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Biological control of the postharvest diseases of citrus fruits using lyophilized antagonistic yeasts

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Abstract

The antagonistic yeast *Pichia guilliermondii* Wickerham, selected for its performance as a biological control agent, was used in this research work for the development of biocontrol methods of the postharvest spoilage fungi on citrus fruits stored in semi-commercial conditions. The antagonistic activity of the yeast *P. guilliermondii*, in an experimental lyophilized product obtained by fermentation system composed of two units of 75 and 500 litres, was tested *in vivo* against the plant pathogenic fungus *Penicillium digitatum* (Pers.) Sacc. in association with one synthetic fungicide (Imazalil) at low dosage and a cold treatment (4 ± 2°C) for one week on artificially inoculated citrus fruits. The results showed which the efficacy of the lyophilized product of *P. guilliermondii* against *P. digitatum* on the oranges and lemons significantly increases when the fruits were treated with imazalil and successively they were submitted a cold treatment for one week, after an artificial inoculation with the pathogen.

Keywords: Biocontrol; Cold treatment; *Penicillium digitatum*; *Pichia guilliermondii*; Pilot plant; Storage semi-commercial conditions.

Introduction

The agents of citrus fruits decay are mainly plant pathogenic or opportunistic fungi and their control is usually conducted by application of synthetic fungicides. The concern related to the toxic effect of the chemical residues on the fruits has enhanced the research on biological control of the postharvest spoilage fungi using antagonistic yeasts (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004). Several strains of the yeast *Pichia guilliermondii* Wickerham, showing antagonistic activity against the spoilage fungi, were tested for the postharvest biological control on citrus fruits (Arras *et al*., 1998; Arras *et al*., 2006). The development of the commercial products based on antagonistic yeasts requires: ability to control a broad range of pathogens under different storage conditions of the fruits, availability of inexpensive systems of multiplication on industrial scale, wide compatibility with the fungicides and easiness of the distribution (Jelusic, 2000).

The strain CTR_2 of the antagonistic yeast *P. guilliermondii*, selected for its performance as a postharvest biological control agent, was used in this work for the development of biocontrol methods of the green mould on citrus fruits in storage semi-commercial conditions. The antagonistic activity of *P. guilliermondii* in an experimental lyophilized product obtained by a pilot plant located in ENEA – Trisaia Research Centre (De Corato *et al.*, 2006; Di Bonito *et al*., 2006), was tested *in vivo* against the fungus *Penicillium digitatum* (Pers.) Sacc. on

artificially inoculated citrus fruits, in association with a synthetic fungicide (imazalil) and a cold treatment.

Materials and Methods

Lyophilized yeast production

A pure culture of the strain CTR_2, grown on the media Potato-Dextrose-Agar (PDA) in Petri dishes for 7 days at 25°C, was used for the biomass production on the liquid minimal medium composed by Yeast Extract, Saccarose, $(NH_4)_2SO_4$, $MgSO_4 \times 7H_2O$, KH_2PO_4 using a bench-top bioreactor of volume 5 litres and successively an industrial system composed of two bioreactors of volumes 75 and 500 litres. A batch culture of 50 litres obtained from the 1st bioreactor was used to inoculate a volume of 350 litres on the 2nd bioreactor. The biomass produced was evaluated at the end of the production cycle, separated from the liquid by centrifugation, washed with sterile water and lyophilized using an industrial unit. The lyophilization unit allowed the processing of 10 litres of the biomass with a cycle of freezing at – 30°C for 90 minutes followed by vacuum – heating at 30°C for 3 hours.

The original cultures of the yeast and the colonies obtained from the lyophilized product were identified by evaluation of the single carbon source metabolism using the BIOLOG YT MicroPlates™ and the software MicroLog™3 (ML3).

The lyophilized product was stored at two different temperatures (25°C and 4°C) in aluminium envelopes or bottles for 4 months. The lyophilized was suspended in a sterile water and plated on media Nutrient-Agar (NA), Tryptic-Soy-Agar (TSA) and PDA in order to detect bacterial or fungal contaminants after the production cycle and during the storage. The vitality of the lyophilized was monitored immediately after the production cycle and during the storage by evaluation of CFU on PDA before their use in a biological tests on citrus fruits.

Biological tests

The trials were carried out on orange (*Citrus sinensis* L. Osbeck) fruits cv Navel and lemon (*Citrus limon* L. Burn.) fruits cv Femminello untreated with synthetic fungicides and harvested from commercial fields located in Policoro and Montescaglioso areas (Basilicata, Southern Italy). Each fruit was washed under running tap water, surface sterilized by dipping in NaClO 0,1% for 1 minute and wounded in two points on the surface by a sterile scalpel.

