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Antagonistic yeasts for the biological control of *Penicillium digitatum* on lemons stored under export conditions

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ABSTRACT

The main phytopathogen that affects lemons in the postharvest stage is *Penicillium digitatum*. Currently, chemical fungicides which have been shown to have negative impacts on both, the environment and human health are used to combat fungus decay. As alternatives to the use of these fungicides killer yeasts which were previously isolated from citrus plants, showed an effective biocontrol effect against *Penicillium* spp. In order to determine the efficacy of selected killer yeasts throughout the harvest period, *in vivo* protection trials were performed at both room and low temperature; these trials were carried out under similar conditions to those applied for export to overseas markets. In addition, tolerance to commonly used postharvest fungicides was also tested. *Clavispora lusitaniae* 146 and *Pichia fermentans* 27 showed high and consistent protection during the whole harvest period in both temperature conditions. Even their protection efficiencies were superior to a commercial product based on *Candida oleophila*. Strains 146 and 27 were tolerant to fungicides; therefore; combined application of chemical agents along with the biocontrol agent could be employed in postharvest stages.

1. Introduction

Penicillium digitatum is one of the main pathogens that causes postharvest fungal diseases in lemons (Pitt & Hocking, 2009), causing important economical loses. Wounds on fruits, which can be produced by factors such as wind, hail and insects, or during the process of harvest, transport and subsequent treatments, are the entrance doors for infections. Currently, fungicides such as imazalil and thiabendazole are used to control mold decays, but the strains that are resistant to them often occur, and therefore, they lose effectiveness (Sánchez-Torres & Tuset, 2011). Furthermore, trade barriers restrict their use, since they have proven to be toxic to health and the environment (Palou et al., 2008; Tripathi & Dubey, 2004). For these reasons, efficient and safer biological alternatives have arisen.

In recent years, biological control has emerged as one of the most promising alternatives to synthetic fungicides (Wilson & Wisniewski, 1989). Yeasts have proven to be efficient biological control agents for postharvest fungal diseases (Droby et al., 2002; Liu et al., 2010; Taqarort et al., 2008; Zhang et al., 2005). Among them, there is an interesting group, known as "killer" yeasts, which have the ability to secrete protein toxins or low molecular mass glycoproteins that are lethal to other yeasts, and even to filamentous fungi and bacteria (Bajaj et al., 2013; de Lima et al., 2013; Platania et al., 2012). In our previous work, in order to look for killer yeasts as candidates for biocontrol agents, 437 epiphytic yeasts were isolated from citrus plants. 8.5% of the strains showed a killer phenotype and they were challenged regarding their *in vitro* antagonistic activity against fungal citrus pathogens. The best antagonists were selected for further *in vivo* tests. As a result of these test, two strains belonging to the genus *Pichia* and one to the genus *Wickerhamomyces* showed the significant ability to inhibit the development of *P. digitatum* in lemons at room temperature (Perez et al., 2016).

In the case of lemons for export, fruits are shipped in containers at low temperature, where they remain approximately 30–40 days in transit until they reach the destination markets. Cold storage allows the extension of their commercial shelf life and fruit preservation (Sui et al., 2015). However, loses by fungal phytopathogens are still a problem, especially for those exported lemons which, by market requirements,

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the application of fungicides is not allowed. Under the above temperature conditions, the ability of killer yeasts to colonize and control fungal postharvest decays in citrus is unknown. Furthermore, there is no study regarding the control consistency of killer agents throughout an entire lemon harvest period, which in Tucumán Province (Argentina) lasts normally 5–6 months, starting in March.

The aim of this study was to test the biological control efficiency and consistency of native killer yeasts against *P. digitatum* on lemons, under cold and room temperature storage conditions, during a complete harvest period.

2. Materials and methods

2.1. Killer yeasts and phytopathogens

The killer yeasts used in this study were previously isolated from citrus fruits from the province of Tucumán (Argentina) (Perez et al., 2016). They were identified as belonging to the species *Pichia fermentans* (27 and 28), *Wickerhamomyces anomalus* (56), *Kazachstania exigua* (120), *Candida pararugosa* (123), *Saccharomyces cerevisiae* (125 and 137), *Clavispora lusitaniae* (146), and *Candida catenulata* (M1.4 and M1.6) (Perez et al., 2016). The strain *P. digitatum* belongs to the citrus pathogen culture collections from the Phytopathology Lab of the citrus company San Miguel SA (Tucumán, Argentina).

