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Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*)

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ABSTRACT

Killer *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* yeast strains were tested as biocontrol agents against *Penicillium digitatum*, one the most important causes of postharvest decay in orange fruits.

W. anomalus, grown on acidified medium, demonstrated micocinogenic activity against *P. digitatum*, as indicated by large inhibition halos and hyphal damage resulting from β-glucanase activity.

Oranges that had been deliberately inoculated with pathogens were protected from decay by *W.* anomalus. Inoculation of oranges with *W. anomalus* strains BS 91 and BS 92 reduced disease severity to 1 and 4%, respectively, for up to 10 days in storage.

1. Introduction

In Italy, about 40% of the sweet orange production is represented by a pigmented (blood) cultivar, called "Tarocco". The cultivation of this variety is mainly found in certain areas of Eastern Sicily, which have the most suitable soil and climate conditions to produce fruits of high quality (Tribulato and La Rosa, 1994). The success of the Tarocco orange in domestic and international markets is the result of its exceptional sensorial characteristics, such as the brilliant red flesh colour, the distinctive and agreeable fragrance, the large size, and the balanced levels of sugar and acids. In addition, this fruit contains high levels of antioxidant compounds, including vitamin C, flavanones, hydroxycinnamic acids, and anthocyanins (Rapisarda et al., 2006; Rapisarda and Russo, 2003). The green mould caused by *Penicillium digitatum* is one the major postharvest diseases of citrus fruits, which limits the commercial life of harvested fruits. In addition, *P. digitatum* can cause an allergic response due to the enormous numbers of dry air-borne spores it produces (Moss, 2008). This fungus is usually controlled by chemical fungicides, such as imazalil or thiabendazol. However, resistance to the fungicide often occurs in the biologically dynamic fungal population, which limits the effectiveness of these fungicides (Sánchez-Torres and Tuset, 2011). Moreover, growing public health and environmental concerns have resulted in the de-registration of some of the more effective synthetic fungicides, and Tarozzi et al. (2006) clearly demonstrated that organic Tarocco oranges have a higher phytochemical content (i.e., phenolics, anthocyanins and ascorbic acid), total antioxidant activity, and bioactivity than integrated red oranges. Thus, there is a growing interest in the development of safer, effective alternative compounds to control postharvest fungal diseases, especially for citrus fruits, the peels of which are widely used by confectioners and often have high concentrations of pesticide residues. Several reports have proposed the use of microbial antagonists for controlling the postharvest fruit infections caused by *Penicillium* (Chalutz and Wilson, 1990; Cirvilleri et al., 2005; El-Ghaouth et al., 2000; Lahlali et al., 2004; Petersson et al., 1998; Restuccia et al., 2006; Taqarort et al., 2008). Among the different approaches, the discovery of naturally occurring antifungal proteins, which have no environmental impact or mammalian toxicity and a low tendency to elicit resistance, are attracting increased attention (Bobek and Situ, 2003). Antifungal proteins are naturally produced by microorganisms, including bacteria and fungi (Selitrennikoff, 2001). Among these proteins, the yeast killer proteins are promising as potential antifungal agents. Certain yeast strains with a killer phenotype (K+) produce extracellular protein toxins designated as killer proteins or killer toxins, which are lethal to sensitive microbial cells (Hemandez et al., 2008; Schmitt and Breinig, 2002). Killer protein production appears to be a widespread characteristic of yeast species from

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different genera, including *Saccharomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and several others. The killer proteins confer an ecological advantage to yeast cells over their competitors (Comitini et al., 2009; Magliani et al., 2008). Generally, the killer toxins are active at pH values from 3 to 5.5 (Izgu and Altinbay, 2004; Marquina et al., 2002), which could present an advantage because the reduction of the pH value of wounded or injured fruits reduces the effectiveness of traditional fungicides (López-García et al., 2003).

In *Saccharomyces cerevisiae*, three different killer toxins, K1, K2 and K28, have been clearly identified. K1 and K2 have virtually identical toxic activities; both of them bind to β-1-6-D-Glucan components in the walls of sensitive cells and then disrupt the ion exclusion barrier of the target cell plasma membrane. The third killer toxin, K28, binds to α-1-3 linked mannose residues in the mono protein of the walls of sensitive strains and reversibly inhibits DNA synthesis (Schmitt and Breinig, 2002). Very recently, Rodríguez-Cousiño et al. (2011) isolated wine *S. cerevisiae* strains that produce a new killer toxin (Klus), which killed all of the previously known *S. cerevisiae* killer strains in addition to other yeast species, including *Kluyveromyces lactis* and *Candida albicans*.

