The ability of the antagonist yeast *Pichia guilliermondii* strain Z1 to suppress green mould infection in citrus fruit

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Abstract

In previous studies it was shown that *Pichia guilliermondii* strain Z1, isolated from healthy Moroccan citrus Valencia-Late oranges, was effective against *Penicillium italicum.* Here the effectiveness of strain Z1 was assessed against *Penicillium digitatum*, the causal agent of green mould, under different temperature (5-25°C) and relative humidity (RH) (45-100%) regimes for its reliable and large-scale application in packinghouse. All main effects and interactions were significant (P<0.0001). In the pathogen control, the largest lesion diameter was at an RH range between 98 and 100%, regardless of the incubation temperature. The efficacy of strain Z1 was not dependent on the environment and reduced disease incidence by >80%. Its applications as a formulated product significantly reduced the incidence of infected fruit (55%) and the percentage of infected wounds (47%) compared to the only pathogen control treatment. However, disease control with formulated product was significantly less than that obtained with thiabendazole (30%) or strain Z1 culturable cells (35%). These results highlight that strain Z1 is an effective biological control agent for control of green mould under varying environmental conditions, and control may be optimized by combining its use with other environmentally-safe post-harvest treatments or improved formulation.

Introduction

Morocco is one of the largest citrus-producing countries in the world with about 76,500 ha and an estimated annual yield of 1.5 million tons (Talibi *et al.*, 2012). Blue, caused by *Pencillium italicum,* and green mould, caused by *Penicillium digitatum* Sacc., are the major postharvest diseases of citrus fruits in Morocco and worldwide (Lahlali *et al.*, 2006). In Moroccan packing-houses, these pathogens are commonly managed by synthetic chemicals fungicides such as sodium ortho-phenylphenate, thiabendazole (TBZ), and imzalil. Since fungicides have different modes of action, they could be used alone, combined in mixtures, or applied separately in sequence, and they have been considered as the primary method of controlling citrus fruit decay during storage and packinghouse for more than 25 years (Ismail and Zhang, 2004). These synthetic chemicals are known to act quickly and effectively against postharvest fruit decays (Ballester *et al.*, 2013). However, the appearance of resistant strains and the growing public concern over health and environmental risks associated with the excessive use of pesticides in fruits have led to investigate developing alternative methods of control (Lahlali *et al.*, 2011; Jijakli and Lepoivre, 1998). Biological control was proposed as an interesting alternative to chemical control of diseases of fruits caused by pathogenic micro-organisms (Jijakli and Lepoivre, 1998; Lahlali *et al.*, 2005).

Several reports have underlined a successful control of postharvest diseases using biological control agents (BCAs) on citrus fruit (Kinay and Yildiz, 2008; Lahlali *et al.*, 2004, 2005). Consequently, different biofungicides based on bacteria and yeasts are now commercially available (Lahlali *et al.*, 2011). In Morocco, different attempts were explored to find alternative strategies to synthetic fungicides in postharvest fruits storage, particularly with fungicides residues restriction imposed by importing countries. Over the last ten years, different strategies have been assayed. Lahlali *et al.* (2004, 2005) reported that Belgian yeast *Pichia anomala* strain K and *Candida oleophila* strain O were effective against green and blue moulds. Recently, Moroccan researchers have screened different effective medicinal plants against major postharvest pathogens of citrus (Ameziane *et al.*, 2007; Talibi *et al.*, 2012).

Among yeasts screened as BCAs for postharvest diseases of fruits, *Pichia guilliermondii* strain Z1 has proven effective against blue mould caused by *P. italicum* at different temperatures and relative humidities (Lahlali *et al.*, 2011). This strain also may act against *P. italicum* by rapid colonization of fruit wounds and competition for nutrients (Arras *et al.*, 1998; Lahlali *et al.*, 2011). For a large-scale application of this antagonistic yeast in Moroccan packinghouse, this strain should demonstrate higher effectiveness toward green mould and other postharvest diseases of citrus fruit. However, little is known about its inhibitory capacity against green mould, *P. digitatum*. Therefore, the main objectives of this study were: i) to evaluate its ability to control *P. digitatum* under different temperatures and relative humidities; and ii) to compare its efficacy as culturable cell and as formulated products or TBZ.

