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BIOLOGICAL CONTROL OF APPLES BLUE MOLD BY ISOLATES OF *SACCHAROMYCES CEREVISIAE*

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Abstract: Yeasts (52, 51, 69, and 04) were received from Biotechnology Center of Karaj and *Penicillium expansum* isolates P_{11} and P_{12} isolated from Golden Delicious. Isolates were evaluated as a potential biological control agents of apple blue mold caused by *P. expansum*. Dual culture, cell free metabolites and volatile test were used *in vitro* assay. All tested of yeast isolates inhibited growth of *P. expansum*. The inhibition varied among isolates of yeasts and ranged from 19.81% to 40.73%, in dual culture, from 43.16% to 66.44% in volatile metabolite and from 22.16% to 50.23% in cell free metabolite test.
Apple fruit wounds were inoculated with 40 µl of yeast cell suspension (10^7cell/ml) followed 48 h later by *P. expansum* (10^5 conidia/ml). The apples were then incubated at 25°C. Four isolates of *Saccharomyces cerevisiae* reduced, the decay area from 13.46 to 24.92 cm^2 compared to 32.18 cm^2 in control after incubation for 14 days at 25°C. At 5°C, the lesion size ranged from 13.58 to 24.68 cm^2 for the antagonist treatments compared to 22 cm^2 for the control treatments after 32 days. The isolate 69 of *S. cervisiae* was the most effective isolate at both tempetures in this assay and could be one of important new biological control agents for apple blue mold.

Key words: biological control, *Saccharomyces cerevisiae, Penicillium expansum*, apple

INTRODUCTION

Fruits and vegetables suffer significant losses from fungal diseases after harvest (Eckert 1988; Filonow *et al*. 1998). Postharvest losses of fruits and vegetables are high, ranging from 10 and 40% depending on the species and technologies used in the packinghouses

Postharvest fungal diseases of apple are mainly caused by *Penicillium expansum* (Romano *et al*. 1983). Traditionally, this disease is controlled by the application of synthetic fungicides. However, the potential impact on environment as well as human health largely limits their application (Eckert *et al*. 1994). It is reported that some pathogens become fungicide resistant, and thus a fungicide's effect on controlling fungal growth may be greatly reduced (Spotts and Cervantes 1986). Considering the human health and pollution risks, some fungicides are prohibited to use in many developed countries (e.g. America, England).

Recently, biological control was developed as an alternative to synthetic fungicides (Wilson *et al*. 1993), and considerable success was achieved by utilizing antagonistic microorganisms for controlling postharvest diseases. Ever since some authors reported that *Bacillus subtilis* was antagonistic toward fruit pathogens, many studies involving antagonistic microorganisms to control postharvest diseases of fruits and vegetables were done (Wisniewski *et al*. 1991; Ippolito *et al.* 2000). Roberts (1990) discovered that *Cryptococcus laurentii* has antagonistic activity against many postharvest pathogens. The competition for nutrients may play a role in the antagonism of *C. laurentii*. Decay caused by *Rhizopus* sp. was reduced to 70% when strawberries were treated with *Aureobasiduim pullulans* before storage (Lima *et al.* 1997). Calvente found that studies on postharvest biocontrol of fruits and vegetables became an important new area in the world research (Calvente *et al*. 1999).

Advantages of using antagonistic microorganisms include reducing environmental pollutation, effectively controlling postharvest diseases, and producing high quality and safe food. Unfortunately, only a few studies in this field were reported in Iran.

In this paper the capability of yeasts to control apple postharvest diseases caused by *P. expansum* was studied. Conditions for antagonistic yeast and the type of antagonism were also discussed.

MATERIALS AND METHODS

Fruit

Golden Delicious apples of uniform size and maturity without wounds or rot, were used in this study. Apples were harvested at commercial maturity and kept at 0°C until used.