There is a considerable amount of published information on the widely intergeneric killing spectrum of *Pichia* toxins. Among the species with a killer phenotype, *W. anomalus* (formerly *Pichia anomala*) NCYC 434 has been extensively studied, and its killer protein, Panomycocin, has been suggested as a potential antifungal agent (Izgu and Altinbay, 2004; Izgu et al., 2011). Panomycocin is a glycosylated monomeric protein with a molecular mass of 49 kDa. It belongs to the exo β-1,3 glucanase group and exerts its cytocidal activity by hydrolysing the β-1,3-glucans, which are the primary polymers within the fungal cell wall (Izgu et al., 2005). Hydrolysis of this polymer leads to leakage of cytoplasmic components and ultimately cell death. Exo β-1,3 glucanases have been shown to contribute to the mechanism of action of the antagonistic yeast *Wickerhamomyces anomalus* (strain K) against *Botrytis cinerea* and *Penicillium expansum* on the apple (Friel et al., 2007; Jijakli et al., 1993; Jijakli, 2011), *B. cinerea* on grapevine plants (Masih et al., 2000), crown rot on bananas (Lassois et al., 2008) and *Penicillium roqueforti* on stored cereal grain (Druvefors et al., 2002). Its performance might also be connected to its ability to grow and survive in different and stressful environments (Walker, 2011). Furthermore, *W. anomalus* currently has QPS (Qualified Presumption of Safety) status from the EFSA (European Food Safety Authority), and this has benefits in terms of public perspectives on food biotechnology and the acceptability of novel microorganisms in food (Sundh and Melin, 2011).

The aim of this work was to investigate the effectiveness of different killer *S. cerevisiae* and *W. anomalus* strains, which were isolated from olive brine, as biocontrol agents of the citrus pathogenic mould *P. digitatum in vitro* and *in vivo*.

2. Materials and methods

2.1. Fungal strains

The yeasts used in this study, belonging to DISPA (DIpartimento di Scienze delle Produzioni Agrarie ed Alimentari, University of Catania) collection, were isolated from naturally fermented olives. The yeasts were identified as *S. cerevisiae* BS 46, *S. cerevisiae* BCA 61, *W. anomalus* BCU 24, *W. anomalus* BS 91, *W. anomalus* BS 92 and *W. anomalus* BCA 15, by sequencing the D1/D2 region of the 26S rRNA gene. These strains were selected based on a high killing capacity against a sensitive strain (5 × 47) of *S. cerevisiae*. Moreover, for the *W. anomalus* strains, the toxic mechanism was identified as a β-glucanase (Muccilli et al., 2010).

The inhibition activity of the strains was tested against a *P. digitatum* strain isolated from diseased citrus fruits in Sicily, Italy, and scored by the key system reported by Pitt (2000).

The yeast and mould stock cultures were stored at 4 °C on YPDA (g/l distilled water: yeast extract, 10; peptone, 10; dextrose, 20; agar, 20; Oxoid, Basingstoke, UK) or Potato-Dextrose Agar (PDA, CM0139, Oxoid, Basingstoke, UK) plates, respectively.

2.2. Total DNA extraction

To determine the intraspecies genetic variability by RAPD analysis, DNA was isolated using the following protocol. Pure cultures were grown in YPD liquid medium (g/l of distilled water: yeast extract, 10, peptone, 10, and dextrose, 20; Oxoid, Basingstoke, UK) at 25 °C for 18 h with a rotation of 250 rpm on an orbital shaker. Two millilitres of cell culture was then centrifuged at 9000×*g* for 2 min at room temperature. The cells were resuspended in 0.5 ml of 1 M sorbitol-0.1 M EDTA, pH 7.5. Subsequently, they were transferred to a 1.5-ml microcentrifuge tube, to which 0.02 ml of a solution of Zymolyase 100 (2.5 mg/ml) was added. The tubes were incubated at 37 °C for 30 min. The spheroplasts were pelleted for 3 min in a microcentrifuge and suspended in 0.5 ml of 50 mM Tris–HCl-20 mM EDTA, pH 7.4. After suspension, 0.02 ml of 10% sodium dodecyl sulfate was added and the mixture was incubated at 65 °C for 15 min. Immediately thereafter, 0.2 ml of 5 M potassium acetate was added, and the tubes were incubated on ice for 30 min. The tubes were then centrifuged at 14 000×*g* for 10 min at 4 °C. The supernatant was transferred to a fresh microcentrifuge tube, and the DNA was precipitated by adding 1 volume of isopropanol. After incubation at room temperature for 5 min, the tubes were centrifuged for 10 min. The DNA was washed with 70% ethanol, dried, and dissolved in 50 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5).

