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SCIENTIFIC NOTE

INHIBITORY ACTIVITY OF YEASTS AGAINST SOYBEAN PATHOGENIC FUNGI

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The objective of this study was to evaluate the inhibitory activity of yeasts (*Saccharomyces cerevisiae* and *Pichia fermentans*) against soybean pathogenic fungi. The inhibitory activity of *Pichia fermentans* was previously confirmed against *Candida* spp. and a sensitive strain of *S. cerevisiae* to confirm its killer activity. Simple competition plate bioassays were performed using five soybean pathogenic fungi (*Alternaria* sp., *Cercospora kikuchi, Colletotrichum dematium* var. *truncata*, *Fusarium oxysporum*, and *Macrophomina* sp.) with a killer isolate of *P. fermentans* and a non-killer strain of *S. cerevisiae*. The yeasts tested inhibited 52 to 65% the growth of at least *Alternaria* sp., *Cercospora kikuchi* and *Colletotrichum dematium* var. *truncata*. This is the first record of the inhibition of *Cercospora kikuchi* and *Colletotrichum dematium* var. *truncata* using yeast strains.

Key words: Biocontrol, *Glycine max*, killer yeasts, *Pichia fermentans*, *Saccharomyces cerevisiae*.

Atividade inibitória de leveduras contra fungos patogênicos da soja. O objetivo deste estudo foi avaliar a atividade inibitória de leveduras (*Saccharomyces cerevisiae* e *Pichia fermentans*) contra fungos patogênicos da soja. A atividade inibitória de *Pichia fermentans* foi primeiramente confirmada contra linhagens de *Candida* spp. e uma linhagem sensível de *S. cerevisiae* para confirmar sua atividade *killer.* Um bioensaio de competição simples em placa foi conduzido usando cinco fungos patogênicos da soja (*Alternaria* sp., *Cercospora kikuchi* and *Colletotrichum dematium* var. *truncata*, *Fusarium oxysporum* e *Macrophomina* sp.) com um isolado *killer* de *P. fermentans* e um isolado não-*killer* de *S. cerevisiae*. As leveduras testadas inibiram de 52 a 65% o crescimento de pelo menos *Alternaria* sp., *Cercospora kikuchi* e *Colletotrichum dematium* var. *truncata*. Esse é o primeiro registro da inibição de *Cercospora kikuchi* e *Colletotrichum dematium* var. *truncata* usando linhagens de leveduras.

Palavras-chave: Biocontrole, *Glycine max*, leveduras *killer*, *Pichia fermentans*, *Saccharomyces cerevisiae*.

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Yeasts and their toxins have had several applications for the fungal control in areas of medical (Walker, McLeod and Hodgson, 1995; Conti et al., 2000, 2002; Guyard et al., 2002; Buzzini et al., 2004; Magliani et al., 2014; Travassos et al., 2004) and agronomical sciences (Polonelli and Morace, 1986; Walker, McLeod and Hodgson, 1995; Santos and Marquina, 2004), as well as in the food and fermentation industries for the control of fungal contaminating during the production of wine (Boone et al., 1990; Hara, Imura and Otsuka, 1990; Musmanno, Di Maggio and Coratza, 1999), beer (Young, 1981) and bread (Bortol et al., 1986), or also for biocontrol of plant diseases in field (El-Tarabily, 2004).

A wide range of antimicrobial substances or hydrolytic enzymes has been characterized from yeasts, so that the inhibition of other microorganisms by yeasts could be related to a variety of metabolic products (Polonelli and Morace, 1986; Masih and Paul, 2002; Urquhart and Punja, 2002) and also due to simple competition for space and nutrients (Valdebenito-Sanhueza, 2000). The killer phenomenon represents one of these various mechanisms and corresponds to the ability of certain yeast strains to inhibit by exotoxins the growth of sensitive strains (Bevan and Makower, 1963) and to control antagonistic microorganisms in the same environment, generating a selective advantage (Gill, 1974).