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Materials and Methods

Microorganisms

Yeast strain Z1 was isolated from healthy Moroccan Valencia-Late oranges by the laboratory of Phytopathology of INRA-El Menzeh (Kenitra, Morocco) and identified as *Pichia guilliermondii* by Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). This yeast was selected for its high and reliable protective activity against *P. italicum* (Lahlali *et al.*, 2011). Prior to experiments, it was sub-cultured at 25°C for three successive generations on potato dextrose agar (PDA; Merck, Darmstadt, Germany) for 24 h. The yeast was washed from the agar media in 10 mL of saline solution (0.85% NaCl) supplemented with 0.01% Tween 80%. The final concentration was determined by spectrophotometer by the equation (OD-0.0958/0.03=CFU/mL×10^6). For the following experiments, this strain was used at a higher concentration such as 1×10^8 CFU/mL.

P. digitatum strain PDRGBH used in this study was originally isolated from decayed orange fruits from Gharb region of Morocco (Lahlali *et al.*, 2006). For long term storage, *P.*

digitatum was maintained in 25% glycerol at -80°C. This fungus was recovered from glycerol and grown on PDA when needed for experiments. Its conidial suspension was fixed at 1×10^5 spores/mL for subsequent experiments.

Fruit and inoculation procedures

Citrus fruits (cv. Valencia-Late) (*Citrus sinensis* [L] Osbeck) were harvested from commercial orchards of the region Elgharb Chrarda Beni Hssen, Morocco. Before the experiments, these fruits were stored at 4°C for up to 7 days and only healthy fruits were selected for the subsequent experiments. Fruits were disinfected by soaking in sodium hypochlorite solution (10%) for 2 min and then rinsed twice in sterile distilled water (SDW). After drying for one hour, the orange fruits were injured in two equidistant points on the equatorial site. Each wound was 5 mm in diameter and 4 mm in depth.

Biocontrol efficiency under influence of temperature and relative humidity

To investigate the main effect of temperature and relative humidity (RH) on the lesion diameter of *P. digitatum,* disinfested fruits were treated with 50 µL of strain Z1 and then inoculated 24 h later with 50 µL of *P. digitatum*. Fifty microliter of SDW was applied to the control before pathogen inoculation. Fruits were kept in smaller desiccators with different relative humidities and then stored at each temperature. With the exception of 100% RH, in which the treated fruits were incubated in plastic bags containing moistened paper with water, the different approximate values of equilibrium relative humidity (98±1, 85±1, 75±1 and 45±1%) inside the desiccators were controlled using the saturated salt solutions (Lahlali *et al.*, 2011). Desiccators with different relative humidity regimes were incubated for 48 h at different experimental temperatures (5, 10, 15, 20 or 25°C) before introducing the wounded oranges inoculated with strain Z1 and *P. digitatum*. The relative humidity in each desiccator was checked daily by means of a thermo-hygrometer. There were three biological replicates per treatment, with each replicate consisting of four fruits (eight wounds) in a single desiccator. The experiment lasted 30 days for temperature tests ranging from 5 to 15°C, and only 8 days for temperatures ranging from 20-25°C. At the end of the experiment, the lesion diameters (mm) were recorded for the nontreated and BCA-treated fruits at each temperature and RH combination.

Effectiveness of 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 Melsungen AG, Melsungen, 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, Free University of Bruxelles, 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 fruits were prepared as described above. Each wound was inoculated with 50 µL of antagonist yeast strain Z1 (fresh cells) at 1×10^8 CFU/mL, or strain Z1 formulated product (1 g/3 L) or chemical fungicide TBZ (50% a.i., Tecto 500 SC, RODA, Morocco) at 1g/L. Fruit that received SDW served as controls. Fruit were stored separately in moistened plastic boxes at ambient temperature for 24 h and then inoculated with *P. digitatum* (50 µL/wound). Fruit were stored at 24°C for 7 days before evaluation. There were four biological replicates of 10 fruits (20 wounds) for each treatment and the result was expressed as the percentage of infected fruit and that of infected wounds in each treatment.