The Qubit® Fluorometer (Invitrogen-Life Technologies, Carlsbad, CA, USA) was used to determine the amount of extracted DNA.

2.3. RAPD analysis of yeast strains

For the PCR, the core sequence of the phage M13 and the microsatellite primer (GTG)5 were used as single primers (Meyer et al., 1993). All reactions were performed in 50-μl reaction volumes containing 25 μl of MyTaq Mix, 2× (Bioline, London, UK.), 5 μl of each primer (5 μM), 100 ng of genomic DNA, and water up to 50 μl. The amplification was performed in a Personal Thermocycler (Whatman Biometra, Göttingen, Germany) using the following amplification conditions:

M13: initial denaturation at 95 °C for 5 min; 40 s at 95 °C for denaturing, annealing for 60 s at 45 °C, and extension for 60 s at 72 °C, which was repeated for 40 cycles; and a final extension step of 5 min at 72 °C.

GTG5: initial denaturation at 95 °C for 5 min; 15 s at 95 °C for denaturing, annealing for 45 s at 55 °C, and extension for 60 s at 72 °C, which was repeated for 35 cycles; and a final extension step of 4 min at 72 °C.

Aliquots of 5 μl of the amplified product were separated electrophoretically on a 1.4% (wt/vol) Certified Molecular Biology Agarose (Bio-Rad Hercules, CA, USA) gel, containing GelRed™ Nucleic Acid Gel Stain 10,000× in Water (Biotium, Hayward, CA, USA) in a 0.5× TBE (Tris-Borate-EDTA) buffer. A 1 kb DNA ladder (Fermentas GMBH, Leon-Rot, Germany) was used for the DNA molecular weight marker. The DNA migration patterns were visualised by UV light, recorded and visually analysed. The reproducibility of the RAPD–PCR patterns was assessed by performing the RAPD analysis on the yeast strains at least three times.

2.4. In vitro biocontrol assay

The yeast and mould strains to be tested were grown on YPDA for 48–72 h at 25 °C or on PDA for 7–14 days at 25 °C, until sporulation occurred, respectively. To evaluate mould growth biocontrol by the yeast isolates, both fungi were grown side-by-side. In triplicate, Petri dishes with YPDA at pH 7 or pH 4.5, which were buffered with citrate-phosphate, were inoculated with a 5-mm square plug of an actively growing fungal mycelium near the dish edge. A sterile loop containing the yeast cells was used to inoculate the opposite edge. A control dish inoculated only with the mould was also prepared. The experiment was performed by incubating the dishes at 25 °C. The mycelial growth was measured after 2, 4, 7 and 14 days of incubation and is expressed as the distance (cm) from the plug to the side edge of the actively growing mould. The colonies originating from spores carried upward from the agar were not considered.

The fungal mycelium was microscopically observed after 4 days to assess any hyphal damage caused by the killer toxin. Mycelium disks (5 mm in diameter), which were obtained from the dish in which the yeast had inhibited the mould growth, were removed from an area proximate to the yeast cells. The excess of culture medium was longitudinally cut to obtain thin mycelia layers, which were studied by an upright optical microscope (Olympus, Hamburg, Germany). The hyphae were photographed, and their morphologies were compared with those of the control.

2.5. Fruit decay test

Tarocco orange fruits (*Citrus sinensis*) were obtained from commercial orchards in Sicily, Italy. The fruit had not received any pre-harvest fungicide treatment. The oranges were sorted by hand to remove any with apparent injuries or infections, and, immediately, the selected oranges were randomly assigned to different treatments. Before each experiment, six orange fruits were washed with tap water, surface-disinfected by dipping for 2 min in 2% (wt/vol) of sodium hypochlorite solution, rinsed with sterile distilled water and then air-dried. The fruits were cut with a sterile needle to make four 2-mm deep and 5-mm wide wounds on their peels along their equators (four wounds for each fruit). Yeast suspensions were prepared separately from cells grown on YPD for 24 h at 25 °C. The yeasts cultures were centrifuged at 8000×*g* for 10 min and resuspended in sterile water; the concentrations were adjusted to a cell density corresponding to 2×10^9 cfu ml^{-1} at 595 nm, as determined by a spectrophotometer. The absorbance values were found to have a linear relationship with the number of cells counted by a hemocytometer.

