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Control of citrus blue mold by the antagonist yeast *Pichia guilliermondii* Z1: Compatibility with commercial fruit waxes and putative mechanisms of action

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ABSTRACT

Pichia guilliermondii strain Z1, which was previously proven to be effective against blue mold of citrus fruit, has been further tested in controlled conditions to determine whether the yeast, as an alternative for synthetic fungicides, would be compatible with other postharvest practices used commercially. In particular, commercial fruit waxes can reduce their survival and effectiveness. The commercial fruit waxes tested, in combination at 20% with strain Z1, included microcrystalline, ester gum, candelilla, beeswax, montan, paraffin, rice bran, rosin maleic, carnauba, shellac, and one mixture shellac plus carnauba. Beeswax, paraffin, rosin maleic, carnauba, and shellac increased significantly strain Z1 survival in Petri dish assays. Candelilla, beeswax, rice bran, rosin maleic, carnauba, shellac, and shellac–carnauba mixture did not significantly reduce the strain Z1 yield on orange fruit surfaces compared to other waxes. With the exception of rosin maleic wax, none of the commercial fruit waxes or mixture increased significantly the ability of the formulated product of strain Z1 to control the postharvest pathogen *Penicillium italicum* on wounded orange fruit. When the formulated product of strain Z1 was used in combination with beeswax, strain Z1 retained the same efficacy. In contrast, microcrystalline, ester gum, candelilla, montan, paraffin, rice bran, carnauba, shellac, and shellac-carnauba mixture significantly reduced the effectiveness of the formulated product of strain Z1. No antibiosis was detected for strain Z1 against *P. italicum*. Strain Z1 inhibited the spore germination for the low juice concentration (up to 5%) when compared to the control. However, the addition of fresh juice after antagonist removal allowed the restoration of the germination of *P. italicum* spores; suggesting the possibility of competition for nutrients in the biocontrol activity of strain Z1. The study demonstrates the potential commercial application of strain Z1 with beeswax and rosin maleic wax for postharvest control of citrus blue mold. As biocontrol relies on competition for nutrients, an enriched formulation with nutrients is needed for reliable antifungal activity of this yeast strain.

1. Introduction

During the last decade, one of the most important problems facing the Moroccan citrus industry is postharvest diseases. Postharvest green and blue molds and sour rot, caused respectively by *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum candidum*, are responsible for important economic losses on the citrus industry worldwide (Askarne et al., 2012; Droby, Chalutz, Wislon, & Wisniewski, 1989). In Morocco, losses of citrus fruit due to postharvest diseases in packinghouse and storage conditions are estimated to be approximately 60% (Lahlali, Hamadi, El Guilli, & Jijakli, 2011; Talibi et al., 2012). Control of these pathogens relies mainly on the use of fungicide treatments such as thiabendazole (TBZ), imazalil (IMZ) or sodium ortho-phenylphenate (SOPP) sprayed on fruits during packinghouse operations and during storage (Eckert & Ogawa, 1985; Ismail & Zhang, 2004). However, the use of fungicides is being limited due to the appearance of pathogen resistant strains caused by the frequent overuse of these chemical substances (Eckert, Sievert, & Ratnayake, 1994; Vinas, Vallverdun,

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Monallao, Usall, & Sanchis, 1993), the increase of public concern for human health safety and environmental hazards associated with high levels of chemical pesticides used in fruit orchards (Smilanick, 1994; Wisniewski & Wilson, 1992), and the growing public demand of fruits free from residues impose a necessity for the development of new technologies for the control of postharvest diseases as alternative methods to synthetic fungicides (Jijakli & Lepoivre, 1998; Kinay & Yildiz, 2008).

Biological control using microbial antagonists has emerged as a desirable and rapidly developing alternative, either on its own or as part of an integrated control strategy to reduce fungicide input (Karabulut & Baykal, 2004; Lahlali, Serrhini, & Jijakli, 2004, 2005; Teixido, Vinas, Usall, & Magan, 1998). Many factors have contributed to the successful use of microbial antagonists as realistic alternatives to common synthetic substances. The important known factors are: the application sites which are limited to the fruits; the environmental factors are defined and stable during the storage rooms; and the harvested commodities are of high value; which supports the higher costs of such biological treatments (Fokkema, 1991; Jijakli, Lepoivre, & Grevesse, 1999; Wilson and Wisniewski, 1992). Effective antagonists have been screened to control postharvest diseases in many fruit commodities, such as citrus (Chalutz & Wilson, 1990; El-Ghaout, Smilanick, Wisniewski, & Wilson, 2000; Lahlali et al., 2011; Sharma, Singh, & Singh, 2009), apples and pears (Janisiewicz, 1987; Jijakli & Lepoivre, 1998) and other fruits (Lima, Ippolito, Nigro, & Salerno, 1997). The mechanism of this biological control is based on ecological interactions, such as competition for space and nutrients, mycoparasitism, antibiosis, and induction of host defenses (Janisiewicz & Korsten, 2002; Massart & Jijakli, 2007). Lahlali et al. (2011) have screened *Pichia guilliermondii* strain Z1 to protect harvested oranges from infection by *P. italicum*. Known to protect a wide range of hosts from major postharvest pathogens (Lahlali et al., 2011; Zhang, Spadaro, Garibaldi, & Gullino, 2011; Zhao, Tu, Shao, Jing, & Su, 2008), *P. guilliermondii* may be considered as a potential biocontrol agent (BCA) for plant diseases. However, a good understanding of the mechanisms underlying its biocontrol activity is a prerequisite to identify pivotal biocontrol features and to develop an adequate formulation allowing their expression (Yu, Wang, Yin, Wang, & Zheng, 2008). Moreover, knowledge of such mechanisms is very important to establish specific criteria for a better screening of new potential BCAs as well as to facilitate registration procedures. The modes of action of *P. guilliermondii* appear to rely essentially on rapid colonization of fruit injuries and competition for nutrients but also on induction of defense responses and on production of lytic enzymes (Arras, De Cicco, Arru, & Lima, 1998; Yu et al., 2008).

