Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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The papers contained in this book report some of the peer reviewed Proceedings of the International Conference "Environmentally friendly and safe technologies for quality of fruit and vegetables", but also other papers related with the subject were included. The manuscripts were reviewed by the Editor and Editorial Board, and only those papers judged suitable for publication were accepted. The Editor wish to thank to all the reviewers and authors for their contribution.

Authors are responsible for content and accuracy of their papers.

Proceedings of the International Conference “Environmentally friendly and safe technologies for quality of fruit and vegetables”, held in Universidade do Algarve, Faro, Portugal, on January 14-16, 2009. This Conference was a join activity with COST Action 924.

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SECTION 4. ENVIRONMENTALLY FRIENDLY AND SAFE METHODS TO CONTROL POSTHARVEST LOSSES

23. STUDY OF MODES OF ACTION OF THE BIOCONTROL AGENT *METSCHNIKOWIA ANDAUENSIS* PBC-2

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Abstract

Metschnikowia andauensis NCYC 3728 (PBC-2) is an effective antagonist against the postharvest pathogens *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolofiner* on pome fruits, however its mode of action is unknown. The ability of this strain to produce inhibitory compounds in 4 distinct media (PDA, NYDA, YPDA, CJA) at 3 temperatures (1, 25, 30 ºC), was investigated. It was also assayed the competition for iron in media with different iron concentrations and characterized the capability of PBC-2 produce and secrete fungal cell wall lytic enzymes, like chitinase, protease, and glucanase in a culture media with fungal pathogen cell wall as unique carbon source. *M. andauensis* PBC-2 did not show any inhibition zone to cope pathogens in any of the tested media. The results obtained in this study suggest that the production and secretion of lytic enzymes is not the main or more important mode of action of the new biocontrol agent PBC-2, since the production of chitinase was observed only past 5 and 7 d of incubation, and the production of β-1.3-glucanases and proteases was not observed, which mean that the biocontrol agent PBC-2 have more than one mechanism of action.

Keywords: biocontrol agent, inhibitory compounds, iron competition, lytic enzymes, mode of action

Introduction

Fruits and vegetables are highly perishable products, especially during the postharvest phase, when considerable losses, due to microbiological diseases, disorders, transpiration and senescence, can occur. Traditionally, postharvest diseases are often controlled by the application of synthetic fungicides, however and during the last decade the application of microorganisms for the biocontrol of postharvest diseases has received increasing attention (Droby 2006; Nunes *et al.* 2009). Several yeasts and bacteria have been shown to protect against a number of postharvest pathogens on a variety of harvested commodities (Janisiewicz *et al.* 1994; Chand-Goyal & Spots 1996; Ippolito *et al.* 2000; Nunes *et al.* 2001; Kurtzman & Droby 2002; Vero *et al.* 2002). Antagonistic yeasts have received particular attention, as their activity usually does not depend on the production of antibiotics or other toxic secondary metabolites, which could have a negative environmental or toxicological impact (Sipiczki 2006). Different species of genus *Metschnikowia* have been described as an effective biocontrol agents (Kurtzmann & Droby 2002; Spadaro *et al*. 2002; Karabulut *et al* 2004; Kinay & Yildz 2008). The yeast *Metschnikowia andauensis* NCYC 3728 (PBC-2) (Nunes & Manso 2010), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer* in pome and *Penicillium digitatum* and *Penicillium italicum* in citrus fruits.

The knowledge of the mechanisms involved in biological control play an essential role in the development and registration of a biocontrol agent and in maximizing the efficacy of this control system (Janisiewicz *et al.* 2001). Attempts to characterize those mechanisms has resulted in a variety of studies like antibiosis with volatile and non-volatile compounds, competition for nutrients and space, induction of resistance in fruits (Calvente *et al.* 1999; Janisiewicz *et al.* 2000; Poppe *et al.* 2003; Santos & Marquina, 2004; Saravanakumar *et al.* 2008). The direct interaction of the biocontrol agent with the pathogen, for example, by the involvement of fungal cell wall degrading enzymes, like chitinase, protease and glucanase, is also suggested to play a role in the mechanisms of action (Berto *et al.* 2001; Castoria *et al*. 1997; Mahadevan *et al*. 1997; Saravanakumar *et al.* 2009).

The aim of this study was to determine the mechanisms of biocontrol activity by *M. andauensis* PBC-2. The ability of this strain to produce *in vitro* antagonism, siderophes and produce and secrete fungal cell wall lytic enzymes was investigated.

Material & Methods

Biocontrol Agent

Metschnikowia andauensis NCYC 3728 (PBC-2) previously isolated from the carposphere of 'Bravo de Esmolfe' apples from Portugal and characterized as a biocontrol agent for postharvest diseases of apples and citrus was used in this work. It was stored as a cell suspension in 20% (v/v) glycerol at -80 °C. When required, *M. andauensis* PBC-2 was streaked on NYDA medium (8 g L^{-1} nutrient broth; 6 g L^{-1} yeast extract; 10 g L^{-1} glucose; 15 g L^{-1} agar) and incubated at 25 °C.