Statistical analysis

General linear model procedure of SAS software (SAS Institute, version 8.2, Cary, NC, USA) was employed to analyze the main effect of temperature, relative humidity, and BCA and their interactions on lesion diameter of *P. digitatum*. A one way ANOVA was performed to compare the effectiveness of strain Z1 cultur-

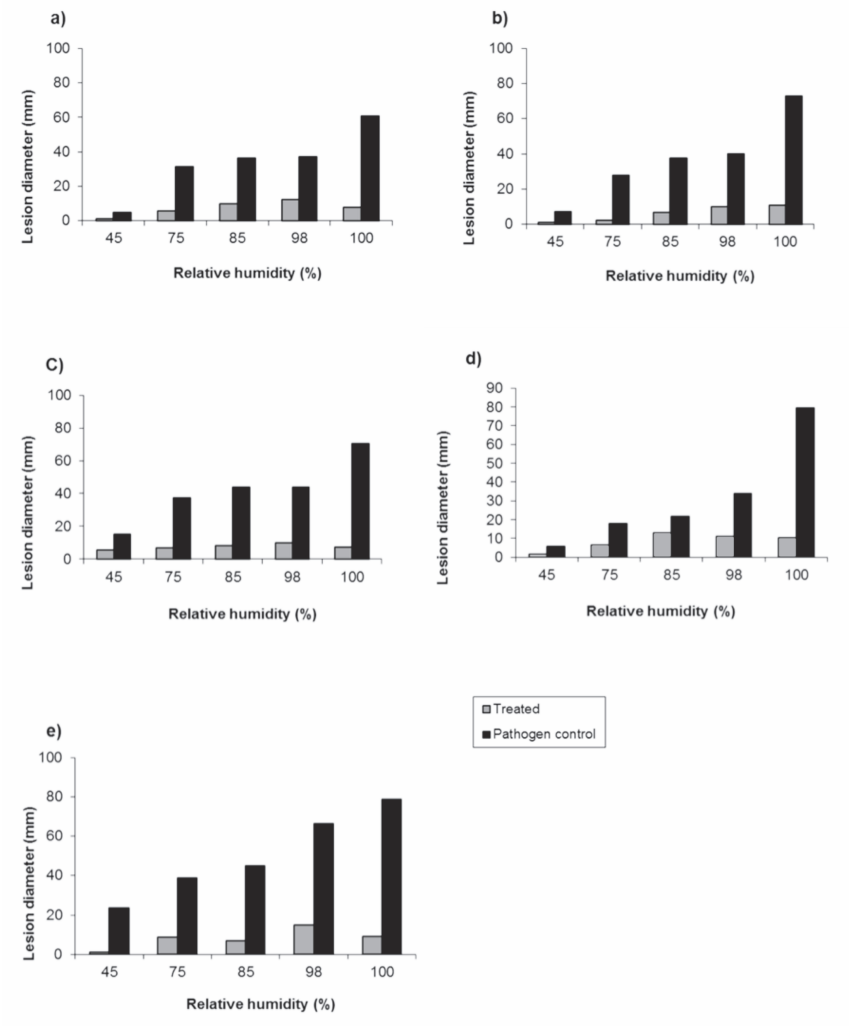

***Figure 1. Inhibition effect of* Pichia guilliermondii strain Z1 (1×10^8 CFU/mL) against Penicillium digitatum (1×10^5 spores/mL) on Valencia-Late fruit at different temperatures (a=5°C; b=10°C; c=15°C; d=20°C; e=25°C) and different relative humidities. The values are the mean of three biological replicates over time (4 fruits per replicate).**

able cells and the formulated product of strain Z1 with that of the commercial fungicide TBZ. Treatment means were separated using $LSD_{0.05}$ if analysis was significant ($P \leq 0.05$).

