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Characteristics of Wild Yeast Killer from Nahuel Huapi National Park (Patagonia, Argentina)

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Summary

The aim of this study was the survey of killer yeasts in natural environments at the Nahuel Huapi National Park in the Subantartic Forest, Northwestern Patagonia. One hundred and twenty three yeast isolates were analysed. Yeasts associated with nectarine flowers, sugary wild fruits and sporangia of the fungus Cyttaria spp., and glacial-origin lakes freshwater samples were screened in their killing activity against the sensitive yeast tester strain Candida glabrata NCYC 388. The sensitivity to known toxins (K_1 to K_{10}) was tested against yeast isolates. Three of the 28 cultures isolated from nectar showed killer activity, whereas none was detected in those derived from fruits or freshwater samples. The 38% of all tested strains were sensitive to one or more toxins. The broadest spectrum of sensitivity was observed in cultures from nectar samples, while 57% of the yeasts isolated from fruits were neutral. The latter were also prevalent among yeasts from freshwater samples.

Keywords: killer yeasts, terrestrial environments, aquatic environments

Introduction

The killer activity was first reported in *Saccharomyces cerevisiae (1)*. Since then the killer character was detected among many yeast genera and species in culture collections, industrial strains, and in ascomycetous and basidiomycetous yeasts colonising different substrates *(2–4)*. Killer yeasts produce extracellular proteins or glycoproteins lethal towards sensitive yeasts being themselves immune to their own toxins. Neutral strains are resistant to killer factors and are not killed as are sensitive strains. This phenomenon was observed in wine-making yeasts *(2)* and its biotechnological significance led to the study of the killer trait in spontaneous fermenting musts *(5,6)* as well as to the selection and/or to construction of the strains carrying this ability *(7)*. The potential therapeutic effect of killer toxins in the treatment of pityriasis versicolor and pneumonia caused by *Pneumocystis carinii* has been evaluated experimentally *(8,9)*. On the other hand killer activity of *Kluyveromyces* spp. strains against the halotolerant fermenting yeast *Zygosaccharomyces rouxii* has been tested in order to select them as natural preservatives for the fermentation industry *(10,11)*.

The ecological significance of killer toxins is a matter of current study *(3,12)*. Ganter and Starmer *(13)* have demonstrated that killer yeasts can change the cactophilic yeasts community structure (species number and distribution). The killing pattern could be used as a taxonomic tool. Broad spectrum toxins may be useful in obtaining phylogenetic affiliations among strains, whereas narrow spectrum toxins may be used for the classification and taxonomic studies of related organisms *(14)*. Recently, Vaughan Martini *et al. (15)*, have suggested the usefulness of differential killer sensitivity in the fingerprinting of wine-yeast strains of *Sacch. cerevisiae*.

In Argentina, studies of killer yeasts are scarce, dealing mainly with musts and grapes *(16,17)*. This is the first report on these yeasts in natural environments. The aim of this work was to test the killer behaviour of wild yeasts isolated from terrestrial-soil interfaces, and from hydrological-soil interfaces of the Nahuel Huapi National Park (NHNP) located in Northwestern Patagonia, Argentina.

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Materials and Methods

Mature and healthy fruits of *Rubus idaeus, Pernettya mucronata*, and sporangia (»llao-llao«) of the *Nothofagus* spp. parasitic fungus *Cyttaria hariotti* were collected aseptically in the field, and stored individually in boxes to avoid cross-contamination or crushing. Yeasts were isolated by placing whole fruits or halves directly on the surface of YEPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar) plates containing amoxicylin (1500 IU/L) to prevent bacterial growth.

Nectar samples from healthy and open flowers of *Desfontainia spinosa* (»taique«), *Embothrium coccineum* (»notro«), *Tristerix tetrandrus* (»quintral«), *Berberis* spp. (»calafate«) and *Asteranthera ovata* (»estrellita«), obtained with sterile Pasteur pipettes, were dropped over sterile filter paper disks aseptically kept in Petri plates (one for each species and sampling place). Once in the laboratory, the disks were placed onto the surface of YEPD agar plates.

Water samples were collected either by submerging sterile glass bottles just below the surface or using a Ruttner bottle to get a mixed sample at different depths (0.30 to 32 m) except for Toncek Lake samples, which were obtained individually at each depth and filtered »in situ«. Filtration of the samples (500–1000 mL) was performed through sterile filter membranes (Millipore, 0.47 μm) with a manual Nalgene vacuum pump. The filter membranes were placed on the surface of YEPD plates as described. All plates were incubated at 22 °C until colonies appeared. Pure cultures of isolated yeasts were transferred to YEPD agar slants, incubated for 48–72 h at 22 °C, and stored at 5 °C for further studies.