In a commercial citrus-packing operation, fruit are generally coated with waxes to improve fruit appearance, reduce moisture loss and serve as a barrier for fungicides. The fungicides usually used for postharvest green and blue molds control are commonly incorporated into wax. The packed Valencia fruit are transported quickly and are generally stored in cold storage for longer periods in order to satisfy consumer needs during the year. Few studies have been undertaken on the compatibility of postharvest BCA with commercial fruit waxes (Droby et al., 1993; Pusey, Wilson, Hotchkiss, & Fraklin, 1986). To our knowledge, there are no reports about the compatibility of this antagonistic yeast strain with commonly used fruit waxes. Therefore, the main objectives of this study were to determine whether the antagonist *P. guilliermondii* strain Z1, when applied to substitute the use of imazalil and thiabendazole or other synthetic substances employed in citrus industry, would be compatible with commercial fruits waxes under laboratory trials and on orange fruit surfaces, and whether it would retain its ability to control blue mold under controlled conditions. In order to maximize its potential use as BCAs, the mechanisms of action by which this new strain of *P. guilliermondii* controls postharvest citrus molds would be investigated.

2. Materials and methods

2.1. Microorganisms

P. guilliermondii strain (Z1), originally isolated from the healthy moroccan oranges "Citrus reticulate Blanco cv.Valencia-late", was selected for its strong inhibitory action against postharvest diseases of citrus fruits (Lahlali et al., 2011). The yeast strain was grown on Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) at 25 °C for three successive subcultures under the same conditions in 24-h intervals. Before use, yeast colonies were flooded with sterile distilled water (SDW) containing NaCl at 0.85% and scraped from Petri dishes. The yeast was washed from the agar medium in 10 ml of saline solution (0.85% NaCl) supplemented with 0.01% Tween 80%. The final concentration was determined according to optical density measurements with a photospectrometer at 595 nm (ONCIFU Version 9 PRIM Type No. 9434. SECOMAN) using the following equation (OD − 0.0958)/0.03 = CFU/mL × 10^6).

P. italicum strain PIRBM1 was isolated from decayed 'clementine' fruits harvested from Tadla plain in Morocco by the laboratory of Plant Pathology (ENA-Meknes) and stored onto PDA medium at 4 °C in a darkroom (Lahlali, Serrhini, Friel, & Jijakli, 2006). The conidial suspension was prepared from 7 ± 2 day-old cultures of pathogen grown on PDA medium at 25 ± 2 °C by scraping the surface of the colonies flooded with tween 20 (0.05%), then transferred to sterile distilled water (SDW) and finally filtered through nylon mesh. Spores were adjusted to desired concentration with a Burker's cell counter in SDW.

2.2. Fruit preparations

Non-treated oranges fruit 'cv. Valencia-late' used in the in vivo and biological control tests were bought from a commercial market and stored for no more than 15 days in a cold room at 4 °C before being used for the bioassays. The selected fruits were free of wounds and rots and were as homogeneous as possible in maturity and size. Healthy oranges were disinfected by soaking in a sodium hypochlorite bath (10%) for 2 min, rinsed twice in SDW and then dried under laminar flow for a maximum of 2 h (Lahlali et al., 2011).

2.3. Wax preparation procedure

Different commercial waxes were tested in this study. The most important types of wax used in the citrus industry are beeswax (Poth Hille, UK), montan wax (Poth Hille, UK), rice Bran wax (*Oryza sativa*, Poth Hille, UK), shellac wax (Sigma–Aldrich), carnauba wax No.1 (Sigma–Aldrich), rosin maleic wax (Strahl & Pitsch Inc., W. Babylon, NY), paraffin wax (Strahl & Pitsch Inc., W. Babylon, NY), ester gum wax (Strahl & Pitsch Inc., W. Babylon, NY), microcrystalline wax (Poth Hille, UK) and candelilla wax (Poth Hille, UK). The wax suspension was prepared at 20% in buffer containing [oleic acid 1% (v/v), ethanol 2% (v/v), carboxyl-methylcellulose (4 g/L), Tween 80% 5 mL/L]. Stock solutions of the oleic acid and ethanol were prepared individually and in combination at concentration of 1% and 2% in 15 mL Falcon tubes containing 10 ml of SDW and sterilized by autoclaving at 120 °C for 20 min. Different formulations of these waxes were placed in metal beakers (stainless steel), melted at 70 °C, and heated continuously to reach 80–90 °C of liquid molten wax. During wax emulsion, water was added continuously to reach a final volume of 100 mL.

