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Polyphasic characterization of *Epicoccum sorghinum*: A tenuazonic acid producer isolated from sorghum grain

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ABSTRACT

Epicoccum sorghinum isolates from sorghum grain grown in Brazil were characterized using a polyphasic approach based on morphological, genetic and physiologic features. A considerable intraspecific variability was observed. The phylogenetic analysis showed that the 53 isolates studied formed four distinct clades, and their morphological features revealed a high variability in culture. The four clades could not be distinguished clearly by morphology or enzyme production patterns. The tenuazonic acid (TeA) production capability of 11 isolates was also determined and revealed a good agreement with the phylogeny results. All of the 11 isolates were revealed as TeA producers, indicating a potential toxicological risk to sorghum crops. This is the first study to provide a detailed morphological description of *E. sorghinum* isolates from sorghum grains in Brazil and it clearly confirms the wide genetic and phenotypic variability previously reported for this species in other countries.

1. Introduction

Sorghum (*Sorghum bicolor* L.) is a drought-resistant crop with increasing global importance for food, feed and industrial uses (Codex Alimentarius Commission, 2012). In Brazil, sorghum grain production has increased significantly during the last decade, becoming an attractive alternative energy source to the national feed agroindustry (EMBRAPA. Empresa Brasileira de Pesquisa Agropecuária, 2015). However, one of the major biotic constraints to sorghum improvement and productivity is fungal contamination, especially if toxigenic species occur during both pre and post-harvest stage (Waliyar et al., 2007).

Among them, *Epicoccum sorghinum* (formerly *Phoma sorghina*) has been reported as one of the most important fungi in the sorghum grain-mold complex and has been commonly isolated in some sorghum-producing countries, such as Nigeria (Makun et al., 2009), Argentina (González et al., 1997) and Brazil (Oliveira et al., 2017; da Silva et al., 2000). According to Boerema et al. (2004) this species is a widespread anamorphic ascomycete that behaves as a weak plant pathogen and seed-borne fungus with preference for members of *Poaceae*, particularly in tropical regions, where it was also reported as a cause of foliar diseases (Do Amaral et al., 2004).

For a long time, the taxonomy of *Phoma* s.l. has been considered a challenge. Difficulties in morphological identification have resulted in a poor understanding of the generic and species boundaries in the genus (de Gruyter et al., 2009). However, during the last decade, studies with emphasis in a multilocus phylogenetic analysis, have been used to clarify the boundaries between *Phoma* and closely related genera (Aveskamp et al., 2009; Aveskamp et al., 2010). These studies revealed that strains of '*Phoma sorghina*' are phylogenetically distant from the generic type, *P. herbarum*, but cluster with species of *Epicoccum*. Therefore, the new combination *Epicoccum sorghinum* was proposed (Aveskamp et al., 2010).

Epicoccum sorghinum has shown considerable morphological and genetic intraspecific variation (Aveskamp et al., 2009; Pažoutová, 2009) and according to Aveskamp et al. (2009), this high variability probably indicates cryptic speciation. However, species limits within this complex have not been properly assessed and previous studies have only included isolates from Africa and North America (Pažoutová, 2009).

The ability of *E. sorghinum* to produce mycotoxins has also been reported and together with *Alternaria* spp. and *Pyricularia oryzae*, has been described producing tenuazonic acid (TeA), a potent inhibitor of protein biosynthesis (Meronuck et al., 1972; Steyn and Rabie, 1976). Tenuazonic acid has been reported to cause acute toxic effects in

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mammals (Smith et al., 1968), lesions in various tissues of treated young chickens (Giambrone et al., 1978; Griffin and Chu, 1983), as well as precancerous changes in esophageal mucosa of treated mice (Yekeler et al., 2001). In a recent discussion paper on fungi and mycotoxins in sorghum, the Codex Alimentarius Commission (2012) recommended that special attention should be paid to the occurrence of *E. sorghinum* in sorghum grain, especially because it was associated with the hemorrhagic human disorder "onyalay".

In Brazil, *Epicoccum sorghinum* is one of the main fungal species associated with sorghum grains (Oliveira et al., 2017). Also, natural occurrence of TeA has been recently reported in freshly harvested sorghum grain samples. Oliveira et al. (2017) carried out a survey of Brazilian sorghum grain at different maturity stages and found that 100% of the samples showed TeA contamination with levels ranging from 20 to 1234 μg/kg. Moreover, evaluating the effects of environmental factors on TeA contamination, Oliveira et al. (2018) have showed that sorghum grains grown under summer season presented higher TeA average level (587.8 μg/kg) when compared to level found in the autumn season (440.5 μg/kg).

In the present study, a set of *E. sorghinum* isolates from Brazil was characterized using a polyphasic approach that included morphology, physiology and multilocus phylogenetic analyses. Moreover, due to the mycotoxigenic potential of this species and its high incidence in Brazilian sorghum grain, we also evaluated the TeA production of selected *E. sorghinum* isolates. The main objectives of this investigation were to characterize isolates of *E. sorghinum* associated with Brazilian sorghum grains and to determine if the phenotypic and genotypic variability of Brazilian isolates are comparable to those previously reported from other countries.