The identification tests were performed following the keys of Kreger van-Rij *(18)*.

Killer activity was assessed using the method described by Starmer *et al. (3)* and *Candida glabrata* Y55 (NCYC 388) was employed as sensitive strain (ss). A 0.1 mL aliquot of a suspension of the ss, in sterile distilled water, was placed as a layer onto the surface of YM agar

Table 1. Killer profile of yeasts from nectar at the Nahuel Huapi National Park

Source	Nr	Genera/Species	Killer [(a)]	Neutral	Sensitive to
Embothrium coccineum [(b)]	167	*Ambrosiozyma* spp.	–	+	–
	168	*Candida colliculosa*	–	–	K_{10}
	170	*Candida famata*	–	–	K_9, K_{10}
	171	(n.i.)	–	+	–
	172	*Candida versatilis*	–	–	K_6
	173	*Rhodotorula* spp.	–	+	–
	174	*Issatchenkia* spp. (l)	–	–	K_5, K_6
	175	*Issatchenkia* spp.(l) *(ascosporógena)*	–	–	K_5
Fuchsia magellanica [(b)]	159	*Candida versatilis*	–	–	K_4, K_6
	160	*Candida colliculosa*	–	+	–
	161	*Candida* spp. G I	–	+	–
	5	*Candida famata*	–	+	–
	7	*Candida pulcherrima* (l)	–	–	K_5
	8	*Candida colliculosa*	–	–	K_{10}
	9	*Candida* spp. G VII	–	+	–
	158	*Saccharomyces* spp.	–	–	K_4, K_6
	162	*Saccharomyces* spp.	–	–	K_8, K_9, K_{10}
Desfontainia spinosa [(b)]	1	*Candida pulcherrima* (l)	–	–	K_5, K_8, K_{10}
	3	*Candida dattila* (l)	–	–	K_5, K_6, K_9, K_{10}
Asteranthera ovata [(b)]	163	*Kloeckera* spp.	–	–	K_8, K_9, K_{10}
Berberis spp. [(c)]	180	*Candida* spp. G VI/VII	–	–	K_8
	181	*Candida* spp. G VI/VII	–	–	K_5, K_8, K_9, K_{10}
Tristerix tetrandrus [(c)]	176	*Taphrina* (y.l.f.)	+	–	K_5, K_9
	177	*Candida* spp. G X	–	+	–
	178	*Candida* spp. G III	–	+	–
Berberis buxifolia [(d)]	187	*Torulaspora* spp.	+	–	K_5, K_8, K_9, K_{10}
	188	*Candida* spp.	–	–	K_5, K_8, K_9, K_{10}
	189	*Torulaspora* spp.	+	–	K_5, K_8, K_9, K_{10}

(a) killer activity against *C. glabrata* NCYC 388. Sampling sites: (b) Puerto Blest, (c) Villa Tacul, (d) Cerro Otto. (n.i.) = not identified. G I...G X = yeast taxonomic groups *(18)*; (y.l.f.) = yeast-like form; (l) = like.

Table 2. Killer profile of yeasts from fruits and *Cyttaria* spp. sporangia at the Nahuel Huapi National Park

