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Maize-associated *Meyerozyma* from the Brazilian semiarid region are effective plant growth-promoting yeasts

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ABSTRACT

Yeasts are potential plant-growth-promoting microbes; however, this resource is underexploited, mainly in the semiarid regions worldwide. In the present study, we isolated potentially endophytic yeasts from roots of field-grown maize and characterized them at molecular, biochemical, and associative levels. Thirteen yeasts were retrieved, and the ITS1-5.8 S-ITS2 region sequences identified them within *the Meyerozyma* genus. The phylogenetic analyses showed that 12 out of 13 strains were closely related to *M. guilliermondii* CBS 2030^{T} and ESA 35 to *M. caribbica* CBS 9966^{T}. All yeasts synthesized indolic compounds and were favorable for siderophore production, and eleven proteolytic and seven amylolytic *Mayerozyma* spp. were identified. The principal component analysis positively correlated the *in vitro* characteristics and the maize growth promotion, highlighting six promising strains. Our results indicated that the maize-associated yeasts from the genus *Meyerozyma* are potential tools for inoculant production for plant growth promotion.

Yeast communities in soil are genetically diverse and play an essential role in maintaining the ecological functioning of the soil, promoting plant growth by several direct and indirect mechanisms (Fu et al., 2016; Hernández-Fernández et al., 2021). Thus, due to their ubiquity and plant growth-promoting characteristics, yeasts are potential microbes to be selected as plant growth-promoting microorganisms (PGPM) for application in sustainable agriculture (Fernandez-San Millan et al., 2020; Fu et al., 2016; Mukherjee and Sen, 2015; Nakayan et al., 2013; Nassar et al., 2005; Suryanarayanan and Shaanker, 2021). However, studies on the diversity and selection of plant stimulating yeasts in tropical Brazilian soils are still scarce (de Oliveira et al., 2019; Marques et al., 2021), especially for the semiarid conditions.

Maize (*Zea mays* L.) is an important staple food, oil source, and forage widely cropped in Brazil, where the maize fields covered almost 20 million ha in Brazilian lands in the 2020/2021 crop season. However, despite the high overall yield, in the northeast region, where the semiarid climate is predominant, the productivity was 35% lower than the national average (CONAB, 2022). Therefore, the maximization of biological interactions in the field is needed to overcome these adverse conditions. Thus, research efforts to select plant-associated microbes, such as yeasts, are still lacking.

The selection of maize-associated cultivable PGPM has been focused on the bacteria (rhizobacteria and endophytes) over the years (Alves et al., 2014, 2021; de Sousa et al., 2021; Hungria et al., 2010; Ikeda et al., 2020; Nascimento et al., 2021; Singh et al., 2015). Significant technological improvements are already achieved, and some strains are now available in the market (de Sousa et al., 2021; Hungria et al., 2010). Nevertheless, the extent of biological prospection targeting non-traditional PGPM (such as yeasts) should reveal important genetic resources with outstanding biotechnological potential. In addition, the use of yeasts in plant stimulating inoculants opens a new window of opportunity for the industry of yeast-based products in agriculture (such as those biological control products carrying *Beauveria bassiana* and other yeasts).

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This study aimed to obtain potentially endophytic yeasts (associative yeasts) from the roots of field-grown maize and characterize them at molecular, biochemical, and associative levels. From these targets, we hypothesized that the field-grown maize in the Brazilian semiarid is colonized by yeasts that can be efficiently used as PGPM.

Maize cv BRS Gorutuba was sowed at the Bebedouro Experimental Field (Embrapa Semiárido, Municipality of Petrolina, Pernambuco state. Latitude: -9.3941, Longitude: -40.5097) in a sandy loam Ultisol. The experiment was implemented in January 2015. The site had no recent (past 5 years) history of maize cultivation and was previously cultivated with annual crops. Soils were fertilized with 20 kg of P_2O_5 ha^{-1} as simple superphosphate, 20 kg of K_2O ha^{-1} as potassium chloride, and 40 kg of N-Urea ha^{-1}. The harvest was made 52 days after sowing, at the flowering stage of the plants.