2.2. Biocontrol tests

The efficiency of the yeasts in the protection of wounds against *P. digitatum* in lemons was studied according to Perez et al. (2016) with modifications. Sixty lemons without any postharvest treatment were used for each yeast strain. Disinfected fruits were wounded in the equatorial zone and then immersed in each yeast suspension. After 24 h of incubation at 24 °C and high humidity (about 90%), the wounded and yeast-treated fruits were immersed in 10 L of a spore suspension of *P. digitatum* (1×10^6 spores/mL) for 1 min using net bags and incubated for 5 days in the same conditions. 20 fruits were taken as a control; they were wounded and only submerged in the fungal spore suspension. Likewise, biocontrol assays at low temperature were also performed, but the lemons were stored at 7 °C for 40 days after immersion in the spore suspension of the pathogen.

Both tests were repeated during three times of the harvest: at the beginning (April), at the middle (June) and at the end (September). Normally, the harvest period in Tucumán lasts for approximately six months, from the end of March to the beginning of September. A commercially available product based on *Candida oleophila* was also used and compared with the efficacy of wild native strains. The yeast and fungal spore suspensions preparation were performed according to Perez et al. (2016).

Before packing and processing, lemons receive treatments by immersion in solutions of hypochlorite and bicarbonate, used as disinfection and healing agents respectively. In order to see if such pretreatments affected yeast control activity, an *in vivo* test at room temperature was performed using the above conditions. Fruits were immersed in a hypochlorite solution (200 ppm) and in hypochlorite (200 ppm) plus sodium bicarbonate (3%), both for 90 s. Afterwards, fruits were washed by immersion in water for 10 s and then, treated with the yeast suspension as explained above.

The data were analyzed by ANOVA, and the mean values were compared with Tukey's test at the 5% significance level. The InfoStat/L software (Di Rienzo et al., 2016) was used for the statistical analysis.

2.3. Yeasts growth at low temperatures

The yeast growth at low temperature was studied according to Robiglio et al. (2011) with modifications. Serial dilutions of each yeast were prepared from fresh cultures in YEPD and aliquots (5 μL) of each dilution were spotted on YEPD agar plates. Plates were incubated at 7–8 °C for 30 days, estimating the growth every 10 days. We evaluated whether there was growth (normal or weak) or not.

2.4. Yeasts sensitivity to fungicides

Yeasts sensitivity to fungicides was tested according to Robiglio et al. (2011) on YEPD agar added with increasing concentrations of thiabendazole (TBZ) or imazalil (IMZ). Serial dilutions of each yeast were prepared from fresh cultures in YEPD and aliquots (5 μL) of each dilution were spotted on YEPD agar plates containing either IMZ or TBZ. IMZ concentrations used were 0 ppm, 0.5 ppm, 1 ppm, 1.5 ppm, 2 ppm, 2.5 ppm, 3 ppm and, 3.5 ppm; and TBZ, 0 ppm, 1 ppm, 2.5 ppm, 5 ppm, 7.5 ppm, 8 ppm, and, 10 ppm.

After 48 h of incubation at 25 °C, yeast growth was determined.

3. Results

3.1. Biocontrol tests

To select the best suitable candidates, ten native killer strains were firstly evaluated regarding their *in vivo* biocontrol activity against *P. digitatum* at 24 °C at the beginning of the local harvest period (March). Infected and healthy lemons were counted and efficiencies regarding wound protection against *P. digitatum* were estimated for each killer. The strains *C. lusitaniae* 146, *P. fermentans* 27, and *C. catenulata* M1.4 were found to have high protection efficiencies similar to the commercial product (83.3%) used as a reference: 98.3%, 90% and 85%, respectively (Fig. 1). Therefore, such killers were selected to perform further *in vivo* tests at low (7 °C) and room (24 °C) temperatures during three times of the lemon harvest period.

3.1.1. Room temperature storage

At the beginning of the harvest, strains 146 and 27 showed higher protection efficiency than the commercial strain. The best result was shown by strain 146, which showed an efficiency of 98.3% (Fig. 2). M1.4 did not show significant difference regarding CS. In the middle of the harvest, although protection efficiencies of wild strains remained high, no significant difference was found compared to CS (Fig. 2).

