Assessment of Orange Fruit Colonization by Biocontrol Yeasts

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Abstract

A scanning electron microscope study of citrus fruits was performed to assess the colonization process of antagonistic yeasts to green mould of Tarocco oranges under artificial inoculation. Yeast suspensions of *Debaryomyces hansenii* DBVPG 4025, *Pichia guilliermondii* NRRL Y 18134, *P. anomala* J121 and *Saccharomyces cerevisiae* P1.6 with or without addition of 1% $CaCl_2$ + 0.1% Tween 80 were evaluated. *Penicillium digitatum* was sprayed one hour later. Observation of fruit surface waxes revealed marked morphological diversity. Yeast cells and conidia were mostly localized on epicuticular waxes, particularly in naturally occurring small pits. The size of the wound affected the number of yeasts and conidia penetrating inside. Treatment with $CaCl_2$ did not affect the waxes and/or the behaviour of the yeasts and pathogen.

Keywords: *Penicillium digitatum*; orange; Scanning Electron Microscopy; yeast; colonization

INTRODUCTION

This note reports the results of a scanning electron microscope (SEM) study that was carried out to investigate the surface morphological features of orange (*Citrus sinensis* (L.) Osbeck) cv. Tarocco fruits collected when mature and ready for post harvesting and to detect which sites of unwounded and artificially wounded rind could be colonized by applied yeasts.

MATERIALS AND METHODS

Three types of wounds were produced around the button of separate fruits: simple puncture by conical needle (1.5 mm wide and 3 mm deep); large puncture (3 × 3 mm), produced by puncturing and simultaneously rotating the needle; and scalpel cutting (4 mm dept and 10 mm in length). Fruits, micro-organisms and incubation conditions were as described in STRANO *et al.* (2002). Yeast species and strains used were: *Debaryomyces hansenii* DBVPG 4025, *Pichia guilliermondii* NRRL Y 18134, *P. anomala* J121 and *Saccharomyces cerevisiae* P1.6. Wounded fruits were dipped in a suspension containing 10^8 cfu/ml of the yeast in water or in 1% $CaCl_2$ + 0.1% Tween 80 and air dried. After one hour fruits were sprayed with the spore suspension (10^5 conidia/ml) of *Penicillium digitatum*. Samples were incubated in the dark at 22°C with 95% R.H. Small pieces were collected by cutting thin slices through the fruit peel, promptly fixed with 3% glutaraldehyde in 0.1M phosphate buffer, pH 6.8, dehydrated in ethanol, critical point dried and observed using a SEM (Zeiss, DSM 940).

RESULTS AND DISCUSSION

The rind showed many isolated areas covered by wax deposits resembling "crusts" that very often determined cracking and lifting from the surface, leaving the cuticolar layer uncovered. Converging and buckling upward of the wax platelets were also observed (Figure 1). Soon after the application, yeast cells were detected in differently sized areas of the rind. These microorganisms were constantly stuck

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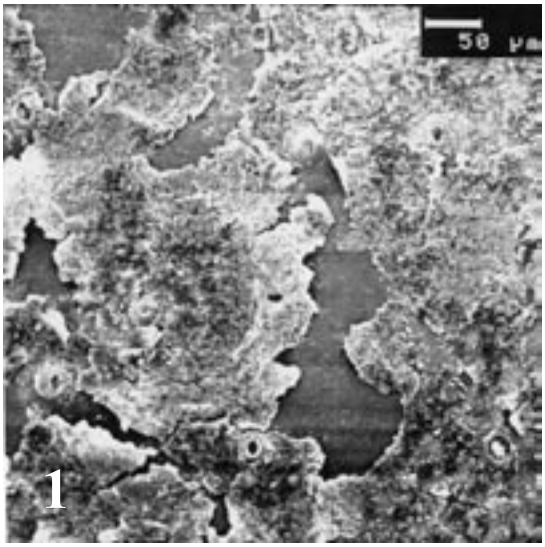

