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Yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil

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Summary Yeast communities associated with sugarcane leaves, stems and rhizosphere during different phases of plant development were studied near Campos, in Rio de Janeiro, Brazil. Atmospheric temperature, soil granulometry and pH, and sugar cane juice °Brix and pH were determined. Yeast communities associated with sugarcane were obtained after cellular extraction by shaking, blending and shaking plus sonication, and cultured on Yeast Nitrogen Base Agar plus glucose (0.5%) and Yeast Extract-Malt Extract Agar. No significant differences in yeast counts were found among the cellular extraction treatments and culture media. 230 yeast cultures were identified according to standard methods, and distinct yeast communities were found for each substrate studied. The prevalent species isolated from sugarcane were *Cryptococcus laurentii*, *Cryptococcus albidus*, *Rhodotorula mucilaginosa* and *Debaryomyces hansenii*.

Key words *Cryptococcus* · *Debaryomyces* · *Rhodotorula* · Sugarcane (*Saccharum officinarum*) · Yeast communities

Introduction

Plant surfaces are considered an important habitat with epiphytic, endophytic and ectomycorhizal microorganisms functioning as growth promoting and phytodefense agents [29]. Leaves support a diverse and dense microbiota composed of yeasts, molds, bacteria and protozoa during their various development stages [17]. The microbiota associated with sugarcane, a major crop in Brazil, have been given considerable attention in the search for more sustainable agricultural practices [2, 15]. Studies on nitrogen fixing bacteria associated with sugarcane have been contributing to a more productive agriculture with less harm to the environment, and to stimulate the development of biofuel programs [8, 22].

The associations between yeasts and habitats supporting their development, are still obscure [6, 20, 21, 23]. Plants are important habitats for yeast communities, and few studies have been made addressing the yeasts associated with sugarcane. Yeast species are associated with microhabitats such as plants, decaying tissues, flowers, nectars, fruits and tree saps, which can be highly interrelated with insects vectors [11, 16, 24, 25]. They can be strongly retained by gummy and mucous secretions on the surface of these materials. *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and, in some areas, *Candida*, are the non-fermentative anamorphic yeasts commonly isolated from leaf surface [21]. The genera *Candida*, *Lipomyces*, *Cryptococcus* and *Rhodotorula* have been found in soils of widely different texture, chemical composition, humidity and pH, at diverse geographic locations and climatic conditions [3, 7, 21]. In this study we describe the yeast communities associated with leaf, stem and rhizosphere microhabitats during the different stages of sugarcane plant development.

Materials and methods

Samples of a predominant variety of sugarcane (*Saccharum officinarum* L.) (CB 45-3) planted in November 1994, were collected in April, June, August and October 1995 at a plantation of an alcohol distillery (Usina Cupim) near Campos, Rio de Janeiro, Brazil. Sugarcane was grown in sandy clay soil fertilized with NPK, conditioned with composted sugarcane processing and ethanol distillation wastes, and cultivated with irrigation and without using pesticides. The soil parameters, granulometry, pH and nutrients were kindly determined by Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) using standard methods. The juice °Brix was measured with a saccharometer and the pH with Merck indicator strips.

Four collections each of leaf, stem and rhizosphere were placed aseptically in sterile plastic bags, 152, 216, 279, 342 days after

