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MODE OF ACTION OF *KLUYVEROMYCES LACTIS* IN BIOCONTROL OF *PENICILLIUM EXPANSUM*

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Numerous yeasts are reported as being effective in controlling plant pathogenic moulds. By selecting new biocontrol agents, knowledge about the mode of action of mould inhibition is important. In our study, mode of action of *Kluyveromyces lactis* – successfully applied against *Penicillium expansum* in former studies – was investigated. According to the results, volatile compounds are supposed to play a role in restriction of mould growth. Antibiotic substances and killer toxins produced by the tested *Kl. lactis* strain were not detected.

Keywords: biocontrol, mode of action, *Penicillium expansum*, *Kluyveromyces lactis*

Biological control – applying antagonistic yeasts in order to inhibit mould growth – is one of the recent possibilities in postharvest treatment for decreasing disease occurrence, e.g. blue mould rot, caused by *Penicillium expansum* (JANISIEWICZ et al., 1994; 2001; USALL et al., 2001; VERO et al., 2002).

Emphasis is set on the investigation of the mechanism of action of yeasts involved in biocontrol. According to CASTORIA and co-workers (2001), the knowledge about the mode of action is useful for easier registration procedures for commercialisation and for the identification of pivotal traits in antagonistic activity that could be enhanced by selection-based or molecular-based genetic tools.

Mode of action of yeasts is mainly a complex effect explained by numerous factors. The main factor in most cases is competition for nutrients and space (BJÖRNBERG & SCHNÜRER, 1993; PIANO et al., 1997; FAN & TIAN, 2001). *Candida guillermondii* was reported to compete for nitrogen sources in biocontrol of *P. expansum* on apple (ORTU et al., 2003). Controversially, the main sources of competition between *Pichia anomala* and *P. roqueforti* are sugars (DRUVEFORS et al., 2003). According to DROBY and co-workers (1991), some antagonists may induce wound healing process and other defence reactions of the host tissue. Direct interaction with the pathogen can also play a role in mode of action, e.g. in case of *C. famata* (ARRAS, 1996).

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In our former studies (TACZMAN-BRÜCKNER et al., 2005), strains of *Kl. lactis* showed inhibitory effect similar to three yeast species reported as biocontrol agents: *Pich. anomala* (BJÖRNBERG & SCHNÜRER, 1993; PETERSSON & SCHNÜRER, 1995), *Metschnikowia pulcherrima* (JANISIEWICZ et al., 2001) and *Sporobolomyces roseus* (JANISIEWICZ et al., 1994). In this work mode of action of *Kl. lactis* was studied.

1. Material and methods

1.1. Microorganisms

Two strains of *Penicillium expansum* NCAIM F00811 and NCAIM F00601, three strains of *Kl. lactis* (NCAIM Y00251, NCAIM Y00260 and NCAIM Y01080) were obtained from the Hungarian National Collection of Agricultural and Industrial Microorganisms. The cultures were grown on malt-extract-glucose slopes (MG, malt extract 17 g l^{-1}, Merck; glucose 5 g l^{-1}, Reanal) at 25 °C. Conidia of *P. expansum* were collected from colonies incubated for 6–7 days.

Concentration of conidia suspension and yeast suspension were adjusted/determined in all cases with hemacytometer.

Inhibition experiments were carried out either on MG or on PDA (potato dextrose agar 39 g l^{-1}, Merck) medium.

1.2. Production of antibiotic substances

In order to investigate antibiotic substances produced by *Kl. lactis* Y00260, cell free culture "filtrate" was prepared. Hundred ml of YEPD broth was inoculated with 1 ml of *Kl. lactis* Y00260 suspension. The concentration of the yeast suspension was adjusted to 10^8 cells ml^{-1}. The inoculated broth was incubated at 30 °C for 2 days on a rotary shaker (180 r.p.m.). The suspension was centrifuged at 4000 r.p.m. for 5 min, and the cell free supernatant was used.

