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Evaluation of population density of *Pichia anomala* strain K and *Candida oleophila* strain O and their protection against *Penicillium expansum* Link on apples

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Abstract: The application of *Pichia anomala* (strain K) and *Candida oleophila* (strain O) in practical conditions had previously offered high protection against *Penicillium expansum* only when an antagonist density of 10^4 cfu/cm^2 was detected on the intact surface of fruit. According to this, the initial concentration of application in controlled conditions for each antagonistic yeast was defined in order to obtain this level. Whatever the strain studied, the initial application concentration of 10^8 cfu/ml allowed to obtain a density of yeast on the intact surface of apple higher than 10^4 cfu/cm^2 after 24 hours, whereas a density lower than 10^4 cfu/cm^2 was observed when an initial application concentration of 10^5 cfu/ml on apple surface is used. Three scenarios reflecting the practical conditions of biological application were then tested with different periods of incubation between biological treatment, wounding of fruit surface and pathogen inoculation. Whatever the strain, the initial application concentration of 10^8 cfu/ml allowed to obtain the highest densities of yeast's per wound and the highest protective levels in comparison to the use of initial application concentration of 10^5 cfu/ml. The protective levels were positively correlated with the density of yeast determined in the wounded sites. Furthermore, these protective levels were influenced by wound wetness. The protective levels registered on wet wounds ranged between 52 and 100% while those observed on dry wounds did not exceed 30%. Whatever the scenario used the yeast density per wound and the protective levels induced by strain O were higher than those observed for strain K.

Key words: Biological control, *Candida oleophila* strain O, *Pichia anomala* strain K, apple, *P. expansum* Link, density of yeast by wound, protective levels

Introduction

Pichia anomala (strain K) and *Candida oleophila* (strain O) were previously selected for their high antagonistic activity (even after their mass production and drying) against *Botrytis cinerea*, *Penicillium expansum*, two wound pathogens causing economically important losses of Golden Delicious apples on storage rooms (Jijakli et al., 1999). The use of antagonistic yeasts in orchard conditions in order to control post-harvest diseases is still very limited, while their application just before harvest should allow the precolonisation of the fruit surface before wounding that occur during harvest and thus before the deposit of conidia of most wound pathogens (Ippolito & Nigro, 2000). Trials efficacy carried out under practical conditions with *P. anomala* strain K on apples showed that the high protective levels were always associated with a population density of strain K superior to 10^4 cfu/cm^2 of surface fruit just after harvest (Jijakli et al., 2002).

In this context, our objectives consisted of 1) evaluating the antagonistic population density on fruit surface and 2) assessing the efficacy of both antagonistic yeasts in relation to their population densities in three scenarios reflecting practical conditions.

Materials and methods

Measurement of antagonistic population density on intact fruit surface

Apples fruits 'Golden delicious' were disinfected by soaking during two minutes in sodium hypochlorite solution (10%) then rinsed twice in sterile distilled water. After drying for one hour, fruits were treated by various concentrations of *C. oleophila* (strain O) or *Pichia anomala* (strain K) (10^5, 10^6, 10^7 and 10^8 cfu/ml) by dipping in a suspension of 350 ml during 2 minutes. Treated fruits were kept in ambient temperature. After 24 hours, the recovery of yeast on intact fruit surface was performed. Apples were introduced into plastic bags of 3000 ml. Each bag contained 4 apples and 1000 ml of washing KBPT buffer [6.8 g of KH2PO4 (0.05 M), 8,71 K2HPO4 (0.05 M) and 500 µl of Tween 80] (one plastic bag per treatment). Plastic bags were centrifuged during 20 minutes at 120 rounds/minute in order to yield the population of yeast strains from apple surface. After agitation, washing waters were diluted before plating onto Potato Dextrose Agar (PDA) medium. Three plates were used per dilution and per yeast concentration. The enumeration of cfu was carried out after 72 hours of incubation at 25°C. The mean surface of the apples was evaluated by means of as previously described (De Clercq et al., 2003) linear relationship between the surface of apples and their volume measured by water displacement [Surface (cm^2) = 0.488 x volume displaced water (ml) + 66.1 with r = 0.99)]. Three trials were carried out over time and each treatment contained 3 replicates per trial.

Efficacy assessment of both antagonistic yeasts in relation to their population densities in three scenarios reflecting practical conditions

Ten-day-old colony of *P. expansum* grown on Potato Dextrose Agar (PDA) was used to obtain spore suspensions in sterile distilled water containing 0.05% of Tween 20 per litre. Spore suspensions were adjusted to 1 x 10^5 spores/ml using a Bürker cell. A volume of 10 µl of this suspension was used to infect wounded apple fruits.