For the yeasts' isolation, the roots were separated from the shoots, the soil adhering to the roots was removed, and the roots were washed abundantly with tap water. The roots were disinfected with NaClO 1% (w/v) for 10 min and washed ten times with distilled and autoclaved water (dH_2O). Root fragments (0.5 cm) were placed in PDA (Potato-Dextrose-Agar) dishes with chloramphenicol (500 μg L^{-1}) and calcium propionate (300 mg L^{-1}). Dishes were incubated at 28 °C in the dark for five days. Then, the colonies were purified in the same medium and morphologically characterized, according to Dias and Schwan (2010).

The yeasts grew in a liquid YPD (Yeast Extract-Potato-Dextrose) medium, and their DNA was extracted using the Wizard® Genomic DNA Purification System (Promega, USA) following the manufacturer's instructions. The yeasts were fingerprinted (genotyped) with microsatellite marker $(GTG)_5$ by PCR with the ISSR primer GTGGTGGTGGTGGTG, according to Gadanho and Sampaio (2002). All 13 yeasts were selected and identified using partial sequencing of the ITS1-5.8 S-ITS2 region. The PCR was carried out using the primers ITS1 (TCCGTAGGTGAACCTGCGG) e ITS4 (TCCTCCGCTTATTGATTGC), applying the conditions described by White et al. (1990). Purified PCR products were sequenced in a 3037 *xl* Genetic Analyzer (Applied Biosystems, USA) in Macrogen (Seoul, South Korea). The sequences were trimmed, compared with the type strains in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), and deposited in GenBank under the accession codes KY024402 to KY024414. The phylogenetic reconstruction was carried out in MEGA X software (Kumar et al., 2018) using the sequences of 13 maize yeasts and other 8 type strains.

The *in vitro* production of indolic compounds was evaluated according to the colorimetric method described by Sarwar and Kremer (1995), modified by Limtong and Koowadjanakul (2012), in the YPD liquid medium supplemented or not with 350 μg L^{-1} of L-tryptophan (L-Trp). Siderophore produced *in vitro* by the yeasts was quantified in a liquid YPD medium with Chromo-Azurol (CAS) (Schwyn and Neilands, 1987), and the siderophore quantification was obtained by comparing the sample data with those obtained in a standard curve made with known concentrations of EDTA (Arora and Verma, 2017; Ribeiro and Cardoso, 2012).

The extracellular hydrolysis of two substrates was evaluated in the YPD medium replacing the original carbon source with 10 g per liter of starch and skimmed milk powder to evaluate the amylolytic (de Oliveira et al., 2007), and proteolytic (Pailin et al., 2001) activities, respectively. The dishes were incubated at room temperature for five days, and the starch degradation was revealed with a 1% (v v^{-1}) iodine solution, and the protein degradation was observed in the surrounding colonies' halo. All evaluations of the hydrolytic activities were carried out with three replications in a completely randomized design.

A pot experiment was set up with maize plants to select plant growth-promoting yeasts. A topsoil sample from a Red-Yellow Ultisol was used as a substrate. The chemical characteristics of this soil sample were evaluated (Teixeira et al., 2017), and the data are shown in Table S1. The yeasts were grown previously in YPD liquid medium for experiment setup. Pots (7 L) were filled with the soil, and four seeds were sowed per pot. The inoculation was done by applying 1 mL of the yeast broth to each seed (10^9 cells mL^{-1}). The plants received water daily, and seven days after the emergence of plants (DAEP), two plants were thinned per pot. The experiment was carried out up to the 45th DAEP. The roots and shoots were placed separately in paper bags and put into the air circulation chamber at 65 °C until constant weight. The experiment was set up within a completely randomized design, with four replications.