Figure 1. Marked formation of epicuticolar waxes in "crusts" in mature Tarocco fruit (bar = 50 μm)

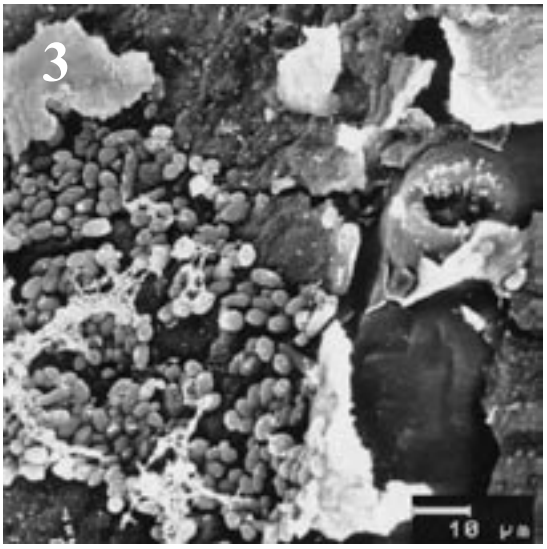

Figure 3. *P. anomala* cells occurring on epicuticolar waxes, but never on smooth cuticle (after 6 days) (bar = 10 μm)

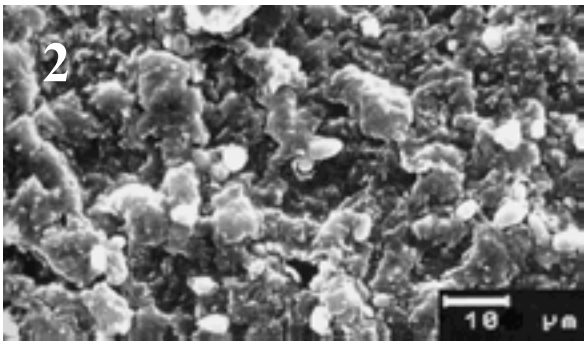

Figure 2. *D. hansenii* cells settle bridled on rough areas (after 5 days) (bar = 10 μm)

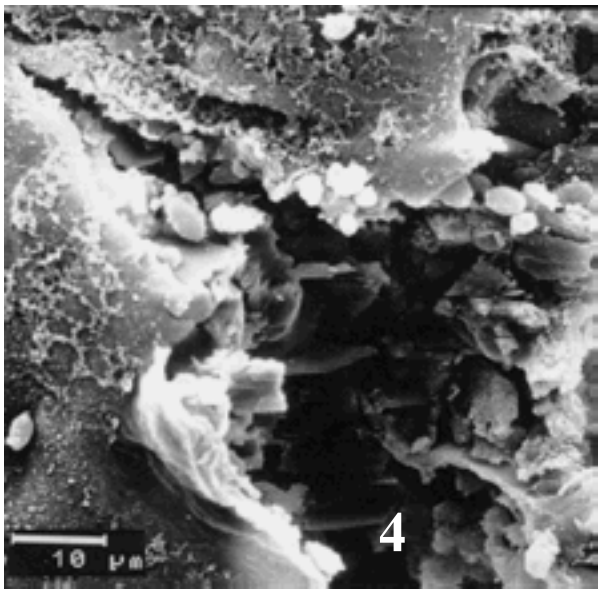

Figure 4. Yeast cells located solely on the uppermost part of the wound (soon after application) (bar = 10 μm)

on the rind epicuticular waxes, mainly in depressed, wrinkled, split, and rough areas (Figures 2 and 3). Observations revealed that yeast distribution on fruit rind was not uniform, and the same fruit showed areas containing large number of these microorganisms close to other areas completely free of them. Yeasts were also found sticking onto different material, including fungal hyphae. Very occasionally the small artificially produced punctures presented yeast cells only in the uppermost part of the wound. This finding was also detected on heavily colonized fruits and, as expected, up to six days after the treatments (Figure 4). Similarly, sprayed penicillium conidia were not uniformly spread over the rind, and this was probably due to the crusts and debris on the fruit surface. Only very few conidia were found on the uppermost side of the small wounds (Figure 5). Three days after treatment, hyphae had grown on and around epicuticular waxes, but very few were found close to one side of the wounds (Figure 6). At the same time, the hyphae produced by the conidia that had remained on the irregular waxes, started to colonize the smooth cuticular layer. The fruit colonization by yeasts and pathogen was not modified when both micro-organisms were applied to the same fruit. Micro-organisms tended to reach and colonize the wider wounds, always in the

