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The Inter-generic Fungicidal Activity of *Xanthophyllomyces dendrorhous*

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In this study, the existence of intra-specific and inter-generic fungicidal activity in *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* strains isolated from different regions of the earth was examined. Assays were performed under several culture conditions, showing that all the analyzed *X. dendrorhous* and *P. rhodozyma* strains have killing activity against *Kloeckera apiculata*, *Rhodotorula sloffiae*, and *R. minuta*. This activity was greater in rich media at a pH from 4.6 to 5.0. Extracellular protein extracts with fungicidal activity were obtained from cultures of all strains, and their characterization suggested that a protein of ~33 kDa is the antifungal factor. According to peptide mass fingerprinting and an analysis of the results with the MASCOT search engine, this protein was identified as an aspartic protease. Additionally, extrachromosomal double-stranded DNA elements (dsDNAs) were observed in all *X. dendrorhous* and *P. rhodozyma* strains. Although there is a high variability, two dsDNAs of 5.4 and 6.8 kb are present in all strains.

***Keywords*:** *X. dendrorhous*, mycocin, killer yeast, fungicidal activity

Killer or mycocinogenic yeasts produce proteinous toxins that kill other yeasts of the same genus and, less frequently, those of different genera (Magliani *et al*., 1997; Marquina *et al*., 2002; Schmitt and Breinig, 2002). Ecological studies have suggested that mycocin production could be a mechanism of interference competition, giving the mycocinogenic yeast an advantage over sensitive competing microorganisms (Morais *et al*., 1995; Conti *et al*., 1996). This phenomenon was discovered in 1963 in strains of *Saccharomyces cerevisiae*, and several studies have reported mycocinogenic strains that belong to a wide range of yeast genera (Koltin and Kandel, 1978; Young and Yagiu, 1978; Middelbeek *et al*., 1980; Golubev and Nakase, 1997; Golubev *et al.*, 2002, 2006; Fuentefria *et al*., 2008; Peng *et al*., 2009). The genetic basis for a mycocinogenic phenotype is variable and can be encoded by the chromosome or by cytoplasmic inherited extrachromosomal genetic elements (EGEs). EGEs can be linear double-stranded DNA (dsDNA) plasmids or double-stranded RNA (dsRNA) elements that are encapsulated in Virus-Like Particles (VLPs) (Magliani *et al*., 1997). Double-stranded DNA plasmids have been associated with a killer phenotype in *Pichia inositovora*, *P. acaciae*, and *Kluyveromyces lactis* (Gunge, 1986; Ligon *et al.,* 1989; Worsham and Bolen 1990; Hayman and Bolen, 1991). In *K. lactis*, two plasmids of 8.9 kb (pGKL1) and 13.4 kb (pGKL2) have been sequenced and characterized in relation to their organization, replication, and expression. These studies found that the killer protein is encoded in the pGKL1 plasmid (Volkert *et al*., 1989; Stark *et al*., 1990; Fukuhara, 1995). The most characterized dsRNA elements are those found in killer strains of *Ustilago maydis* and *Saccharomyces cerevisiae* (Tipper and Schmitt, 1991; Kinal *et al*., 1995; Bruenn, 2001; Kang *et al*., 2001; Wickner *et al*., 2002; Schmitt and Breinig, 2006). Most strains of *S. cerevisiae* carry the L-A dsRNA virus (4.6 kb), and the mycocinogenic strains additionally carry the M dsRNA (1.6 to 1.8 kb). The L-A dsRNA virus belongs to the *Totiviridae* virus family, and its genome contains two overlapping ORFs encoding the proteins necessary for its encapsidation and replication (Field *et al*., 1982; Icho and Wickner, 1989; Fujimura *et al*., 1990; Dinman *et al*., 1991). The genome of M dsRNA contains only one ORF encoding a mycotoxin and self-immunity (Schmitt and Tipper, 1990; Dignard *et al*., 1991; Schmitt and Tipper, 1992; Schmitt, 1995). The M dsRNA is referred to as a "satellite" virus because it uses the viral proteins encoded by L-A, the "helper" virus, for its own encapsidation and replication. Double-stranded RNA elements have been found in several members of the *Cystofilobasidiales clade* and associated fungicidal activity has been described (Karamysheva *et al*., 1993; Pfeiffer *et al*., 1998, 2004b; Fell *et al*., 1999; Golubev *et al*., 2002, 2003) in each strain except for the astaxanthin-producing yeast *Xanthophyllomyces dendrorhous*. The EGEs described in this yeast are variable, and the strains can have dsRNA elements, linear dsDNA plasmids or both; in *Phaffia rhodozyma* (the imperfect phase of *X. dendrorhous*), the presence of only dsDNA plasmids has been reported (Castillo and Cifuentes, 1994; Pfeiffer *et al*., 1996, 2001; Kucsera *et al*., 2000; Baeza *et al*., 2009). In previous studies about the potential functions of dsRNAs, a minor effect on the reproduction and fitness of *X. dendrorhous* was observed, and the results regarding fungicidal activity were inconsistent (Castillo and Cifuentes, 1994; Pfeiffer *et al*., 1996, 2001).