Pathogen

Two isolates of *P. expansum* isolated from rotted apples of the cv. Golden Delicious were used during this study. They were selected for their virulence by inoculation of artificially wounded apples. the mixture of isolates was used throughout this study, to ensure a higher level of the

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disease. After 2 weeks incubation at 25°C, spores from the two isolates of pathogen were collected and suspended in sterile water. Spores were counted and brought to a final concentration of 10^5 conidia/ml.

Biocontrol agents

The yeast were recieved from the Biotechnology Center Institute of Kraj. Then were four isolates of *Saccharomyces cerevisiae* (69, 04,52 and 51). Identification of selected isolates was carried out by our Institute. Cultures were stored at −20°C as cell suspension in 65% v/v of glycerol and 35% v/v of solution of 100 mM MgSO4 and 25mM Tris (pH 8.0) (Sparado *et al*. 2001). Yeast cultures were also maintained at YMA medium in the refrigator in 5°C.

Inocula of the antagonists for all experiments were prepared by subculturing in 250 ml Erlenmeyer flasks containing 75 ml of NYDB: (8 g/l of Nutrient broth; 5 g/l of extract of yeast (Granulated Merck); 10 g/l of D(+)-glucose (Merck); 20 g/l agar), and incubating on a rotary shaker (200 rpm) at 25°C for 24 h (Usall *et al*. 2000)

***In vitro* biological control studies**

The dual culture method was carried out according to Dennis and Webster (1971) to evaluate the antagonistic activity of four isolates of *S. cerevisiae* againist the *Penicillium* isolates. Four 90 mm diameter plates containting 10–15 ml of PDA were used for each treatment. Yeast suspension (200 μl; 10^7CFU/ml culture) was streaked on half plates incubated at 25°C for 24 h prior depositing a plug of *P. expansum* culture on the other side of each plate. The plates were incubated at 25°C for 18 days and then colony diameters and inhibition zones were measured. The diameter of each colony was measured as the average of 3 independent measurements taken with digital caliper (Mitutoy Canada Inc., Toronto, Ont.), and the area was caculated from the diameter measurements. The per cent growth inhibition was calculated using the formula $n = (a–b)/a \times 100$, where n is % growth inhibition; a is the colony diameter of uninhibited *P.expansum*; and b is the colony diameter of yeast treated *P. expansum* (Etebarian *et al*. 2005)

The volatile metabolite assesment was carried out according to Lillbro (2005). Antifungal activity of volatile compounds from yeast isolates was assessed by inoculating a Petri plate containing PDA medium with a plug of *P.expansum* and a second Petri plate containing PDA cultured by yeast isolates. The two plates were wrapped together with parafilm (Fisher Scientific, Ottawa, Ontario) and incubated at 25°C for 7 days. The percent growth inhibition was determined as above. The control treatment consisted of a pure culture of *P.expansum* without yeast (Fiddaman and Rossall 1993).

The cell free metabolite method was based on the method of Weller (1988). A drop of the yeast cell suspension was stripped on the medium in 90 mm diameter Petri dishes and incubated at 25°C for 72 h. Then yeasts were washed out of medium and a piece of cotton soaked in chloroform was placed on the Petri dish for 30 min. After this time, a disk of pathogen culture was placed in the center of the plate and incubated at 25°C. As described above, the surface areas of the colonies were recorded and compared with the control treatments, and the per cent growth inhibition was calculated.

***In vivo* test**

Golden Delicious apples were surface-disinfected with sodium hypochlorite (NaClO, 2.0% as chlorine) for 2 min and then rinsed with strelizied water twice. After air-drying, fruits were treated with 70% ethanol (Dan He *et al*. 2000). Apples were punctured with a sterile needle at the equatorial region (3 mm depth and 2.5 m width; three wounds per fruit). Four apples were used in a treatment. The antagonistic yeast cell suspension (40 μl) was pipetted into the wound. After each treatment, four fruits were placed into a 190×135×50 mm plastic tray wrapped with poly-ethylene sleeve in order to retain high humidity(about 95%), (Spadaro *et al*. 2001).