Data were analyzed throughout the variance analysis (ANOVA), and the means were compared by applying the Scott-Knot mean range test ($p < 0.05$). Before the ANOVA, the normal distribution of the data was verified using the Shapiro-Wilk test. Next, analysis was carried out using SISVAR 5.0 (Ferreira, 2011). Finally, the principal component analysis (PCA) was carried out with data from *in vitro* plant-growth promoting mechanisms, enzymatic activities, and potted-maize experiment. PCA was built with the correlation's matrices using the software PaSt 4.10 (Hammer et al., 2011).

Thirteen mucoid colonies were retrieved from surface-disinfested maize roots. Their morphological characteristics' evaluation showed that classified these putative yeasts showed different colony morphologies at the PDA medium.

The molecular profile obtained by the marker $(GTG)_5$ revealed no yeasts with 100% similarity, showing that all are distinct strains (Fig. 1). Comparisons of the ITS1-5.8 S-ITS2 region sequences with the sequences deposited at the GenBank database indicated that all strains belong to the *Meyerozyma* genus. According to the phylogenetic tree based on the sequencing of the ITS1-5.8 S-ITS2 region, with 13 strains and closest type strains of *Meyerozyma* species, the strains were clustered in two groups, separated from all described *Meyerozyma* species (Fig. 2). The ESA 35 strain was not grouped with any other strains analyzed. However, it exhibited a closer relationship with *M. caribbica* CBS 9966^T (99.64%) and *M. carpophila* CBS 5256^T (99.45%). The 12 strains belonging to two groups were closely related to *M. guilliermondii* CBS 2030^T (98.31–99.80%).

The isolated yeasts produced indolic compounds in YPD liquid medium, supplemented with 350 μg L^{-1} of L-Trp (ranging from 18.47 to 265.61 mg L^{-1}) and without L-Trp (ranging from 37.04 to 206.33 mg L^{-1}) (Table 1). ESA 34 (265.61 μg mL^{-1}) and ESA 37 (224.90 μg mL^{-1}) produced higher concentrations when L-Trp was supplemented in the medium. ESA 35 produced a higher concentration in the medium without L-Trp. ESA 35 stood out because it produced a high indolic compounds concentration in both the presence (195.14 μg mL^{-1}) and absence (206.33 μg mL^{-1}) of L-Trp. The ability of yeasts to produce siderophores was equivalent to the EDTA chelating capacity at the concentrations of 0.49–2.34 mmol L^{-1}. The isolates ESA 37, ESA 38, ESA 39, ESA 40, ESA 41, and ESA 43 were superior to the other yeasts.

The enzymatic activity for the yeast strains showed a very variable

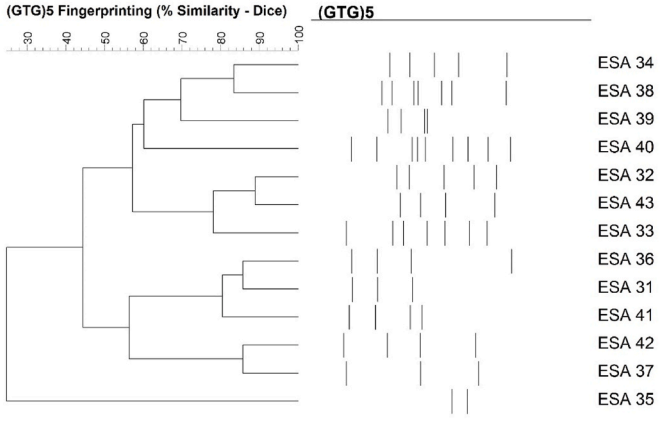

Fig. 1. Dendrogram of similarity based on the molecular fingerprinting applying the $(GTG)_5$ marker on 13 strains isolated from maize (*Zea mays*) roots. The UPGMA method and Dice coefficient were applied. The profiles were analyzed using the BioNumerics 7.6 software (Applied Maths, Belgium).