Assays to test for fungicidal activity are relatively simple to perform. The key factor in these assays is the yeast that is used as the sensitive or target cell. Other experimental conditions, such as the pH, media salinity and incubation temperature, are also very important. For example, most known killer toxins

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Table 1. Yeast strains used in this study

Strain	Source/Reference
Xanthophyllomyces dendrorhous	CBS 6938
	UCD 67-385
	UCD 67-383
	UCD 68-653C
	CBS 5908
	VKM Y-2266
	VKM Y-2786
	VKM Y-2059
	UCD 67-202
	385(S2)-40 (Baeza *et al*., 2009)
Phaffia rhodozyma	CBS 5905
Saccharomyces cerevisiae AH22	ATCC 38626
Bulleromyces albus	CBS 500
Candida albicans 1001	ATCC 64385
Candida albicans 5314	Laboratory collection
Cryptococcus humicola	CBS 571
Debaryomyces castelli	CBS 2923
Kluyveromyces lactis	Laboratory collection
Pichia anomala A4	(Reyes *et al.*, 2004)
Pichia anomala A5	(Reyes *et al.*, 2004)
Kloeckera apiculata	Laboratory collection
Rhodotorula minuta	CBS 319
Rhodotorula sloffiae	CBS 7095
Rhodotorula mucilaginosa	Laboratory collection
Trichosporon mucoides	Laboratory collection

CBS, Centraalbureau voor Schimmelcultures; ATCC, American Type Culture Collection; UCD, University of California Davis; VKM, All-Russian Collection of Microorganisms

are active at pH values from 3 to 5.5 (Golubev and Shabalin, 1994; Marquina *et al*., 2002). In the present study, we tested the intra-specific and inter-generic fungicidal activity of *X. dendrorhous* and *P. rhodozyma* strains isolated from various geographical regions.

Materials and Methods

Yeast strains and culture conditions

All strains used in this study are listed in Table 1. The yeast were routinely grown in YM medium (0.3% yeast extract, 0.3% malt extract and 0.5% peptone) supplemented with 2% glucose at 22°C. The yeast extract was omitted from the liquid media used for obtaining extracellular proteins. Agar (1.5%) was added to make the semisolid media. For assays low in nutrients, minimal Vogel's medium N (Vogel, 1956; Retamales *et al*., 2002) was supplemented with the required nutrients.

Fungicidal activity determination

The assays were performed on "sensitive lawns", which were made by mixing 25 ml of molten medium (40°C), 250 µl of 0.3% methylene blue and 2.5 ml of fresh "sensitive" yeast culture (~10^7 cells/ml) poured into a Petri plate (Salek *et al*., 1990). The pH of the medium was adjusted with a citrate-phosphate buffer as needed. Two kinds of assays were performed: i) The "colony screening" method: *X. dendrorhous* strain colonies were picked from fresh YM plates, seeded onto the sensitive lawns and incubated at different temperatures until death halos appeared (visualized by precipitation of methylene blue). ii) The well test method: aliquots of 100 to 200 µl of samples were deposited into wells (10 mm in diameter) cut into the sensitive lawns. The diameter (D) of the death halo was recorded, and the killer activity in UA/ml (A) was calculated according to the formula D=5 logA × 10 (Schmitt and Tipper, 1990; Gulbiniene *et al*., 2004).