A 20-μl drop of yeast was placed on each wound and allowed to dry. The control fruits were treated with sterile water only. After the inoculation, the fruits were placed on plastic packaging trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The trays were stored at 20 °C.

After three days, the citrus fruit was inoculated with a 20-μl drop of 1×10^6 conidia ml^{-1} of *P. digitatum* (absorbance of 0.1 at 420 nm, as determined by a spectrophotometer) and air dried. The citrus fruits were stored at 20 °C and observed 3, 5 and 10 days after inoculation of the pathogen. Each experiment was performed in triplicate.

The wounds showing symptoms of infection were counted, and the incidence of disease was calculated. Data concerning the percentages of decayed wounds (incidence of disease) were transformed into arcsine square-root values to normalise the distribution before performing the analysis of variance. Subsequently, the data were analysed by a one-way analysis of variance (ANOVA) using CoStat software (CoHort Software, Monterey, CA, U.S.A). Mean separations were performed using Student-Newman Keul's mean separation test with $P = 0.05$.

The disease severity, which is often considered a more useful measure of disease intensity than incidence for determining the effectiveness of disease management strategies, was evaluated with an empiric scale (1 = no visible symptoms; 2 = soft rot; 3 = mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percent midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65%, and 4 = 90%. The percentages of disease severity ratings were subjected to an arcsine square-root transformation before running the analysis of variance. Subsequently, a ANOVA was performed. The means were separated using the Student-Newman-Keul's mean separation test, at $P \leq 0.05$.

3. Results

3.1. RAPD–PCR analysis

RAPD–PCR analysis of the killer strains of the species *S. cerevisiae* and *W. anomalus* included in this study yielded distinctive patterns that permitted clear intraspecies differentiation of the isolates, although primer M13 allowed discrimination among *S. cerevisiae* and *W. anomalus* strains with higher polymorphism and reproducibility (Fig. 1).

3.2. In vitro tests

The *in vitro* antifungal activity of *W. anomalus* and *S. cerevisiae*, which were able to produce killer toxins, was determined against *P. digitatum* at two different pH levels. As expected, no fungal growth inhibition was detected at a neutral pH. At pH 4.5, significant fungal growth inhibition with a clear inhibition halo was observed only for *W. anomalus* isolates (Fig. 2a). The mycelial growth in the Petri dishes was significantly reduced, although different effects were observed among strains (Fig. 3). Slight

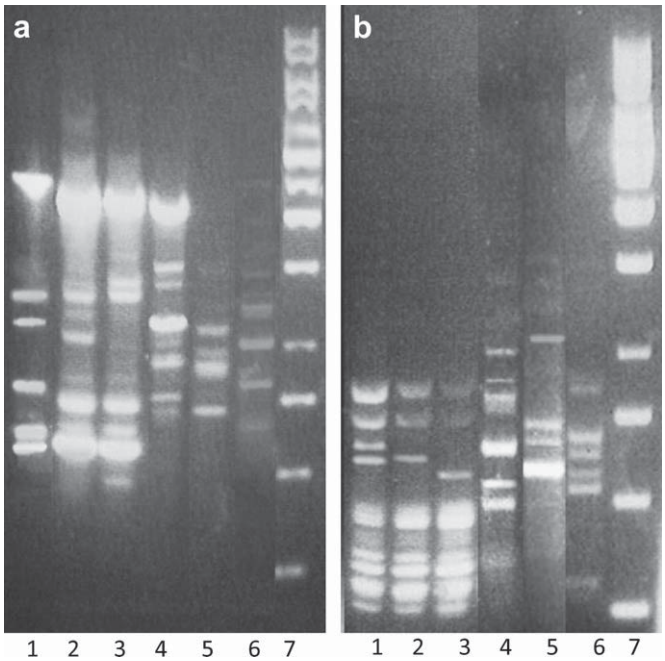

Fig. 1. RAPD profiles of *W. anomalus* and *S. cerevisiae* isolates with M13 (a) and GTG5 (b) primers. Lanes are as follows: 1: BS 91, 2: BS 92, 3: BCA 15, 4: BCU 24, 5: BS 46, 6: BCA 61, and 7: 1 kb DNA Ladder, Fermentas.