2.4. In vitro effect of fruit waxes on antagonist survival

Thirteen 100 mL Erlenmeyer flasks, each containing 50 mL of prepared wax at 20% (v/v), were inoculated with 1×10^7 CFU/mL of strain Z1. The Erlenmeyers containing waxes were placed on a rotatory shaker for 20 min at 120 rpm and maintained in darkness at 25 °C until the time of the measurements. After incubation times of 0, 0.16, 0.5, 1, 4, 8, and 24 h, 1 mL of each strain Z1-wax combination was taken and serially 10-fold diluted until reaching a final concentration of 1×10^3 CFU/mL. An aliquot of 100 μL from each prepared strain Z1-wax mixture was spread over the PDA medium. The Petri dishes were sealed with the polyethylene bags and incubated for 72 h at 25 °C in a growth chamber before the colonies were counted. There were four replicate dishes for each strain Z1-wax combination, and the experiment was conducted twice over time.

2.5. In vivo effect of fruit waxes on antagonist survival

Disinfected oranges were treated by dipping into strain Z1 alone or strain Z1-wax combination suspensions (1×10^8 CFU/mL) for 2 min, and then kept at room temperature. After an incubation period of 7 days, four oranges per treatment were washed in 1 L KBP buffer [KH_2PO_4 (0.05 M), K_2HPO_4 (0.05 M) and 0.05% (wt/v) Tween 80, pH 6.5] (one plastic bag per treatment) on a rotary shaker for 20 min at 120 rpm. Four serial 10-fold dilutions were prepared from 1 mL washing buffer and were plated in triplicate on PDA medium. Petri dishes were kept at 25 °C for 2–3 days. This experiment was repeated twice. The surface area of the orange fruit was calculated as follows: [Area (cm^2) = 1.024 × Weight + 27.66 ($R^2 = 0.96$)] (Lahlali, Hamadi, El Guilli, & Jijakli, 2013). Population sizes were expressed as CFU/cm^2.

2.6. Effectiveness of antagonist yeast in combination with fruit waxes

This experiment trial was conducted by using the formulated product of strain Z1. The formulated product of strain Z1 was conducted at 28 °C in a 10 L Biostat® ED Bioreactor (B. Braun Biotech. Germany). The culture medium used contained 50% glucose (w/w) as a carbon source, amino acids (30 g yeast extract and 30 g soy peptone), and either 5 mL of mineral salts concentrated medium or 5 mL of sterilized concentrated vitamin solution for growth. To increase the biomass production in relation to the batch system, feed-batch technology was used (Biotechnology Unit, Laboratory of Microbiology, ULB, Belgium). The biomass produced in the feed-batch was dried in a fluid bed dryer. Maize starch was used as a loading agent (30%). Air temperature in the bed was maintained at 30 °C and air inflow at 150 m^3/h throughout the drying process. Orange fruit were prepared as described above. Disinfected oranges were wounded at two equidistant points of equatorial zone as previously described. Formulated product was adjusted to 1×10^8 CFU/mL (33 mg/mL) in a suspension of 100 mL of each prepared wax treatment at 20% (v/v) or in SDW. Each wound was treated with 50 μL of the formulated product-wax suspension preparation or the formulated product of strain Z1 alone (prepared only in SDW). The control treatment was only inoculated with the same suspension of SDW. For postharvest application purpose, inoculation with *P. italicum* was carried out 24 h after biological treatment. Each wound received an aliquot of 50 μL from a pathogen suspension of 1×10^5 spores/mL. Treated fruits were stored at a temperature of 24 °C for 7 days. There were ten oranges per treatment. Two trials were conducted over time with three replicates per treatment.

2.7. Evaluation of mode of action of P. guilliermondii: antibiois and competition

P. guilliermondii strain Z1 and citrus blue mold pathogen *P. italicum*, were cultured for 7 days at 28 °C on PDA medium. For each experimental unit (10-cm-diameter Petri dish), and prior to pathogen inoculation, an aliquot of 10 μL of the antagonist yeast *P. guilliermondii* concentrated at 1×10^7 spores/mL was placed top-down on fresh PDA. After 48 h, a 6 mm diameter mycelial disk from the growing edge of *P. italicum* was placed 5 cm away from the antagonist yeast strain Z1. PDA inoculated with a pathogen disk only served as controls. After 10 days of incubation at 28 °C, the inhibition zone around the antagonist colony was measured and the percentages of growth inhibition of the pathogen were calculated in relation to the control. The study was conducted in a completely randomized design with three replicates per combination and the experiment was conducted twice.