DNA isolation

The nucleic acids were purified from yeast protoplasts by a method adapted from a previous study (Santopietro and Kula, 2001). Briefly, 25 ml of yeast culture grown in YM medium at 22°C was centrifuged at 6,000×g for 10 min. The cellular pellet was washed with 12.5 ml of 0.8 M KCl and suspended in 4 ml of 0.8 M KCl with 4 mg/ml lysing enzymes (catalog # L-1412, Sigma, USA). The samples were incubated at 22°C for 4 h with gentle agitation and then centrifuged at 6,000×g for 10 min; and the cellular pellet was washed with 50 ml of 0.8 M KCl. The protoplasts were suspended in 5 ml of 1% SDS, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0 with 100 µg/ml Proteinase K and incubated at 65°C for 30 min. Subsequently, 1.5 ml of 3 M potassium acetate was added, and the sample was incubated on ice for 1 h and then centrifuged at 12,000×g for 10 min. Two volumes of isopropanol were added to the supernatant, and the sample was incubated at -20°C for 2 h. The nucleic acids were obtained by centrifugation at 12,000×g for 10 min at 4°C, washed with 5 ml of 70% ethanol, dried and suspended in 500 µl of nuclease free water. The samples were stored at -20°C.

Enzymatic treatments

The samples were digested with DNase I, Nuclease S1, and RNase H according to standard protocols (Muthukrishnan and Shatkin, 1975; Sambrook *et al.*, 1989). Digestions with RNase A were performed in 0.01× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) using 1 enzymatic unit per microgram of total nucleic acids (Pryor and Boelen, 1987; Castillo and Cifuentes, 1994).

Extracellular protein extraction

The yeast culture (100 to 500 ml) was centrifuged at 7,000×g for 5 min at 4°C, and the supernatant was filtered through a sterile 0.45-µm pore size polyvinylidene fluoride membrane (Millipore, USA). The cell-free supernatant was adjusted to a 45% (v/v) final concentration with ethanol and incubated on ice for 30 min. After centrifugation at 8,000×g for 15 min, the supernatant was recovered, and ethanol was added to reach a final concentration of 75% (v/v). The sample was incubated on ice for 30 min, centrifuged at 8,000×g for 15 min, and the pellet was dried and dissolved in H_2O or citrate/phosphate buffer at pH 4.6. The total protein content was estimated spectrophotometrically (Aitken and Learmonth, 1996).

Electrophoresis and peptide mass fingerprinting (PMF)

Protein samples were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue G-250 (Vetec Química Fina LTDA, Brazil). The protein bands were excised from the Coomassie gels, digested with trypsin, and subjected to peptide mass fingerprinting via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at the Cambridge Centre for Proteomics (University of Cambridge, UK). The data obtained were analyzed by Mascot and only the results with a score greater than 54 ($P<0.05$) were considered statistically significant (Perkins *et al*., 1999).

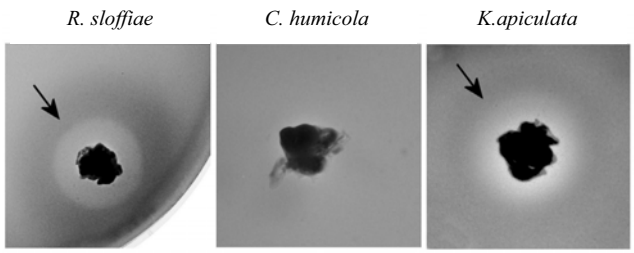

Fig. 1. Antifungal activity of *X. dendrorhous*. Assays were performed by the colony screening method on yeast lawns made in YM medium, as described in the 'Materials and Methods', and incubated at 22°C. Arrows indicate the methylene blue precipitate (death halo).