At the end, the efficiency of the commercial strain fell and it was widely surpassed by the wild strains 146 and 27. Again, the strain 146 presented the best result with protection efficiency of 91.6%, compared to 42% of commercial strain (Fig. 2). Regarding strain 27, it also showed rather good protection efficiencies in the three harvest periods (90%, 83.3% and 80%) (Fig. 2). On the other hand, yeast M1.4 showed the lowest efficiencies during the whole harvest period. In all cases, efficiency in the protection depicted a decreasing trend during the development of the harvest, as it can be seen in Fig. 2.

3.1.2. Low temperature storage

At the beginning of the harvest, killer 146 showed greater protection effectiveness than the commercial strain (Fig. 3), and the results were similar to that seen for the *in vivo* test at 24 °C in the same phase. Strain 146 showed a protection efficiency of 83.3% (Figs. 3 and 4), while 27, M1.4 and commercial strain reached 75%, 78.3% and 58.3%, respectively.

In the middle of the harvest, the best performance for the commercial reference strain was obtained (76.6%). The value was higher than that observed for the wild yeasts, although, there were no significant differences according to Tukey's test ($p < 0.05$).

Likewise, as for the room temperature assay, the efficiency of the commercial strain fell again to 53.7% at the end of the harvest and it was overcome by the strains 146 and 27 during the whole storage period (Fig. 3). In this case, strains 146 and 27 showed good protection efficiency, being 83.3% after 40 days of store at 7 °C.

Although infected lemons were covered by the white mycelium of

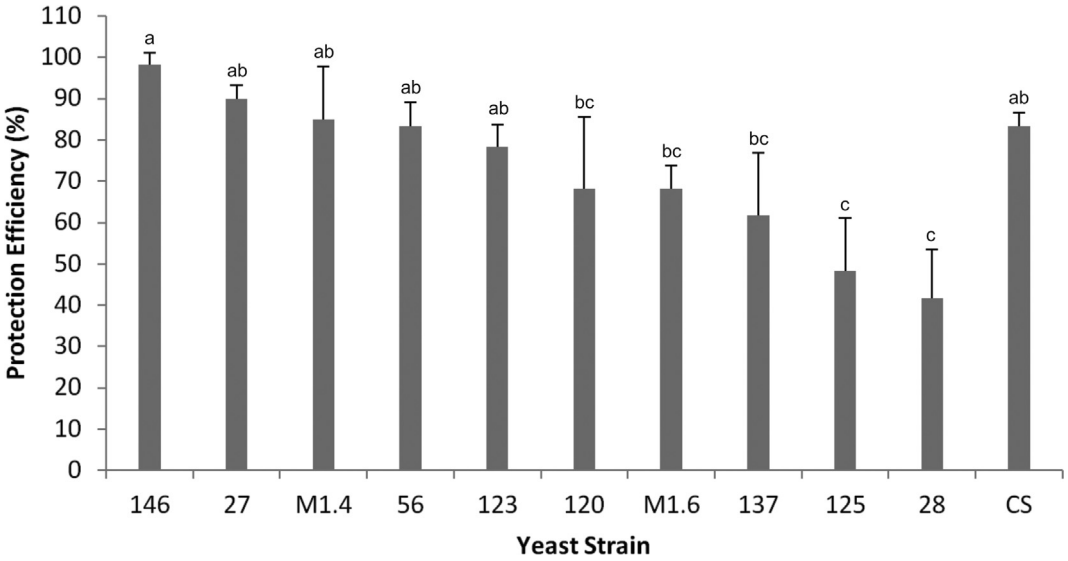

Fig. 1. Protection efficiencies of killer yeast strains against *P. digitatum* at 24 °C. 146: *C. lusitaniae*; 27: *P. fermentans*; M1.4: *C. catenulata*; 56: *W. anomalus*; 123: *C. pararugosa*; 120: *K. exigua*; M1.6: *C. catenulata*; 137: *S. cerevisiae*; 125: *S. cerevisiae*; 28: *P. fermentans*; CS: commercial product. Error bars indicate standard deviations. Mean values marked with the same lowercase letter are not significantly different according to Tukey's test ($p < 0.05$).

the phytopathogen, infected fruits did not become "green" (as for the typical infection by *P. digitatum*) but remained white, presumably because the fungus, under such temperatures, did not reach the sporulation stage (Fig. 4).