The effect of concentrated supernatant was also investigated. Cell free supernatant was prepared as previously described. Fifteen–fifteen ml of the supernatant was pipetted into sterile Petri dishes then they were frozen at –18 °C. Higher concentration was achieved by lyophilising at 45 °C. The same procedure was carried out with sterile YEPD broth which served as control. The lyophilised samples were rehydrated in 1.5 ml of sterile distilled water.

Agar well diffusion assay was carried out to study the effect of the cell free culture filtrate of the yeast on strains of *P. expansum*. Hundred ml of conidia suspension (10^6 conidia ml^{-1}) of *P. expansum* F00811 and F00601, respectively, were spread plated on MG and PDA media. Three wells (diameter of 6 mm) were prepared in the inoculated media. The wells were filled with 100–100 μl of the cell free supernatant. Two replicates were prepared for each set of conditions. Mould development around the wells was investigated.

1.3. Volatile and gaseous compounds produced by Kluyveromyces lactis

The effect of volatile and gaseous compounds produced by *Kl. lactis* was investigated with two agar plates facing each other ("mouth-to-mouth assay"). This method was developed in the laboratory of Professor Johan Schnürer, Department of Microbiology, Swedish University of Agricultural Sciences, Sweden (Ulrika Druvefors, personal communication). The upper agar plate was inoculated on three spots with 10–10 μl of (10^5 conidia ml^{-1}) conidia suspension of *P. expansum* strains F00811 and F00601, respectively. On the lower agar plate 100–100 μl of (10^7 cells ml^{-1}) suspension of *Kl. lactis* strains Y00260, Y00251 and Y01080, respectively, were spread plated (Fig. 1). Mouth-to-mouth plates without yeast inoculum on the lower plate served as controls. Inhibition was determined by measuring mould colony diameter. The trials were carried out on MG and PDA media at 22 °C and 5 °C with two replicates.

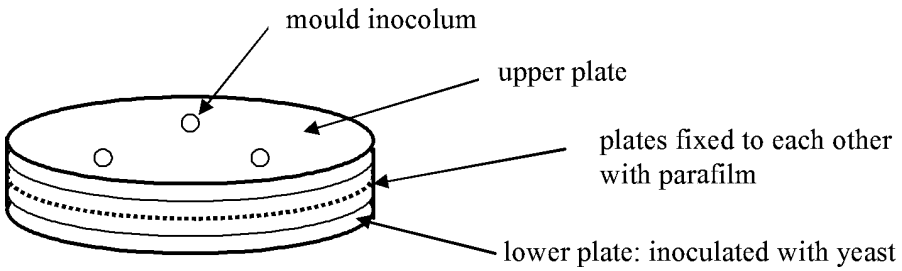

Fig. 1. Mouth-to-mouth assay for investigating the inhibitory effect of volatile compounds produced by antagonistic yeasts against moulds

Changes in CO_2 concentration – caused by metabolism of *Kl. lactis* Y00260 – were measured in mouth-to-mouth plates not inoculated with mould by PBI DANSENSOR gas analyser (Combi Check 9800-1). The sampling syringe of the gas analyser was driven between the two plates. Samples were taken on days 2 and 5 at 1 min intervals from 5 mouth-to-mouth plates respectively, and the O_2/CO_2 concentration was determined.

Main volatile compounds produced by *Kl. lactis* and present in the headspace of the mouth-to-mouth plates were identified by GC-MS analysis. Measurements were carried out on a chromatograph type HP 5890A (Hewlett-Packard Company, Avondale, PA) connected with a quadrupole mass spectrometer type VG-TRIO-2 (VG Masslab Ltd., Altrincham, UK). The GC conditions were as follows: 25 m×0.2 mm Ultra-2 capillary column with methyl-silicon coating (0.33 μm) (Hewlett-Packard Company, Avondale, PA), column temperature from 60 °C to 280 °C at a rate of 15 °C min^{-1}, injector temperature 280 °C. The MS conditions were as follows: EI scan mode,

electron energy 70 eV, interface and source temperature 280 °C. Measurements were carried out in selected ion recording mode (SIR) by the following mass numbers m/z 70, 71 and 73. Components were identified on the basis of retention times of standards and the ion intensity ratios measured at different mass numbers.