Results

Determination of fungicidal activity by the colony screening method

Because mycocinogenic yeast are typically able to kill other yeast that belong to the same or closely related species or genera, we initially performed cross tests of fungicidal activity among the strains of *X. dendrorhous* and *P. rhodozyma* listed in Table 1. The sensitive lawns were prepared in rich (YM) and minimal (Vogel's medium N) media at various pH (4.2, 4.6, 5.0, 5.4, and 5.8) and supplemented with 2% glucose. The plates were incubated at 15 or 22°C and were inspected daily to determine yeast growth and monitor the development of death halos (noticeable as precipitation of methylene blue). Through repeated experiments, no fungicidal activity was observed among any of the *X. dendrorhous* and *P. rhodozyma* strains even after 15 days incubation (data not shown). The assays to determine the inter-generic antifungal activity were performed using several yeasts as sensitive cells, including some belonging to genera that cohabit with *X. dendrorhous* and *P. rhodozyma* in the colonizing of birch tree spring sap-flows (Golubev *et al*., 2002; Weber, 2006), and are therefore potential targets. As shown in Fig. 1, UCD 67-385 developed a clear death halo on lawns of *Rhodotorula sloffiae* and *Kloeckera apiculata*, indicative of antifungal activity; no halo was observed on lawns of *C. humicola*. In Table 2, the antifungal activities of *X. dendrorhous* and *P. rhodozyma* strains are summarized. All strains displayed killing activity against *R. sloffiae*, *R. minuta*, and *K. apiculata*. The *P. rhodozyma* strain also has activity on *R. mucilaginosa* lawns, but it is important to mention that these experiments were difficult to interpret because of the mucosal growth of *R. mucilaginosa*. The killing activity against *R. sloffiae* was higher at 22°C and pH 4.6 (Table 3), and similar results were obtained on *K. apiculata* and *R. minuta* lawns. The fungicidal assays were also performed on lawns of *K. apiculata* and *R. sloffiae* grown on minimal Vogel's medium N supplemented with 2% glucose. Contrary to the results observed with the rich media, all strains of *X. dendrorhous* and *P. rhodozyma* displayed very low or no fungicidal activity with this medium (data not shown).

Fungicidal activity and characterization of extracellular protein extracts

To determine if the fungicidal activity corresponds to a secreted protein, the *X. dendrorhous* and *P. rhodozyma* strains were cultivated in YM broth and the total proteins were obtained from the cell-free supernatants. The fungicidal activity of these protein extracts was determined by the well test method, and the protein profiles were assessed by SDS-PAGE. Unexpectedly, protein extract obtained from sterile YM medium (used as control) showed some low fungicidal activity on lawns of *R. sloffiae* and *K. apiculata*. We analyzed each component of the medium and found that the yeast extract was responsible for this activity, which was later omitted from the liquid media. In the colony screening method, the results

Table 2. Inter-generic killing patterns of *X. dendrorhous* and *P. rhodozyma* strains

Strain lawn	CBS 6938	UCD 67-385	UCD 68-653C	CBS 5908	VKM Y-2266	VKM Y-2786	VKM Y-2059	UCD 67-202	CBS 5905[a]
Saccharomyces cerevisiae AH22	-	-	-	-	-	-	-	-	-
Bulleromyces albus	-	-	-	-	-	-	-	-	-
Candida albicans 1001	-	-	-	-	-	-	-	-	-
Candida albicans 5314	-	-	-	-	-	-	-	-	-
Kloeckera apiculata	+	++++	++	++++	++++	++++	+++	+	++++
Debaryomyces castelli	-	-	-	-	-	-	-	-	-
Kluyveromyces lactis	-	-	-	-	-	-	-	-	-
Pichia anomala A4	-	-	-	-	-	-	-	-	-
Pichia anomala A5	-	-	-	-	-	-	-	-	-
Cryptococcus humicola	-	-	-	-	-	-	-	-	-
Rhodotorula minuta	+	++	++	++	++	+	+	+	++
Rhodotorula sloffiae	+	++++	++	++++	+++	++++	+++	+	++++
Rhodotorula mucilaginosa	-	±	±	-	±	-	±	-	+
Trichosporon mucoides	-	-	-	-	-	-	-	-	-

The assays were performed by the colony screening method at pH values of 4.2, 4.6, 5.0, and 5.4 and incubation temperatures of 15 and 22°C. Results obtained at pH 4.6 and 22°C are shown. The development of death halos was observed after 5-7 days incubation except for *R. mucilaginosa* and *R. minuta* lawns, in which the halos appeared after 10-13 days incubation. The assays were performed in YM medium with or without yeast extract and the same results were obtained. (–) represents no fungicidal activity; (+) to (++++), low to high fungicidal activity; (±), uncertain fungicidal activity.

