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BIOLOGICAL CONTROL OF Botrytis cinerea AND Penicillium sp. ON POST-HARVEST APPLES BY TWO ANTAGONISTIC YEASTS

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SUMMARY.

Two yeasts, Pichia anomala (strain K) and Candida sake (strain O) were selected for their protective activity against B. cinerea and Penicillium sp. on wounded Golden Delicious, among 329 epiphytic microorganisms isolated from apple fruit surface. Treatment of wounded sites on apple fruit with 50 µl of yeast suspension (10^7 cfu/ml) was sufficient to inhibit rot development induced by 50 µl (10^6 spores/ml) of either B. cinerea or Penicillium sp. The antagonists were active at 5° C and 25 °C. Similar results were obtained with the application of K or O strains produced in fermentor. Ratio between inoculum concentration of antagonists and pathogens, and time elapsing between application of the protective yeast, and inoculation of the pathogen, appeared to be the two major factors in controlling the level of protection. The density of yeast population and the level of protection increased with the incubation time of apples between yeast treatment and inoculation by B. cinerea, indicating that colonization is a prerequisite to disease control. Whatever the incubation time between yeast application and pathogen inoculation, and whatever the level of protection, in situ spore germination of B. cinerea was markedly reduced on K or O-treated wounded sites even when pathogen and yeast were applied simultaneously with no subsequent protection. This suggested that other factor(s) than inhibition of germination may interact in biocontrol effectiveness.

INTRODUCTION.

Control measures applicable to postharvest diseases include fungicide application, but the development of resistant fungal strains and the toxicological problems linked to indiscriminate use of chemical pesticides, explain the growing interest for biological control. Because of the regulation of environmental parameters during storage, and the high value of postharvest products, such alternative approach seems to be realistic, so that progress has been achieved in the

biocontrol of fruits postharvest diseases (Janisiewicz, 1991 ; Wilson & Wisniewsky, 1989).
Our objectives were the biological and technological assessment of microorganisms for their protective properties against postharvest diseases of apples. Major experimental parameters controlling protection level were identified and the activity of biological control agents (BCA) was studied, in relation with colonization of infection sites. A short report of these results was presented (Jijakli & Lepoivre, in press).

MATERIAL AND METHODS.

Source of fruits.

Apple fruits (cultivar Golden Delicious) used for bioassays of potential antagonistic microorganisms, were harvested and maintained in a cold room (3° C) at the "Station de Culture Fruitière et Maraîchère" (Gembloux). Further experiments with selected protective strains were carried out with apples harvested at Fouron-le-Comte, and stored in a commercially operated room with controlled atmosphere.

Antagonistic microorganisms and pathogen strains.

The potential epiphytic antagonists (yeasts and bacteria) were isolated from the surface of apple fruits (cv Golden Delicious and Jonagold). The fruits were washed in 200 ml of sterile 0.1 % pepton-water on a rotary shaker at 100 rpm. Washes were diluted, plated on Potato Dextrose Agar (PDA), and incubated for at least 48 hours at 25° C. Colonies differing in appearance on isolation plates were streaked onto PDA, purified and stored at 4° C.
Strains of *Botrytis cinerea* and *Penicillium* sp. were isolated from strawberry and apple respectively and stored onto PDA at 4° C in the dark. Pathogens were cultivated on PDA at 25° C for 12-14 days under a photoperiod of 16 hours fluorescent light. Spore suspensions were prepared by scraping the surface of the colonies recovered with 0.05 % Tween 20 in sterile water and diluting them to the required concentrations, as determined with a Bürker's cell.

Screening for antagonistic properties.

Potentially antagonistic microorganisms were successively plated on PDA with an incubation time of 24 hours. The fourth generation was diluted by transferring a loopful into sterile 0.1 % pepton-water, to give suspensions of 0.5 A at 0.595 nm (about 10^7-10^9 cfu/ml), according to the method of Janisiewicz (1987).
Apples were surface-disinfected with sodium hypoclorite (10 % of commercial product for 2 minutes), and rinsed under sterile water. Fruits were wounded with a cork-borer (2 wounds of 6 mm diameter and 3 mm deep at the equator of each apple), treated with 50 μl of antagonist suspension (or water for control) and incubated on wet filter paper in closed plastic boxes at 25° C

in the dark. After 24 h of incubation, the wounded sites were inoculated by depositing 50 µl of *B. cinerea* or *Penicillium* sp. preparations (10^6 spores/ml).
Inoculated fruits were maintained in plastic boxes for 5 days at 25° C, or 15 days at 5° C, before measuring diameters of decay lesions. This experiment was repeated at least twice.