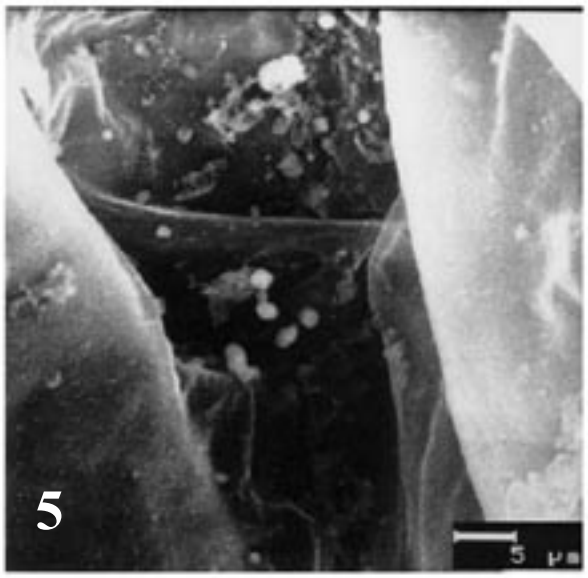

Figure 5. Few *P. digitatum* conidia on the external side of the scalpel-cut wound soon after application (bar = 5 μm)

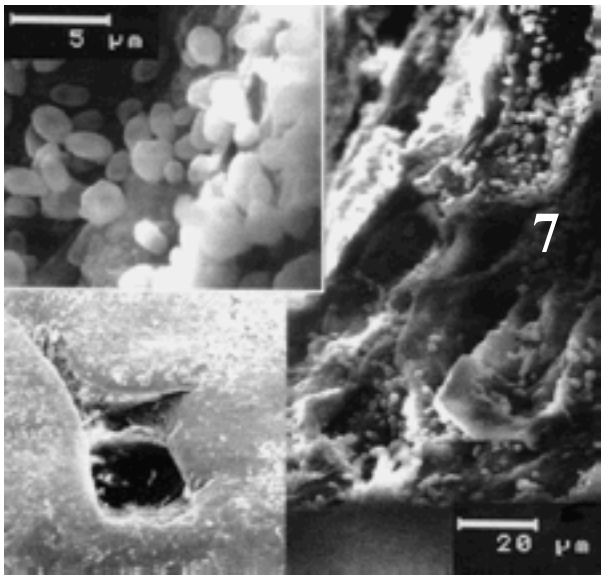

Figure 7. Large puncture and enlargement showing large amount of conidia and yeast cells one day after treatment (bars = 5; 20 and 25 μm)

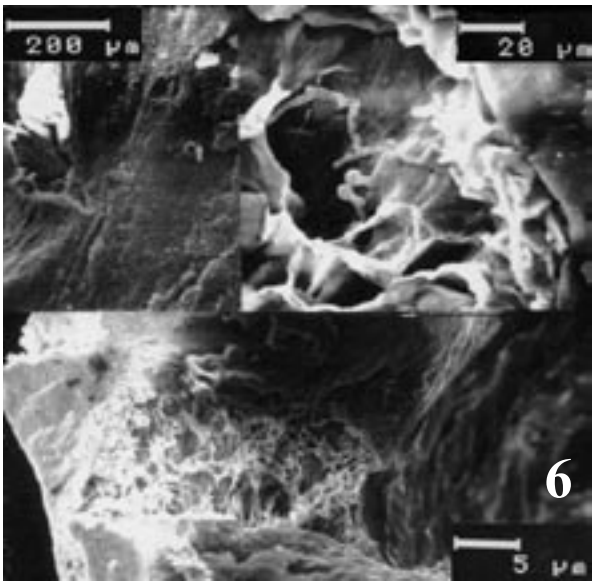

Figure 6. Large puncture on the peel and germinated hyphae inside the uppermost part of the wound three days after inoculation (bars = 5; 20 and 200 μm)