[a] type strain of *P. rhodozyma*.

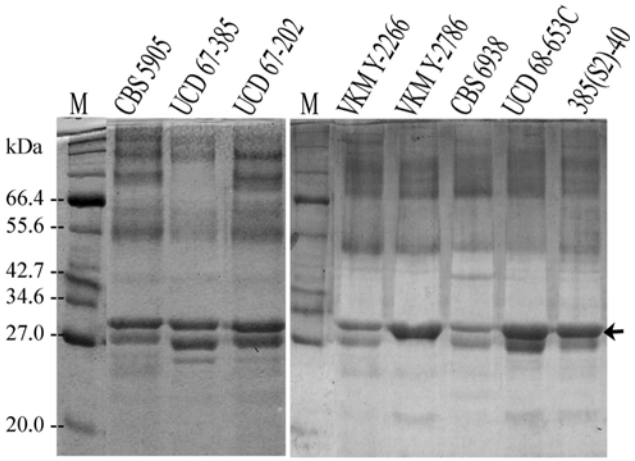

Fig. 2. Protein profiles of extracellular extracts. The total extracellular protein content was obtained from cell-free supernatants of *X. dendrorhous* and *P. rhodozyma* cultures and analyzed by SDS-PAGE. M, protein marker P7702S (New England Biolabs).

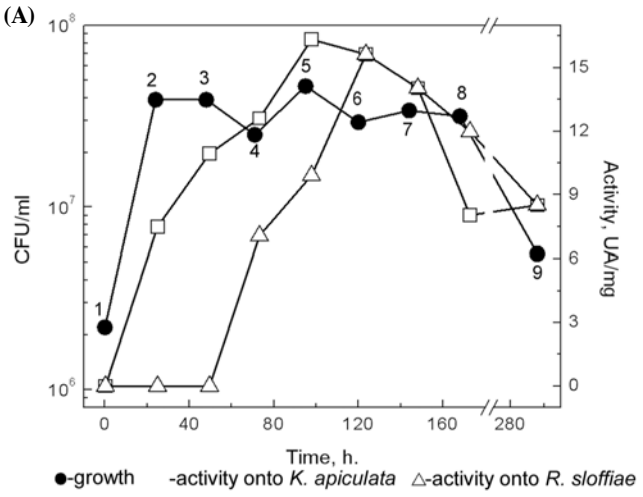

Fig. 3. Antifungal activity and extracellular protein content along the growth curve. *X. dendrorhous* UCD 67-385 was grown in YM medium at 22°C, and samples were collected at the indicated times (points 1 to 9). The extracellular proteins were obtained from each sample, and the fungicidal activity (A) and protein profiles (B) were determined. The activities were normalized by the total protein amount. M, protein marker M4038 (Sigma).

obtained were invariable if the sensitive lawns were made in medium with or without yeast extract. The cell-free extracts obtained from cultures of all strains of *X. dendrorhous* and *P. rhodozyma* showed fungicidal activity on lawns of *R. sloffiae* and *K. apiculata*; this activity was almost completely lost after incubation at 70°C for 15 min. The protein content of the active samples obtained from cultures of the different yeast strains was characterized by SDS-PAGE (Fig. 2). A protein with an estimated mass of 33 kDa (arrow) was the most abundant and common to all protein samples. Furthermore, in fractionation experiments of protein extracts from the UCD 67-385 and CBS 5905 strains using Amicon Ultra-4 centrifugal filter devices (Millipore) of different nominal molecular weight limits, the activity was mainly observed in fractions between 30 and 50 kDa (data not shown). To additionally characterize mycocin production, a growth curve for *X. dendrorhous* UCD 67-385 was performed in YM medium without yeast extract, and the activity and protein profiles of the culture supernatants were determined at different times. As shown in Fig. 3A, the protein extracts obtained at 24 and 48 h have activity against *K. apiculata* but not against *R. sloffiae*. Samples collected at 72+ h showed activity against both yeast lawns. The maximum activity was observed at the stationary phase and diminished at the end of this phase. SDS-PAGE (Fig. 3B) revealed that extracts active only against *K. apiculata* have mainly two protein bands; the band marked with an asterisk (*) was also observed in extracts obtained from sterile medium that had no activity, as was observed for the zero time point in Fig. 3B (point 1). Furthermore, this band was absent in samples obtained later in the curve that still had fungicidal activity. In contrast, the protein with an estimated size of 33 kDa (Fig. 3B, arrow) increased in