Fig. 2. *In vitro* antagonism by the *W. anomalus* and *S. cerevisiae* isolates against the phytopathogenic mould *P. digitatum*. (a) Petri dishes with YPD medium inoculated with BCU 24, BS 91, BS 92 and BCA 15 are shown. (b) Petri dishes with YPD medium inoculated with BS 46 and BCA 61; (c) Petri dish with medium inoculated solely with the mould.

reductions in *P. digitatum* growth were also observed for *S. cerevisiae* BS 46 and BCA 61 isolates, but, in this case, the pathogen grew in proximity to the yeast streaks without overgrowing them for complete colonisation of the plate (Fig. 2b).

Therefore, *in vitro* fungal growth inhibition by *W. anomalus* was analysed microscopically. The growth of the *P. digitatum* tested strain was compared with the pathogen growth control. Hyphal deformities were observed when mycelial samples were removed from the parts of the PDA medium located close to the inhibition halo. The hyphae appeared wilted, folded, and coiled and had granulation (Fig. 4a) when compared with the thin, elongated, well-extended mycelial growth in the control (Fig. 4c). Moreover, microscopic analysis at 100× magnification showed collapsed hyphae with a loss of intracellular content (Fig. 4b).

3.3. *In vivo tests*

After 3 days incubation, *W. anomalus* strains BS 91, BS 92 and BCA 15 completely inhibited the growth of *P. digitatum* (0% of disease incidence) (Figs. 5 and 6) compared to the control (100% of disease incidence). Similarly, the incidence of disease on orange fruits inoculated with strain BCU 24 was significantly lower (13% infected wounds) than the control (100%), whereas strains BCA 61 and BS 46 reduced disease incidence to a lesser extent (50%).

These three strains (*W. anomalus* BCU 24 and *S. cerevisiae* BCA 61 and BS 46) were ineffective at preventing disease by the 5th day of infection (100% disease incidence).

On the opposite, *W. anomalus* BS 91 and BS 92 significantly and strongly inhibited *P. digitatum* growth up to the 10th day (disease incidence of 13 and 17%, respectively), whereas BCA 15 reduced the incidence of disease to a lesser, even if significant, extent (70%) compared to the control (100%).

W. anomalus BS 91, BS 92 and BCA 15 strains significantly reduced disease severity (Fig. 7), compared with the control (90% disease severity). In particular, BS 91 and BS 92 strains showed low disease severity (1% and 4%, respectively), up to 10 days, while BCA 15 reduced the severity of disease to a lesser extent (44%). On the opposite, BCU24 and BCA61 strains slightly reduced disease severity (64% and 70%, respectively) after 5 days, while after 10 days they were entirely ineffective (100% disease severity) and sporulation of the pathogen occurred on infected wounds.

S. cerevisiae BS 46 slightly reduced the severity of disease but only at the first stage of infection (3 days; disease severity of 18%), whereas it failed to reduce the severity of disease beginning 5 days after infection when sporulation was observed on infected wound.

4. Discussion

Oranges are one of the most popular citrus fruits in the world and are cultivated in warmer climates across the globe, such as southern Italy. The primary cause of *P. digitatum* infection in warehouses is a wound on the fruit where nutrients became available to stimulate spore germination. Wounds can be inflicted during harvest and subsequent handling, and the resulting infections are primarily controlled by the application of synthetic fungicides. However, because candied orange peel and essential oils are widely used by confectioners and bakeries, the accumulation of fungicide residues that occurs in the peel is a concern. The development of non-toxic, safer alternatives to control phytopathogenic

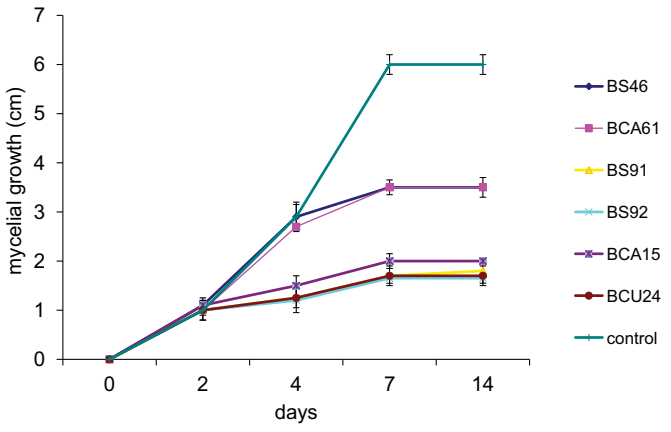

Fig. 3. Mycelial growth (cm) of the mould growing in YPD medium with the *S. cerevisiae* and *W. anomalus* strains compared to the control. The vertical bars represent the standard deviation of the mean of three replicates for each strain.