Disinfected fruits were handled by soaking in a suspension of strain K or strain O at concentration of 10^5 or 10^8 cfu/ml during two minutes then wounded (4 wounds by fruit) at the equatorial site. Each wound was 2 to 3 mm in diameter and 4 mm in the deep. The biological treatment sequence of fruits was organized following three scenarios: 1) wounds were imposed directly after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were made 24 hours after wounding; 2) wounds were realized 24 hours after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were carried out immediately after wounding and 3) wounds were realized 24 hours after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were made 24 hours after wounding.

Whatever the scenario and the treatment combination strain-concentration studied, a set of 4 apples (4 wounds/ apple) was used to analyze the population density in wounds on fruits. The wounded sites were taken by means of a scalpel. Each site was separately placed in a solution of 10 ml of KBPT 'washing' buffer and crushed during 1 minute and 30 seconds by Ultra-thurrax T25. Hundred µl of each treatment wound were plated onto Petri dishes (3 replicates/wound site) containing PDA. Petri dishes were incubated at 25°C during 72 hours and yeast colonies of white colour were counted. The statistical analysis was made by ANOVA and Duncan's test ($P \leq 0.05$) was used to distinguish the mean values.

A set of 20 apples (4 wounds per apple, 4 apples per treatment strain-concentration and 4 apples for the control) was also used for each yeast strain to measure the efficacy against *P. expansum*. After inoculation of the wounded sites, fruits were kept at 20°C during 7 to 11 days. A percentage of protection was calculated based on comparison of lesion diameter of

treated wounded sites and untreated wounded sites but inoculated. Four replicates were carried out per trial. The trial was not repeated.

Results and discussion

Evaluation of population density of both antagonistic yeasts on apples surface fruits

The recovery of yeast on fruit surface was made 24 hours after their application to estimate the population density of yeast per cm^2 of apple surface for each initial yeast concentration of application. The initial concentrations of 10^7 and 10^8 cfu/ml allowed obtaining a density of strain O superior to 10^4 cfu/cm^2 of apple surface. Whereas for strain K, only the initial concentration of 10^8 cfu/ml allowed to reach a similar population density.

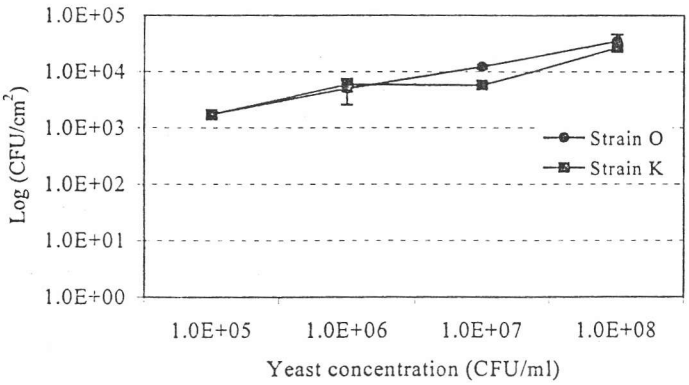

Figure 1. Population density of both yeast strains K and O (cfu/cm^2) on intact surface of apple fruit in relation to their initial concentrations of application (cfu/ml) after 24 hours. Points represent the means values calculated from 2 trials carried over time. Each trial contains 3 replicates per treatment. Bars represent the standard errors corresponding to their respective means.

Evaluation of population density of yeast strains K and O and their efficacy in three scenarios reflecting practical conditions

Two initial concentrations, 10^8 cfu/ml (allowing having a yeast density higher than 10^4 cfu/cm^2 on the intact surface of apple fruit) and 10^5 cfu/ml (allowing having a yeast density lower than 10^4 cfu/cm^2 on intact surface of apple surface fruit) were used in theses experiments.

Scenario I

In the case of wounds made directly after the biocontrol treatment, the recovery of each antagonistic strain in wounded apples showed that the highest levels of populations were detected for strain O at both concentrations of application in comparison with strain K (Fig.2). The highest protective level was attributed to the initial concentration of application of 10^8 cfu/ml whatever the yeast strain. Strain O offered 83.9% and 100% of protection respectively for treatments at 10^5 and 10^8 cfu/ml, while the protective levels due to strain K were 52.5% and 71.1% respectively for the same treatments (Figure 2).

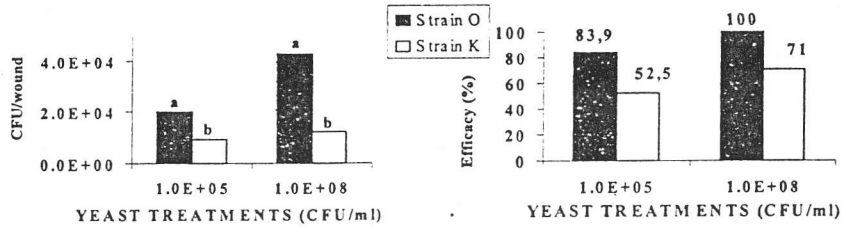

Figure 2. Population densities of two yeast strains and their efficacy against *P. expansum* (10^5 conidia/ml) in wounded sites realized directly after biological treatment of apples by soaking in two initial concentrations of application 10^5 and 10^8 cfu/ml. The recovery of yeast in wounded sites or the pathogen inoculation were made 24 hours after wounding.