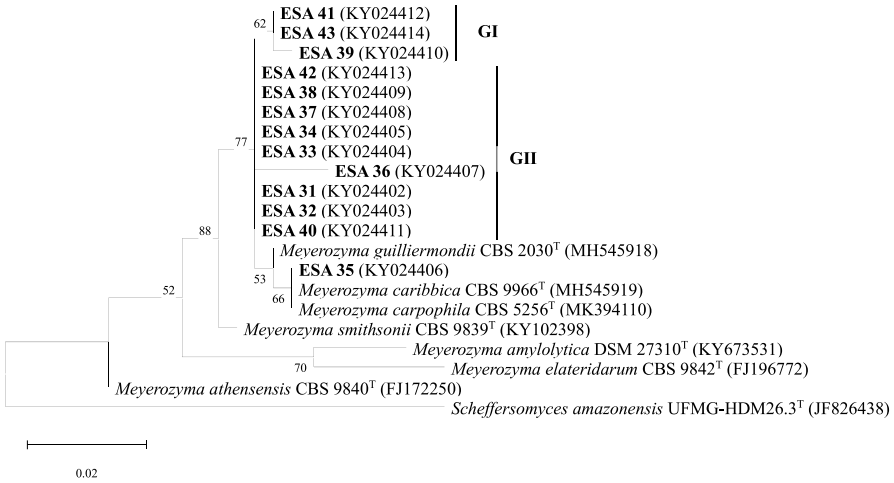

Fig. 2. Maximum-likelihood phylogenetic tree of the ITS1-5.8 S-ITS2 region sequences of 13 *Meyerozyma* spp. and seven type strains isolated from maize (*Zea mays*) roots. The numbers in the branches are the bootstrap values > 50% (1000 replications). *Scheffersomyces amazonensis* UFMG-HDM26.3^T was included as an outgroup. Strains under study are shown in boldface. Jukes-Cantor model was used for phylogenetic reconstruction. In parenthesis: GenBank accession number.

Table 1
In vitro auxin and siderophore production, proteolytic and amylolytic activities of the 13 yeasts isolated from roots of field-grown mayze (*Zea mays*) in the Brazilian semiarid region. Shoot, root, and total dry mass (SDM, RDM, and TDM, respectively), of *Zea mays* at 45 days after emergence inoculated with the same yeasts. For the plant assay, one control treatment (without inoculation) is also shown.

Treatments	Auxin (mg L^{-1})		Siderophore	Enzymatic index		SDM	RDM	TDM
	+ L-Trp	- L-Trp	(mmol of EDTA)	Proteolytic	Amylolytic			
ESA 31	38.47 d	44.42 d	0.49 b	6.44 a	0.00 d	0.75 b	1.01 b	1.76 b
ESA 32	26.33 d	37.04 d	1.20 b	0.00 d	1.70 b	1.72 a	2.16 a	3.88 a
ESA 33	28.47 d	64.19 d	0.79 b	4.73 b	2.37 a	1.61 a	2.12 a	3.73 a
ESA 34	265.61 a	161.80 c	1.23 b	5.20 b	1.73 b	1.36 a	1.78 a	3.14 a
ESA 35	195.14 b	206.33 a	1.61 b	0.00 d	0.00 d	0.77 b	0.96 b	1.73 b
ESA 36	102.28 c	134.90 c	1.39 b	4.48 c	0.00 d	1.26 a	1.36 b	2.62 a
ESA 37	224.90 a	174.42 b	2.22 a	3.25 c	0.00 d	1.56 a	2.55 a	4.11 a
ESA 38	80.61 c	141.80 c	2.21 a	4.73 b	0.85 c	1.09 a	1.15 b	2.24 b
ESA 39	111.80 c	184.42 b	2.32 a	6.69 a	2.54 a	1.87 a	2.30 a	4.17 a
ESA 40	21.09 d	71.57 d	2.20 a	7.80 a	0.00 d	1.51 a	1.54 b	3.05 a
ESA 41	22.76 d	43.23 d	2.01 a	5.34 b	1.49 c	1.02 a	2.03 a	3.05 a
ESA 42	37.28 d	50.20 d	1.36 b	6.36 a	1.33 c	1.04 a	1.44 b	2.48 b
ESA 43	18.47 d	49.42 d	2.34 a	5.56 a	0.00 d	1.11 a	1.25 b	2.36 b
Negative control	–	–	–	–	–	0.47 b	1.13 b	1.60 b
CV%	10.2	11.9	9.1	4.8	7.6	15.6	16.8	16.1