Solid-phase-micro-extraction (SPME) was applied to investigate volatile compounds in the headspace. Adsorption fibre with 100 μm poli-dimethyl-siloxane coating (Supelco, Bellefonte, PA) was used. Adsorption was carried out at 60 °C for 30 min.

2. Results and discussion

2.1. Production of antibiotic substances

Cell free supernatant had no inhibition effect on the *P. expansum* strain F00811 on MG or PDA medium. Very slight changes in conidia formation were observed in a 2–3 mm zone around the wells in case of *P. expansum* F00601 on both media.

Applying the concentrated form of the cell free supernatant no inhibition was detected in case of the *P. expansum* strains. Comparing these with similar investigations carried out with cell free culture filtrate of *M. pulcherrima* against *Botrytis cinerea* (PIANO et al., 1997; SPADARO et al., 2002) or of *Cryptococcus albidus* against *B. cinerea* and *P. expansum* (FAN & TIAN, 2001), our results suggest that no antibiotic substance was produced by *Kl. lactis*. This trait of the yeast is one of the important characteristics of biocontrol agents, because production of antibiotic substances can lead to potential development of pathogen resistance, secondly there may be unpredictable consequences for consumers (CASTORIA et al., 2001).

2.2. Volatile and gaseous compounds produced by Kluyveromyces lactis

Inhibition of *P. expansum* in the mouth-to-mouth assay depended on the inhibited mould strain, the applied culture medium and the incubation temperature (Figs 2 and 3).

P. expansum F00811 was significantly inhibited by all of the tested *Kl. lactis* strains (Y00251; Y01080 and Y00260) at 22 °C on MG medium (Fig. 2a). The same result was achieved on PDA medium only in case of the *Kl. lactis* strain Y00260, the inhibitory effect of the yeast strains Y00251 and Y01080 was less significant (Fig. 2c). The growth of mould – irrespective of the presence of antagonist – was slower at 5 °C than at 22 °C. No significant inhibition was detected on MG medium at 5 °C (Fig. 2b) despite samples grown on PDA medium, where *Kl. lactis* Y00260 and Y01080 showed significant inhibition (Fig. 2d).

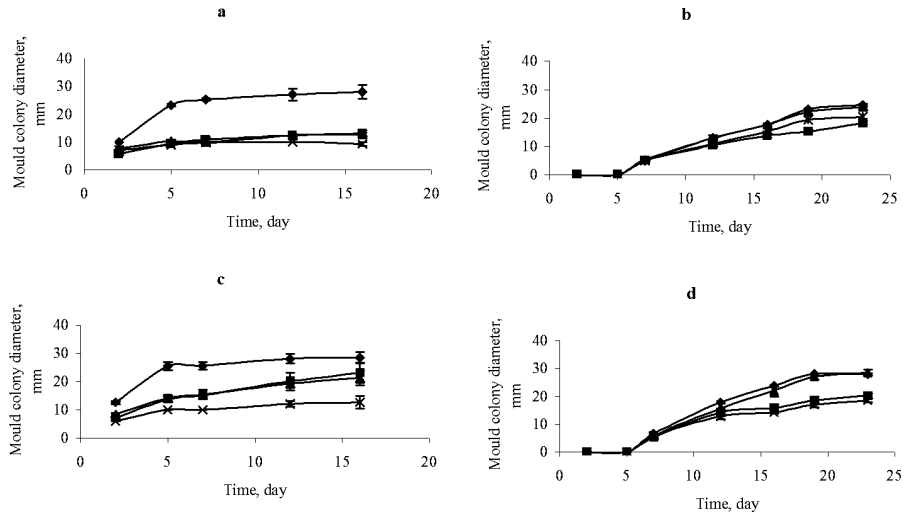

Fig. 2. Inhibitory effect of volatile products produced by *Kluyveromyces lactis* strains on the growth of *Penicillium expansum* NCAIM F00811. (Bars indicate standard error of the means) **a:** MG medium, 22 °C; **b:** MG medium, 5 °C; **c:** PDA medium, 22 °C; **d:** PDA medium, 5 °C; ♦: *P. exp*. F00811 control; ■: mould growth in the presence of *Kl. lactis* Y01080; ▲: mould growth in the presence of *Kl. lactis* Y00251; ×: mould growth in the presence of *Kl. lactis* Y00260