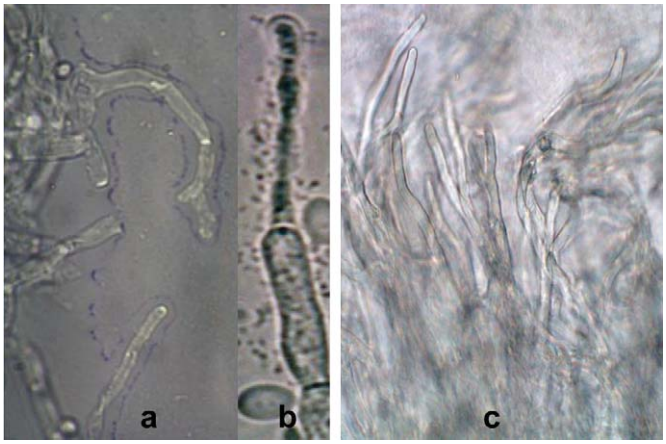

Fig. 4. Hyphal morphology of *P. digitatum* under optical microscopy. (a) A sample taken from the YPD medium inoculated with the BS 91 *W. anomalus* strain is shown at 40× magnification. (b) A sample taken from the YPD medium inoculated with the BS 91 *W. anomalus* strain is shown at 100×. (c) A sample taken from the YPD medium inoculated solely with the mould is shown at 40×.

Fig. 5. Fungicidal activity of killer *W. anomalus* strains BCA 15, BS 91 and BS 92 upon experimental infection of Tarocco orange fruits with *P. digitatum* on. Results 5 days after treatment are shown.