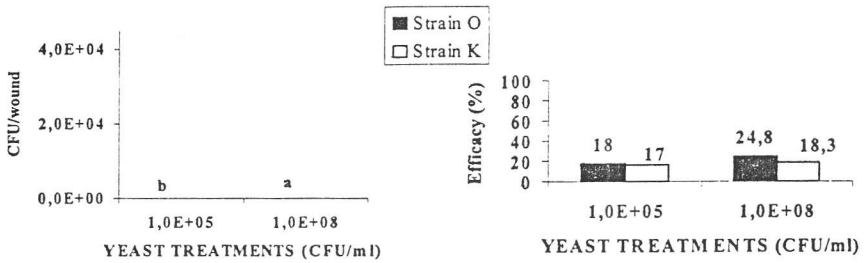

Figure 3. Population densities of two yeast strains and their efficacy against *P. expansum* (10^5 spores/ml) on wounded sites realized 24 hours after biocontrol treatment. The recovery of yeast and the pathogenic inoculation were made immediately after wounding.

Scenario II

The recovery of both strains in wounded sites (applied 24 hours before the creation of wounds) showed the total absence of colonies at treatment of 10^5 cfu/ml (Figure 3). While at treatment 10^8 cfu/ml, the average number of colony by wound was significantly higher and varies between 90 and 120 cfu/wound for both yeast. Whatever the initial concentration of application of each antagonistic strain, the efficacy reported against *P. expansum* did not exceed 30%.

Scenario III

In this scenario, population densities in wounded fruits for both initial concentrations of application were higher with strain O than with strain K. Whatever the strain, an initial concentration of application of 10^8 cfu/ml gave an average number of colonies significantly higher than the number obtained with treatment at 10^5 cfu/ml (Figure 4). The protective levels offered by strain O (*C. oleophila*) against the blue decay were 72.2% and 59.5% respectively for treatments at 10^8 and 10^5 cfu/ml. For strain K, these levels reached 62.1% and 42.8% respectively for both treatments. The higher protective levels observed in scenario I and III were associated with a density of yeast on apple exceeding 10^4 cfu/cm^2 of intact apple surface due to an initial concentration of application of 10^8 cfu/ml whatever the antagonistic yeast strain. This was not the case in scenario II where population densities at the wound site were very low whatever the initial concentration of yeast application.

The scenario III is reflecting the most frequent practical conditions in case of pre-harvest application of yeast one or two days before harvesting and handling. These manipulations created wounds before storage. The protective levels were positively correlated with the population densities whatever the scenario and the yeast [Strain O: 11,20X + 4,77 (r = 0,93) and strain K: 17, 13X – 0,21 (r = 0,96)].

Mercier & Wilson (1995) studied the effect of the humidity on the growth of *C. oleophila* and the protective level against *B. cinerea* on apples. The population of this strain increased quickly when water was periodically applied at the wounded sites of the fruit. These authors supposed that the humidity could be a factor limiting the development of antagonistic microorganisms. Our scenarios reflected also different situations of humidity at the wound site. The protective levels registered on wet wounds ranged between 52 and 100% (scenario I) while those observed on dry wounds did not exceed 30% (scenario II). Our results suggest that the humidity controlled the population densities of yeasts and their protective levels.

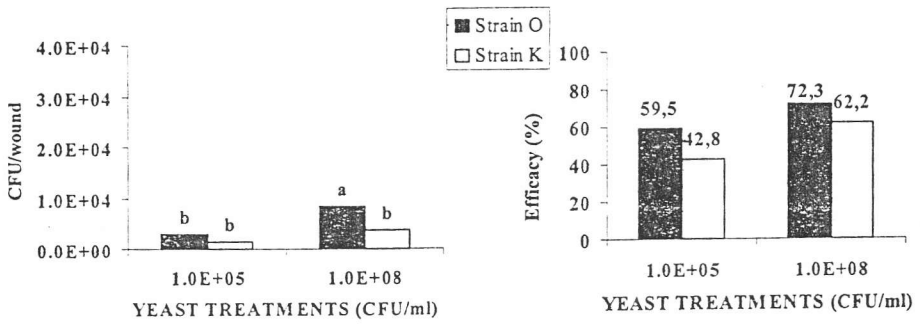

Figure 4. Population densities of both yeast strains and their efficacy against *P. expansum* (10^5 conodia/ml) on wounded sites realized 24 hours after biological treatment. The recovery and pathogenic inoculation were realized 24 hours after wounding.

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