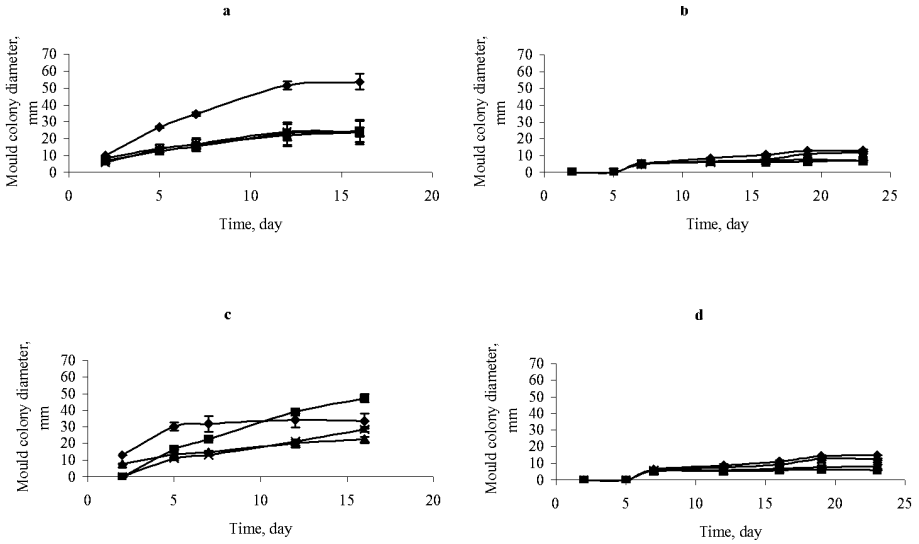

Fig. 3. Inhibitory effect of volatile products produced by *Kluyveromyces lactis* strains on the growth of *Penicillium expansum* NCAIM F00601. (Bars indicate standard error of the means) **a:** MG medium, 22 °C; **b:** MG medium, 5 °C; **c:** PDA medium, 22 °C, **d:** PDA medium, 5 °C; ♦: *P. exp*. NCAIM F00601 control; ■: mould growth in the presence of *Kl. lactis* Y01080; ▲: mould growth in the presence of *Kl. lactis* Y00251; ×: mould growth in the presence of *Kl. lactis* Y00260

The results of the trial were similar in case of *P. expansum* F00601 with some exceptions. The control mould colonies were larger than those of the *P. expansum* F00811 at 22 °C on MG medium (Fig. 3a). Nevertheless, low incubation temperature had a stronger inhibitory effect on growth of the *P. expansum* strain F00601 than on strain F00811 on both culture media (Fig. 3b and d). Difference between the tested mould strains was detected on PDA medium at 22 °C (Fig. 3c). Growth of *P. expansum* was inhibited significantly by *Kl. lactis* Y00260 for 12–15 days. The inhibitory effect of this yeast strain decreased in the last days of storage. Surprisingly, *P. expansum* F00601 colonies grown in the presence of *Kl. lactis* Y01080 were not inhibited, moreover the colonies were more extended at the end of the experiment than control colonies.

SAKSENA and TRIPATHI (1987) achieved similar results when the inhibitory effect of some organic volatile compounds (acetaldehyde, ethyl acetate, ethanol, 2-methyl-butanol, 3-methyl-butanol) was tested on mould (*Alternaria solani*, *Colletotrichum capsici*) growth. It was reported that volatile compounds applied singly or in various combinations suppressed the spore germination of target moulds.

Results of identification of volatile compounds are shown in Fig. 4. Ethyl acetate (short chain ester), isoamyl alcohol and a mixture of isobutyric and isovaleric acid compontents of the culture medium were identified at mass number 70 (Fig. 4a); and 2-phenylethyl isobutyrate was identified at mass number 73 (Fig. 4b).