Antagonistic effect of $CaCl_2$ concentration on biocontrol activity of yeast

Calcum chloride was included in washed out cell suspension of antagonist at concentration of 0 mmol/l, 173 mmol/l (2%), 347 mmol/l (4%), and 521 mmol/l (6%) Ca^{2+}, respectively. Washed cell suspension of the isolate 69 of *S. cerevisiae* was prepared 1×10^7 cells/ml. Wounds of apples were inoculated with a 40 μl of this solution. After 24 h, 20 μl pathogen suspension of P_{11} isolate in concentration of 1×10^5 spores/ml was added to each wound, and the apples were stored at 25°C (Fan and Shiping 2000).

Antagonistic effect at different storage temperatures

Wounds of apples were inoculated with a 40 μl yeast cell suspension at 1×10^7 cells/ml. After 24 h, a 20 μl *P. expansum* suspension at concentration 1×10^5 spores/ml was pipetted to each wound. The apples were stored at 5°C, and 25°C, respectively. Area of spots on apples were measured after 8, and 14 days of storage at 25°C, and after 10, 20, and 32 days at 5°C (Vero *et al*. 2002).

Population dynamics of **S. cerevisiae** *on apple surface*

Population dynamics of the antagonistic yeast on the fruit surface was evaluated for the isolate 69 of *S. cerevisiae*. Wounded apples treated with isolate 69 were stored under the same postharvest conditions, samples were taken at 0, 5, 10 and 15 days of storage in 25°C, and 0, 10, 20, and 30 days for cold storage at 5°C. Population of isolate 69 on the apple surface was quantified using the following technique.

Block of tissue was removed from apples (1 g). The block was shaken in 9 ml of sterile water on a rotatory shaker for 20 min at 150 rpm. Serial dilutions of the washings were made and plated on NYDB containing 0.5 g/l streptomycin sulphate (Merck, 1.10117.0025, Darmstadt, Germany) as a bacteriostat.

After incubation at 25°C for 48 h the isolated viable colonies per gram of fresh weight of fruit (CFU/g) were calculated for each sample. This study was carried out with four replicates, sample unit was four apples. (Teixido *et al.* 2000)

Statistical analysis

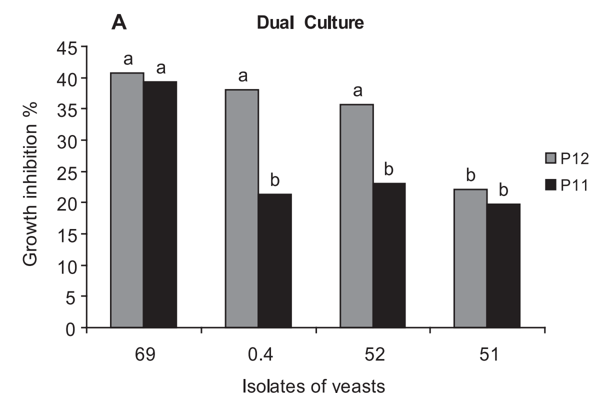
The in vitro and in vivo assays were calculated by the analysis of variance (ANOVA) with SAS Software (SAS Institute, version 9, Cary, NC). Statistical significance was analysed at the level $p < 0.05$. When the analysis was sta-

tistically significant, Duncan's Multiple-Range Test (SSR Test) was used to test mean separation among mean values of each treatment.