In vitro competition for nutrients between the antagonist yeast *P. guilliermondii* and *P. italicum* was studied following the non-destructive method described by Janisiewicz, Tworkoski, and Sharer (2000) and Krimi Bencheqroun et al. (2007). Briefly, tissue culture plates with 24 wells (TC-test plates) and culture plate inserts, equipped with a membrane filter of 0.4 μm pore size (Millicell-CM, Millipore, Belford, MA) attached to the bottom part of the cylinder were used. Such system allows physical separation of the antagonist and the pathogen with possible interchange of media nutrients and metabolites. Two different tests were undertaken. In the first one, the plate wells were filled with 600 μL of orange juice at various concentrations (0, 0.1, 0.5, 1, 5, 10 and 15%) with/out the antagonist (strain Z1 at 1×10^7 CFU/mL). Cylinder inserts were placed in the wells and filled with a conidial suspension of *P. italicum* (2×10^5 conidia/mL) inside the inserts at 400 μL per cylinder. The plates containing the inserts were incubated in the dark at 25 °C for 24 h. Cultures containing *P. italicum* alone at the same orange juice concentrations are controls. After incubation period, inserts were removed from the wells and blotted from the bottom with a tissue paper. For each insert, the membrane was cut with a sharp scalpel, transferred to a glass side, stained with lacto-phenol cotton blue and mounted for optical microscope observation. Germinated conidia on the membrane were counted within a sample of 100 conidia and using two membranes per treatment in each experiment. In the second test, inserts containing inhibited conidia of *P. italicum* in the antagonist suspension at 0.5% orange juice concentration were removed from culture plates and inserted into new ones containing orange juice at concentrations of 0, 0.1, 0.5, 5, and 15%. After an additional 24 h of incubation in the same conditions as above, germinated conidia were recorded for each treatment within a sample of 100 conidia using two membranes per treatment in each trial.

2.8. Statistical analysis

The strain Z1 survival (CFU/mL and CFU/cm^2) and efficacy (%) data were transformed to logarithms to normalize the distribution and processed for statistical analysis using the ANOVA procedure of the statistical analysis system (SAS Institute, version 9.1, Cary, NC, USA). Before the analysis, Bartlett's test was used to check the homogeneity between repeated trials. Means were compared using Fisher's LSD test at statistical significance $P = 0.05$.

3. Results

3.1. In vitro effect of fruit waxes on antagonist survival

Statistical analysis shows a significant effect ($P < 0.0001$) of main factors, which are commercial waxes, incubation time and

Table 1
Variance analysis of effect of commercial waxes (wax), and incubation time (time) on the survival of *P. guilliermondii* strain Z1 (Log (CFU/mL) in PDA medium.

Source	*df*	MS	*F*	Pr > *F*
Wax	11	0.522	141.76	0.0000*
Time	6	15.65	4250.17	0.0000*
Wax × time	66	0.423	115.01	0.0000*
Error	588	0.003		

MS, mean square.
*Significant ($P < 0.0001$).

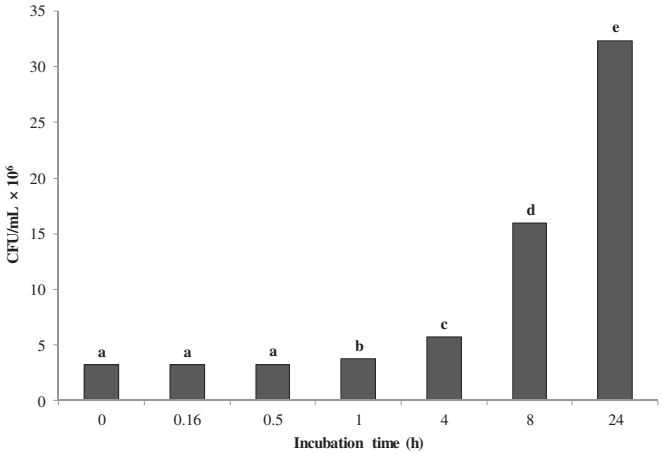

Fig. 1. Effect of incubation time on the *in vitro* growth of *P. guilliermondii* when used alone or in mixture with commercial fruit waxes (20%). Shown are the mean values of combined datasets of two independent experiments with four replicates. Data having the same letter are not significantly different ($P < 0.05$) according to LSD Fisher's test.

their interaction on strain Z1 survival (Table 1). Regardless of the treatment, the number of strain Z1 cells remained statistically similar at the first three times of incubation 0, 0.16, and 0.5 h, but they increased significantly with increasing incubation periods (Fig. 1). The maximum growth (3.25×10^7 CFU/mL) was observed at 24 h of incubation, independent of biological treatment. Variance analysis showed that only beeswax, rosin maleic, paraffin wax, carnauba wax, and shellac wax significantly enhanced the number of strain Z1 cells (Fig. 2). The highest yield was obtained with beeswax and rosin maleic. Microcrystalline and ester gum waxes did not improve the strain Z1 yield, whereas candelilla, montan, rice bran, and formula shellac + carnauba waxes negatively affected the growth of strain Z1.