The biocontrol tests at room temperature using lemons previously treated with hypochlorite and bicarbonate were performed only with strains 27 and 146, and high protection efficiencies (96.7% and 95%) were obtained (data not shown).

3.2. Yeasts growth at low temperatures

Yeast growth at 7 °C on YPED plates for strains 27, 146 and M1.4 was followed during 30 days. As it can be seen in Fig. 5, *P. fermentans* 27 developed more rapidly, since it showed growth for all dilutions. On the other hand, *C. lusitaniae* 146 depicted growth only after 20 days. *C. catenulata* M1.4 did not grow at such conditions.

3.3. Yeasts sensitivity to fungicides

Considering the residual concentrations of fungicides that remain on the treated lemons, according to local citrus companies, the tolerance of killer yeasts to fungicides was performed. From a concentration of 1 ppm IMZ changes in the growth of strains M1.4 and 146 were seen for the 1/10,000 dilution (Fig. 6c), while the decrease in growth for strain 27 was evident from 2 ppm of the fungicide (Fig. 6e). It is important to note that in the concentration of the fungicide which is usually used commercially (2.5 ppm), the three yeast strains depicted growth, although M1.4 showed the weakest growth (Fig. 6f). For the maximum concentration of IMZ tested (3.5 ppm), killer 27 was the most tolerant (Fig. 6 h).

In the case of TBZ, all three strains were tolerant to the commercial concentration (7.5 ppm) (Fig. 7b). Growth was observed in all the strains, even for the maximum concentration of the fungicide tested (10 ppm) (Fig. 7c).

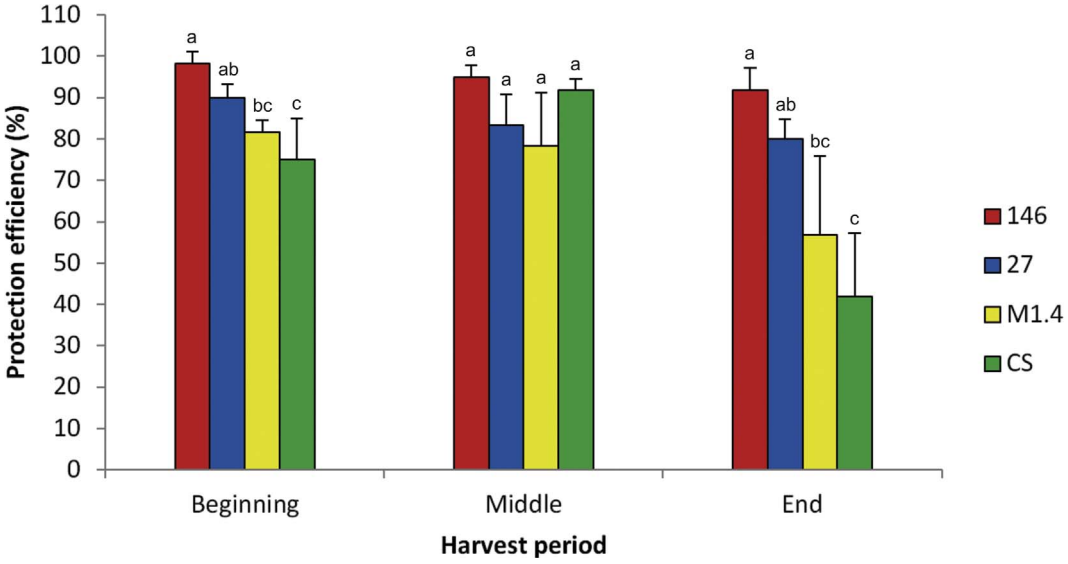

Fig. 2. Efficiencies of killer yeast strains 146, 27 and M1.4 in the protection of wounds against *P. digitatum* at 24 °C at the three harvest times. CS: commercial product. Error bars indicate standard deviations. Mean values were compared with Tukey's test ($p < 0.05$) for each of the harvest periods. Mean values marked with the same lowercase letter are not significantly different.