The fruits wounded (60 fruits per lot) were dipped for 2 minutes in water (Co = control), imazalil 1 g l^{-1} (Th1), a suspension of the lyophilized yeast 10^7 CFU ml^{-1} (Th2) and a mixture of the lyophilized yeast 10^7 CFU ml^{-1} + imazalil 1 g l^{-1} (Th3).

Each fruit was artificially inoculated with *P. digitatum*, grown on PDA for 10 days at 25°C. The conidia of the pathogen were collected and suspended in a sterile water (10^5 conidia ml^{-1}) and each wound on the fruit was inoculated with 20 µl of a conidia suspension.

One set of the fruits tested was submitted at 5 ± 1°C for 7 days into cold room after artificial inoculation. All sets of the fruits (untreated and treated with low temperature) were incubated at 25 ± 2°C and 90 ± 3% R.H. for 4-5 days into climatic room.

The symptoms on fruits were evaluated at the end of incubation into climatic room by the index "Decay Inhibition Percentage" I% = [(Co – Th)/Co] × 100 (Co = number of infected fruits in the control; Th = number of infected fruits in the treatments). The values of the index were statistically analysed using Duncan's test (P = 0,01).

Results and Discussion

Lyophilized yeast production

The original cultures were identified as *P. guilliermondii* after incubation of BIOLOG YT MicroPlates™ at 26°C for 72 hours and the colonies obtained from lyophilized product confirmed their identity as *P. guilliermondii*. Moreover, was observed an absence of bacterial

and fungal contamination in the final product tested after the lyophilization and during the storage. Finally, no significant reduction of survival rate was observed after storage of the lyophilized product for 2 months at 4°C, a slight reduction was observed after 2 months at 25°C and after 4 months the yeast retained a good survival rate only at 4°C (Figure 1).

Biological tests

The results showed which on the orange and lemon were obtained values of decay inhibition index until to 50-58% when the fruits were treated with the lyophilized yeast and successively submitted at low temperature for one week (Figure 2). The same result was obtained when the citrus fruits were treated with a mixture of the lyophilized yeast and imazalil under both temperature conditions (Figure 2). Therefore, the efficacy of the lyophilized product of *P. guilliermondii* against *P. digitatum* increases, on the orange and lemon, when these fruits were treated with imazalil and successively submitted a cold treatment for one week after an artificial inoculation with the pathogen.

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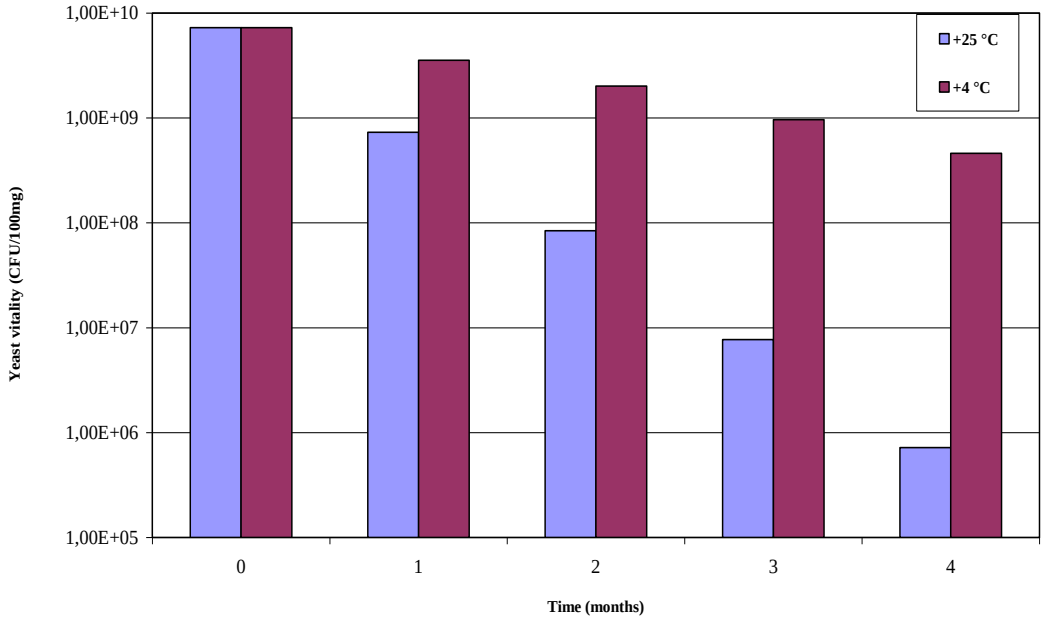

Figure 1: Yeast vitality in the lyophilized product after the production cycle (time = 0) and during the storage for 4 months at two temperatures (25°C and 4°C).

