FORMULATION AND INTEGRATED USE OF TWO ANTAGONISTIC YEASTS TO POSTHARVEST TREATMENTS AGAINST DISEASES ON APPLES.

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ABSTRACT.

Protection against *Botrytis cinerea* and *Penicillium* sp. on wounded Golden Delicious apples was obtained with the application of yeast suspension of *Pichia anomala* (strain K) or *Candida sake* (strain O)(6,7).

Among 29 nutrients, 2-deoxy-D-glucose was selected for its enhancement and stabilization effect of the protective capacity of strains K and O against *B. cinerea*. The application of antagonistic yeast (10^7 cfu/ml of suspension) in a solution of 2-deoxy-D-glucose (0.2%) gave a protection of 95 % against *B. cinerea* which was simultaneously inoculated at 10^6 spores/ml. This treatment increased the protective level of more than 30 % as compared to the protection offered by the antagonistic agent alone.

In order to integrate biological control to others sanitary measures,three treatments consisting of dipping the apples in water at 45°C for 10 min, or in a water suspension of a mixture of strains K and O (10^7 cfu/ml each) for 2 min or in an emulsion of a film-forming antitranspirant (Nu-film-P, 2 %) for 2 min, were carried out separately or in combination on postharvest Golden Delicious. The separate or combined effects of these treatments were studied on fruit quality and incidence of naturally occurring decay caused by different pathogens. The quality parameters (weight, size , skin color, firmness, acidity, refractometric indice) were not affected by any of the treatments. Thermotherapy alone reduced the incidence of *Gloeosporium* spp. infection from 54.4 % (untreated apples) to 4.6 %, but heat treated apples were more sensitive to *Penicillium* spp. The proportion of fruits infected by this pathogen was decreased by the application of yeasts and Nu-Film-P after thermotherapy.

INTRODUCTION.

Biopesticides are generating a great enthusiasm, notwithstanding the still limited relevance of biocontrol agents today. Among the factors which limit the application of biological control, we can point out the low consistency of the protective effect of biocontrol agents and their too high specificity wich limits the market size. The unreliable activity of biocontrol agents can be improved by adjuvants of formulation enhancing and stabilizing the antagonistic activities. On the other hand, the integration of the biological control to other sanitary measures (thermotherapy, gamma and U.V. irradiation, film forming polymers treatments) could be a solution to widen the spectrum of activity.

Two yeasts, *Pichia anomala* (strain K) and *Candida sake* (strain O), were previously found to show the highest protective activity against infection with *Botrytis*

cinerea and *Penicillium* sp. on wounded Golden Delicious apples among 329 epiphytic microbial strains (6). Both yeasts can be produced in fermentor while maintaining their antagonistic activity (7).

The objectives of this work were the selection of nutrient elements which could enhance the protective activity of strains K and O against *B. cinerea* and *Penicillium* sp. (two parasitic fungi of wounds) and in order to enlarge the protection against *Gloeosporium* spp. (fungal pathogen developping post-harvest apple rots from latent infections), we studied the possibility to integrate yeasts application to others control measures such as thermotherapy and film-forming antitranspirant treatments.

MATERIAL AND METHODS.

Source of fruits

Apple fruits (cultivar Golden Delicious) were harvested in 1992 and stored in a commercially operated room with controlled atmosphere at Fouron-le-Comte (Belgium) until april 1993. During experiments, apples were maintained in a cold room (3°C) at Fruit Research Station (Gembloux, Belgium).

Antagonistic microorganisms and pathogen strains.

The strains of *P. anomala* and *C. sake* were respectively isolated from the surface of apple and grape fruits. The two strains were stored at 5°C on PDA. For the experiments, they were plated three times running on PDA and cultivated each time 24 hours at 25°C. Yeast suspensions were prepared by diluting the fourth generation colonies in sterile distilled water to the required concentrations.

Strains of *B. cinerea* and *Penicillium* sp. were isolated from strawberry and apple respectively and stored onto PDA at 5° C in the dark. Pathogens were cultivated on PDA at 25° C for 12-14 days under a 16 h photoperiod 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.

Nutrients screening.