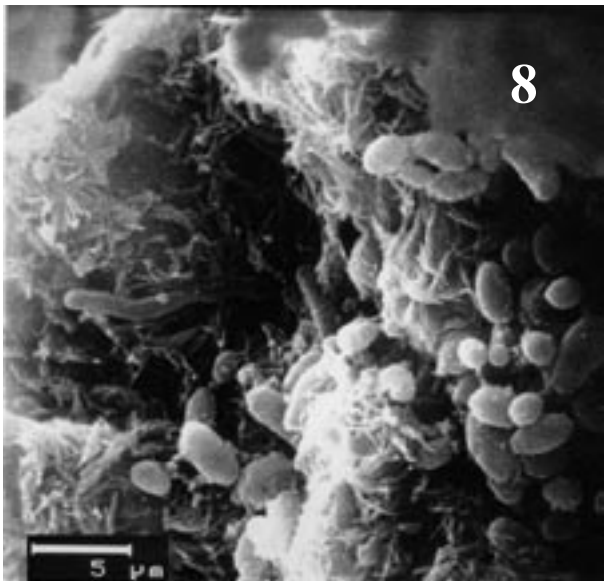

Figure 8. Uppermost side of wall in a scalpel cut wound one day after application. Germinated hyphae, conidia and yeast cells can be observed (bar = 5 μm)

most exposed site, more than the smallest wounds, whereas the latter were scarsely, or not reached, by microorganisms. Conidia started to germinate after over 24 hours from inoculation (Figure 7). The first germinated hyphae, among yeasts, were observed inside the scalpel wounds 24 hours after inoculation (Figure 8). No different behaviour was observed among the four yeast species.

The rind morphological features observed were similar to those previously reported on mature orange fruits (ALBRIGO 1972; FREEMANN *et al.* 1979). Wax wrinkling and cracking started to develop late in the summer and continued as fruit ripened, exposing the underlying smooth cuticular layer. It has been reported that this also occurred in mandarin fruits (SALA 2000). The spreading pattern of yeast cells and *Penicillium* conidia observed on the fruit is caused by the drying of the medium solution utilized that leaves the particles to settle bridled on the more irregular fruit surface areas. According to the observations, the cells

show a better affinity versus the waxes than cuticular layer. SEM investigation allowed us to ascertain the following: (i) the amount of yeast cells entrapped on the fruit is related to rind morphology around the oil glands. Therefore it is affected by citrus species and cultivar features and may modify the treatment effectiveness; (ii) epicuticular waxes are the main, if not the only, sticking site of micro-organisms; (iii) the presence of yeast on fruit is always erratic, very far to produce a continuous protection layer; (iv) the size of the wound affects yeast and conidia penetration and subsequent colonization. The investigation technique was sample destructive and therefore, colonization process of microorganisms on the peel and inside the wounds could not be monitored.

The mechanism of biological control by yeasts is still unclear and different modes of action have been described on the basis of specific tests. It was observed that *Candida oleofila* rapidly colonized wide wounds of apple fruits and presented a high title (MERCIER & WILSON 1994). *C. famata* colonized the wounds of orange fruits presenting competition for *P. digitatum* mycelium (ARRAS 1996). The activity of a *P. guilliermondii* isolate versus *P. italicum* was related to competition for space and nutrients (ARRAS *et al.* 1998). The very low likelihood of yeasts and conidia in reaching fruit wounds indicated that the noteworthy biocontrol activity observed in our experimental conditions could be attributed to mechanisms other than mere competition for space and nutrients.

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