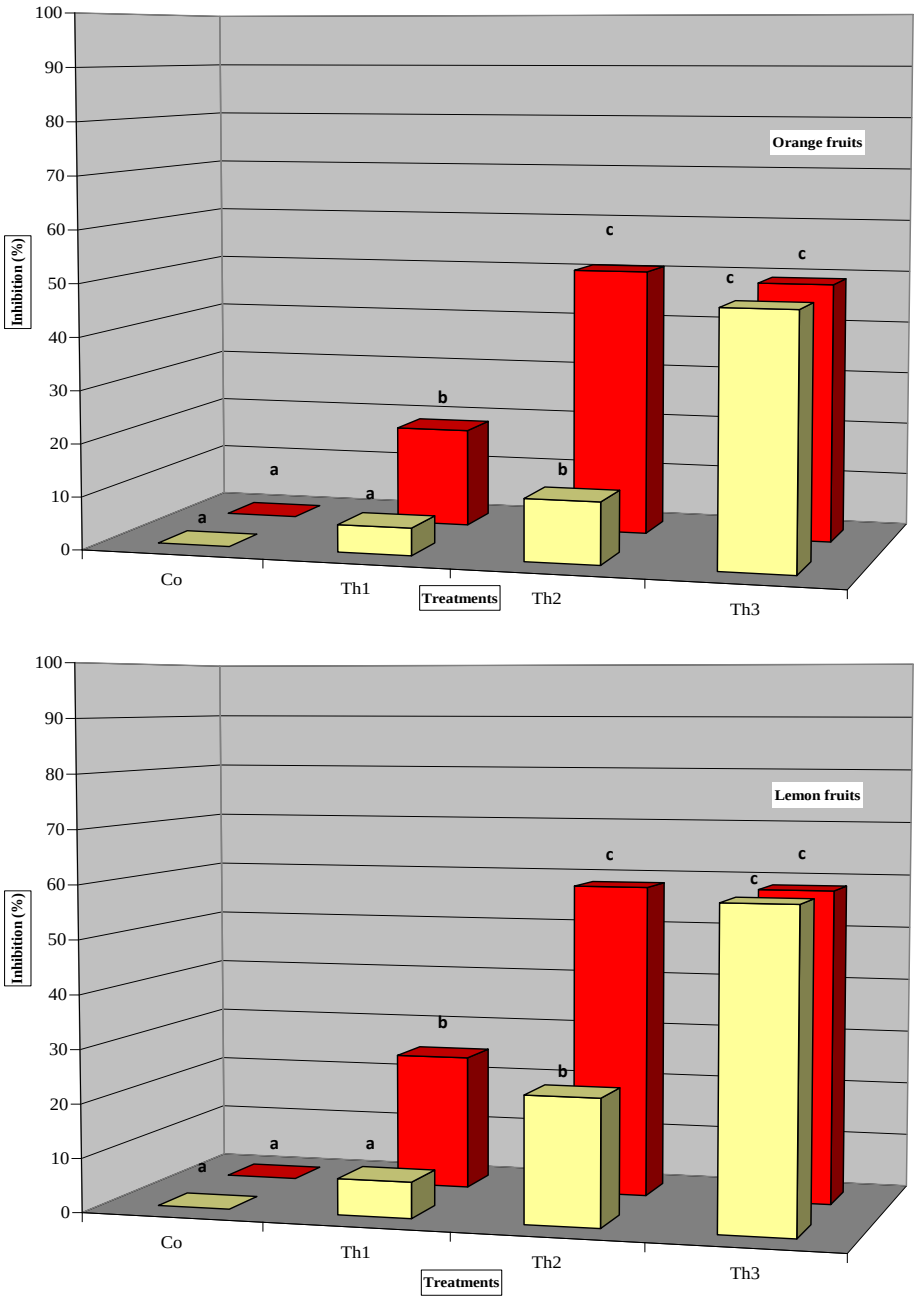

Figure 2: Decay inhibition percentage of the citrus fruits untreated (Co) and treated with imazalil (Th1), yeast (Th2) and a mixture of the yeast and imazalil (Th3). Fruits not submitted at cold treatment (yellow) and treated at 5 ± 1°C for 7 days (red) after an artificial inoculation with the pathogen. Data followed by the same letter were not statistically different at *P=0,01* according to Duncan's test.