Pathogens

P. expansum, *R. stolonifer* and *B. cinerea* strains used were isolated from decayed pome fruits, and selected for their high level of aggressiveness on 'Red Delicious' apples. All the strains were maintained on PDA medium (Potato Dextrose Agar) at 4 °C.

In Vitro Antagonism

The ability of the yeast to inhibit growth of *P. expansum*, *B. cinerea*, *R. stolonifer* was tested on dual cultures on different culture media, at different temperatures. A mycelial disk (diameter 5 mm) from 10 days (d) old cultures of each pathogen, was placed at the center of Petri dishes containing the following media, PDA, NYDA, YPDA (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} glucose; 15 g L^{-1} agar), and CJA (20 g L^{-1} citrus juice by-products with 15 g L^{-1} agar). For each medium tested, the yeast, produced in the respective liquid media, was streaked at two sides of the pathogen. Plates were incubated in different temperatures, 1, 25 and 30 °C. Control plates without antagonist were performed for each pathogen, in each media at all temperatures. After 15 d of incubation diameters of pathogens colonies towards the antagonist were measured in each case and compared to colony diameters in control plates. Three plates constituted a single replicate and the experiment was replicated twice.

Competition for Iron

Petri dishes containing a medium for siderophore production (sucrose 25 g L^{-1}; ammonium sulphate 4 g L^{-1}; potassium dibasic phosphate 3 g L^{-1}; citric acid 1 g L^{-1}; magnesium sulphate 0.08 g L^{-1}; zinc sulphate 0.002 g L^{-1}; agar 20 g L^{-1}) were amended with different concentration of iron (0, 10, 100 and 500 μM of ferric chloride). An agar disk (diameter 5 mm) from 10-d old cultures of *P. expansum* or *B. cinerea* was placed at 30 mm from the edge plate. A single streak of *M. andauensis* PBC-2 was inoculated 40 mm from the pathogen. Plates were incubated at 25 °C and the development of both organisms was observed.

Fungal Cell Wall Production and Induction of Lytic Enzymes

P. expansum strain was grown on YES broth (sucrose 150 g L^{-1}; yeast extract 20 g L^{-1}) at 25 °C, for 7 d. Obtained mycelium was dried with sterile filter paper and ground in a sterile mortar in the presence of liquid nitrogen to get a fine powder. The mycelial powder was suspended in 5 M NaCl, sonicated for 5 min and centrifuged at 5724×*g* for 10 min. The supernatant was discarded and the pellet was washed 3 times with distilled water. Fungal cell wall were dried in Petri dishes at 60 °C for 3 h.

Flasks containing yeast nitrogen base medium (YNB) supplemented with previously prepared fungal cell wall at 1 g L^{-1}, were inoculated with PBC-2 fresh cells at an initial concentration 1×10^5 cfu mL^{-1} and incubated at 25 °C, 150 rpm. Samples were withdrawn aseptically at different times and filtrated by 0.45 μm (Millipore). Filtrates were stored at -20 °C, until enzymes determination. Experiment was conducted in three replicates.

Enzymes Assays

β-1,3 glucanase activity was assayed in 62.5 μL of culture filtrate and 62.5 μL of 0.05 M acetate buffer, pH 5, containing 1% laminarin. The reaction was stopped by adding dinitrosalicylic reagent (DNS) and heating the tubes for 5 min at 100 °C. The net increase of reducing sugar in the reaction mixture was determined by comparing the measured optical densities (540 nm) with those on a standard curve prepared with glucose. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 μmol reduction group min^{-1}.

Chitinase activity was assayed in 90 μL of culture filtrate and 10 μL potassium phosphate buffer, pH 6.1, containing 0.18 mM nitrophenyl N-acetyl β-D glucosamide. The reaction was stopped by adding 10 μL NaOH 1 M. The formation of *p*-nitrophenol was followed by absorbance at 405 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protease activity was measured in a reaction mixture contained 100 μL of culture filtrate, 100 μL 0.05 M acetate buffer, pH 5 and 100 μL azocasein 1%. The reaction was stopped adding 400 μL TCA 10% and after centrifugation 500 μL of NaOH 525 mM was added to the supernatant and the optical density was measured at 450 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protein quantification was determinate using the kit BCA from Pierce, following the fabricant instructions.