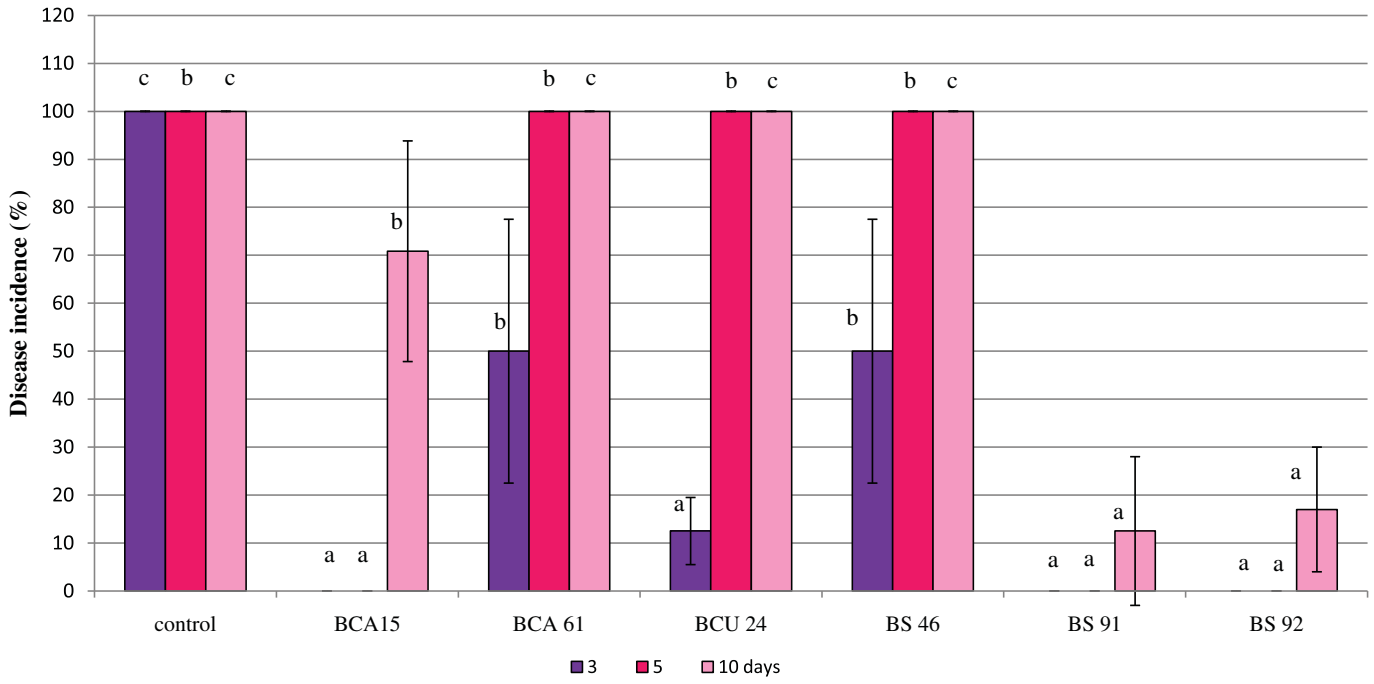

Fig. 6. Disease incidence (percentage of infected wounds) on Tarocco oranges. The oranges were treated with yeast strains *W. anomalus* BCA 15, *S. cerevisiae* BCA 61, *W. anomalus* BCU 24, *S. cerevisiae* BS 46, *W. anomalus* BS 91, or *W. anomalus* BS 92 and 3 days later inoculated with *P. digitatum*. The vertical bars represent one standard deviation of the mean of three replicates for each strain. Means for each time followed by different letters are significantly different at $P < 0.05$ according to a Student-Newman–Keul's one-way ANOVA test for ranks.

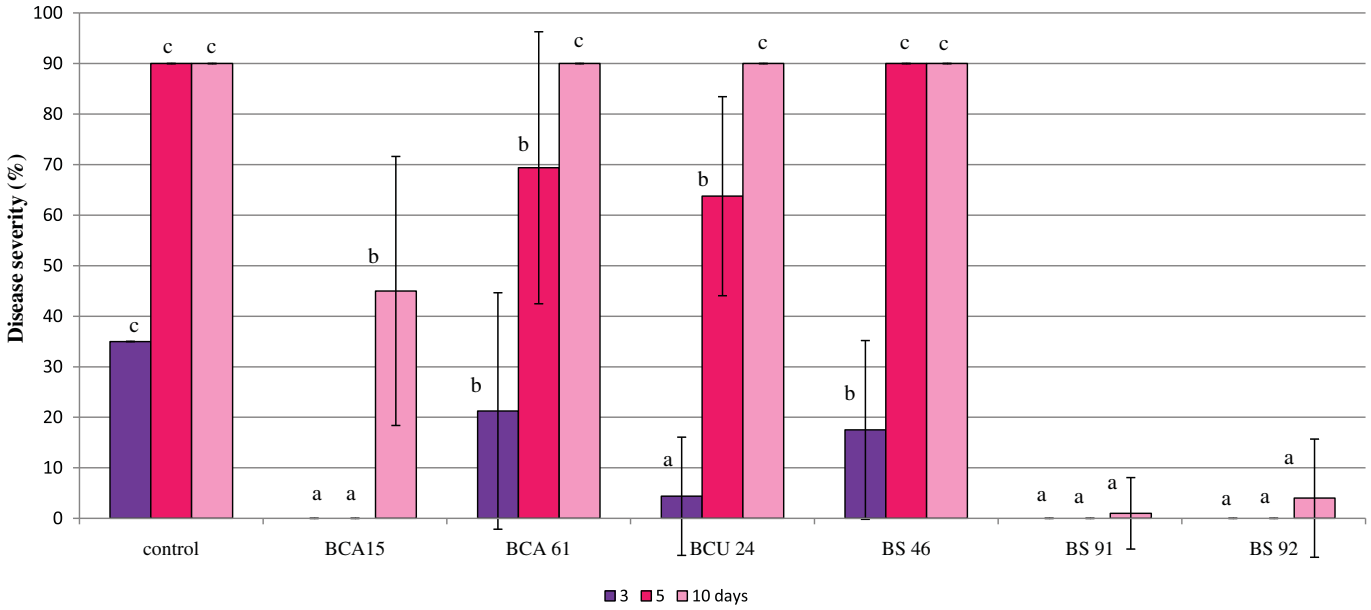

Fig. 7. Severity of disease on Tarocco oranges. Oranges were treated with the yeast strains *W. anomalus* BCA 15, *S. cerevisiae* BCA 61, *W. anomalus* BCU 24, *S. cerevisiae* BS 46, *W. anomalus* BS 91, or *W. anomalus* BS 92 and 3 days later inoculated with *P. digitatum*. The vertical bars represent the standard deviation of the mean of three replicates for each strain. Means for each time followed by different letters are significantly different at $P < 0.05$ according to a Student-Newman–Keul's one-way ANOVA test for ranks.

fungi and prevent postharvest decay is of upmost importance for citrus fruit because removal of the chemical fungicides from the peel by simple washing is very difficult.