The Brazilian soybean production has a global importance because since 2013 it is the world's largest supplier of soybean and their exports represent 26% of agricultural export earnings of the country (OECD-FAO, 2015). Estimative data also suggest the culture of oilseeds, mainly soybean, will continue to dominate the land use in Brazil, taking up almost half of the additional crop area until 2024 (OECD-FAO, 2015).

Among the many factors could decrease the soybean production and affect the culture are the infections caused by various phytopathogenic fungi that can colonize and infect seeds, pods, or flowers prior to harvesting (Roy, Baird and Abney, 2001), including species of *Alternaria*, *Aspergillus*, *Boeremia*, *Cercospora*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Epicoccum*, *Fusarium*, *Gibberella*, *Hannaella*, *Macrophomina*, *Penicillium*, *Periconia*, and *Verticillium* (Peshney, Mahant and Ninawe, 1994; Villarroel et al., 2004; Navi, Rajasab and Yang, 2016; Liu et al., 2017).

Thus, the objective of this study was to evaluate the activity of two yeast strains (*Saccharomyces cerevisiae* and *Pichia fermentans*) in the inhibition of five phytopathogenic fungi with importance for the soybean cultivation (*Alternaria* sp., *Cercospora kikuchi*, Colletotrichum *dematium* var. *truncata*, *Fusarium oxysporum* and *Macrophomina* sp.). The reference strain *S. cerevisiae* NCYC1006 and a wild isolate of *P. fermentans* Y11-E were tested. *Pichia fermentans* Y11-E was previously studied regarding its inhibitory activity against *Trichoderma* sp. from mushroom cultivation (Marques-Marçal, 2005).

The killer activity of *P. fermentans* Y11-E was previously confirmed according to the protocol described by Rosini (1983). An additional pilot experiment was performed using *P. fermentans* Y11-E against *Candida* species according to the methods carried by Polonelli et al. (1983) and Buzzini and Martini (2001) and with the killer strain *S. cerevisiae* NCYC738 for comparison.

The yeast strains utilized as inhibitors and the other fungal strains tested for growth susceptibility or as control are presented in Table 1.

Table 1 - Fungal strains used for inhibitory tests, including the pilote testing

Fungal species tested regarding the inhibition by yeasts	
***Candida* strains (Pilot testing)**	**Origin**
C. albicans Mg06 *C. lusitaniae* 80D *C. tropicalis* 32D	Laboratory of Molecular Biology of Microorganisms, State University of Londrina (Londrina, PR, Brazil)
Soybean pathogenic fungi (Main testing)	**Origin**
Alternaria sp. *Cercospora kikuchi* *Colletotrichum dematium* var. *truncata* *Fusarium oxysporum* *Macrophomina* sp.	Laboratory of Phytopathology, Embrapa Soybean (Londrina, PR, Brazil)
Yeast strains tested as inhibitors	
Non-killer strain (susceptible to killer toxin)	**Origin**
Saccharomyces cerevisiae NCYC1006	André Tosello Foundation (Campinas, SP, Brazil)
Killer strains	**Origin**
Saccharomyces cerevisiae NCYC738	André Tosello Foundation (Campinas, SP, Brazil)
Pichia fermentans Y11-E	Laboratory of Molecular Biology of Microorganisms, State University of Londrina (Londrina, PR, Brazil)

For the inhibitory test against soybean pathogenic fungi, simple competition plate bioassays were performed using PDA (Potato Dextrose Agar) medium with the killer isolate *P. fermentans* Y11-E and the non-killer strain *S. cerevisiae* NCYC1006 as potential inhibitors. On one half of the Petri plate, a small mycelium disc of the phytopathogen was placed whilst on the other half of the plate the both yeast strains were streaked in separated treatments. All plates were incubated at 25°C for 14 days. The assessment was carried out in three repetitions and the inhibition was considered positive when clear zones of inhibitory were apparent between the yeast colony and the filamentous fungal biomass. The percentage of inhibition was calculated by comparing the radial mycelium growth of the phytopathogens when in contact with the yeasts and based on the control plates that lacked yeast inoculum.