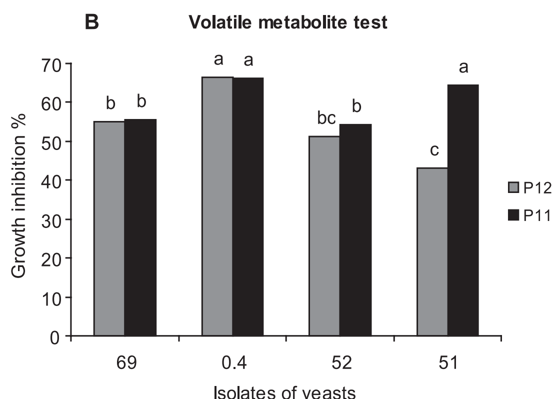
RESULTS

Effects of the antagonist in control of pathogen *in vitro*

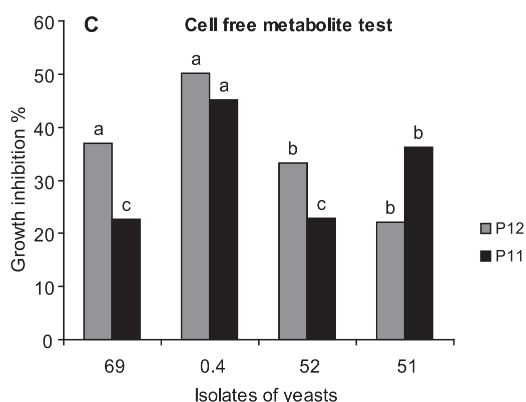
Four tested isolates of *S. cerevisiae* inhibited mycelial growth on average from 22.10 to 40.73% of P_{12} isolate, and from 19.81 to 39.33% of P_{11} isolate in dual culture. The impact of isolate 69 of yeasts on growth of two isolates of *P. expansum* was more than of the other isolates (Fig. 1A).



Growth inhibition percentage of *P. expansum* in dual culture with yeast



Growth inhibition percentage of *P. expansum* in volatile test with yeast



Growth inhibition percentage of *P. expansum* in cells free metabolite assay with yeast

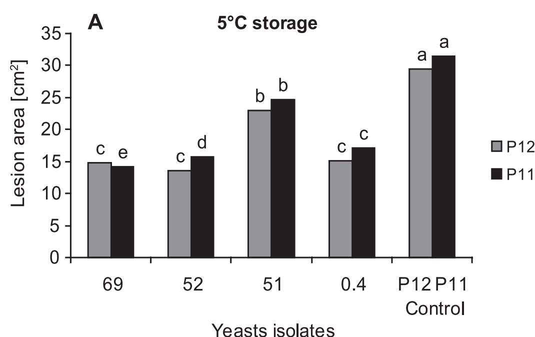
Fig. 1. Growth inhibition percentage invitro assay, dual culture (A), volatile test (B), and cells free metabolite (C). Different letters in the assay indicate significant differences between means according to Duncan's multiple range test ($p \leq 0.05$)

A volatile metabolite produced by four yeast isolates reduced the colony area of P_{12} from 54.3% to 66.30% and 43.16% to 66.44% for P_{11}. The impact of 04 isolate on growth of two isolates of *P. expansum* was more than for the other isolates. Regarrdless of the treatment in this experiment, of sinificant inhibition in the growth of two isolates of *P. expansum* was observed, suggesting that one or more volatile metabolites were responsible for biocontrol effect exhibited by these yeast isolates (Fig. 2B).

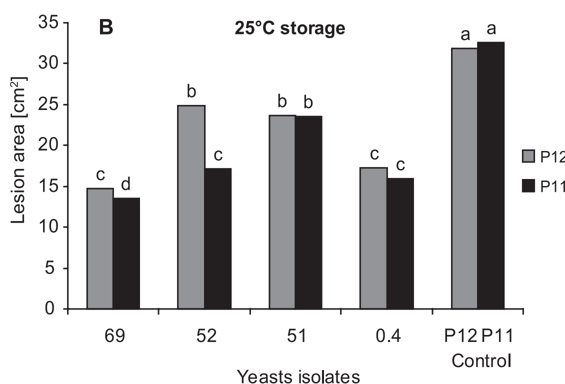
Cell-free metabolite produced by isolates of *S. cerevisiae* reduced the colony area of P_{12} isolate of *P. expansum* from 22.16 to 50.23% and 22.66 to 45.05% of P_{11}. Isolates 04 of the tested yeast was the most effective isolate in this assay (Fig. 3C).