Table 3. Fungicidal activity of *X. dendrorhous* strains against *R. sloffiae*

Strain	Temp	15°C				22°C			
	pH	4.2	4.6	5.0	5.4	4.2	4.6	5.0	5.4
UCD 67-385		+	++	++	±	++	+++	++	+
CBS 5905[a]		±	++	++	±	+++	+++	++	+
VKM Y-2786		+	+	±	-	++	+++	++	+
CBS 6938		-	-	-	-	±	++	-	-
CBS 5908		++	++	++	±	+	+++	+	±

The lawns of *R. sloffiae* were made in YM buffered at different pH.
[a] type strain of *P. rhodozyma*

Table 4. Extrachromosomal dsDNA plasmids in *X. dendrorhous* strains

Strain	dsDNA (kb)						
	7.5	6.8	5.4	4.7	4.4	3.4	2.7
UCD 67-202	+	+	+	+	+	+	+
VKM Y-2786	-	+	+	+	-	+	+
VKM Y-2266	-	+	+	+	-	+	-
VKM Y-2059	-	+	+	+	-	+	-
CBS 5905[a]	-	+	+	+	-	+	-
UCD 68-653C	-	+	+	-	-	+	-
UCD 67-385	-	+	+	-	-	-	-
UCD 67-383	-	+	+	-	-	-	-
CBS 6938	-	+	+	-	-	-	-

[a] type strain of *P. rhodozyma*

intensity and remained relatively constant in concentration in all of the samples with fungicidal activity. Similar results were obtained from cultures of *P. rhodozyma* CBS 5905 (data not shown). These results suggest that the ~33 kDa protein is the antifungal factor; therefore, the band corresponding to this protein was excised from the Coomassie gels, digested with trypsin and subjected to peptide mass fingerprinting (PMF) via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at the Cambridge Centre for Proteomics (University of Cambridge, UK). The results were analyzed using the Mascot search engine and three peptides of 11 (KYTLTSTGKKQ), 24 (KQSGTFSISYGDGSSASGPVYKD) and 32 (RLATTNSELYLGGINSAHYTGAITYTPVTQKA) residues showed similarity to a precursor of an aspartic protease in *X. dendrorhous* for all protein samples. The protein bands under 33 kDa that appear late in the growth curve (clasp in Fig. 3B) were also analyzed by PMF, and the same three peptides with similarity to aspartic protease were obtained, suggesting that these are degradation products derived from the ~33 kDa protein.

The yeast fungicidal phenotype can be encoded by the chromosomal DNA, linear dsDNA plasmids or viral dsRNA elements. Most strains of *X. dendrorhous* have dsRNA elements that vary in number and size (Castillo and Cifuentes, 1994; Pfeiffer *et al*., 1996; Baeza *et al*., 2009). From strain UCD 67-385, which has four dsRNAs of 5.0 (L1), 3.7 (L2), 0.9 (S1), and 0.8 (S2) kb, we previously obtained a clone lacking the S2 dsRNA [385(S2)-40] by treatment with the peptidyl transferase inhibitor anisomycin (Baeza *et al*., 2009). This cured strain displayed fungicidal activity against *R. Sloffiae*, *R. minuta*, and *K. apiculata*, as the parental uncured strain does (not shown). Thus, at least the S2 dsRNA does not encode for the fungicidal phenotype.