In the pilot experiment we verified that the three *Candida* strains had their growth inhibited from 80 to 90% by the action of both killer yeasts, with values of inhibition lower and significantly different (two-way ANOVA, $F = 7.7020 > 3.2389$, $p < 0.05$; Tukey-Kramer test $p < 0.05$) from those verified to the sensitive strain *S. cerevisiae* NCYC1006, which was inhibited from 96 to 100% by *S. cerevisiae* NCYC738 and *P. fermentans* Y11-E, respectively (Figure 1). No significative difference of inhibition was verified by the action of the same killer yeast among different *Candida* strains (Tukey-Kramer test $p < 0.05$). The inhibition of each *Candida* strains was not significantly different in function of the killer strain tested (two-way ANOVA, $F = 0.9532 < 4.4940$, $p < 0.05$).

On the main testing using yeasts against soybean pathogenic fungi, the yeasts tested inhibited 52 to 65% the growth of at least *Alternaria* sp., *Cercospora kikuchi* and *Colletotrichum dematium* var. *truncata* (Figure 2). The inhibition of each phytopathogen was not significantly different (T test, $p < 0.05$) regarding the yeast tested, except for *C. dematium* var. *truncata*, which was inhibited only by *P. fermentans* Y11-E. Among the three phytopathogens inhibited by the yeast strains, *Alternaria* sp. was the most susceptible to the inhibitory activity, with inhibition values significantly higher (T test, $p < 0.05$; one-way ANOVA, $F = 13.26$, $p < 0.01$) than those verified for *C. dematium* var. *truncata* (Tukey-Kramer test, $p < 0.05$) and *C. kikuchi* (Tukey-Kramer test, $p < 0.01$).

In the competition plate bioassays including *Alternaria* sp. and *Cercospora kikuchi* it was possible to observe a zone of inhibition between the yeasts and

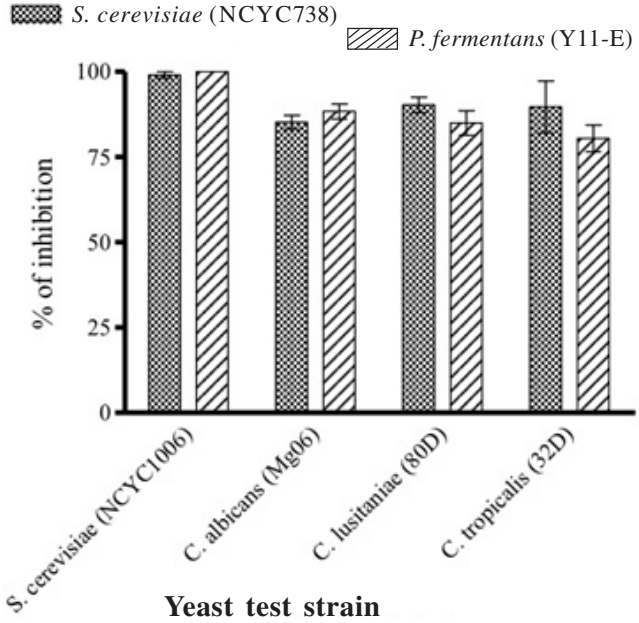

Figure 1 - Inhibition of *S. cerevisiae* NCYC1006 (control) and *Candida* species by the killer strains *S. cerevisiae* NCYC 738 and *P. fermentans* Y11-E.