3.2. *In vivo* effect of fruit waxes on antagonist survival

Relative to the control (strain Z1 alone), microcrystalline wax and montan wax significantly reduced the number of strain Z1 cells when the yeast was applied in mixture on the citrus fruit surface (Fig. 3). Ester gum and paraffin wax reduced the strain Z1 survival on the citrus fruit surface, but not significantly. Commercial waxes candelilla, beeswax, rice bran wax, rosin maleic, carnauba wax, shellac, and shellac–carnauba wax did not significantly affect the survival of strain Z1 on the orange surface. The highest strain Z1 cell density was obtained with beeswax followed by rosin maleic, candelilla wax, carnauba wax, shellac–carnauba wax, and shellac respectively (Fig. 3).

3.3. *Effectiveness of antagonist yeast in combination with fruit waxes on blue mold*

The lesion mean diameters of *P. italicum* on orange fruit were generally lower for the formulated product of strain Z1 and the formulated product of strain Z1 applied in combination with wax compared to the water control treatment (Table 2). Strain Z1 significantly reduced the incidence of decayed citrus fruit due to *P. italicum* by 65%. Significant differences ($P < 0.05$) in the number of decayed fruit were shown in all wax treatments compared to the water control treatment. Many commercial waxes, including microcrystalline, ester gum, candelilla, montan, paraffin, rice bran, carnauba, shellac, shellac-carnauba, significantly reduced the efficacy of the formulated product of *P. guilliermondii* strain Z1. The combination of the formulated product of strain Z1 with beeswax demonstrated similar efficacy to that obtained with the formulated product of strain Z1 alone and only the strain Z1-rosein maleic mixture increased significantly the efficacy of disease control relative to the formulated product of strain Z1 alone (Table 2).

3.4. *Mechanisms of action of P. guilliermondii strain Z1: antibiosis and competition for nutrients*

To elucidate the potential antibiosis of strain Z1 against *P. italicum*, an *in vitro* dual culture assay was performed. Results

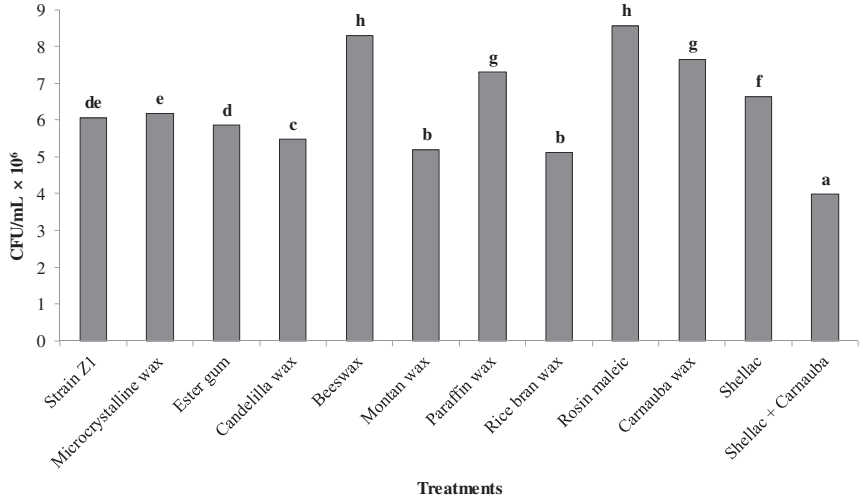

Fig. 2. Effect of fruit waxes, applied in mixture with antagonist yeast *P. guilliermondii* at 20%, on its *in vitro* growth. Shown are the mean values of combined datasets of two independent experiments with four replicates. Data having the same letter are not significantly different ($P < 0.05$) according to LSD Fisher's test.

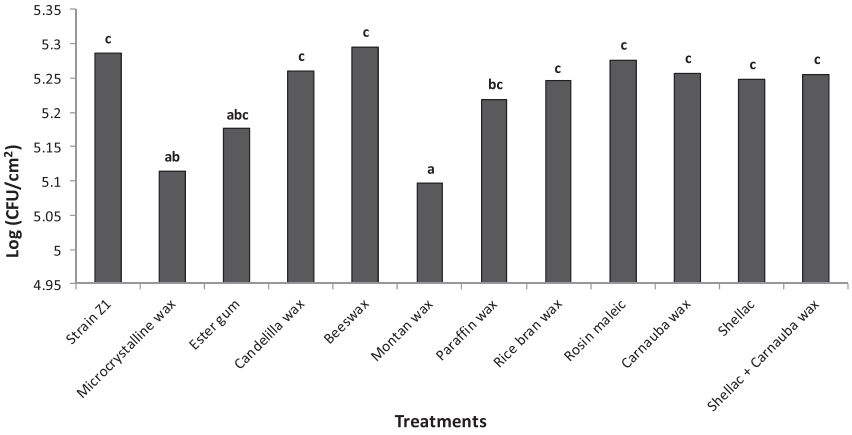

Fig. 3. *P. guilliermondii* strain Z1 survival on citrus fruit surface when applied in combination with commercial fruit waxes after an incubation period of 7 days. Shown are the mean values of combined datasets of two independent experiments with four replicates. Data having the same letter are not significantly different ($P < 0.05$) according to LSD Fisher's test.

show no inhibition zone in dual culture assay of strain Z1 with *P. italicum* (data not shown).