Technological screening.

The *in vivo* selected yeast strains were tested for their technological properties. They were produced in fermentor by CWBI (Centre Wallon de Bio-Industrie) at the University of Liège and fermentation products were dried by lyophilisation. The protective activity of microbial preparations grown in Petri dish or in fermentor (media 863) was compared.
Surface-sterilized wounded apples were inoculated with 50 µl of antagonist suspension (10^7 cfu/ml in sterile 0.1 % pepton-water). Final dilutions of strains grown on Petri dish were adjusted according to a standard curve, using a spectrophotometer, whereas suspensions of the fermented strains were prepared according to the concentration of the fermentation product expressed as cfu/g of dried final product. One day after treatment, the fruits were inoculated with 50 µl of spore suspension of *Botrytis cinerea* or *Penicillium* sp. (10^6 spores/ml) and incubated at 25° C for 5 days. This experiment was repeated twice.

Major parameters controlling the level of protection.

To determine the effect of incubation time between yeast treatment and inoculation by the pathogen, Golden Delicious apples were inoculated with 50 µl of conidial suspensions of *B. cinerea* or *Penicillium* sp. at 10^6 spores/ml at 0, 12, 24 and 48 hours after pretreatment with 50 µl of antagonistic strain suspensions (10^7 cfu/ml)
The second investigated parameter was the respective concentrations of yeast and pathogen. Suspensions of antagonists were serially diluted from 10^8 to 10^5 cfu/ml and 50 µl of each dilution were applied onto wounded sites. The apple fruits were incubated for 24 hours at 25° C and for each concentration, were inoculated with 50 µl of conidial suspension of *B. cinerea* or *Penicillium* sp. diluted from 10^7 to 10^4 spores/ml.
Fours apples (2 wounds per apples i.e. 8 replicates) were used for each incubation time between yeast treatment and inoculation by the pathogen, and for each possible combination between yeast and pathogen concentrations. These experiments were repeated twice.

Effect of biocontrol agents on *B. cinerea* spore germination in relation with colonization and protection level.

The ability of the antagonist to colonize the inoculation site was investigated by inoculating wounded fruits (one wound of 20 mm in diameter, 1 mm deep per apple) with 50 µl of antagonist suspension (10^8 cfu/ml) and maintaining them in moist plastic boxes at 25° C. Wounds were sampled 0, 4, 8, 12, 24, and 48 hours after inoculation by excising the entire

wounded-tissue and placing in 100 ml of sterile 0.1 % pepton water. The tissues were blended and dilution-plated in triplicate onto PDA for assay of colony forming units (cfu). There were two replicates (two wounded fruits) for each incubation time and the trial was repeated three times. Population densities were expressed as $\log_{10}$ cfu per wound.

Parallel with population-kinetic studies, the level of protection activity against *B. cinerea* (10^6 spores/ml) was measured according to the same experimental protocol. Each treatment was replicated 6 times and two trials were carried on.

In order to determine the effect of BCA on germination of *B. cinerea* on apple, inoculum of the pathogen was applied after growing times on a transparent membrane filter (Millipore, 0.2 µm) placed in the antagonist-pretreated wound sites (10^8 cfu/ml of yeast suspension). After 24 hours following pathogen inoculation, the membrane filters were colored with cotton blue and the percentage of germination of *B. cinerea* was determined under ligth microscopy. Twenty fields were observed per filter and a spore was considered as germinated when the germinating tube was longer than the conidia. One membrane filter was observed per treatment and the experiment was repeated twice.

Statistical analyses.

Factorial variance analyses for the results concerning the two parameters controlling the level of protection, were performed with the models procedure of SAS. Mean separations were calculated by Dunnett's method (p=0.001).

RESULTS.

In vivo **screening.**

Preliminary screening (results not shown) at 25 °C, allowed to select 40 strains among 329 microorganisms tested. The protective level of each selected strains was evaluated by a mean percentage of protection as compared to untreated control. Seven strains (all yeasts) were finally selected for their protective level ranging from 90% to 100 % at 5° and 25° C against *B. cinerea* whereas only two yeasts (K and O) among these seven strains gave a protection between 80 % and 90 % against *Penicillium* sp.

Technological screening.

The application of antagonist suspension (10^7 cfu/ml) onto wound sites gave a similar protection level at 25° C against *B. cinerea* or *Penicillium* sp. but that strains K and O were produced either in fermentor or in Petri dish (table 1). The lyophilised preparations of strains K and O contained 6.27 10^8 and 1.85 10^9 cfu/g respectively. The fermented preparation of

the other selected strains did not reach a high enough concentration to inhibit rot development (results not shown).