Source	Nr	Genera/Species	Killer [a]	Neutral	Sensitive to
Rubus idaeus [b]	13	*Kloeckera* spp.	–	+	–
	90	*Kloeckera* spp.	–	+	–
	91	*Kloeckera* spp.	–	+	–
	92	*Kloeckera* spp.	–	+	–
	95	*Kloeckera* spp.	–	+	–
	96	*Kloeckera* spp.	–	+	–
	97	*Kloeckera* spp.	–	+	–
	14	*Rhodotorula araucariae* (l)	–	–	K_{10}
Rubus idaeus [c]	16	*Taphrina* (y.l.f.)	–	+	–
	98	*Candida* G VII	–	–	K_5, K_8, K_9
	99	*Candida* G II	–	–	K_8, K_9
	15	*Candida pulcherrima* (l)	–	–	K_5
	17	*Candida pulcherrima* (l)	–	–	K_5, K_8, K_9, K_{10}
	101	*Candida* spp. G III	–	–	K_8
	102	*Candida* spp. G VII	–	–	K_5, K_8
	114	*Candida* spp. G VII	–	–	K_5, K_8, K_9
	100	*Cryptococcus albidus*	–	+	–
	104	*Cryptococcus kuetzingii*	–	+	–
Pernettya mucronata [d]	11	*Candida* spp. G II		+	–
	12	*Sporobolomyces roseus*	–	+	–
	119	*Cryptococcus albidus*	–	+	–
Cyttaria harioti [e]	2	*Cryptococcus laurentii*	–	+	–
	4	*Cryptococcus gastricus* (l)	–	–	K_9
Cyttaria harioti [f]	183	*Candida savonica* (l)	–	–	K_9
	186	*Candida versatilis*	–	+	–
	182	*Saccharomyces* spp.	–	–	K_1, K_2, K_3, K_9–K_{10}
	184	*Saccharomyces* spp.	–	–	K_1, K_2, K_3, K_9, K_{10}
	185	*Saccharomyces* spp.	–	+	–

(a) Killer activity against *C. glabrata* NCYC 388. Sampling sites: (b) cultivar, (c) Puerto Blest, (d) Puerto Frías, (e) Brazo Tristeza, (f) Villa Tacul. (y.l.f.) = yeast-like form. (l) = like. G II, III, VII = taxonomic yeasts groups (18).

(Difco) containing 0.003% methylene blue and buffered to pH = 4.5 with citrate-phosphate buffer. The yeasts under study were heavily streaked over the layer and the plates were incubated at 22 °C. Killer toxin activity appeared as a clear growth inhibition area around the streaks, sometimes surrounded by a blue circle of dead cells.

Sacch. cerevisiae YAT 679 (K_1 type), *Sacch. cerevisiae* NCYC 738 (K_2 type), *Sacch. capensis* NCYC 671 (K_3 type), *Candida glabrata* NCYC 388 (K_4 type), *Hansenula anomala* NCYC 434 (K_5 type), *Kluyveromyces fragilis* NCYC 587 (K_6 type), *C. valida* NCYC 327 (K_7 type), *H. anomala* NCYC 435 (K_8 type), *H. mrakii* NCYC 500 (K_9 type) and *K. drosophilarum* NCYC 575 (K_{10} type), kindly provided by Dr. Isato Kono (Industrial Technology Centre of Okayama Prefecture, Japan), were employed for testing the sensitivity of the isolated yeast strains to known toxins in the same way.

Results and Discussion

Twenty eight strains belonging to eight genera were isolated from flower nectar of seven native species (Table 1). Among them a high proportion (about 80%) of non-pigmented and fermenting yeasts was observed. Non fermenting yeasts of genus *Cryptococcus* and *Rhodotorula* were not detected.

In fruits, fermenting genus *Kloeckera* was found associated mostly to the surface of *Rubus idaeus* from gardens while *R. idaeus* growing freely in areas with low disturbance impact within the NHNP, showed a broader diversity of species (Table 2). Kloeckera was not recovered from these samples. Fermenting species of *Candida* and *Saccharomyces*, as well as the non-fermenting genus *Cryptococcus* have been isolated from *Cyttaria hariotti*, a parasitic fungus growing on *Nothofagus* spp. (19). *Cryptococcus albidus* and *Cr. laurentii* were mostly isolated

Table 3. Killer profile of yeasts from lakes freshwaters at the Nahuel Huapi National Park