15 carbohydrates and 16 nitrogenous compounds were selected as potential adjuvants of yeast formulation. Fruits were surface-desinfected with sodium hypochlorite (10% of commercial product for 2 min), rinsed with sterile water, wounded with a cork-borer (2 wounds of 6 mm diameter and 3 mm deep at the equator of each apple). The wounded sites were treated with 50 µl of a mixture of antagonist suspension (10^7cfu/ml) in 0.2 % nutrient solution (or with 50 µl of either distilled water or antagonist suspension

in distilled water for control). The pathogen inoculation (*B. cinerea* or *Penicillium sp.*) was carried out immediatly after this application by depositing 50 µl of spore suspension (10^6spores/ml) on the wounded sites. Fruits were incubated on wet filter paper in closed plastic boxes at 25° C in the dark for 5 days before measuring diameters of decay lesions and calculating a mean percentage of protection as compared to the control (inoculated with the pathogen).

To determine the optimal cobditions of application of the selected nutrients, different parameters such as nutrient and antagonist concentrations, elapsing time between biological treatment and pathogen inoculation, were studied by following the same *in vivo* protocole. Two trials were made for each experiment and 4 apples (2 wounds per apples i.e. 8 replicates) were used per kind of treatment.

Integration of thermotherapy to a film-forming antitranspirant and/or antagonistic treatments.

8 batches of 160 apples (unwounded and uninoculated) were used. The first batch served as control, the three following batches were respectively dipped for 2 min in a tap water suspension of strains K and O (10^7 cfu/ml for each yeast), in a tap water emulsion of 2 % Nu-Film-P (NFP, 96 % of poly-1-P-Menthen, Miller laboratory) or in a suspension of yeasts in a 2 % NFP emulsion. The fifth batch was plunged in a thermostatic water bath (45° C) during 10 min. This thermotherapy was followed by yeasts and/or NFP treatment for the three last batches. After these different treatments, each batch was stored in a cold room (3° C) during one month before being transferred in a room at 25° C for another month. The different pathogens were identified, scored and a percentage of infection was calculated for each agent.

For each batch, 6 parameters reflecting the fruit maturity were evaluated after the first (3° C) and the second month (25° C) of storage. The diameter and weight of 30 apples were measured per batch. Juice was extracted and filtered from 20 of the 30 fruits and a percentage of dry soluble matter was evaluated by measuring the refractometric indice (or Brix degree). 10 ml of apple juice was titrated with NaOH solution (0.1 N) and the equivalent quantity of malic acid (g/l) was estimated by the formula n x 0.67 (n=ml number of NaOH). The 10 left fruits were used to evaluate the apple skin color with "Lange" engine and the firmness following the Taylor Magness method with "Instron" engine.

RESULTS.

Nutrients screening.

Enhancement of the protective effect of strain K or O against *B. cinerea* and *Penicillium* sp. in wounded sites of apples was evaluated by adding different nutrients to the yeast suspension. Among the 15 carbohydrates and 16 nitrogenous compounds

tested (see list 1), only the 2-deoxy-D-glucose (2-gluc) brought a significant increase of protective level of both antagonistic yeasts (*P. anomala* or *C. sake*) against *B. cinerea* (results not shown). The protective level of strain K or O in suspension with 2-gluc (0.2 %) was respectively 96.3 % or 94.1 % whereas the application of yeasts on their own gave 64.7 % (strain K) and 48.5 % (strain O) of protection.

List 1 : Nutrive elements tested for their capacity to enhance the biological control.

NITROGENOUS COMPOUNDS	CARBOHYDRATES
L-aspartic acid	Arabinose
L-glutamic acid	D-cellobiose
L-asparagin	2-deoxy-D-glucose
L-cystein	D-fucose
Glycin	D-galactose
Hydroxy-L-prolin	Glycerol
L-leucin	Maltose
Lysin	L-mannose
L-methionin	Mannitol
Pepton	Melezitose
L-prolin	Meso-erythritol
L-serin	Raffinose
L-threonin	D-ribose
L-thryptophan	D-sorbitol
L-valin	

In order to define the optimal application of 2-gluc with yeast suspension, the wounded apples were treated with different combinations of concentrations of 2-gluc solution (0.4, 0.2, 0.05 %) and of antagonistic suspensions (10^5 and 10^7 cfu/ml). Elapsing time between antagonistic treatment and pathogen inoculation was also studied. The percentage of protection decreased with the concentration of both 2-gluc and yeast (see table 1). A protective level ranging from 96.9 and 100 % was obtained against *B. cinerea* when the 2-gluc concentration was 0.4 % whatever the elapsing time between yeast application and pathogen inoculation and whatever the yeast concentration.