Major parameters controlling the level of protection.

Protection level by yeasts against *B. cinerea* and *Penicillium* sp. increased with time between application of the antagonist and inoculation of the pathogen (table 2). The most efficient strains (K and O) reduced significantly the diameter of decay lesion, even when inoculation of the pathogen and application of the yeast were performed simultaneously. Selected strains showed a lower protective activity against *Penicillium* sp. Application of strains K and O gave 90-100% of inhibition of lesions for both pathogen when inoculated 48h after the antagonist application (table 2).
Because of their protection properties and technological ability, strains K and O were selected for further experiments. Strain K was identified as *Pichia anomala* and O as *Candida sake* by CWBI (Centre Wallon de Bio-Industrie) and MUCL (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve).

There was a quantitative relationship between spore concentration of *B. cinerea* and the amount of antagonist required for disease control (tables 3). The application of 10^8 cfu/ml of strain K was necessary to completely inhibit the lesions of *B. cinerea* upon inoculation with 50 μl of 10^6 spores/ml whereas 10^8 cfu/ml of strain O protected fruits against inoculation with 50 μl of 10^5 spores/ml of *B. cinerea*. Cell concentration of the antagonist had also an impact on the biocontrol efficacy against *Penicillium* sp., while the level of inhibition of decay lesions was little influenced by the spore concentration of the pathogen.

Effect of biocontrol agents on spore germination of *B. cinerea*, in relation with colonization and protection level.

Population of K and O strains in wounds increased at 25°C to reach a maximum after 12h of incubation (fig. 1) ; after 72h , the population level of the antagonist was approximately 1 log unit over the initial population level. A close relationship appeared between population of the biocontrol agent and level of protection. The protection levels when *B. cinerea* was inoculated 0, 4, 8, 12, 24 and 48h after the antagonist application, were 21.4, 27.2, 38.7, 72.5, 97.3 or 98.1 %, respectively, on K-treated fruits, and 30.9, 35.3, 44.8, 81.8, 98.3 or 100.0 % on O-treated fruit.
Spore germination of *B. cinerea* was reduced on K or O-treated-wound sites (fig. 1). After 0, 4, 8, 12 or 24 h of apples incubation between treatment of strain K and inoculation of pathogen, spore germinations of *B. cinerea* on membrane filters were 14, 19, 14, 10, 6 % respectively. After the same times between application of strain O and inoculation of the pathogen, spore germination was 20, 15, 15, 12 or 9 %, respectively. The spore germination of control (untreated fruit), after the same incubation times of wound sites before *B. cinerea* deposit, were 58, 73, 83, 60 or 45 %, respectively.

Table 1: Protection level of *Pichia anomala* (K) or *Candida sake* (0) produced in fermentor or in Petri dish against *Botrytis cinerea* and *Penicillium* sp.

Strains (grown in)	*Botrytis cinerea*	*Penicillium* sp.
K (Fermentor)	93,3%[1] ± 6,70[2]	62,7% ± 22,10
K (Petri dish)	96,8% ± 3,15	81,2% ± 4,45
0 (Fermentor)	91,4% ± 8,60	75,1% ± 18,50
0 (Petri dish)	94,2% ± 5,85	88,0% ± 3,30

1: Mean percentage of protection as compared to the untreated control
2: Standard error.

Table 2: Lesion development (mm) on wounded Golden Delicious apples treated with 50 l of antagonistic yeast suspension (about 10^7 CFU/ml), and then inoculated with 50 l of pathogen suspension (10^6 spores/ml) after different incubation times of the antagonist.

	Botrytis cinerea[b]				*Penicillium* sp.[b]			
Incubation time	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.13[c]	21,6[a]	9,4[d]	6,9[d]	0,5	20,2	15,6[d]	6,9[d]	5,4[d]
1.58	24,9	12,2[d]	11,2[d]	9,1	16,4[d]	15,6[d]	14,2	9,0
9C5	24,7	19,5	7,0[d]	3,1[d]	20,2	22,6	19,1	10,4
5F2	27,0	13,9	7,1[d]	19,7[d]	18,5	16,7[d]	15,5	4,1[d]
K	10,7[d]	8,6[d]	0,0[d]	0,1[d]	10,6[d]	12,2[d]	3,7[d]	0,0[d]
0	15,2[d]	5,7[d]	4,7[d]	3,0[d]	19,2	18,1	3,1[d]	2,0[d]
9A4	22,0	19,5	14,1[d]	5,4[d]	25,4	22,6	19,2	14,2
control[e]	33,8	25,1	31,9	29,4	23,6	23,4	22,6	19,0

a: Data represent average lesion diameter (mm) measured 5 days after pathogen inoculation.
b: Pathogen
c: Antagonistic strains.
d: Means of lesion diameters of the antagonist-treated apples are significantly different ($p = 0,001$) from the control mean (in the same column) according to Dunnett's procedure.
e : Untreated apples inoculated with the pathogen only
Remark: Data shown for 1 of the 2 trials (Data of separate trials were not pooled because variances differed significantly

Table 3: Lesion development (mm) on wounded Golden Delicious apples inoculated with various spores concentrations of *B. cinerea* or *Penicillium* sp. 24 h after treatment of different concentrations of *Pichia anomala* or *Candida sake*.