Source	Nr	Genera/Species	Killer[(a)]	Neutral	Sensitive to
Nahuel Huapi	27	*Rhodotorula glutinis*	–	+	–
	32	*Rhodotorula rubra*	–	+	–
	34	*Rhodotorula rubra*	–	+	–
	40	*Rhodotorula glutinis*	–	–	K_{10}
	47	*Rhodotorula rubra*	–	+	–
	48	*Rhodotorula rubra*	–	+	–
	49	*Rhodotorula rubra*	–	+	–
	37	*Candida pseudointermedia*	–	–	K_5
	39	*Candida* spp. G III	–	–	K_5
Gutiérrez	55	*Rhodotorula rubra*	–	+	–
	56	*Rhodotorula rubra*	–	+	–
	57	*Rhodotorula rubra*	–	+	–
	58	*Rhodotorula rubra*	–	+	–
	59	*Rhodotorula rubra*	–	+	–
	50	*Candida* spp. G VII	–	+	–
	51	*Candida* spp. G VII	–	+	–
	52	*Candida* spp.G VII	–	–	K_5
Mascardi	23	*Rhodotorula rubra*	–	+	–
	25	*Rhodotorula minuta*	–	+	–
	70	*Rhodotorula rubra*	–	+	–
	71	*Rhodotorula rubra*	–	+	–
	72	*Rhodotorula rubra*	–	+	–
	73	*Rhodotorula rubra*	–	+	–
	76	*Rhodotorula* spp.	–	–	K_5, K_{10}
	26	*Torulaspora* spp.	–	–	K_1, K_2, K_3, K_5, K_8, K_9, K_{10}
	20	*Cryptococcus* spp.	–	–	K_9
	75	*Cryptococcus* spp.	–	–	K_6
Escondido	18	*Rhodotorula minuta*	–	+	–
	60	*Rhodotorula rubra*	–	+	–
	61	*Rhodotorula rubra*	–	+	–
	64	*Rhodotorula rubra*	–	+	–
	66	*Rhodotorula rubra*	–	+	–
	69	*Rhodotorula rubra*	–	+	–
	68	*Torulaspora* spp.	–	–	K_1, K_2, K_3, K_5, K_8, K_9, K_{10}
	62	*Candida famata* (I)	–	+	–
	67	*Candida* spp.	–	–	K_9, K_{10}
Cántaros	22	*Rhodotorula rubra*	–	+	–
	45	*Rhodotorula rubra*	–	+	–
	46	*Rhodotorula rubra*	–	+	–
	21	*Cryptococcus* spp.	–	+	–
	42	*Cryptococcus laurentii*	–	+	–
Toncek	120	*Rhodotorula* spp.	–	+	–
	121	*Rhodotorula rubra*	–	+	–
	124	*Rhodotorula rubra*	–	+	–
	126	*Rhodotorula rubra*	–	+	–
	127	*Rhodotorula graminis*	–	+	–
	128	*Rhodotorula rubra*	–	+	–
	134	*Rhodotorula* spp.	–	+	–
	137	*Rhodotorula lactosa*	–	–	K_{10}
	138	*Rhodotorula lactosa*	–	+	–

	142	*Rhodotorula* spp.	–	+	–
	148	*Rhodotorula* spp.	–	–	–
	149	*Rhodotorula* spp.	–	–	K_{10}
	150	*Rhodotorula rubra*	–	+	–
	151	*Rhodotorula rubra*	–	+	–
	154	*Rhodotorula rubra*	–	–	K_6
	123	*Cryptococcus albidus*	–	+	–
	125	*Cryptococcus* spp.	–	+	–
	130	*Cryptococcus albidus*	–	+	–
	139	*Candida* spp.	–	+	–
	140	*Cryptococcus albidus*	–	–	K_{10}
	147	*Cryptococcus elinovii* (l)	–	–	K_5, K_6, K_9, K_{10}
	153	*Candida* spp.	–	+	–
	157	*Candida* spp.	–	–	K_9
	155	(n.i.)	–	–	K_{10}

(a) killer activity against *C. glabrata* NCYC 388; (n.i.) = not identified, (l) = like, G III–VII = yeast taxonomic groups *(18)*

from the surface of healthy strawberries. These non fermenting species are not considered as spoilage yeasts, and probably come from soil *(20)*. *Kloeckera apiculata*, known as strawberry decay agent and used as a starter culture in winemaking, was recovered from the surface of grapes. During spontaneous grape must fermentation process *K. apiculata* is rapidly replaced by *Sacch. cerevisiae* almost absent on grape surface *(17,20,21)*.

A high prevalence of fermenting species in fruit surfaces, although not excluding aerobic ones, is reported here. *Metchnikowia* spp., *Torulopsis* spp., *Hanseniaspora* spp. and *Aureobasidium* spp. (known as »black yeasts«) recorded in these substrates *(20)*, were not isolated.

Strains of the genus *Rhodotorula* were present in all water samples and accounted for the 66% of the isolates, *Rhodotorula rubra* being the most frequent. Non fermenting genus *Cryptococcus* was recovered from 3 out of 6 lakes under study. *Candida* species were also isolated in low proportion, none of them associated to anthropogenic impact *(22)*. This statement is in agreement with the oligotrophic and low disturbance condition of the NHNP lakes (Table 3). As in samples from nectar or fruits, black yeasts failed to be isolated. Current reports on the subject state that red yeasts are frequently found in aquatic environments comprising more than 50% of yeast population, especially in oligotrophic marine or freshwater. *Rh. rubra, Rh. glutinis; Debaryomyces hansenii* and its anamorph *Candida famata* are ubiquitous in fresh and marine waters, as well as *Cryptococcus*, mainly *Cr. albidus* and *Cr. laurentii (22)*.