The germination percentages of *P. italicum* conidia after 24 h of incubation at different orange juice concentrations with/out the antagonist are shown in Fig. 4. The smallest percentages were obtained at 0% orange juice in the absence as well as in the presence of strain Z1. In control treatments (orange juice alone) at different orange juice concentrations, conidial germination was observed with percentages ranging from 8 to 98.5%. However, the presence of the antagonist significantly reduced germination of conidia in orange juice except for both highest concentrations 10 and 15% of orange juice (Fig. 4). When cylinders containing conidia previously incubated with the antagonist for 24 h in 0.5% orange juice were moved to new wells with new nutritional conditions (0, 0.1, 0.5, 1, 5% orange juice) and in the absence of strain Z1, germinated conidia were 0, 16, 19, 56, and 100%, respectively, after an additional 24 h of incubation (Table 3).

4. Discussion

The yeast *P. guilliermondii* strain Z1 was isolated from the surface of oranges and selected for its high and reliable biocontrol activity against *P. italicum* and *P. digitatum*, two serious pathogens affecting stored citrus fruit, worldwide (Lahlali et al., 2006 and Lahlali et al., 2011). The underlying mechanisms responsible for its biocontrol activity were unclear and it was hypothesized around competition for space and nutrients (Papon et al., 2013). To be successful in controlling molds under storage conditions, its effectiveness and viability when applied in combination with commercial waxes must be determined. In addition, its mode of action should be explored for formulation development, registration process, and commercial marketing.

Table 2
Compatibility of the formulated product of *Pichia guilliermondii* (strain Z1) for citrus blue mold control with commercial postharvest waxes.

Treatment	Lesion diameter (mm)[a]	Infected fruit (%)[b]
Water control	54.2	100.0a
Formulated product (FP)	3.7	35.0f
FP + Microcrystalline wax	7.9	56.7de
FP + Ester gum	6.9	58.3cd
FP + Candelilla wax	5.1	58.3cd
FP + Beeswax	2.8	35.0f
FP + Montan wax	14.4	75.0b
FP + Paraffin wax	6.2	50.0de
FP + Rice bran wax	6.2	75.0b
FP + Rosin maleic	4.6	25.0g
FP + Carnauba wax	5.5	48.3e
FP + Shellac	12.3	68.3bc
FP + (Shellac + Carnauba wax)	10.9	88.3a

[a] Data were averaged over two replicates at separate times with three biological replicates each (10 oranges fruit).
[b] Mean in column with the same online letter do not differ significantly (Fisher's LSD, $P = 0.05$).

Films and coatings have received much attention in recent years because they extend shelf-life and improve food quality by providing a barrier to mass transfer, carry food ingredients, and/or improve mechanical integrity or handling characteristics of a food (Krochta, 1997). Waxing of citrus is a normal practice in today's packinghouses, aimed at replacing natural waxes on fruit surface that are removed during washing. Application of waxes therefore, serves to reduce fruit shrinkage and improve their appearance (Martinez-Javega, Cuquerella, Del-Rio, & Navarro, 1989). Most wax coatings applied to citrus are applied as micro emulsions. Performance of various wax micro emulsions as fruit coatings depend on the quality of coating emulsion and presence of ingredients in the formula (Hagenmaier, 1998). The research presented here is the first detailed investigation on the compatibility of the antagonist yeast strain Z1 for the control of citrus blue mold with commercial fruit waxes. Our results show that the cell number of strain Z1, applied alone or in combination with commercial fruit waxes, was affected significantly by incubation time and fruit waxes (Table 1). Cell number increased significantly with increasing incubation time, with the exception of the 1 h of incubation during which its population remained stable around 3.2×10^6 CFU/mL. This result confirmed our previous findings (Lahlali et al., 2011) and those of other investigators who reported an increase of antagonistic yeast survival in petri dishes with increasing incubation time (Chanchaichaovivat, Panijpan, & Ruenwongsa, 2008; Lahlali et al., 2005, and Zhao et al., 2008).