	B. cinerea spores concentration (spores/ml)				*Penicillium* sp. spores concentration (spores/ml)			
Yeast concentration (CFU/ml)	10^7	10^6	10^5	10^4	10^7	10^6	10^5	10^4
Pichia anomala								
10^8	3,5[ad]	0,0[d]	0,0[d]	0,0[d]	4,6[d]	0,0[d]	1,1[d]	0,0[d]
10^7	4,7[d]	0,7[d]	1,4[d]	1,6[d]	9,4[d]	2,6[d]	0,0[d]	3,7[d]
10^6	17,0	5,2[d]	10,2[d]	0,0[d]	17,2	18,0	14,1	20,2
10^5	24,4	11,2[d]	5,9[d]	5,0[d]	18,1	12,9[d]	15,4	4,9[d]
control[b]	24,5	28,1	25,0	22,1	21,0	21,4	21,6	19,1
Candida sake								
10^8	9,5[ad]	2,9[d]	0,0[d]	0,0[d]	4,7[d]	6,2[d]	8,5[d]	4,6[d]
10^7	14,2	2,6[d]	3,4[d]	0,0[d]	15,5	4,0[d]	2,1[d]	0,0[d]
10^6	17,1	18,6[d]	9,1[d]	6,7[d]	11,0	15,2	10,1[d]	4,7[d]
10^5	13,2	7,9[d]	2,6[d]	0,9[d]	14,9	16,1	8,4[d]	9,2[d]
control[b]	24,5	28,1	25,6	22,1	21,0	21,4	21,6	19,1

a: Data are the average lesion diameter (mm) measured 5 days after pathogen inoculation.
b: Untreated apples inoculated with the pathogen only.
c: Means of lesion diameters of the antagonist-treated apples are significantly different to the control mean (in the same column) according to Dunnett's procedures (P = 0,001)

Remark: Data shown for 1 of the 2 trials (data of separate trials were not pooled because variances differed significantly).

