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# Non-toxicity of some antifungal yeasts (*Pichia guilliermondii, Rhodotorula glutinis* and *Candida oleophila*) in laboratory animals*

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**Abstract -** The pathogenic characteristics of three yeasts, *Pichia guilliermondii, Candida oleophila* and *Rhodotorula glutinis* antagonistic to *Penicillium digitatum, Penicillium italicum* and *Botrytis cinerea,* were tested in guinea pigs and outbred Swiss mice. The results show that the yeasts are not pathogenic to these experimental animals. Histological examination of the liver, kidneys, lungs and spleen of the subjects inoculated with the yeasts revealed a clinical picture that was not significantly different from that of the controls.

**Keywords**: yeasts, toxicology, biological control, postharvest diseases.

## INTRODUCTION

Losses due to postharvest diseases of fruit and vegetables can reach proportions of 10-30% according to the species and kind of technology used. This has a considerable economic impact world-wide, of particular concern in Third World countries (Jeffries and Jeger, 1990). Fungi (*Penicillium digitatum, Penicillium italicum* and *Botrytis cinerea*) which attack the fruits through wounds or latent preharvest infections generally cause disease.

Synthetic chemical compounds are usually employed in the prevention of alterations but their use has determined the selection of resistant pathogen strains, and thus a reduction in their efficacy (Dave *et al*., 1990). The main problem regarding treatment in the postharvest period is the presence of toxic residues on fruit, due to the short time elapsing between treatment and consumption. Italian laws are becoming stricter in this area, and only a limited number of plant protection products are used after harvest on a few plant species (Gorini, 1990).

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This problem has led to the need to find new methods of controlling microbiological alterations by using the natural microflora present on fruits which play an important role in the control of fungal pathogens.

It is very important therefore to isolate and characterise microorganisms with antagonistic activity so that biological control can be put into practice (Droby *et al*.,1989; El Ghaouth, 1997; Lima *et al*., 1997 and 1998).

We isolated more than 900 microorganisms from a variety of fruit species and found mainly bacteria (Arras, 1993) and yeasts (Arras *et al.,* 1996b and 1997; Arras and Arru, 1997a). Yeasts are more effective than bacteria and they do not act by antibiosis, but by competing with the pathogen for space and nutrients. In contrast, the bacteria isolated synthesize antibiotics or toxic compounds which could be harmful to the consumer (Arras and Arru, 1997b). Furthermore, the biosynthesis of antibiotics could determine the appearance in humans of resistant pathogen strains.

The following are the most effective yeasts tested against *P. digitatum*, *P. italicum* and *B. cinerea* on artificially wounded citrus fruits: *Pichia guilliermondii* Wickerham (strain 5A, isolated from figs, inhibition of between 92 and 100%), *Rhodotorula glutinis* (Fres.) Harrison (strain 21A, isolated from tomatoes, 89-96% inhibition), *Candida oleophila* Montrocher (strain 13L, isolated from mandarins, 96 - 98% inhibition). The main mechanisms of action of yeasts are: rapid colonization of the fruit, wounds and fungal hyphae; degradation of hyphal cell walls; synthesizing lytic enzymes (unpublished results); elicitation of phytoalexins (scoparone and scopoletin) in the wound sites of citrus fruits (Arras *et al*., 1998a). The yeasts did not produce toxic compounds *in vitro*. Moreover, *Pichia* and *Rhodotorula* were found to be viable at low temperatures (0 °C), to grow at 5 °C, and to be resistant to fungicides: tiabendazole (TBZ) and imazalil (IMZ) up to 5 g/l, allowing integrated control (Arras *et al.,* 1996a). Interspecific activity of the yeasts on different fruit species against several pathogens was observed; they were non-toxic to the fruits (Lima *et al*., 1997).

The karyotype of these yeasts was characterized using pulsed field gel electrophoresis (PFGE). For their classification nDNA/nDNA reassociation tests and traditional taxonomic assays were performed (Arras *et al*., 1996a and 1998b).

Semicommercial trials, carried out on large quantities of fruit, confirmed the strong inhibitory activity of *Pichia guilliermondii* (Arras and Arru, 1999). Further assays are in progress in packinghouses to put the method into practice.

We again studied the antagonistic activity of the 3 yeasts and carried out tests on outbred Swiss mice and guinea pigs to acquire new data on their toxicity.