Results

In Vitro Antagonism

M. andauensis NCYC 3728 (PBC-2) did not show any inhibition zone to cope pathogens. In YPDA and CJA media, pathogens developed without sporulation. Optimal growth of pathogens, in the absence or in the presence of the biocontrol agent, was observed in PDA medium (Fig 1). Growth rate of all pathogens was higher at 25 °C, when compared to 1 and 30 °C. Although with some differences in growth between the media, the pathogens in the presence of the biocontrol agent, developed without inhibition, when compared with the pathogen inoculated separately.

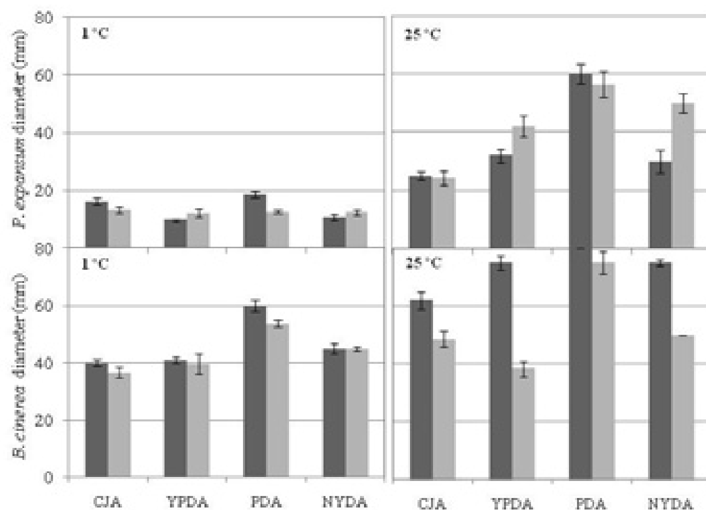

Fig 1. Diameter development of *P. expansum* and *B. cinerea* grown in 4 different culture media, *in vitro* assays, incubated at 1 and 25 °C. ■ Control treatment, inoculation only the pathogen. ■ Simultaneous inoculation of the pathogen and *M. andauensis* PBC-2. Bars represent standard deviation.

The high diffusion of pulcherrim produced by the biocontrol agent was observed in PDA and NYDA (data not shown).

Competition for Iron

At the tested conditions no inhibition zones were observed in the presence or in the absence of iron. *M. andauensis* PBC-2 produced pale pink to dark red colonies under different iron conditions. The intensity of the colour increased with increasing iron concentrations. The antagonistic yeast produced wider pigmented halos in the medium without iron and an increase in ferric chloride concentration decreased the pigmented halo (Fig 2).

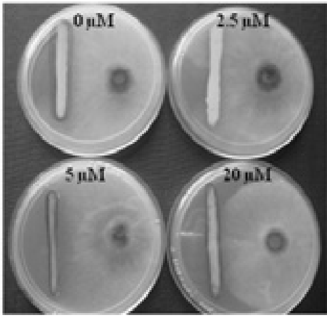

Fig 2. Dual cultures of *M. andauensis* PBC-2 and *B. cinerea* in media with different concentrations of ferric chloride (0, 2.5, 5, 20 μM).

Fungal Cell Wall Production and Induction of Lytic Enzymes

The biocontrol agent PBC-2 showed a slight growth in the YNB media supplemented with *P. expansum* cell wall. Growth was more evident after 5 d of incubation even so no more than 10 fold of the initial population was achieved.

Enzymes Assays

The production of glucanase and protease was not observed at the tested conditions. The maximal level of protein was reached after 5 d of incubation (Fig 3), reaching 113 μg mL^{-1}, after which the production of chitinase increased until the 7th day (Fig 4).

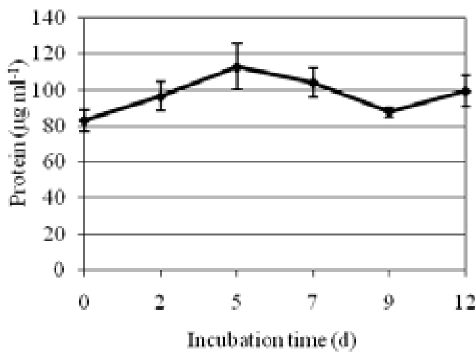

Fig 3. Time course of changes in concentration of protein during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

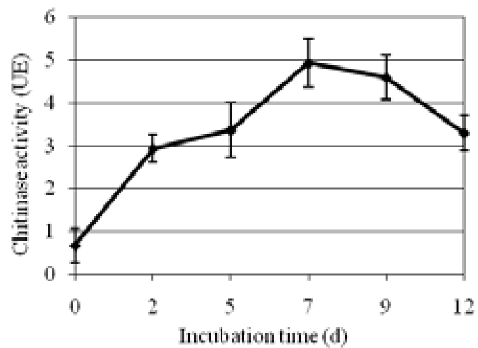

Fig 4. Time course of changes in chitinase activity during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