The survival of strain Z1 in combination with commercial waxes was largely different between petri dish assays and orange fruit experiments. In the Petri dish assays, population density of strain Z1 significantly increased with beeswax, paraffin wax, rosin maleic, carnauba wax, and shellac 24 h after incubation whereas it was still unaffected by commercial fruit waxes on orange fruit surfaces. Candelilla wax, beeswax, rice bran wax, rosin maleic, carnauba wax, shellac, and formula shellac–carnauba were the only waxes that did not alter significantly the population density of strain Z1. It is possible that hydrophobic interactions between the epidermal layer of orange fruit and strain Z1 with or without commercial waxes played a role in this difference. Indeed, beeswax, paraffin wax, rosin maleic, carnauba wax, and shellac may act as additive compounds for strain Z1 that may explain its higher increase in

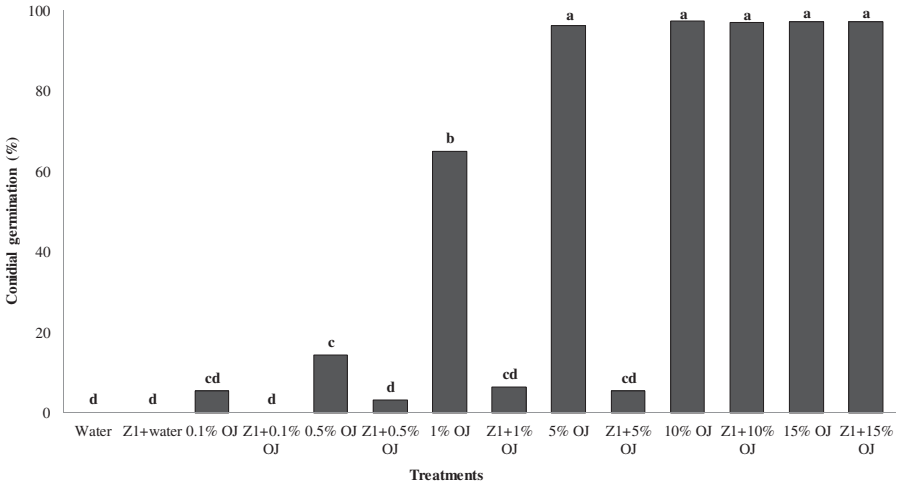

Fig. 4. The germination percentages of *P. italicum* conidia after 24 h of incubation at different orange juice (OJ) concentrations with/out strain Z1. Shown are the mean values of combined datasets of two independent experiments with four replicates. Data having the same letter are not significantly different ($P < 0.05$) according to LSD Fisher's test.

density on petri dishes compared to strain Z1 alone. Theses waxes are highly used in the citrus industry as fruit wax coatings during storage or before shipment for many reasons such as preventing fruit from spoilages, reducing fruit weight loss, preserving fruit quality and providing carrier for fungicides and growth regulators (Petracek, Dou, Mourer, & Davis, 1998, 2000).

In the biocontrol experiment with wounded orange fruit, the formulated product of strain Z1 promoted significant control (i.e., 65%) against *P. italicum*. In previous studies, this yeast provided 60–100% control of postharvest citrus blue and green molds, depending on the concentration of applied yeast, the time elapsed after its application, and the specifics of pathogen inoculation and pathogen pressure (El Guilli, Achbani, Fahad, & Jijakli, 2009; Lahlali et al., 2011). Even better control in the current study could have been achieved from the use of freshly harvested orange fruit or lower pathogen pressure (1×10^5 spores/mL). Microcrystalline wax, ester gum, candelilla, montan, paraffin, rice bran, carnauba, shellac, and formula shellac plus carnauba negatively affected the performance of strain Z1 on wounded orange fruit. Why these commercial fruit waxes increased survival rates but did not enhance biocontrol warrants further investigation. Maybe these waxes only enhance biocontrol when the yeast is exposed to environmental stresses or when they are used separately from yeast. It is unclear if rosin maleic and beeswax can increase strain Z1 performance. Experiments with lower levels of strain Z1 performance may allow the separation of mean lesions of *P. italicum* and, thus, help clarify this point. Many examples are reported in the literature. For example, the biocontrol agent *Bacillus subtilis* proved to be effective with commercial fruit waxes at suppressing peach brown rot (Pusey et al., 1986). Applying *Pseudomonas fluorescens* in combination with different application methods including the addition of adhesive material, peptone, fruit wax or sucrose polyester reduced significantly anthracnose, caused by *Colletotrichum gloeosporioides*, on mango (Kooman & Jeffries, 1993). The lower efficacy obtained with strain Z1 with/out fruit waxes compared to that previously reported for *P. italicum* could be the result of several factors related to the initial quality of the fruit used in each trial and the higher aggressiveness and rapid development of green mold.