Results and Discussion

Biocontrol efficiency under influence of temperature and relative humidity

Effective suppression of plant diseases by BCA is largely influenced by environmental conditions. Environment affects the establishment, survival and activity of the BCAs (Benbow and Sugar, 1999; Lahlali *et al.*, 2011). Therefore, the effect of temperature and RH on the ability of strain Z1 to suppress green mould was evaluated. Variance analysis (ANOVA) showed a highly significant effect of strain Z1, temperature, and RH, and their interactions on the lesion diameter of *P. digitatum* on Valencia-Late oranges (Table 1). Figure 1 shows that strain Z1 reduced the green mould lesion diameter by more than 80% on wounded citrus fruits under different temperature and RH regimes compared to the pathogen control. The *P. digitatum* lesion diameter declined with decreasing temperature and RH values, with the exception of 10 and 15°C where a lower growth difference was observed at RH values from 85 to 98%. The lesion diameter was largest at a temperature of 25°C and 100% RH, and the lowest at 45% RH independent of temperature (Figure 1). Using the same approach, we have found the same results on lesion diameter of *P. italicum* (Lahlali *et al.*, 2011). Like *P. italicum*, the lesion diameter of *P. digitatum* dropped with decreasing temperature and RH values with a maximum growth at 25°C and 100% RH. This result corroborated those previously reported by Lahlali *et al.* (2006, 2011), Plaza *et al.* (2003), and Hannusch and Boland (1996) on *Botrytis cinerea*. Lahlali *et al.* (2008) indicated that temperature and RH significantly affect the population density of BCA on fruit surface and may have reduced the biocontrol efficacy in postharvest conditions. Furthermore, the effect of temperature on strain Z1 survival was evaluated (Lahlali *et al.*, 2013). They found that the highest density of Z1 was recorded at 25°C.

Effectiveness of the formulated product of strain Z1

The effectiveness of strain Z1, produced on industrial scale, was evaluated against *P. digitatum* and compared with strain Z1 culturable cells or TBZ (1 g/L) (Figure 2). This experiment was of great importance for the large-scale application of this antagonistic yeast (Lahlali *et al.*, 2011). All treatments significantly reduced the incidence of decayed fruits caused by *P. digitatum* relative to the non-treated fruits (pathogen control). No significant difference in disease incidence was observed between TBZ and strain Z1 culturable cells, whereas the percentage of infected fruit was slightly higher in the formulated strain Z1 product (Figure 2). The incidence of infected fruits was reduced by 70, 65 and 45% in TBZ, strain Z1 culturable cells and formulated product, respectively. Also, all treatments were sig-

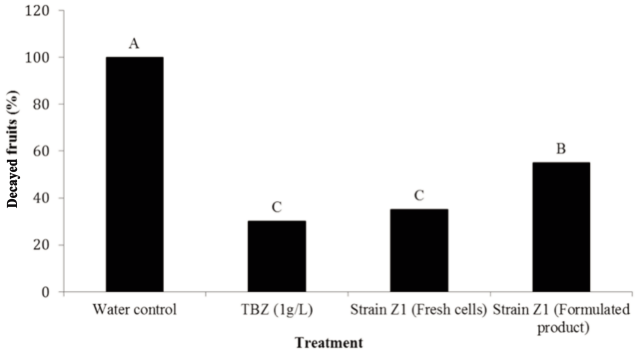

Figure 2. Percentage of decayed Valencia-Late citrus fruit treated with *Penicillium guilliermomdii* strain Z1 produced in Petri dishes (1×10^8 CFU/mL) on industrial scale (1 g/3 L), or TBZ (1 g/L) and then inoculated 24 later with *Penicillium digitatum* (1×10^5 spores/mL). The values are the means of four biological replicates over time (10 fruits per replicate).