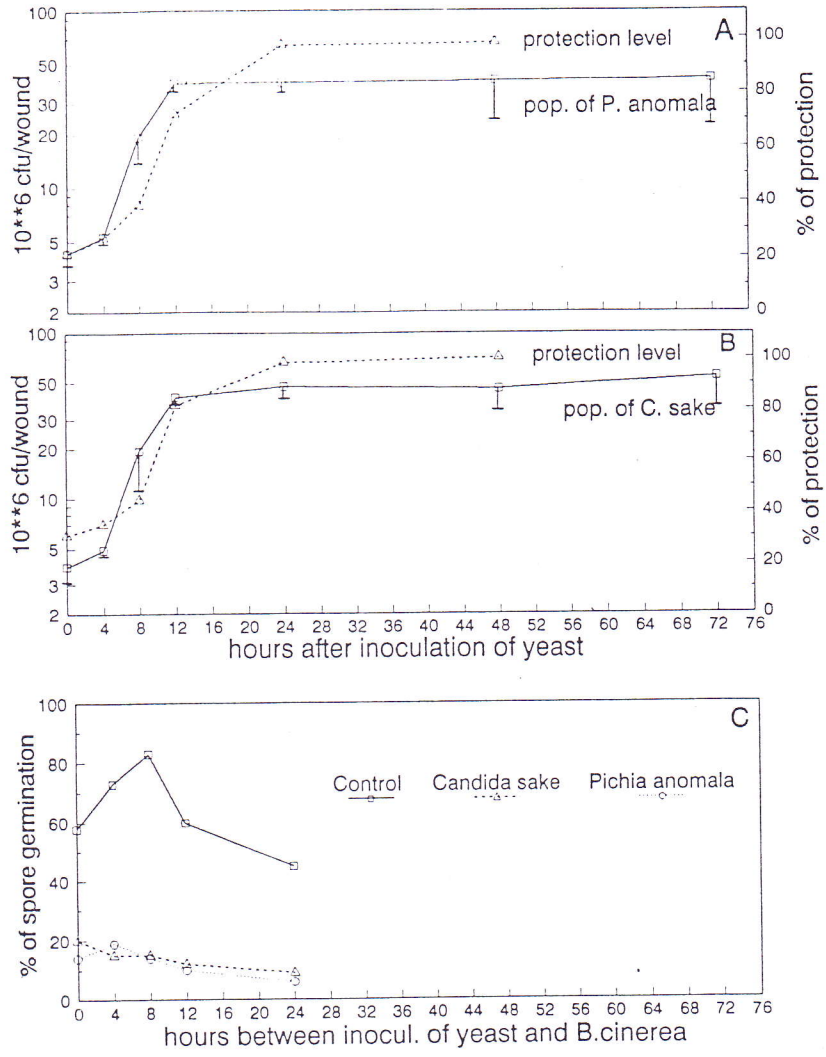

fig 1 : - Effect of population densities of *Pichia anomala* (A) and *Candida sake* (B) on level of protective activity against *B. cinerea*. Data from population densities represent mean colony from three trials (one wound site/trial). Each wound was triplicate-plated. Data from protection level represent the mean % of protection (as compared to the control wich was not treated with yeast before inoculation of *B. cinerea* from two trials (6 wounds/trial). Bars represent standart error of the mean.

- Effect of *P. anomala* or *C. sake* on spore germination (C). Data represent the mean % of spore germination from one of the two trials (1 replicate per trial). twenty fields were observed per replicate and a spore was considered as germinated when the germinating tube was longer than the spore.

DISCUSSION.

Among 329 epiphytic microorganisms (yeasts and bacteria) screened for their protection properties, yeasts K (*Pichia anomala*) and O (*Candida sake*) appeared to be the most effective biocontrol agents against *Botrytis cinerea* and *Penicillium* sp. during *in vivo* screening. Both strains were produced in fermentor and the dried preparation appeared to keep their efficacy against both pathogens when applied as suspensions onto apple wounds.
Yeasts have been reported as effective BCA of postharvest diseases. Roberts (1990) selected *Cryptoccocus laurentii* as BCA of *B. cinerea* on apples. After screening, both *Trichosporon* sp. and *Candida* sp. were found to be the most active antagonists against *B. cinerea* and *P. expansum* (Gullino *et al.*,1991), *Acromenium breve* was also selected to control blue mold on apples (Janisiewicz, 1987), *and Debaryomyces hansenii* exhibited the highest efficacy for controlling *P. digitatum* on grapefruit (Droby *et al.*, 1989). Protection properties were related to the fact that yeasts appear to be the major component of flora of fruit surfaces (Janisiewicz, 1991), although selected bacteria appeared also effective for the bioprotection on apples (Janisciewicz, 1987, Janisciewicz, 1988)

Results of our work demonstrated that antagonistic activity of yeasts was dependent on the incubation time before inoculation of *B. cinerea* or *Penicillium* sp. and on the ratio of concentrations between pathogen and antagonist, thus suggesting that colonization of the wounded sites is a prerequisite to protection. The same parameters controlling the level of protection were already identified by other authors (Gullino *et al.*, 1991 ; Janisiewicz, 1987 ; Roberts , 1990 ; Mc Laughlin *et al.*, 1990).

The curves of yeast population in apple wounds incubated at 25° C indicated rapid multiplication to reach a maximum density after 12 hours of incubation. Protection level also increased progressively to reach a maximum after 12 hours of yeast incubation. The ability of strains K and O to rapidly colonize the wounded sites could be implied in biocontrol by nutrient competition, as spore germination of some strains of *B. cinerea* are known to be affected by the carbohydrates and nitrogen status of their environment (Blakeman & Brodie, 1977). In this respect, *Pichia anomala* and *Candida sake* can assimilate or ferment different carbohydrates or nitrogen compounds, and were found in water, soft drinks, fruit juices and mushrooms, frozen salmon, etc. *Candida sake* was also commonly found in man and other mammals (Van Uden and Buckley, 1971 ; Wickerham, 1971).

In situ spore germination of *B. cinerea* was markedly reduced on K- or O-treated wounded sites, even when pathogen and yeast were applied simultaneously with no subsequent protection (fig 1), thus indicating that biocontrol effectiveness cannot be explained only in term of reduction of spore germination.

The biological efficacy of strains K and O against *B. cinerea* and *Penicillium* sp., and the possibility to produce both yeasts in fermentor, are a prerequisite to practical applications. Additional studies related to the formulation, shelf-life, easier handling and toxicology of the product must be carried on while the mechanisms of action wich are involved in the antagonistic relationship should be studied further.

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