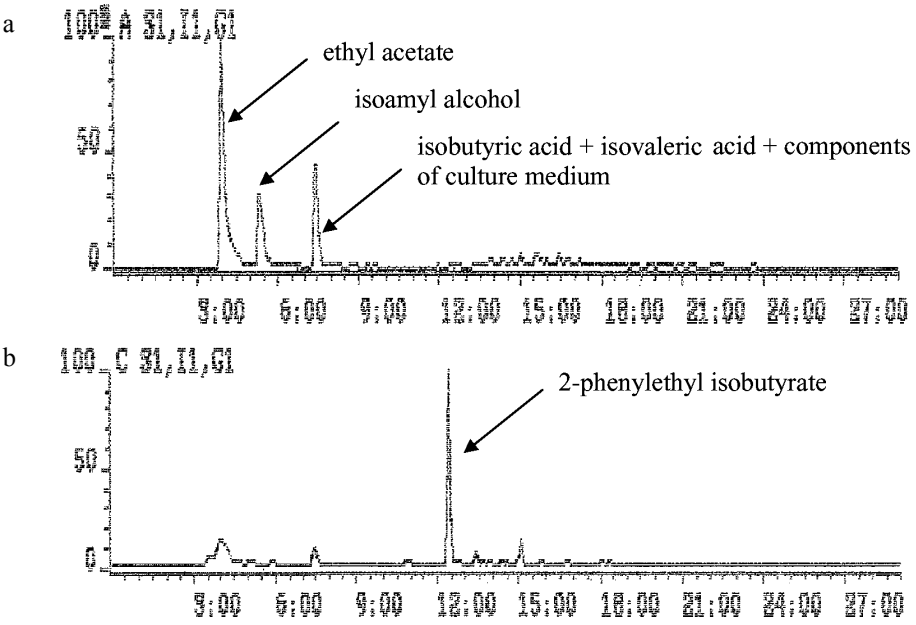

Fig. 4. Chromatogram of GC/MS analysis of volatile compounds produced by *Kluyveromyces lactis* NCAIM Y00260. **a:** Mass number m/z 70; **b:** Mass number m/z 73

JIANG (1995) identified 52 compounds from liquid culture of *Kl. lactis*. Among these compounds, isoamyl alcohol and phenylmethanol were detected in relatively high amounts. *Kl. lactis* produces also short-chain acids, especially ethyl acetate in large quantities (HANSSEN et al., 1984). Comparing these facts with the results of our study, volatile compounds produced by *Kl. lactis* may play a role in the mode of action in inhibiting *P. expansum*.

Due to metabolism of *Kl. lactis* Y00260 significant changes were observed in the gas composition of the area between the two plates facing each other (Table 1). CO_2 level increased from 0.1% up to 8–10% and O_2 concentration decreased from 20.5% to 13–14%.

Table 1. Changes in O_2/CO_2 concentration caused by *Kluyveromyces lactis* NCAIM Y00260 in mouth-to-mouth plates

Days of incubation	Control mouth-to-mouth plates without microorgansim inoculation		Mouth to mouth plates inoculated with *Kl. lactis*	
	O_2 (%)	CO_2 (%)	O_2 (%)	CO_2 (%)
2	20.3±0.3	0	14.0±0.5	10.0±0.6
5	20.5±0.06	0.1	14.6±0.9	7.9±0.9

The increased CO_2 concentration may contribute to the inhibitory effect of *Kl. lactis*, however, former studies showed (not published) that application of modified atmosphere storage with 10% CO_2 has not resulted in such inhibition of *P. expansum* as it was found in mouth-to-mouth assay. This is in agreement with the study of YACKEL and co-workers (1971) which shows that controlled atmosphere storage with 10.5% CO_2 and 2.0% O_2 concentration had no effect on appearance of *P. expansum* at 21 °C compared to samples grown in normal gas atmosphere of the air.

3. Conclusions

This study shows that volatile and/or gaseous substances produced by *Kluyveromyces lactis* cause inhibition of *Penicillium expansum*. Further investigations are needed to examine the effect of the different compounds separately and combined.

Competition for nutrients, which is the major factor of mode of action of biocontrol yeasts, has to be also investigated in future.

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