Citrus fruit is a perishable crop and is not only prone to postharvest decays but also to water loss following transpiration and respiration after the fruit has been harvested. Coating is one of the major applications done on fruit to prevent undesirable changes and extend shelf life (Tugwell, 1980). The purposes of coating the fruit are to give a good shiny attractive appearance that will last throughout the whole marketing process, to reduce fruit weight loss, to preserve fruit quality and to also provide a carrier for fungicides and growth regulators (Hall, 1981; Kaplan, 1986; Mannheim & Soffer, 1996; Petracek et al., 1998, 2000). Applying strain Z1 in wax coatings may result in lower efficacy due to the encapsulation by wax as previously demonstrated for Imazalil (Brown, 1984). For this reason, workers suggested increasing concentration of fungicides when used in wax coating (Brown, Nagy, & Maraulja, 1983). Accordingly, strain Z1 must be applied in higher concentration in wax coatings with beeswax or rosin maleic wax to avoid the lack of its efficiency during long term storage. To our knowledge, there are two wax coating categories applied to fruit before marketing: storage coatings and shipment wax (Hall & Sorenson, 2006). Therefore, strain Z1 must be applied in wax coatings during storage so that it could have enough time to multiply and colonize the fruit surface before the arrival of the pathogen. Some fruit waxes could act as nutrient additives to strain Z1 promoting better growth on fruit surface. Using IMZ in wax coatings to control *P. digitatum* on degreened orange fruit, Brown (1984) demonstrated less green mold control than IMZ applied as aqueous solution and this was due to the inability of less viscous wax to penetrate the wounded fruit. It has been reported that IMZ performs better when applied as aqueous formulation rather than with water-based wax fungicide sprays (Njombolwana et al., 2013; Similanick, 1997). Consequently, further investigations are required to determine the practical feasibility of applying strain Z1 in wax coating and the impact of such practice on fruit quality and shelf life.

Knowledge on modes of action is essential for enhancing the chance that this yeast will be successfully used for biological control. For example, understanding mode of action can facilitate registration

Table 3
Germination percentages of *P. italicum* conidia previously exposed to strain Z1 in 0.5% orange juice (OJ) for 24 h and then submitted to new nutritional conditions in the absence of strain Z1 for an additional 24 h.

Treatment	Germination rate (%)*
Water control	0[a]
0.1% OJ	16[b]
0.5% OJ	19[b]
1% OJ	56[c]
5% OJ	100[d]

*Mean in column with the same letter do not differ significantly (Fisher's LSD, $P = 0.05$).

for commercial use and can be useful for optimizing the formulation and delivery systems (Andrews, 1992; Chanchaichaovivat et al., 2008). Competition for nutrient is one of the most important mechanisms of action, mainly against wound pathogens that are typically dependent on exogenous nutrients for their development. The experimental demonstration of this mechanism is difficult to achieve, given primarily to the lack of appropriate methods for studying this type of interaction. And in the literature, most studies on this subject have used approaches *in vitro* or *in situ* and do not differentiate between the part of the involvement of competition for nutrition and competition for space (Castoria, De Curtis, Lima, & De Cicco, 1997, Castoria et al., 2001; Guetsky, Shitienberg, Elad, Fischerr, & Dinoor, 2002; Janisiewicz et al., 2000; Lima et al., 1997; Vero, Mondino, Burgueno, Soubes, & Wisniewski, 2002). In the absence of the antagonist, higher *in vitro* germination percentages of *P. italicum* conidia were recorded in the presence than in the absence of orange juice (Table 2). This confirms that *P. italicum* is nutrient-dependent and, as a necrotrophic pathogen, requires sufficient nutrients for its conidial germination and hyphal development. In the presence of the antagonist, however, *in vitro* germination of the pathogen conidia was inhibited except at the highest orange juice concentration (Table 2). Moreover, inhibited conidia were still able to germinate when submitted to high orange juice concentrations with as well as without the antagonist (Table 3). These data suggest that strain Z1 outcompetes *P. italicum* conidia for orange juice nutrients without affecting their viability. Current results are consistent with previous investigations with other strains of *P. guilliermondii* (Liu, Luo, & Long, 2013; Zhao et al., 2008; Zhang et al., 2011) and other antagonistic yeasts indicated that competition for nutrients is an important mechanism of action (Castoria et al., 2001; Krimi et al., 2007; Lima et al., 1997; Liu et al., 2013). As better efficiency in biocontrol was obtained with strain when fruit are treated with yeast 24 h before the arrival of pathogen, this suggested that induction of plant defense is related to its mode of action in controlling *P. italicum* as previously shown by Liu et al. (2013). Other modes of action, which have not been determined and could not be excluded, for this strain Z1will be under investigation and includes: competition for carbon source, nitrogen sources, vitamins and production of lytic enzymes. The latter may explain lower germination rate of *P. italicum* in the presence of strain Z1 at lower orange juice concentration.

In summary, this study has determined the impact of commercial fruit waxes on the *in vitro* and *in vivo* strain Z1 survival and on its performance in controlling blue decay on orange fruit when it was applied in combination with commercial waxes as formulated product. We have shown that the formulated product of this yeast strain is compatible with rosin maleic and beeswax waxes. Whether both commercial fruit waxes rosin maleic and beeswax can increase the efficacy of the formulated product of strain Z1 is still unclear; however, some commercial fruit waxes increased the survival of strain Z1 on orange fruit without visible enhancement of protection. Because these conclusions are drawn from data obtained under controlled conditions, therefore, rosin maleic and beeswax should be further investigated in combination with the formulated product of strain Z1 in simulated commercial packinghouse. The biocontrol of this tested yeast may rely on competition for nutrients. Accordingly, further commercial formulations of this strain must take into account this aspect.

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