The protection due to the application of 2-gluc alone on wounded apples was also studied when *B. cinerea* was silmultaneously inoculated (table 2). The percentage of protection increased with the 2-gluc concentration to reach 100 % of protection against *B. cinerea* with 0.4 % 2-gluc solution.

Table 1 : Effect of yeasts and 2-deoxy-D-glucose concentrations and incubation time between yeast/nutrient treatment and inoculation of B. cinerea on the protection of wounded sites of apples.

	DELAY° = 0 hour				DELAY° = 24 hours			
	K.10^{7}*	K.10^{5}*	O.10^{7}*	O.10^{5}*	K.10^{7}*	K.10^{5}*	O.10^{7}*	O.10^{5}*
2-deoxy-D-glucose 0,4%	100 % ± 0	98,65 % ± 1,35	100 % ± 0	100 % ± 0	99,52 % ± 0,48	96,90 % ± 3,10	100 % ± 0	100 % ± 0
2-deoxy-D-glucose 0,2%	91,00 % ± 9,00	57,85 % ± 15,75	91,05 % ± 3,65	69,35 % ± 9,55	97,90 % ± 2,10	87,30 % ± 5,00	100 % ± 0	85,10 % ± 4,2
2-deoxy-D-glucose 0,05%	67,55 % ± 3,95	31,90 % ± 9,50	51,80 % ± 3,10	26,15 % ± 2,55	75,54 % ± 16,73	66,40 % ± 18,10	83,70 % ± 11,50	62,90 % ±0,19
yeast alone	38,00 % ± 3,50	9,60 % ± 1,10	43,35 % ± 1,45	12,95 % ± 3,75	82,10 % ± 16,10	48,95 % ± 8,75	69,45 % ± 4,35	53,25 % ± 11,65
2-deoxy-D-glucose 0,2% alone	53,60 % ± 11,80				11,15 % ± 17,05			

a = mean percentage of protection calculated from 2 trials (8 repetitions per trial); the percentage is calculated as compared to the untreated control (apples just inoculated with the pathogen).
b = standard error.
* = strain K = *Pichia anomala* and strain O = *Candida sake*.
° = elapsing time between yeast application in suspension with nutrient solution and inoculation by the pathogen.

Table 2 : Protection of wounded sites of apples against *Botrytis cinerea* (inoculated at 10^{6}spores/ml) in terms of 2-deoxy-D-glucose concentrations.

	Protection*	Standard error
2-deoxy-D-glucose 0,4%	100 %	± 0
2-deoxy-D-glucose 0,2%	34,62 %	± 0,92
2-deoxy-D-glucose 0,05%	12,17 %	± 2,62

* = mean protection calculated from 2 trials (8 repetitions per trial); the percentage is calculated as compared to the untreated control (apples just inoculated with the pathogen).

Integration of thermotherapy to a film-forming antitranspirant and/or antagonistic treatments.

Our objectives were to evaluated the separate and combined effects of thermotherapy, application of strains K and O mixture and Nu-Film-P (NFP) treatment on the incidence of naturally occuring infections and of some parameters reflecting the quality of fruit.

After one month at 3° C and another month at 25°C, the untreated batch showed 78 % of infected fruits (table 3). This total percentage of infection decreased whatever the applied treatment on apples ; best protection level was obtained with heat treatment followed by yeasts application in NFP (48 % of infection).

54 % of untreated fruits were infected by *Gloeosporium* spp. This percentage was reduced to less than 5 % for all treatments including thermotherapy whereas the level of *Gloeosporium* infection ranged from 34 to 41 % for batches treated only with the mixture of yeasts alone or in suspension with NFP. Nervertheless, when the development of *Gloeosporium* spp. was reduced with a heat treatment, percentage of infection caused by *Alternaria* spp., *Fusarium* spp., and particularly *Penicillium* spp. increased. Treatments including yeasts application were more efficient against *Penicillium* spp. After application of heat treatment, control of this pathogen was restored with a bath of yeast suspension in NFP emulsion.

We observed a normal variation of acidity, Brix degree, skin color and firmness of the control batch between the first and second month of storage. Whatever the incubation time of apples, none significative effect were found on the physico-chemical parameters of fruit maturity between the different treatments with regard to their respective control batch (table 4).

DISCUSSION.