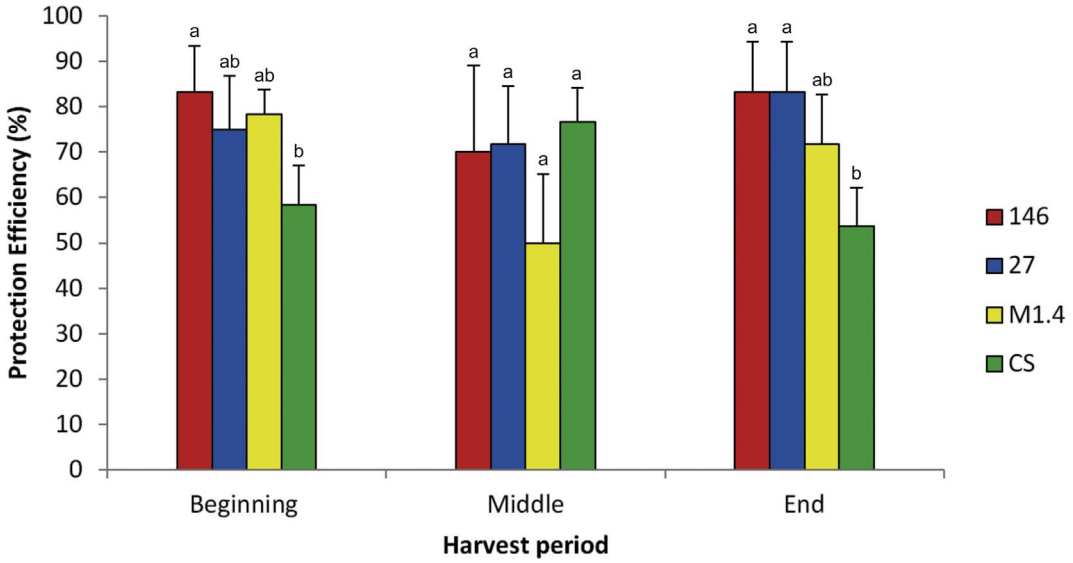

Fig. 3. Protection efficiencies of killer yeast strains 146, 27 and M1.4 against *P. digitatum* during 40 days of incubation at 7 °C for each harvest time. CS: commercial product. Error bars indicate standard deviations. Mean values were compared with Tukey's test ($p < 0.05$) for each of the harvest periods. Mean values marked with the same lowercase letter are not significantly different.

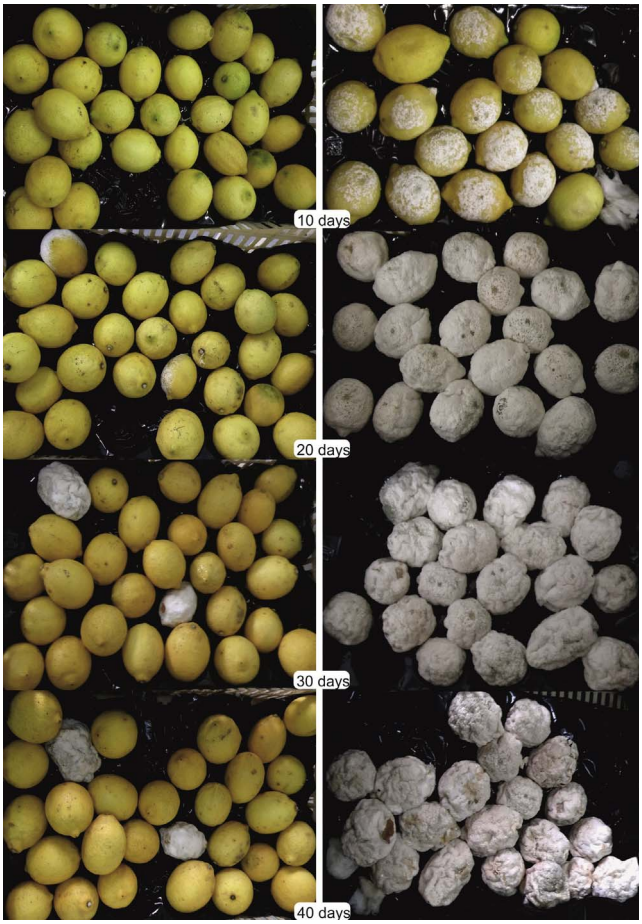

Fig. 4. Low temperature wound protection test against *P. digitatum* at the beginning of the harvest. To the left, lemons treated with *C. lusitaniae* 146. To the right, lemons used as control, only inoculated with the pathogen. Fruits were stored at 7 °C during 40 days.