planting. Samples were transported on ice to the laboratory and processed within 24 h. Each of the four (10 g) leaf samples was suspended in 90 ml of sterile wash solution (0.85% NaCl, 0.1% Tween 40), and agitated on a rotatory shaker (250 rpm) for 30 min [23]. Decimal dilutions, using the same sterile wash solution, were spread on Yeast Extract-Malt Extract (Y-M) Agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar, 0.01% chloramphenicol, pH 3.7), and on Yeast Nitrogen Base (YNB, Difco) with 0.5% glucose, 1.7% agar and 0.02% chloramphenicol. The remainder of each sample was sonified at low level for 30 s and plated out as noted above. Each 10 g rhizosphere sample was treated and processed as noted for leaves, but using 90 ml of a different wash solution (0.5% Tween 80, pH 7.0 potassium phosphate buffer) and 15 min of shaking [23]. Each one of the 10 g stem samples was placed in 90 ml sterile water, blended for 1 to 2 min at maximum speed and plated out on Y-M Agar and YNB-glucose Agar as noted above. Plates were incubated for 5 days at 17 ± 1°C and yeast counts reported as mean of colony forming units (cfu) per gram dry weight from the four samples of each habitat studied per collection. The ANOVA and Hypothesis Statistical tests ($\alpha = 5\%$) were used to evaluate the extraction methods employed to release yeasts from samples, and counts on the culture media. Colonies from selected primary isolation plates were streaked out on Y-M Agar to obtain axenic cultures, and were maintained on GYMP Agar [25] stored under sterile mineral oil at 8 ± 4°C. Isolates were characterized and identified following standard methods [4, 5, 10, 13, 14].

Results and Discussion

Sugarcane crops depend on a humid climate with high temperature, which can vary between 16 to 33°C. The best soil characteristics for cane development are high percentage of clay and a pH ranging from 4.0 to 8.3. The introduction of nutrients not present in adequate quantities in soil is required for sugarcane plantation [2, 15]. The hot humid region of Campos, with mean temperatures ranging from 20 to 26°C, is known as one of the best areas in the south of Brazil for sugarcane cultivation [2]. The atmospheric temperatures found during the collections ranged from 23 to 32°C. The soil samples from Campos experimental fields were acidic, high in clay content and had appropriate nutrient levels for successful sugarcane cultivation. The sugarcane juice °Brix increased with the plant development (7.7–18.6), and the pH ranged from 4.5 to 5.2. The increase in °Brix value and the acidification of sugarcane juice reflected the sucrose accumulation in the stem during plant development until mature.

Cell extraction with aggressive treatment (sonication) did not result in notably higher yeast counts than the agitation method, but it resulted in different proportions of the yeast species isolated (data not shown). The yeast populations and colony morphologies were similar on both Y-M Agar and YNB-glucose Agar. However, less mold grew on YNB-glucose Agar, making this medium more adequate for yeast counts and isolation. Robbs et al. [23] also observed similar results for yeast enumeration on YNB-glucose Agar with plant materials, air and soil samples of a pineapple plantation in Rio de Janeiro. No significant statistical differences were found among the media and treatments used. The yeast counts for the leaf, rhizosphere and stem, obtained with the agitation method followed by plating on Y-M Agar, are presented in Table 1. No rhizosphere yeast counts were obtained for the first collection due to intense mold growth.

Table 1 Mean yeast counts (cfu/g dry wt.) from sugarcane plated on Y-M Agar

Days after planting	Processing method: Leaf[a] (n = 4)[c]	Rhizosphere[a] (n = 4)	Stem[b] (n = 4)
152	$3.5 (\pm 0.72) \times 10^7$	–[d]	$29.9 (\pm 5.11) \times 10^5$
216	$3.5 (\pm 0.36) \times 10^7$	$1.5 (\pm 0.27) \times 10^6$	$9.7 (\pm 0.88) \times 10^5$
279	$1.3 (\pm 0.24) \times 10^7$	$6.1 (\pm 1.40) \times 10^6$	$2.1 (\pm 0.66) \times 10^5$
342	$0.9 (\pm 0.21) \times 10^7$	$10.23 (\pm 5.83) \times 10^6$	$7.3 (\pm 0.25) \times 10^5$

[a]Samples treated with shaking.
[b]Samples treated with blending.
[c]Number of samples.
[d]Intense mold growth.