Among the 29 nutrients tested, only one sugar (2-deoxy-D-glucose) showed a protective effect against *B. cinerea* and increased the level of protection from about 60 % to 90-95 % when added to the yeast suspension. Janisiewicz *et al.* (4,5) achieved *in vitro* inhibition of *Penicillium expansum* spore germination and radial growth mycelium reduction with this analogue of glucose. They also observed an inhibition of this pathogen on apple fruits by combining treatment of 2-gluc and *Pseudomonas syringae* application. Contrary to these results, we did not observe any significant protective effect against *Penicillium* sp (results not shown). None of the other nutrients (L-asparagine, L-proline, galactose, mannitol, ribose and sorbitol) selected for their *in vitro* and/or *in vivo* antagonist stimulation or pathogen inhibition, either by Janisiewicz *et al.* (4) or Harper *et al.* (3), enhanced the protective activity of strain K and O in our experiments. These different results show that an effect observed in a specific plant-antagonist-pathogen combination is not transposable in another system. The application

Table 3 : Separate and combined effects of thermotherapy, biological control and antitranspirant application on post-harvest diseases of apples.

	Control	45°C[b]	45°C+NFP[c]	45°+K+O[d]	45°C+NFP+K+O	K+O	NFP	NFP+K+O
total infection	78,20[a]%	59,20%	67,70%	50,00%	47,70%	60,00%	61,50%	68,50%
Gloesporium spp.	54,40%	4,60%	4,60%	2,30%	1,50%	37,70%	33,80%	41,50%
Penicillium spp.	8,20%	20,00%	32,30%	19,20%	11,50%	3,10%	10,00%	6,10%
Alternaria spp.	4,80%	13,10%	10,00%	10,80%	11,50%	4,60%	5,40%	7,70%
Fusarium spp.	3,40%	7,70%	5,40%	3,10%	6,90%	0,80%	0,80%	2,30%
Cylindrocarpon	3,40%	3,80%	3,80%	5,40%	6,90%	3,80%	1,50%	3,80%
Rhysopus spp.	0%	1,50%	0%	0%	0%	0%	0%	0%
Non determined	4,10%	8,50%	11,50%	9,20%%	9,20%	10,00%	10,00%	6,90%

a = mean percentage of infection from 130 apples. b = apples were dipped in heat water (45° C) for 10 min. c = apples were dipped in Nu-Film-P (2 %) for 2 min. c = apples were dipped in strain K and O suspension (107 of each strain/ml).

Table 4 : Separate and combined effects of thermotherapy, biological control and antitranspirant application on physico-chemical parameters of apples maturity.

Fruits maturity after a first month of storage at 3° C.

	ACIDITY	BRIX	COLOR	FIRMNESS	WEIGHT	Ø[a]	H[b]
Control	2.40[c]	12.5 %[d]	L = 56.93[e] ± 3.47[f] A = -0.21 ± 3.21 B = 25.21 ± 1.90	74.25[g] ± 6.19[h]	184.14[i] ± 9.95[j]	75.66[k] ± 3.24[l]	73.57[m] ± 2.75[n]
NFP*	2.49	12.2 %	L = 58.20 ± 5.42 A = 4.60 ± 7.23 B = 25.04 ± 3.43	83.08 ± 7.72	148.15* ± 21.9	70.63 ± 3.49	68.28 ± 5.02
K + O*	2.21	12.2 %	L = 58.45 ± 2.04 A = -0.39 ± 2.90 B = 25.94 ± 1.14	76.08 ± 6.91	168.67 ± 26.2	73.95 ± 4.5	70.85 ± 4.96
K + O + NFP*	2.50	12.3 %	L = 58.09 ± 3.40 A = 1.25 ± 4.90 B = 24.44 ± 2.11	70.19 ± 6.5	162.34 ± 25.3	73.48 ± 3.77	71.21 ± 5.22
45°	2.68	13.3 %	L = 56.71 ± 8.15 A = 1.89 ± 4.63 B = 26.20 ± 2.23	70.19 ± 9.62	146.15* ± 20.5	71.15 ± 3.02	67.86 ± 4.95
45° + NFP*	2.37	11.9 %	L = 58.87 ± 2.74 A = 1.10 ± 3.36 B = 25.41 ± 1.63	75.11 ± 6.74	136.83* ± 13.8	68.78 ± 3.72	64.20* ± 4.46
45°+K+O*	2.10	12.0 %	L = 58.79 ± 2.65 A = 0.13 ± 2.80 B = 26.05 ± 1.24	75.13 ± 11.57	157.57 ± 22.2	71.6 ± 4.31	69.18 ± 4.77
45°+K+O+NFP*	2.48	12.4 %	L = 57.59 ± 2.74 A = 2.09 ± 4.17 B = 25.63 ± 1.76	72.99 ± 7.28	176.34 ± 12.3	75.18 ± 2.37	73.56 ± 3.24

Fruits maturity after a second month of storage at 25° C.