Data are averages of three replicates for auxin, siderophore and enzymatic activities, and four replicates SDM, RDM and TDM.
Averages followed by the same letter (within the same variable) do not differ using the Scott-Knott average range test ($p < 0.05$).
CV= Coefficent of variation.

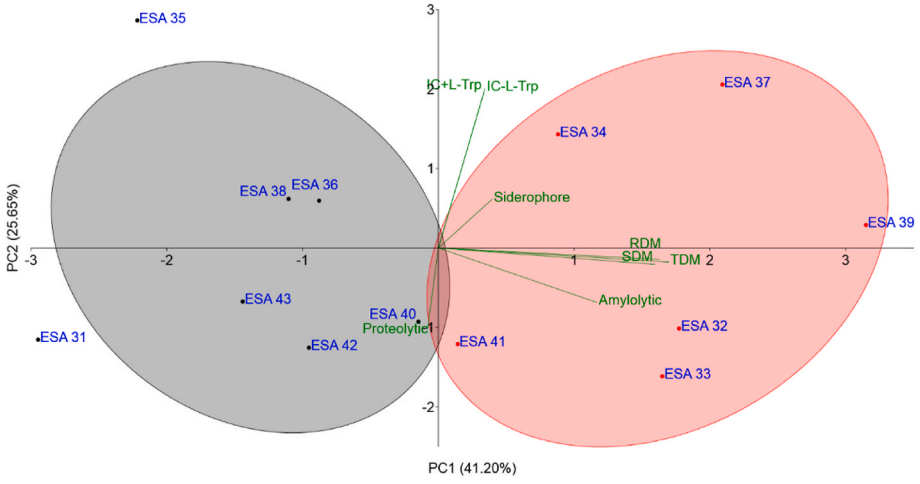

Fig. 3. Principal component analysis (PCA) of *in vitro* the plant growth promotion traits, enzymatic activity, and maize growth promotion by 13 yeasts strains isolated from maize roots. Red and black dots present the most and the less efficient plant-growth promoting yeasts, respectively. PC1 and PC2 are then principal components 1 and 2, respectively. IC + L-Trp = production of indolic compounds in the medium with L-tryptophan; IC-L-Trp = production of indolic compounds in the medium without L-tryptophan; RDM = root dry mass; SDM = shoot dry mass; TDM = total dry mass; Amylolytic = amylolytic activity; Proteolytic = proteolytic activity.

profile. Among the 13 isolates, eleven were proteolytic, and seven were amylolytic strains. ESA 39 was the highest enzymatic index for proteolytic and amylolytic activities. Among the 13 isolates, eleven, six, and eight yeasts induced the growth of shoot, root, and whole maize plants, respectively. The strains ESA 32, ESA 33, ESA 34, ESA 37, ESA 39, and ESA 41 stood out regarding the growth parameters of plants. Compared to the uninoculated control, these strains improved maize growth parameters in 44–74% under non-sterile conditions.

The PCA analysis indicated that the principal components 1 and 2 explained 66.85% of the observed variance (Fig. 3). The *in vitro* plant-growth promoting mechanisms and the amylolytic activity were correlated with the plant growth promotion traits in the potted maize assay. The six strains mentioned above clustered closely related to the mechanisms and maize growth promotion.