Effect of different storage temperatures on control of blue mold

All four isolates of *S. cerevisiae* appeared to be good antagonists of blue mold on apples at 25 and 5°C ($p \leq 0.05$; Fig. 2). Four isolates *S. cerevisiae* reduced decay area from 14.77 to 24.92 cm^2 compared to 31 cm^2 in the control after incubation for 14 days at 25°C, and to 13.47 to 23.57 cm^2 compared to 32.57 cm^2 in the control. The isolate 69 was the most effective at both tempetures in this assay.



Effect of yeasts on apple decay in storage at 5°C in cold storage



Effect of yeasts on apple decay in storage at 25°C

Fig. 2. Biocontrol efficacy of four antagonistic yeasts on decay of apple fruit caused by *P. expansum* at 5°C (A), and 25°C (B). The data were based on four replicates of each treatment. Values followed by different letters are significantly different according to Duncan's multiple range test at $p \leq 0.05$

Effectiveness of Ca^{2+} on biocontrol activity of *S. cerevisiae*

There was a significant reduction of the disease with the Ca^{2+} yeast treatments, in comparision to yeast treatments alone ($p \leq 0.05$) (Fig. 3). The control of pathogen was correlated concentrations of Ca^2; $CaCl_2$ at 521 mmol/l provided a better control than 347 mmol/l but was not significant. Finally, 347 mmol/l was the best concentration of Ca^{2+}. The results showed that all concentrations of Ca^{2+} had a significant effect on decay diameter.

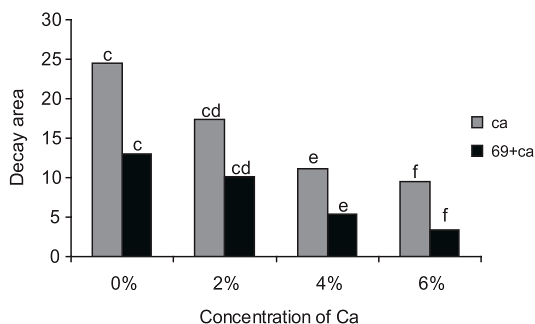
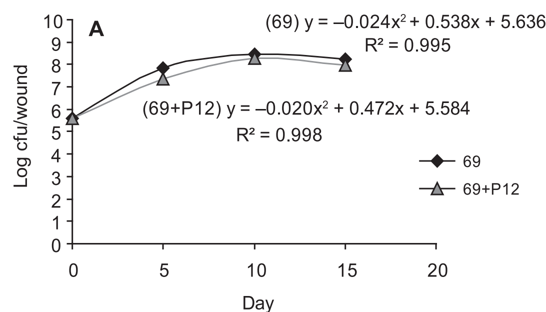
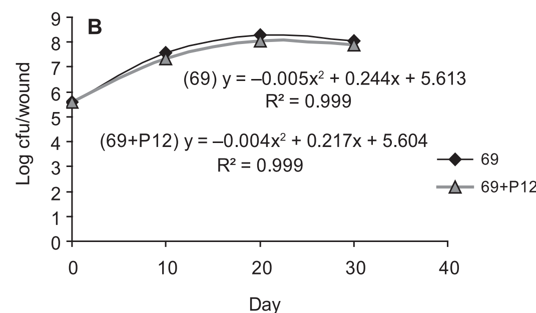


Fig. 3. Effect of different concentrations of Ca^{2+} and yeast on the decay area on apple fruits. Pathogen *P. expansum* concentrations was 10^5spore/ml lesion area was determined 14 days after storage at 25°C. Values followed by different letters are significantly different according to Duncan's multiple range test at $p \leq 0.05$