Various factors may influence epiphytic microbial populations, such as leaf age and size, shade, direct contact between leaf and soil, and transfers of microbial cells from leaves to the soil by rainfall [12], resulting in a decrease of the yeast numbers on the leaves and their increase on the rhizosphere (Table 1). Yeast populations on most plant surfaces are dominated by basidiomycetous forms and their anamorphs, especially of the genera *Cryptococcus*, *Rhodothorula* and *Trichosporon*. The ascomycetous yeast *Debaryomyces hansenii* has been isolated frequently from leaves in the arid climate of the Canary Islands [18] and from tank waters [1] and leaves (unpublished data) of bromeliads growing in shade but not in direct sunlight in Rio de Janeiro. Frequently *Candida* spp. and black yeasts, such as the ascomycetous yeast like-fungus *Aureobasidium pullulans*, are also found [20, 21, 23, 26].

The 230 yeast isolates were included in 19 genera and 41 species, and 79.6% of the cultures isolated had basidiomycetous affinity. Basidiomycetous yeasts and their anamorphs were prevalent on the leaf (92%), in the stem (66%) and rhizosphere (74%) samples during this study with *Cryptococcus* and *Rhodotorula* being the prevalent genera (Table 2). *Cryptococcus albidus*, *Cryptococcus laurentii* and *D. hansenii* were prevalent in all three of the microhabitats. *Rhodotorula mucilaginosa* and *Rhodotorula minuta* were prevalent for the leaf and stem microhabitats but not in the rhizosphere. The genera *Cryptococcus*, *Rhodotorula* and *Trichosporon*, commonly isolated from plant surfaces [3, 21, 26], were the predominant yeasts found on sugarcane leaves. A prevalence of *Cryptococcus, Rhodotorula*, and black yeast isolates was noted by Robbs et al. [23] for leaves, flowers and fruit of pineapple near this same region. The ballistospore forming species *Sporobolomyces roseus* was present

Table 2 Yeast species and number of isolates in four leaf, stem and rhizosphere samples of sugarcane at Usina Cupim, near Campos, Rio de Janeiro, Brazil

Yeast species			
	L[a]	S	R
BASIDIOMYCETES			
Bullera variabilis-like[b]	4	3	2
Cryptococcus albidus	18	7	5
Cryptococcus humiculus	1	–	–
Cryptococcus hungaricus	7	2	–
Cryptococcus laurentii	14	12	14
Cryptococcus macerans	1	–	–
Cryptococcus sp. L	1	–	–
Cryptococcus sp. R	–	–	1
Cystofilobasidium infirmo-miniatum	2	–	–
Fellomyces horovitziae-like[b]	1	–	9
Filobasidiella neoformans	–	1	–
Leucosporidium scotii	–	–	1
Rhodosporidium toruloides	1	–	–
Rhodotorula glutinis	2	2	2
Rhodotorula lactosa	–	1	–
Rhodotorula minuta	5	6	1
Rhodotorula sp. S	–	1	–
Rhodotorula sp. R	–	–	1
Rhodotorula mucilaginosa	9	9	1
Sporobolomyces roseus	9	1	–
Sporidiobolus pararoseus-like[b]	1	–	–
Tremella aurantia-like[b]	1	–	–
Tremella foliacea	–	1	–
Tremella mesenterica	–	–	1
Trichosporon cutaneum-like[b]	11	2	–
Trichosporon dulcitum-like[b]	1	–	–
Trichosporon pullulans-like[b]	6	–	–
Trichosporon sp.	–	–	2
Subtotal of basidiomycetous yeasts	95	48	40
ASCOMYCETES			
Candida austromarina ?	–	1	–
Candida azyma	1	2	2
Candida diddensiae-like[b]	–	–	1
Candida guilliermondii	–	2	–
Candida maltosa	–	–	1
Candida sp.	–	1	–
Candida zeylanoides	–	1	–
Clavispora lusitaneae	–	1	–
Debaryomyces hansenii	4	13	7
Pichia guilliermondii	–	–	1
Saccharomyces cerevisiae	1	2	1
Torulaspora delbrueckii	–	2	1
Zyogoascus hellenicus	2	–	–
Subtotal of ascomycetous yeasts	8	25	14