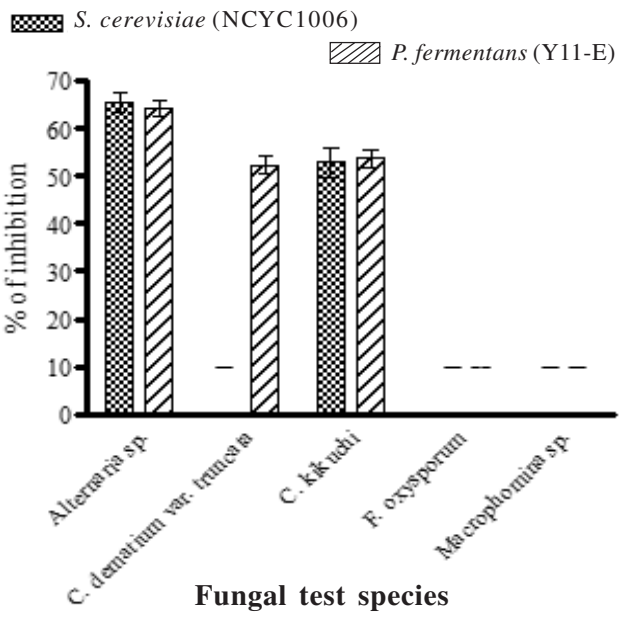

Figure 2 - Inhibition of soybean pathogenic fungi by the yeast strains *S. cerevisiae* (NCYC1006) and *P. fermentans* Y11-E. (-) negative inhibition.

the pathogenic fungi (Figure 3), which could be an indicative of inhibition by antibiosis. However, different action modes could be occurring because living cells were used to demonstrate this inhibitory activity and the non-killer strain *S. cerevisiae* NCYC1006 also have had inhibitory activity under two of the five soybean pathogenic fungi tested. In the inhibition of *Colletotrichum dematium* var. *truncata* by *P. fermentans* Y11-E it was not observed a zone of inhibition and the mycelium of the phytopathogenic fungi grew weakly until to the limit of contact with the yeasts, most likely representing a kind of competition for space between both fungal species.

Although *S. cerevisiae* NCYC1006 has been described as sensitive to the killer toxin, variations in external factors such as temperature, nutritional conditions and mainly pH could affect the expression of the killer toxins or the sensitivity to the strains exposed to the factor (Polonelli et al., 1983). Furthermore, the inhibition of phytopathogens by a non-killer strain most likely is not because the killer toxins but to a variety of different metabolic products that have antagonistic activity on the growth of other fungi, as suggested by Polonelli and Morace (1986), or also by competition for space and nutrients between the fungal species, which has been the most common investigated way for biocontrol (Valdebenito-Sanhueza, 2000).

The same range of mechanisms could be applied for the inhibition by the killer isolate *P. fermentans* Y11-E, because, in this method, living cells were used to demonstrate the inhibitory activity. However, the applicability of this method was previously demonstrated by Walker, McLeod and Hodgson (1995) that showed the activity of *S. cerevisiae* killer strains and other *Pichia* yeasts against phytopathogenic fungi with activity varying up to 100 % inhibition in some cases.

The activity of yeast and its toxins in the control of microorganisms could represent a great potential for