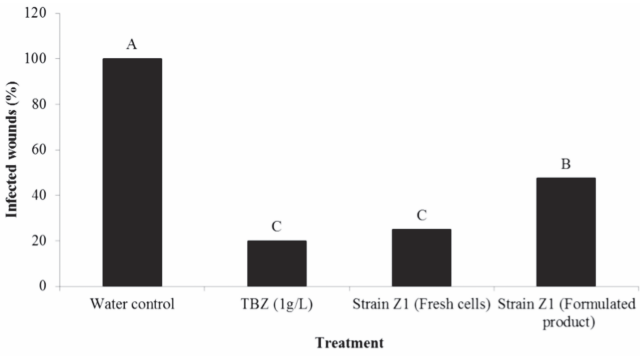

Figure 3. Percentage of infected wounds of Valencia-Late citrus fruit treated with *Penicillium digitatum* (1×10^5 spores/mL), after 7 days of incubation at 24°C as affected by P*ichia guilliermomdii* strain Z1 produced in Petri dishes (1×10^8 CFU/mL) on industrial scale (1 g/3 L), or TBZ (1 g/L). The values are the means of four biological replicates over time (10 fruits per replicate).

Table 1. Analysis of variance of the effect of *Pichia guilliermondii* strain Z1, relative humidity and temperature on lesion diameter of *Penicillium digitatum*.

Source of variation	DF	SS	MS	F	P
A	1	48,846.9	48,846.9	1517.0	0.000*
B	4	24,198.9	6049.7	187.8	0.000*
C	4	2313.7	578.4	17.9	0.000*
A×B	4	16,409.1	4102.2	127.4	0.000*
A×C	4	2151.1	537.8	16.7	0.000*
B×C	16	1653.7	103.3	3.2	0.000*
A×B×C	16	1773.5	110.8	3.4	0.000*
Error	150	4829.7	32.2	-	-

A, biocontrol agent; B, relative humidity; C, temperature; DF, degree of freedom; SS, sum of squares; MS, mean square. *P<0.0001.

nificantly different from the non-treated control (pathogen control) in terms of percentage of infected wounds. The percentage of infected wounds was significantly lower with TBZ, followed by strain Z1 culturable cells and formulated product with 20, 25, and 47.5%, respectively (Figure 3). These results are adequately in agreement with our previous findings on *P. italicum* by using strain Z1 as BCA (Lahlali *et al.*, 2011). Similar results for other screened antagonists *Bacillus* spp. (Obagwu and Korsten, 2003), and *Candida oleophila* strain O (Lahlali *et al.*, 2005) were reported.

Understanding the mechanism of this antagonistic yeast is essential for developing appropriate commercial formulation, application methods, and for maximizing the effectiveness of BCAs (Zhang *et al.*, 2011). Several modes of action have been identified for this antagonistic yeast, including rapid colonization of wounds, competition for nutrients (Zhang *et al.*, 2011), induction of defense responses (Yu *et al.*, 2008), and production of lytic enzymes (Zhang *et al.*, 2011). Accordingly, the inclusion of wetting agents, secondary metabolites or additives into strain Z1 formulation may lead it to exhibit its proper mechanisms of action, thus allowing a complete control of moulds infection on citrus fruit.

Our results emphasize a significant effect of strain Z1, RH and temperature on the lesion diameter of *P. digitatum* on orange fruits. The biocontrol efficiency of strain Z1 is not substantially affected by temperature and RH regimes. However, its application as formulated product for the protection of wounded fruits was significantly lower than the fresh cell or the standard chemical TBZ. This suggests the need of an improving formulation that may contain some wetting agents or compounds capable of boosting the efficiency of strain Z1 making it comparable to that provided by chemical or strain Z1 fresh cells.

Conclusions

Results presented here, together with our previous finding (Lahlali *et al.*, 2011), highlight a high potential of this strain as a promising BCA for green and blue mould of citrus fruit over a wide range of temperature and RH combinations. However, these factors could present a real handicap for its use before storage in controlling postharvest decay of citrus fruit by limiting its survival on fruit surface and should be taken into account for further improvement of its formulation. As biological control, the use of bio-pesticide based on *P. guilliermondii* against green mould can be the most effective and environment-friendly method and best alternative to chemicals.

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