## MATERIALS AND METHODS

**Antagonistic activity of the yeasts and SEM observations.** The antagonistic activity of the yeasts *P. guilliermondii* 5A, *R. glutinis* 21A and *C. oleophila* 13L, previously described, was evaluated on artificially wounded orange fruits cv Valencia late (30 fruits, wounded in four points with a total of 120 wounds per lot) according to the methodology previously reported (Arras *et al*., 1998a). Each wound was inoculated with 20 $\mu$l of a yeast suspension ($10^8$ cells $ml^{-1}$), and, one hour later, with the pathogens (*P. digitatum*, *P. italicum* and *B. cinerea* $10^5$ conidia $ml^{-1}$).

TABLE 1 – Inhibitory activity of yeast strain against pathogens on orange "Valencia late" fruits 5 days after treatment

| Antagonist ($10^8$ cells $ml^{-1}$) | Pathogen ($10^5$ conidia $ml^{-1}$) | | |
|---|---|---|---|
| | *P. digitatum* | *P. italicum* | *B. cinerea* |
| *P. guilliermondii* 5A | 98.3 a* | 94.2a | 94.1a |
| *R. glutinis* 21A | 90.4b | 89.2a | 94.0a |
| *C. oleophila* 13L | 88.4b | 93.0a | 95.4a |
| Control (no yeast inoculation) | 0.0c | 0.0b | 0.0b |

*Data are expressed in percentages of inhibition. In each column, data followed by the same letters were not statistically different at P = 0.01 according to Duncan's test.

Wounds were assessed daily and data transformed into percentage inhibition (I%) as follows: I% = [(T-A)/T] x 100, where T = number of infected wounds in the test (*P. italicum* only); A = number of infected wounds in the yeast and pathogens treatment. The values range from 0 (no inhibition) to 100% (total inhibition) (Table 1). Data were analysed using Duncan's test ( P= 0.01).

Mechanisms of action were studied by scanning electron microscope observations.

**Toxicity tests on mice and guinea pigs.** The tests were done to determine whether the yeasts *Candida oleophyla, Rhodotorula glutinis* and *Pichia guilliermondii* had any toxic effects, using outbred Swiss mice and guinea pigs in the Experimental Zooprophylactic Institute of Sardinia (Decreto legislativo 27/1/1992 n. 116).

Yeasts were grown in NYDB (Nutrient Yeast Dextrose Broth, Difco, USA), then washed and resuspended in sterile physiological saline solution; the latter suspension was administered to 3 groups of 5 guinea pigs and 3 groups of 5 Swiss mice in the following doses: 100 $\mu$l per mouse and 500 $\mu$l per guinea pig orally (*per os*)*;* 500 $\mu$l per mouse and 1000 $\mu$l per guinea pig subcutaneously and the same doses intraperitoneally (Scalvini and Guaitani, 1994; Institute of Laboratory Animal Resource, 1995). The titres of the suspension were as follows: 7 x $10^7$ cells $ml^{-1}$ for *C. oleophila*, 1.4 x $10^8$ cells $ml^{-1}$ for *P. guilliermondii* and $10^8$ cells $ml^{-1}$ for *R. glutinis*.

Each group contained 3 control animals that was given of physiological saline solution in the same doses.

Guinea pigs and mice were examined twice a day for signs of illness or abnormal behaviour, distress or other deviations from normal**.** After one week, 3 animals from each group were euthanized and anatomo-pathological and bacteriological examinations immediately performed (Commission of European Communities, 1993). Spleen, kidney, liver and lung specimens were seeded by impression in plates of antibiotic-treated NYDA (Nutrient Yeast Dextrose Agar, Difco, USA) and left for 5 days at 20° C. For histological examination specimens

of the same organs were fixed in 70% buffered formalin, embedded in paraffin, cut in 5 $\mu$ sections using a microtome, and stained with haematoxylin-eosin by the usual methods. The remaining animals, 2 in each group, were euthanized 4 weeks after inoculation of the suspension. *Post mortem* anatomo-pathological and bacteriological examinations were carried out as previously described.

## RESULTS

**Antagonistic activity of the yeasts and SEM observations.**
The results show that overall a high percentage of inhibition was produced by the yeasts (Table 1). The isolate 5A of *Pichia guilliermondii* showed particularly good antagonistic activity against the pathogens, confirming the results of several previous tests (Arras *et al*.,1998a). SEM observations of the isolate 5A of *Pichia* revealed its ability to colonize the wounds and adhere to the hyphae of *P. digitatum* (Fig. 1).