Antifungal proteins have attracted attention because of their potential application in plant disease control. Among the antifungal proteins with known mechanisms of action, glucanases are under investigation as a promising group in providing protection against diseases.

In this work, we have investigated the biocontrol ability of killer *S. cerevisiae* and *W. anomalus* strains against *P. digitatum* infection.

The intraspecies genetic variability of the killer strains was determined by RAPD-PCR. RAPD is one of the simplest molecular typing methods, and, although problems with reproducibility may compromise the reliability of the results, it has been recommended for food yeast typing (Andrighetto et al., 2000; Gomes et al., 2000). The (GTG)5 and M13 primers were tested for their ability to differentiate killer strains of *S. cerevisiae* and *W. anomalus* isolated from the same source, olive brine. The level of polymorphism obtained with both primers was high within the two species; however, considering the patterns produced by the strains belonging to the same species, RAPD analysis with the M13 primer has been more effective in typing strains isolated from the oleic environment.

Data obtained from the present study showed that only *W. anomalus* isolates exerted various levels of antifungal activity towards the tested pathogen. The yeast inoculum in the *in vitro* assay performed at pH 4.5 caused abnormal morphological changes and the collapse of *P. digitatum* hyphae as has been previously reported by Wisniewski et al. (1991) for *Pichia guillermondii*. The effect of the *W. anomalus* killer protein on hyphae is crucial in terms of plant pathogenesis because the loss of rigidity leads to unsuccessful penetration of the plant cell wall (Mendgen et al., 1996). A slight reduction in *P. digitatum* growth was also observed with the *S. cerevisiae* isolates, but, in this case, the pathogen grew in the proximity of the yeast streaks without overgrowing the yeast biomass for complete colonisation of the plate. Because no inhibition halo was detectable, direct competition for nutrients or space with the pathogen was proposed as the mechanism of biocontrol for the *S. cerevisiae* species.

The controlled *in vivo* fruit decay investigations also demonstrated that treating pathogen-inoculated orange fruits with a drop of the *W. anomalus* strains significantly reduced the decay of orange fruits, and this effect lasted for 10 days. These results are in agreement with those obtained by Izgu et al. (2011) on lemon fruits; however, the ability to grow and survive in different, stressful environments (Walker, 2011), the high viability in both liquid and desiccated formulations for environmental biocontrol applications (Melin et al., 2007; Mokiou and Magan, 2008) and the high level of efficacy seen in this study lead us to suggest that the β-glucanase activity of *W. anomalus* could be more cheaply exploited without any expensive concentration and purification steps. Citrus fruits are the first fruit crop in international trade in terms of value, and oranges constitute the bulk of citrus fruit production. Considering that about 20–25% of the harvested fruits and vegetables are decayed by pathogens during postharvest handling, even in developed countries (Sharma et al., 2009), this potential biotechnology could protect the fruit and allow the crop to net a very high value, especially in developing countries where postharvest losses are often more severe due to inadequate storage and transportation facilities.

Furthermore, because the killer activity was seen *in vitro* at pH 4.5, and it is well known that the surface pH of the injured or wounded citrus fruit decreases (the natural pH of an orange within 2 mm deep is around 5), it could be of particular importance in providing protection towards pathogens under acidic conditions (pH 3–5.5). It is well known that changes in the pH value of citrus fruit reduces the effectiveness of traditional fungicides (López-García et al., 2003).

Studies on the efficacy and mechanism of action of new antifungal compounds are always important to provide access to several biocontrol yeast species in order to take advantage of their different biological properties or avoid resistance phenomena, thus making biological control measures more effective. Although further investigations using scaled-up conditions are necessary, the results of this study highlight the potential application of killer *W. anomalus* strains as biological agents for postharvest protection of Tarocco orange fruits against *P. digitatum*, both to increase the storage time of the fruit and to reduce the economic losses due to green mould decay.

Finally, with regard to both ecological and human health concerns, the selectivity of *W. anomalus* glucanases towards β-1,3-glucans, which are not present in the structure of mammalian cells, together with the QPS status recently obtained by EFSA for this species, makes the killer toxin of this species an attractive candidate for a future antifungal compound to protect fresh citrus fruit.

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