The present study classified 13 yeasts isolated from maize roots from Brazilian semiarid region soils within the genus *Meyerozyma*. The molecular fingerprinting analysis of these yeasts using the $(GTG)_5$ marker showed non-redundant profiles, even with the strains belonging to the same species. Thus, all strains in this study are distinct isolates and do not contain any clones. Twelve out of the thirteen strains were closest to *M. guilliermondii* CBS 2030^T and ESA 35 to *M. caribbica* CBS 9966^T (99.64%), and *M. carpophila* CBS 5256^T. *Meyerozyma* yeasts are ubiquitously distributed in the environment, inhabiting rhizospheres, bulk soils, and plant tissues (Moreira et al., 2020; Moreira and Vale, 2018; Nakayan et al., 2013; Peng et al., 2018). Despite the absence of reports for Brazil, in C4 grasses this genus was already isolated from maize rhizosphere in Mexico (Sarabia et al., 2018), in addition to maize and sugarcane (*Saccharum* spp.) leaves in Thailand (Khunnamwong et al., 2018; Limtong et al., 2014).

Overall, in the present study, the production of the indolic compound by *Meyerozyma* spp. in the YPD medium was higher without L-Trp medium than in the L-Trp-amended one. Since tryptophan is the major precursor of other indolic substance, such as auxins, for example. Therefore, the supplementation of culture medium with L-Trp amino-acid generally induces auxin-like substances production by the microorganisms; however, L-Trp-free auxin production, as observed in the present study, indicated different pathways to produce auxin within the yeasts collection (Fernandez-San Millan et al., 2020; Fu et al., 2016; Marques et al., 2021; Mestre et al., 2021).

In our study, all yeast strains were positive for siderophore production. *Meyerozyma* have been previously reported as positive for siderophore production (Fernandez-San Millan et al., 2020; Marques et al., 2021). In Brazil, *Meyerozyma* strains isolated from bromeliads (Marques et al., 2021) were assigned as siderophore producers, but there are no results of maize-associated Brazilian *Meyerozyma* as siderophore producers. The siderophore-produced yeasts play an essential role in the yeasts' micronutrient nutrition (Lesuisse et al., 2001) and mediate iron uptake by plants (El-Maraghy et al., 2020). Our results indicate that yeasts within our culture collection can induce plant growth by a siderophore-mediated mechanism.

Our study demonstrates differences in the enzymes produced by the strains. Six out of 13 strains were positive for the two enzymes tested. Only strain ESA 39 was highlighted both for high proteolytic and amylolytic activity. The protease activity was the most widely expressed extracellular enzymatic activity (positive for 11 out of 13 isolates tested), followed by amylase (positive for seven out of 13 yeasts). This result corroborates the findings of the other research, in which for yeasts identified in different genera, protease production is more frequent than amylase production (Gomes et al., 2015; Jaiboon et al., 2016; Yang et al., 2013).

Overall, few studies on the selection and use of plant-growth promoting yeasts in Brazil are available (de Oliveira et al., 2019; Marques et al., 2021). Regarding maize crops, little is known about the functional diversity of maize-yeasts association worldwide (Kandar et al., 2018; Nakayan et al., 2013; Nassar et al., 2005; Sarabia et al., 2018). In the present study, the strains ESA 32, ESA 33, ESA 34, ESA 37, ESA 39, and ESA 41 stood out regarding maize growth parameters. In addition, some of these isolates also stood out in the high production of auxin in medium supplemented with L-Trp (ESA 34 and ESA 37) and siderophore production (ESA 37, ESA 39 and ESA 41), proteolytic (ESA 39), and amylolytic (ESA 33 and ESA 39) enzymatic activities *in vitro*.

The present study is the first in Brazil to demonstrate the potential of associative yeasts to promote maize growth. The yeasts ESA 32, ESA 33, ESA 34, ESA 37, ESA 39, and ESA 41 emerged with high potential for agricultural application, evidenced by having shown plant growth-promoting traits *in vitro* and experimental data of maize performance under non-axenic conditions. Therefore, it may be assumed that these isolates can be a good candidate for use as agricultural inoculants. Thus, yeasts from the Brazilian Semiarid region are promising resources for biotechnological exploitation as microbial products in the maize crop. However, more studies are needed in field conditions to assess the ecological and agricultural benefits of maize-associated *Meyerozyma* yeasts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100538.

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