**Anatomo-histopathological examination.**
An anatomo-pathological examination of the subjects inoculated with *C. oleophila* showed the presence of a 2-3 mm structured abscessed nodule, non attributable to the experimental infection, in a mouse inoculated *per os*. Histological examination revealed nidi of interstitial hepatitis in the liver of a guinea pig inoculated *per os*, due to excess in proteinic diet. No significant differences were found between either case and the control animals.

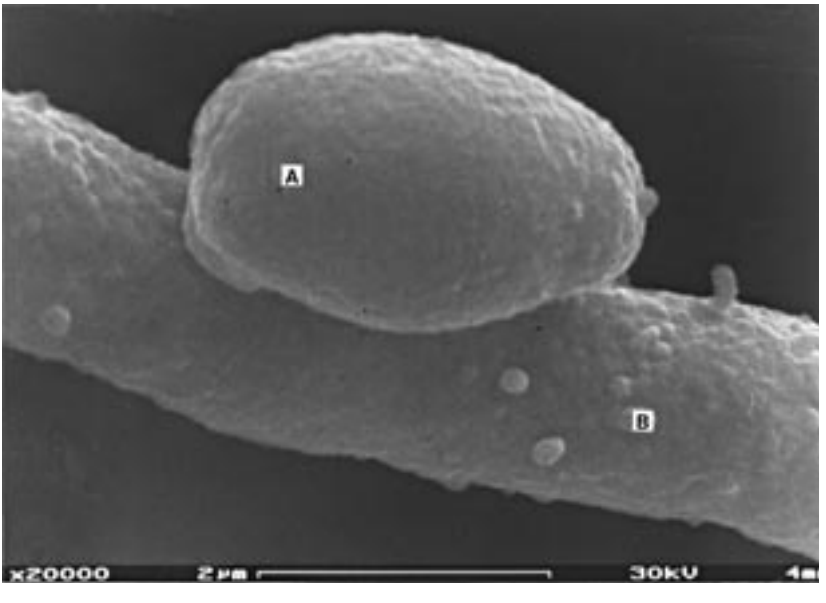


FIG. 1 – *Pichia guilliermondii* cell (A) adhering to *Penicillium digitatum* hypha (B).

With regard to the subjects inoculated with *P. guilliermondii,* at anatomopathological examination a 4 mm structured abscessed nodule was seen in the liver of a mouse inoculated *per os*. No significant lesions were revealed by histological examination, and this was confirmed by the culture, which did not show any etiology attributable to the 5A strain.

In the subjects inoculated with *R. glutinis,* mild interstitial hepatitis was found in the liver of a guinea pig inoculated subcutaneously. An ovoidal abscess, 3.2 x 2.7 cm in size, was seen in the liver of a guinea pig inoculated intraperitoneally. The culture tests of all subjects were negative in NYDA.

Anatomo-histopathological examination of the control animals revealed some cases of hepatic abscesses and interstitial hepatitis nidi, caused by inappropriate diet.

## DISCUSSION

The yeasts tested against the pathogens in our study showed interspecific antifungal activity in different species of fruits (Lima *et al*., 1997). The mechanisms of action of the yeasts on the fungi are mainly colonization of the wounds and the fungal hyphae, and inhibition of the growth of the fungi by competing for space and nutrients, without producing toxic metabolites (Arras *et al*., 1996b), unlike bacteria such as *Bacillus subtilis*, *Pseudomonas cepacia*, *Pseudomonas fluorescens* (Arras and Arru, 1997b).

The yeasts examined are able to synthesize lytic enzymes like β-1,3-glucanase (unpublished data), determining lysis of the *Botrytis* hypha cell wall, as some authors found in other isolates (Wisniewski *et al*.,1991; Castoria *et al*.,1997). In particular, *Pichia* is able to elicit phytoalexins (scoparone and scopoletin) in the wound sites of citrus fruits, thus enhancing inhibitory activity (Arras *et al*., 1998a).

No animals (either controls or inoculated subjects) showed signs of illness distress or abnormal behaviour. Anatomopathological and histological examinations of the liver, kidneys, lungs and spleen of inoculated subjects did not reveal a picture significantly different from the controls.

The results of pathogenicity tests excluded any possibility of the yeasts *C. oleophila, P. guilliermondii* and *R. glutinis* being toxic to guinea pigs and mice.

These data confirm the non-toxicity of the yeasts tested. Considering the characteristics reported in the introduction, they provide a sound basis for using the yeasts either alone as contact biologicals or combined with low concentrations of a fungicide (TBZ o IMZ, 100 ppm) to prevent postharvest microbiological alterations, as soon as authorization is obtained from the Ministry of Health.

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