	ACIDITY	BRIX	COLOR	FIRMNESS	WEIGHT	Ø[a]	H[b]
Control	1.72[c]	11.7 %[d]	L = 62.11[e] ± 2.85[f] A = 10.34 ± 3.37 B = 49.40 ± 3.03	66.26[g] ± 10.11[h]	140.44[i] ± 26.18[j]	69.13[k] ± 4.52[l]	66.53[m] ± 5.07[n]
NFP*	1.74	11.6 %	L = 62.38 ± 3.62 A = 10.17 ± 4.84 B = 48.74 ± 3.67	66.33 ± 9.39	133.43 ± 16.19	68.70 ± 2.81	67.05 ± 4.04
K + O*	1.61	11.6 %	L = 63.08 ± 3.50 A = 8.07 ± 4.61 B = 51.25 ± 3.83	64.28 ± 7.24	141.66 ± 18.70	70.33 ± 3.24	67.75 ± 3.17
K + O + NFP*	1.54	12.0 %	L = 61.30 ± 3.20 A = 9.67 ± 4.76 B = 49.57 ± 4.96	70.58 ± 6.06	153.95 ± 22.72	71.50 ± 3.97	69.19 ± 4.68
45°	1.51	11.8 %	L = 62.55 ± 1.96 A = 9.53 ± 3.19 B = 49.89 ± 2.24	57.36 ± 15.89	154.58 ± 22.07	71.62 ± 3.99	71.61 ± 4.49
45° + NFP*	1.52	11.9 %	L = 62.46 ± 2.95 A = 9.38 ± 5.01 B = 48.40 ± 4.00	66.57 ± 9.98	130.68 ± 20.17	68.06 ± 4.01	66.79 ± 3.09
45° + K + O*	1.37	11.6 %	L = 63.64 ± 2.38 A = 8.90 ± 2.65 B = 49.35 ± 2.56	63.27 ± 8.53	145.68 ± 25.22	71.20 ± 4.73	67.96 ± 4.59
45°+NFP+K+O*	1.60	11.8 %	L = 62.73 ± 3.26 A = 8.20 ± 4.58 B = 49.57 ± 3.48	69.10 ± 10.62	147.81 ± 21.64	70.91 ± 4.04	69.37 ± 3.52

45° = heat treatment in water at 45° C ; * = strain K = *Pichia anomala* (107cfu/ml), strain O = *Candida sake* (107cfu/ml) and NFP = Nu-Film-P (0.2%) ; L, A, B : components of color. a : Ø = diameter ; b : H = high ; c = mean concentration of malique acid (g/l) in apple juice (2 repetitions). d = mean percentage of dry soluble matiers in apple juice (2 repetitions). e = mean calculated from 10 apples (4 repetitions/apple). g = mean of penetration force (20 repetitions) expressed in Newton. i = mean weight (30 repetitions) expressed in grammes. k, m = mean diameter and high (30 repetitions) expressed in mm. f, h, j, l, n = respective standard error of means e, g, i, k, m.

of 2-gluc on its own reduced or totally inhibited the development of *B. cinerea*. Preliminary assays (results not shown) indicate that this glucose analogue reduced *in vitro* spore germination and hyphal growth of *B. cinerea* without affecting the kinetic yeast colonization of wounded sites.

The thermotherapy constituted a non chemical measure to control plant pathogens (2). Golden Delicious apples seem to be tolerant to a heat water (45° C) bath because such a treatment didn't influence technical characteristic of fruit maturity. This treatment strongly reduced the latent infection caused by *Gloeosporium* spp. thus confirming the results obtained by other authors (1,2). We observed that after heat treatment, a higher percentage of fruits was infected by *Penicillium* spp. Edney and Burchill (2) already noticed a higher sensitivity to *Penicillium* infections after thermotherapy and explained the phenomenon by lenticelle dammages. The combined treatment of heat and antagonistic yeastsapplied in a NFP emulsion (an antitranspirant) allowed to reduce the diseases caused by *Gloeosporium* spp. and *Penicillium* spp. without affecting the parameters related to fruit maturity. In our experiments, the different treatments were compatible and the integrated use of biological control permitted to enlarge the spectrum of controlled pathogens.

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