Total number of isolates in sugarcane: 103 (leaf); 73 (stem); 54 (rhizosphere)
Species richness: 23 (leaf); 22 (stem); 19 (rhizosphere)

[a]L: leaf samples; S: stem samples; R: rhizosphere samples.
[b]Probable new species similar in characteristics to the species indicated.
–: Yeast species not isolated.

in all leaf samples (Table 2). Isolates of the genus *Sporobolomyces* have been relatively rare in the studies of yeasts in tropical habitats, but this may be the result of not using specific methods that favor the isolation of this group. *Sporobolomyces* was found previously in association with morning glories from Brazilian sandy coastal plane (restinga) ecosystems [22]. In rhizosphere samples, 19 different species were identified, with *C. laurentii*, *D. hansenii* and *Fellomyces horovitziae*-like prevalent (Table 2). In spite of *A. pullulans* being usually found associated with plants from temperate and tropical habitats [11, 17, 25], no isolate of this species was found in the sugarcane samples analyzed in the present study. A succession of mostly oxidative basidiomycetous species to fermentative ascomycetous species often occurs during the ripping and deterioration of fruits [9, 19, 26], but no similar succession was found for sugarcane.

Saccharomyces cerevisiae was rarely isolated from leaves, stems and rhizosphere of sugarcane and could be a source of inoculum for natural cachaça rum fermentations. Brazilian fresh sugarcane juice had more species diversity than fermented juice, with the presence of *S. cerevisiae*, *Pichia membranaefaciens*, *Candida krusei*, *Candida stellata*, *Candida guilliermondii*, *Candida intermedia* and *Schizosaccharomyces pombe* as prevalent in fresh juice [27]. The heterogeneous yeast biota of fresh cane juice may be influenced by the presence of soil particles which are commonly present on the cane, since many of these species have been isolated from soil [23]. Studies of the soil yeast community made in parallel with an examination of the plant yeast biota are important to know more about the degree of interchange between these two habitats.

Several cultures isolated from sugarcane showed variations on their response to conventional taxonomic characteristics, representing new biotypes of described species or new species. The *Bullera variabilis*-like cultures fitted with the description of *B. variabilis* except that they assimilated nitrate and lactose, and failed to form mycelium and to produce ballistospores. *Fellomyces horovitziae*-like isolates had physiological profiles typical of this species but sterigma was observed in only one culture. The *Candida diddensiae*-like culture fittted with the description of this species except that it did not assimilate cellobiose and erytritol. *Sporiodiobolus pararoseus*-like and *Tremella aurantia*-like cultures differed from the standard descriptions of these species in being negative for mycelium formation and assimilation of salicin, respectively. The assimilation of galactose, ribose, cellobiose and citrate, as well as arthrospores production, were negative in the *Trichosporon cutaneum*-like cultures, differing from the standard description of this species. The *Trichosporon pullulans*-like cultures fitted with the standard description, except in being negative for assimilation of sucrose and citrate, and positive for galactitol assimilation. The *Trichosporon dulcitum*-like culture differed from the standard description in being negative for arthrospores production.

The presence or growth of certain species in microhabitats may be related to many factors (e.g., deposit of plant or animal residues on a particular soil, organic content, pH) [21]. Some

species, such as *C. laurentii* and *D. hansenii*, have potential application as biocontrol agents for mold postharvest diseases of fruits and vegetables. This inhibitory action functions does not by an antibiose, but possibly by plant resistance mechanisms, competition with pathogens for nutrients and space, or direct interaction among the antagonist and the pathogenic cells [28, 30]. *C. laurentii* and *D. hansenii*, frequently isolated in this study, may have the potential to protect the sugarcane against phytopathogens.

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