Discussion

Metschnikowia andauensis NCYC 3728 (PBC-2), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer*, in pome and *P. digitatum* and *P. italicum* in citrus fruit. However the mechanism by which the yeast reduces decay is not known. In the present work, *in vitro* antagonism, competition for iron and production of extracellular lytic enzymes, was investigated as possible modes of action. No inhibition of any of the tested pathogens was observed in the 4 different media, at the 3 temperatures studied. Results obtained suggest that in our experimental conditions, the production of inhibitory compounds is not the mode of action of this biocontrol agent. Numerous reports in the literature describe the inhibition of postharvest diseases by antibiotics-producing microorganisms, especially in the case of bacteria. *Bacillus subtilis* and *Pseudomonas cepacia* are known to kill pathogens by producing the antibiotic iturin (Gueldner *et al.* 1988). *Pseudomonas cepacia* inhibited the growth of postharvest pathogens like *B. cinerea* and *P. expansum* in apple by producing an antibiotic, pyrrolnitrin (Janisiewicz *et al.* 1991). *Aureobasidium pullulans* produces aureobasidin A, an antifungal cyclic depsipeptide antibiotic that inhibit the development of *P. digitatum*, *P. italicum*, *P. expansum*, *B. cinerea* and *Monilinia fructicola* (Liu *et al.* 2007). However, at present, antibiotic-producing antagonists are not likely to be registered for postharvest use on food products (Nunes *et al.* 2009; Sharma *et al.* 2009). Since the antagonistic activity of *M. andauensis* PBC-2 did not seem to be related to inhibitory substances, competition for iron was tested as a possible mechanism. Iron is essential for the fungal growth and pathogenesis and iron sequestration by non-pathogenic microbes, could be exploited in novel systems for biological control of postharvest pathogens (Calvente *et al.* 1999; Zhang *et al.* 2007). The yeast *M. andauensis* PBC-2 produced a pigmented zone around them, in the media without iron or with low concentrations of this micronutrient. Increasing the ferric chloride concentration the pigmented halo diminished, but the pale pink colour of the colonies became red. Contrasting with the observed by Sipiczki (2006), Saravanakumar *et al.* (2008) and Vero *et al.* (2009), in the present work, no inhibition in the pathogens development was observed, in the presence or in the absence of iron in the media (Fig 2). In the mentioned studies, higher inhibition halos by the antagonist strains in front of the pathogens were observed in lower iron amendments, suggesting the depletion of the micronutrient by the biocontrol agent under low iron conditions. Nevertheless, the colour change of the *M. andauensis* PBC-2 colonies observed with the increase of iron concentration is in agreement with previous findings of Sipiczki (2006), who demonstrated the iron competence between strains of *M. pulcherrima* and pathogenic fungi, suggesting the iron immobilization from the medium and the formation of a red, insoluble pigment called pulcherrimin. By the formation of this insoluble complex, it was suggested that iron remained in the medium but was inaccessible.

Microbial antagonists produce lytic enzymes such as glucanases, chitinases, and proteases that help in the cell wall degradation of the pathogenic fungi (Castoria *et al.* 2001). In the present study, the production of proteases and β-1,3-glucanase was not observed at the tested conditions. The highest level of protein was reached after 5 d of incubation and 2 d later the chitinase activity achieved its maximum level. Chitin, the unbranched homopolymer of N-acetyl glucosamine in a β-1,4 linkage, is a structural component of cell walls in most of the fungi, chitinases hydrolyze this polymer and have been implicated in biocontrol processes (Castoria *et al.* 2001). Although reported in many works, as an important role in the biocontrol activity, the chitinase productivity differs greatly between biocontrol agents. Saravanakumar *et al.* (2009) found a higher production of chitinases by yeast *M. pulcherrima* strain MACH1 in PDB and YPD in the presence of pathogen cell wall indicated the induction of chitinases by biocontrol yeast. Vero *et al.* (2009) demonstrated that *A. pullulans* strain ApB produced both chitinase and β-1,3-glucanase at 5 ºC, in the presence of *P. expansum* cell walls in minimal medium and in apple juice. The production of these enzymes at 25 ºC by an *A. pullulans* isolate has previously been reported by Castoria *et al.* (2001).

The current study demonstrated that *M. andauensis* PBC-2 did not inhibit pathogens development, through the production of inhibitory substances or by iron competition. The determination of lytic enzymes

revealed that this strain, at the tested conditions, did not produced and secrete β-1,3-glucanase and proteases, nevertheless it showed a modest chitinase activity, which can be consider an advantage, once the biocontrol agent PBC-2 have more than one mechanism of action.

Further studies should be performed in order to clarify the modes of action involved in the antagonistic activity of the biocontrol agent *M. andauensis* PBC-2. Competition for nutrients, other than iron, as well as, the ability of the biocontrol agent, to induce host plants to produce antioxidants and synthesis of pathogenesis-related proteins, should be examined.

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