Extrachromosomal dsDNA plasmids

The total nucleic acids were purified from the cultures of *X. dendrorhous* and *P. rhodozyma* strains. Samples were treated with DNase I (for degrading the deoxyribonucleic acids), nuclease S1 (for degrading single-stranded nucleic acids), RNase H (for degrading RNA from a DNA/RNA hybrid), and RNase A (for degrading ribonucleic acids) and then analyzed in agarose gels. In all samples, there were numerous extrachromosomal nucleic acid bands that were identified as dsDNA because they were not degraded by nuclease S1, RNase H or RNase A but were degraded by DNase I. The estimated sizes vary from 2.7 to 7.5 kb, and each strain of *X. dendrorhous* can harbor 2, 3, 4, 5 or 7 dsDNAs (Table 4). When the dsDNA profiles were compared among the strains, two dsDNAs of 6.8 and 5.4 kb were present in all of them.

Discussion

Generally, mycocins (killer toxins) are characterized by their specific activity against organisms related to the mycocinogenic strain (Golubev, 1998). In this study, we did not detect cross-fungicidal activity among *X. dendrorhous* and *P. rhodozyma* strains isolated from various geographic areas. *X. dendrorhous,* together with *Tr. pullulans* and *N. fulvescens* var. *elongate*, are late-colonizing organisms of the spring sap flux. This habitat is initially highly diverse and contains species that belong to fourteen different yeast genera (Golubev *et al*., 2002). Therefore, we performed additional assays to test for fungicidal activity toward these cohabitant members. We found that all strains of *X. dendrorhous* and *P. rhodozyma* have fungicidal activity against *R. sloffiae*, *R. minuta*, and *K. apiculata*. This mycocinogenic characteristic of *X. dendrorhous* could help, together with substrate competition and its freeze-thawing tolerance (Weber, 2006), establish the organisms' dominance during the final phase of birch sap colonization. The fungicidal activity of all *X. dendrorhous* strains is higher in rich media than in minimal media, and in many cases cannot be detected in the later. This finding agrees with previous reports about the competition between killer and non-killer strains of *S. cerevisiae*. These studies found that toxin production is beneficial only when nutritional resources are abundant (Wloch-salamon *et al*., 2008).

Generally, mycocins are encoded by extra-chromosomal viral dsRNAs or linear dsDNA plasmids. The presence of dsRNA viruses was previously described in the three dominant yeasts that colonize spring tree exudates (Castillo and Cifuentes, 1994; Pfeiffer *et al*., 1996, 2004a; Golubev *et al*., 2002). These viral elements were associated with mycocin production in *Tr. pullulans*. No fungicidal activity of *N. fulvescens* against other *Nadsonia* species was detected. The mycocinogenic strains of *X. dendrorhous* have a high degree of polymorphism in dsRNAs with no common elements; one *X. dendrorhous* strain and the *P. rhodozyma* strain have no dsRNA (Baeza *et al.,* 2009). Therefore, the dsRNAs cannot encode for the fungicidal activity of *X. dendrorhous* against *R. sloffiae*, *R. minuta*, and *K. apiculata*. On the other hand, all *X. dendrorhous* and *P. rhodozyma* strains have two dsDNA plasmids of 6.8 and 5.4 kb. Although the presence of DNA plasmids is disseminated over a large variety of yeasts, only the linear plasmids found in *K. lactis* (Gunge, 1986), *P. inositovora* and *Pichia acaciae* (Worsham and Bolen, 1990; Bolen *et al*., 1994; McCracken *et al*., 1994) have been associated with fungicidal activity.

The results obtained from characterizing the growth curves, activity and extracellular proteins of cell-free culture supernatants from *X. dendrorhous* and *P. rhodozyma* strongly suggest that a 33 kDa protein may be the toxin. Based on size estimation and the results from peptide mass fingerprint characterization and mascot analysis, our toxin is remarkably

similar to a previously described exported aspartic protease of *X. dendrorhous* (Bang *et al*., 1999). No previous studies have reported an aspartic protease with antifungal activity in yeast or filamentous fungi. In plants, the production of proteases is believed to be a general defense mechanism against pathogenic fungi. Secretion of aspartic proteases with antimicrobial activity against *Phytophthora infestans* and the conidia of *Fusarium solani* has been described in potato tubers (*Solanum tuberosum*) after infection and damage to the intercellular fluids (Guevara *et al*., 2001, 2002, 2005; Mendieta *et al*., 2006).

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