Population size of *S. cerevisiae* isolate 69 in apple wounds at 25°C

Fig. 4. Population dynamics of *S. cerevisiae*, isolate 69 (♦), and isolate 69 with added pathogen (▲), in wounds of apple fruit at 25°C, and 5°C

Table 1. Inhibition of mycelium growth of *P. expansum* (D) by *S. cerevisiae* isolates 52, 51, 69, and 04 *in vitro*[a]

Isolates of yeast	Percentage of inhibition[b]					
	dual culture		volatile test		cell free metabolite	
	P_{12}	P_{11}	P_{12}	P_{11}	P_{12}	P_{11}
52	35.67 a	23.08 b	51.33 bc	54.33 b	33.3 b	22.87 c
51	22.1 b	19.81 b	43.16 c	64.48 a	22.16 b	36.2 b
04	38.08 a	21.24 b	66.3 a	66.3 a	50.23 a	45.05 a
69	40.73 a	39.33 a	54.93 b	55.66 b	36.94 a	22.66 c

[a]values are mean growth inhibition from four petri-dishes
[b]values in the same row followed in the same letter are not statistically different in Duncan's Multiple Range Test ($p < 0.05$)

Table 2. Effect of yeast isolates on apple decay in storage at 25°C, and 5°C[a]

Treatment	Isolate of pathogen	Inhibition[b] [cm²]				
		52	51	04	69	control
25°C	P_{12}	24.92 b	23.7 b	17.22 c	14.77 c	31.81 a
	P_{11}	17.13 c	23.56 b	15.98 c	13.46 d	32.56 a
5°C	P_{12}	13.58 c	22.98 b	15.07 c	14.75 c	29.48 a
	P_{11}	15.78 d	24.98 b	17.16 c	14.16 e	31.51 a

[a]values are mean lesion area from four apples
[b]values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($p < 0.05$)

Table 3. Effect of different concentrations of Ca^{2+} on decay area diametera on apple

Treatment ($CaCl_2$)	Isolate of pathogen / Yeast isolate	Inhibition (cm²)	
		69+$CaCl_2$	$CaCl_2$ (whitout yeast)
0%	P_{11}	12.97 c	24.52 c
2%		10.09 cd	17.38 cd
4%		5.43 e	11.07 e
6%		3.4 f	9.54 f

[a]values are the mean lesion area from four apples
values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test ($p < 0.05$)

Dynamics of yeast population on wounded apples

Population dynamics of *S. cerevisiae* isolate 69 on wounded apple surface during cold storage period after different treatments are shown in figure 4. Initially, 10 days after the application of *S. cerevisiae*, populations of the antagonist were higher of about 4×10^5 CFU/g than initial 1.7×10^7 CFU/g. Subsequently, they increased progressively to reach maximum populations after 20 days of storage and after that decrease.

In 25°C storage, population dynamics of *S. cerevisiae* on wounded apple surface reached maximum after 10 days and after that decrease.

The effects of each treatment

The effects of *S. cerevisiae* in vitro, different concentrations of Cl^{2+} on wound apples, and storage temperature on decay were analyzed by analysis of variance (ANOVA).

As the effect of every treatment was significant, Duncan's Multiple-Range Test was adopted to compare mean separations among mean values of every treatment. In this analysis, diameter of rot was indicated as mean value. The treatment means were separated at the 5% significance level using Duncan's multiple range tests. We can see from Table 2 that reducing the storage temperature can significantly enhance antagonism. But Ca^{2+} in culture medium should be adjusted to an appropriate concentration to achieve the best antagonism.

DISCUSSION

The use of biocontrol agents to manage postharvest decay of fruit was explored as an alternative to the use of synthetic fungicides. Costs involved in the registration of a product may prevent its widespread availability in several countries. In the present research, we identified four isolates of the antagonist yeast that exhibit biocontrol efficacy against blue mold of the apples caused by *P. expansum*. This is the first report of *S. cerevisiae* as an antagonist of blue mold of apples in Iran.