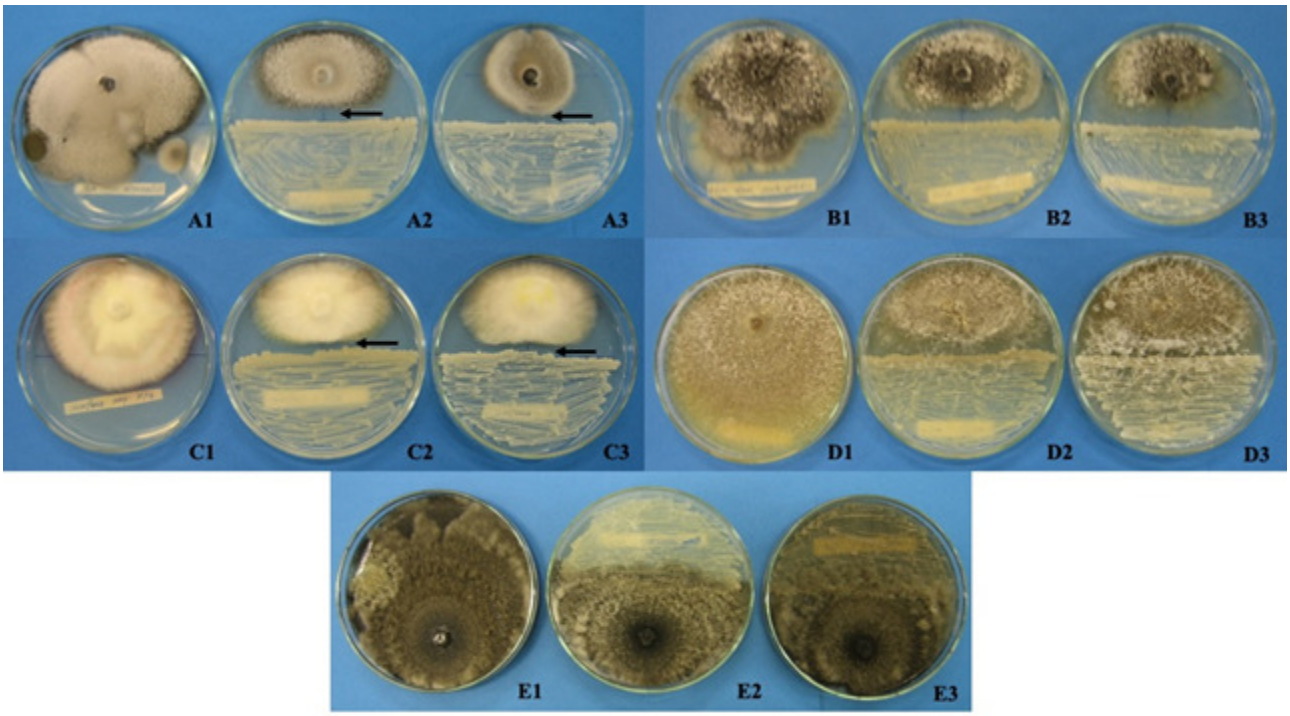

Figure 3 – Competition plate bioassays in PDA (Potato Dextrose Agar) medium on day 14 of incubation at 25°C. Black arrows indicate the zone of inhibition between the yeast and the pathogenic fungi. A1. *Alternaria* sp. (control). A2. *Alternaria* sp. and *P. fermentans* Y11-E. A3. *Alternaria* sp. and *S. cerevisiae* NCYC1006. B1. *Colletotrichum dematium* var. *truncata* (control). B2. *C. dematium* var. *truncata* and *P. fermentans* Y11-E. B3. *C. dematium* var. *truncata* and *S. cerevisiae* NCYC1006. C1. *Cercospora kikuchi* (control). C2. *C. kikuchi* and *P. fermentans* Y11-E. C3. *C. kikuchi* and *S. cerevisiae* NCYC1006. D1. *Fusarium oxysporum* (control). D2. *F. oxysporum* and *P. fermentans* Y11-E. D3. *F. oxysporum* and *S. cerevisiae* NCYC1006. E1. *Macrophomina* sp. (control). E2. *Macrophomina* sp. and *P. fermentans* Y11-E. E3. *Macrophomina* sp. and *S. cerevisiae* NCYC1006.

use as a natural antimycotic in the control of plant fungal infections as showed by many authors (Walker, McLeod and Hodgson 1995; Cabral et al., 2009; Rosa-Magri, Tauk-Tornisielo and Ceccato-Antonini, 2011; Mekbib, Thierry and Regnier, 2011; Portes et al., 2013).

The use of *Pichia* strains as promisor biocontrol agents has been frequently studied and reported (Masih and Paul, 2002; Santos and Marquina, 2004; Santos, Sanchez and Marquina, 2004; Comitini et al., 2004; Druverfors, Passoth and Schnurer, 2005; Portes et al. 2013). The growth inhibiting of *Alternaria* and *Colletrotrichum* species also has been reported using yeast strains (Koomen and Jeffries, 1993; Wang et al., 2008, 2010; Chaisemsaeng, Mongkolthanaruk and Bunyatratchata, 2013). However, the discovery of yeast strains capable of inhibiting the growth of *Cercospora kikuchi* and *Colletotrichum dematium* var. *truncata* is apparently new and no previous reports were found on the literature.

The in vitro inhibitory tests are promising for the utilization of yeasts in biocontrol against these pathogenic fungi as an alternative to traditional treatment methods that utilize chemical compounds and can result in eventual toxicity consequences to the cultivars. The study of the inhibitory mechanisms of the tested yeasts is also encouraged in order to identify the action mode and the means by which the yeast controls the mold growth.

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