Under essay conditions 3 strains out of 28 isolated cultures from nectar samples were killer, whereas no killer activity was detected among isolates from freshwater or fruit samples. It has been stated that most natural isolates apparently lack toxin production *(12)*, but different surveys have demonstrated that killer yeast occurrence can vary widely *(3,4,16,17,23,24)*. The test conditions employed (nutrients and pH of culture media, incubation temperature, sensitive strain) may account for these variations. A low incidence (3/123 or 2.4%) of wild yeasts with killer activity against *C. glabrata* NCYC 388 was found in this survey as a first approach to killer activity in wild isolates. This strain has a broad sensitivity spectrum to known killer toxins and is useful in general screenings of yeast strains for killer activity. *C. glabrata* NCYC 388 is a type K_4 killer yeast and therefore resistant to yeasts carrying this type of toxins *(3)*. There is a slight posibility to get a sensitive tester strain able to detect all known and unknown killer toxins, specially because the latter could be more abundant than the former. Cross-culturing of wild isolates is being performed at this laboratory and may reveal new killer-sensitive patterns. Regarding the sensitive response of the yeasts studied towards known killer toxins, 48 strains out of 123 (38%) had positive reaction to one or more of them. A broader spectrum of sensitivity was observed among yeast cultures from nectar samples (64%). The fraction of 57% of the yeasts isolated from fruits and 75% of yeasts (most of them belonging to the genus *Rhodotorula*) from freshwater samples were of neutral type. A high sensitivity to *Hansenula* killer toxins (K_5, K_8, K_9) and *K. drosophilarum* (K_{10}) was observed mainly in *Candida* strains. Bearing in mind that *Hansenula* and *Kluyveromyces* have their anamorphs in the former genus and that only four strains identified as *Saccharomyces* and *Torulaspora* were sensitive to *Saccharomyces* killer toxins (K_1, K_2, K_3), there could be an enhanced sensitivity to closely related genera. Basidiomycetous yeasts *Rhodotorula* and *Cryptococcus* were mostly neutral to this set of ascomycetous killer type tester strains. Wild yeast strains sensitive response against basidiomycetous killer yeasts is under study and will be further reported.

Conclusions

Non-pigmented fermenting species prevail in samples from terrestrial environments. Pigmented and non fermenting species of genus *Rhodotorula* are associated to hydrological-soil interfaces at the NHNP. A low incidence of killer strains against *C. glabrata* NCYC 388 sensitive tester strain is found in yeasts associated with the terrestrial and hydrological-soil interfaces surveyed. Ascomycetous and basidiomycetous wild yeasts behave towards ascomycetous yeasts carrying killer activity mainly as sensitive or neutral, respectively.

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Osobine divljih ubojitih kvasaca iz Nacionalnog parka Nahuel Huapi (Patagonija, Argentina)

Sažetak

Svrha je ove studije pregled ubojitih kvasaca u prirodnom okolišu Nacionalnog parka Nahuel Huapi u subantarktičkoj šumi sjeverozapadne Patagonije i određivanje njihovih osobina. Analizirana su stodvadesettri izolata kvasaca. Ispitivana je ubilačka aktivnost kvasaca iz cvjetnih nektara, slatkog divljeg voća, sporangija gljive Cyttaria spp. i svježe jezerske vode ledenjaka, koristeći kao test osjetljivi soj kvasca Candida glabrata NCYC 388. Testirana je osjetljivost izolata kvasaca na poznate toksine (K_1–K_{10}). Tri od dvadeset osam kultura, izoliranih iz nektara, pokazale su ubilačku aktivnost, a ni jedna izolirana iz voća ili uzoraka svježe vode nije imala tu sposobnost. Od svih ispitivanih sojeva 38% bilo je osjetljivo na jedan ili više toksina. Najširi spektar osjetljivosti ustanovljen je u kulturama iz uzoraka nektara, dok je 57% kvasaca, izoliranih iz voća, bilo neutralno. Kvasci iz uzoraka svježe vode bili su također pretežno neutralni.