The results of this study indicate that four tested isolates of *S. cerevisiae* have antagonistic biocontrol efficacy agaiinst *P. expansum*. There results of the efficacy trials show that a significant difference in biocontrol capability of the four antagonists did not exist, although isolate 04 gave a better control in in vitro assay conditions, and the isolate 69 was the most effective in vivo. Biocontrol of postharvest decay caused by different pathogens may need different antagonists.

Growth curves of the antagonists demonstrated that all isolates of yeasts could colonize and grow in apples wounds. Even after a period of 32 days at 5°C, and 14 days at 25°C the number of viable cells was greater than that originally introduced into the wound. These data indicate that only one application of the antagonists may be enough to prevent blue mold rot for at least a period of 32 days at 5°C, and 14 days at 25°C.

The reduction of exponential growth in apple wounds after 10 days at 25°C, and 20 days at 5°C indicates that nutrients may have become limited for antagonists and, most likely for the pathogen.

A better control of decay achieved by integration of yeast and $CaCl_2$ may be due to the combination of mechanisms of action displayed by both factor. These mechanisms are complex and not specific, affecting multiple sites in the pathogen; in addition, it is unlikely for a fungal pathogen to develop resistance to a biocontrol yeast.

Yeast are the most common biological control agents. This study showed a valuable method of control evalution of pathogen causing postharvest decay by using antagonistic yeasts. However, the method using yeasts include many aspects and needs further studying. Better methods are needed to achieve decay control and to combine microorganisms with storage of apples.

Currently, very few studies are done in our country on the ability of antagonistic microorganisms to control fresh fruit and vegetable pathogens causing postharvest decay.This area has a great potential and deserves more attention.

CONCLUSIONS

In summary, in this research were identified four isolates of yeast antagonists that exhibited biocontrol efficacy against blue mold of apples. All four isolates of yeast effectively colonized wounds of apple at 5 and 25°C. They have a good effect in vivo assay. They can be applied in storage under commerical conditions.

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POLISH SUMMARY

BIOLOGICZNE ZWALCZANIE NIEBIESKIEJ PLESNI JABŁEK IZOLATAMI *SACCHAROMYCES CEREVISAE*

Drożdże (52, 51, 69 i 04) otrzymano z centrum Biotechnologii Karaj, a *Penicillium expansum* izolowano z Golden Delicious. Izolaty oceniano jako potencjalne czynniki biologicznego zwalczania niebieskiej pleśni jabłek wywoływanej przez *P. expansum*. W testach *in vitro* uwzględniono podwójne kultury, bezkomórkowe metabolity i związki lotne. Wszystkie testowane izolaty drożdży inhibitowały wzrost *P. expansum*. Inhibicja różniła się w ramach testowanych izolatów i wahała się w granicach 19,81 do 40,73% w podwójnych kulturach, wynosiła od 43,16 do 66,44% w teście ze związkami lotnymi i od 22,16 do 50,23% w teście nie uwzględniającym metabolitów.

Rany na jabłkach inokulowano 40 μl zawiesiny komórek drożdzy (10^7 komórek/ml), a po 48 godzinach *P. expansum* (10^5 komórek/ml). Następnie jabłko inkubowano w 25°C. Cztery izolaty *P. expansum* redukowały powierzchnię zgnilizny w zakresie od 13,46 do 24,92 cm^2 w porównaniu do 32,18 cm^2 w kontroli, po inkubacji przez 14 dni w 25°C. Po 32 dniach inkubacji w 5°C wielkość ran wynosiła od 13,58 do 24,68 cm^2 dla kombinacji z traktowaniem antagonistą, w porównaniu do 22 cm^2 w kontroli. Izolat 69 *S. cerevicae* był najbardziej efektywny w obydwóch temperaturach tego testu i mógłby być ważnym, nowym czynnikiem biologicznego zwalczania niebieskiej pleśni jabłek.