu-film	1.3	87 a	83 ab	60 b
Without additives		90 a	80 a	66 bc
[y]MSE_{within}		26	50	19

[z]Data are the mean of three replications. Fifty conidia were counted per replication. Means for each additive followed by the same letter in each column are not significantly different (P=0.005) by Student Newman multiple range test. NT: not tested. F (Fresh) conidia and D (Dried) conidia of *P. frequentans* and *M. laxa* conidia were suspended in the additive ($_x$) solutions for germination assays.

[y]MSE_{within}=error mean square of analysis of variance. Data were subjected to arcsin transformation to improve the homogeneity of variances before analysis, and, in this case, MSE_{within}. comes from transformed data.