4. Discussion

The export of lemons represents an economic activity of great importance in Argentina, being the European Union and Russia the main destination markets. The transport of lemons to such markets is carried out on ships at low temperatures, and in some cases, the fruits are stored for 30–40 days until they reach their destination. The loses caused by mold decays have become a serious issue, even more for the lemons destined to markets which restrict the entrance of fruits with fungicide treatments. For this reason, we have looked for biocontrol agents that act efficiently under these shipping conditions as an alternative to synthetic fungicides. Furthermore, we studied the biological control consistency of native killer yeasts against *P. digitatum* infections during the entire lemon harvest period.

In this work, it was found that *C. lusitaniae* 146 and *P. fermentans* 27 showed remarkable protection efficiencies against infections by *P. digitatum* after 40 days at 7 °C, better than the commercial product based in *C. oleophila*. It is notably the high degree of protection of killers in cold storage conditions, even for strains which were not originally isolated from a cold environment (Perez et al., 2016) where it is expected to find well adapted yeasts to low temperatures. Similar studies at low temperature storage have been done in pears and apples. Lutz et al. (2013) described strains of *Pichia membranifaciens* and *Cryptococcus victoriae* as able to reduce in a significant way the incidence and lesion diameter caused by two postharvest pathogens of pears (*Penicillium expansum* and *Botrytis cinerea*) in trials performed for 120 days at 0 ± 1 °C. Vero et al. (2013) reported a psychrotrophicn strain *Leucosporidium scottii* able to reduce the incidence of blue mold (*P. expansum*) by 88% in apples stored for 3 months at 0–1 °C. Furthermore, *Aureobasidium pullulans* and *Rhodotorula mucilaginosa* reduced the incidence of *P. expansum* to 33% and the lesion diameter in 88% in pears after 60 days of incubation in cold (−1/0 °C) (Robiglio et al., 2011).

On the other hand, for strains 146 and 27 protection efficiencies at room temperature, higher than those obtained by the commercial product, were observed at the beginning and at the end of the harvest. In similar trials at room temperature, Platania et al. (2012) found killer strains of *W. anomalus* efficient in the biocontrol of *P. digitatum* on Tarocco oranges. Meanwhile, Taqarort et al. (2008) reported fifteen yeast strains capable of reducing the incidence of *P. digitatum* by more than 50% after 7 days of incubation at 25 °C. Our trials on fruits were carried out on three stages of the lemon harvest period: beginning, middle and end, as variations in phenology of lemon fruits during harvest were expected. However, the protection activity of wild yeasts was generally consistent on harvested fruits at different stages and temperatures storages, except for strain *C. catenulata* M1.4 which

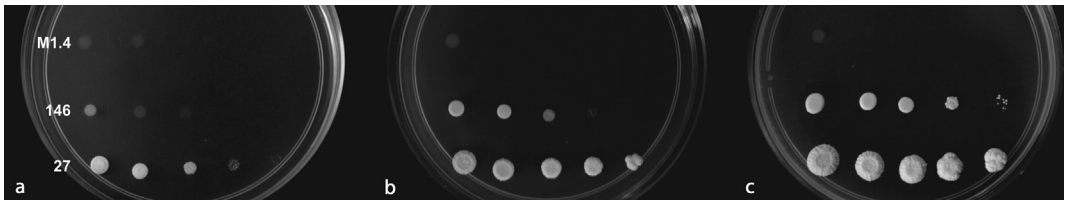

Fig. 5. Growth at low temperature (7–8 °C) of strains *C. catenulata* M1.4, *C. lusitaniae* 146 and *P. fermentans* 27. Growth after 10 (a), 20 (b) and 30 (c) days. Serial dilutions of the yeast cells were spotted on YEPD agar plates.

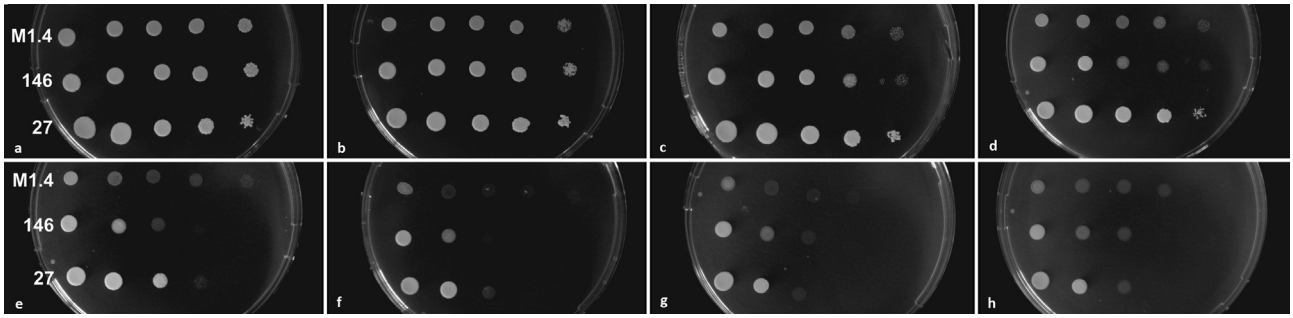

Fig. 6. Yeasts sensitivity to different concentrations of IMZ. Serial dilutions of the yeast cells were spotted on agar medium containing IMZ: 0 ppm (without fungicide) (a), 0.5 ppm (b), 1 ppm (c), 1.5 ppm (d), 2 ppm (e), 2.5 ppm (f), 3 ppm (g), 3.5 ppm (h).

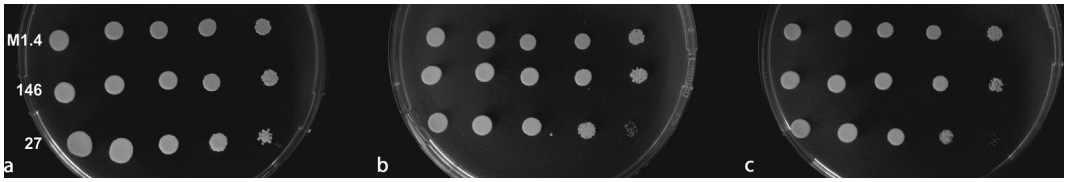

Fig. 7. Yeasts sensitivity to different concentrations of TBZ. Serial dilutions of the yeast cells were spotted on agar medium containing TBZ: 0 ppm (a), 7.5 ppm (b) and 10 ppm (c).

showed the lowest protection (50%) in the middle of the harvest at 7 °C. In the case of the commercial yeast product, it showed a rather inconsistent protection behavior at both stored temperatures, presenting its best efficient only in the middle period of the harvest (Figs. 2 and 3). Robiglio et al. (2011) found differences in the protection efficiency of yeasts against pathogens in two consecutive annual assays, possibly due to small physiological differences in the host (pears).

Strains *C. lusitaniae* 146, *P. fermentans* 27 and *C. catenulata* M1.4 were found to tolerate concentrations of fungicides used commercially (IMZ 2.5 ppm and TBZ 7.5 ppm). Therefore, the use of yeasts in combination with such chemicals would be compatible and lower doses of fungicide on fruits might be feasible. A comparable behavior was also described in *L. scottii* strain, which tolerated standard concentrations of TBZ and IMZ used in pears (Vero et al., 2013).

The results of this study indicate that *C. lusitaniae* 146 and *P. fermentans* 27 showed high levels of protection against infections caused by *P. digitatum*. Their efficiencies, both at low and room temperature, were consistent throughout the harvest period, and superior to the commercial product based on *C. catenulata*. Such a product, in addition to the yeast, has additives that improve its biocontrol action. Thus, it was shown that native yeasts have adaptive advantages over a product based on yeast originally isolated from a different source.

5. Conclusions

Yeasts 146 and 27 are excellent candidates for the formulation of a commercial biological control product for postharvest fungal diseases in lemons. Moreover because of their compatibility with IMZ and TBZ, it could be possible to reduce the fungicide doses by combined use; and consequently the impact on both the environment and human health could be reduced.

Acknowledgments

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