2. Material and methods

2.1. Fungal isolates

The *E. sorghinum* isolates used in this study were recovered from 25 freshly harvested sorghum grains samples collected in an experimental area located in the southeast region of Brazil (Votuporanga, state of São Paulo, latitude 20° 28′ S, longitude 50° 04′ W) (Oliveira et al., 2017). The samples were composed of five panicles randomly collected from the experimental area. The sorghum "DKB 550" cultivar (Dekalb seeds) was sown in February of 2013 with a distance of 0.5 m between rows and a final population of 180,000 plants ha^{-1}.

From each sample, 30 grains were surface-disinfected by immersion in sodium hypochlorite solution (1%) for 3 min, followed by three rinses in sterile distilled water and then plated onto a potato dextrose agar (10 grains per plate) (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (100 mg/l) (Oliveira et al., 2017). The plates were incubated at 25 °C in the dark for five days. *Epicoccum* species developing from the grains were then morphologically identified following the protocol described by Boerema et al. (2004). A total of 53 isolates were selected for further characterization and were maintained cryopreserved in sterile 15% glycerol at the Department of Microbiology, University of São Paulo (USP).

2.2. Molecular characterization

2.2.1. DNA extraction, amplification, and sequencing

The isolates were grown on yeast extract sucrose agar (YES) for 3 days at 25 °C. Genomic DNA was extracted directly from fungal colonies following the PrepMan Ultra® kit protocol (Applied Biosystems, Carlsbad, CA, USA). DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Fragments of two loci were amplified: internal transcribed spacer region (*ITS*) and beta-tubulin gene (*TUB2*) (Table 1). Thermal cycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 52 °C (*TUB2*) and 48 °C (*ITS*) for 1 min and 72 °C for 1 min, followed by a final extension of 7 min at 72 °C. Amplified products were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR products were sequenced in both directions with a 3100 DNA sequencer (Applied Biosystems, USA) using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were edited with Sequencher DNA Sequence Assembly Software 4.1.4 (Gene Codes Corporation, USA) and Complete *ITS* and *TUB2* consensus sequences were used to perform BLASTn searches for species identification at the NCBI (http://blast.ncbi.nlm.nih.gov/) and MycoBank (http://www.mycobank.org/) websites. The nucleotide sequences of representative strains were deposited in GenBank (Table 3).

Table 1

Sequence of oligonucleotide primers used in this study.

Target	Primers	Nucleotide sequence (5′ → 3′)	Ta (°C)	Reference
ITS	V9G	TTACGTCCCTGCCCTTTGTA	48	(Aveskamp et al., 2009)
	ITS4	TCCTCCGCTTATTGATAT GC		
TUB2	BT2Fw	GTBCACCTYCARACCGGYCARTG	52	(White et al., 1990)
	BT4Rd	CCRGAYTGRCCRAARACRAAGTTGTC		

2.2.2. Phylogenetic analysis

Complete *ITS* and *TUB2* consensus sequences were used to conduct phylogenetic analysis, totalizing 108 sequences obtained from *E. sorghinum*, including the *E. sorghinum* reference strain CBS 179.80. Sequences of one phylogenetically related taxon, *E. pimprinum* CBS 246.60, were retrieved from GenBank and included in the present alignment as outgroup.

Phylogenetic analyses were carried out using the combined sequences of *ITS* and *TUB2*. Multiple sequence alignments were carried out using Clustal X2 (Thompson et al., 2002). Maximum parsimony analysis was carried out using PAUP* version 4.0b10 (Swofford, 2002). One hundred heuristic searches were conducted using random sequence addition and tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches, and saving all minimal-length trees (MulTrees) on different sets of data. Gaps were treated as missing data. Support for internal branches was assessed by a heuristic parsimony search of 1000 bootstrapped sets of data. Other measures were also taken, including tree length, consistency index (CI), homoplasy index (HI), and retention index (RI). The combined data set was tested for incongruence with the partition homogeneity test as implemented in PAUP*.

2.3. Phenotypic characterization

Morphological characterization was performed according to the *Phoma* Identification Manual (Boerema et al., 2004), including colony features and micromorphology. All of the 53 isolates (single spore isolation) were grown on oatmeal agar (OA) and malt-extract agar (MEA) (Difco Laboratories, Detroit, Michigan), in complete darkness at 22 °C for 7 days. Subsequently, the plates were maintained for 7 additional days at 22 °C with a day-night regime of 13 h near UV light and 11 h darkness. Colony diameter and descriptions were made after 7 days of incubation. At the 14th day, colony colors were recorded using the Methuen Handbook of Colour (Kornerup and Wanscher, 1978). The microscopic features (pycnidia, conidia and chlamydospores) were studied in lactic acid mounts. The sizes of the structures were determined by averaging the measurements of 5 samples of each structure.

2.4. Physiological characterization

2.4.1. Extracellular enzymatic analysis

Five wide mycelium plugs (5 mm) from each of *E. sorghinum* isolate

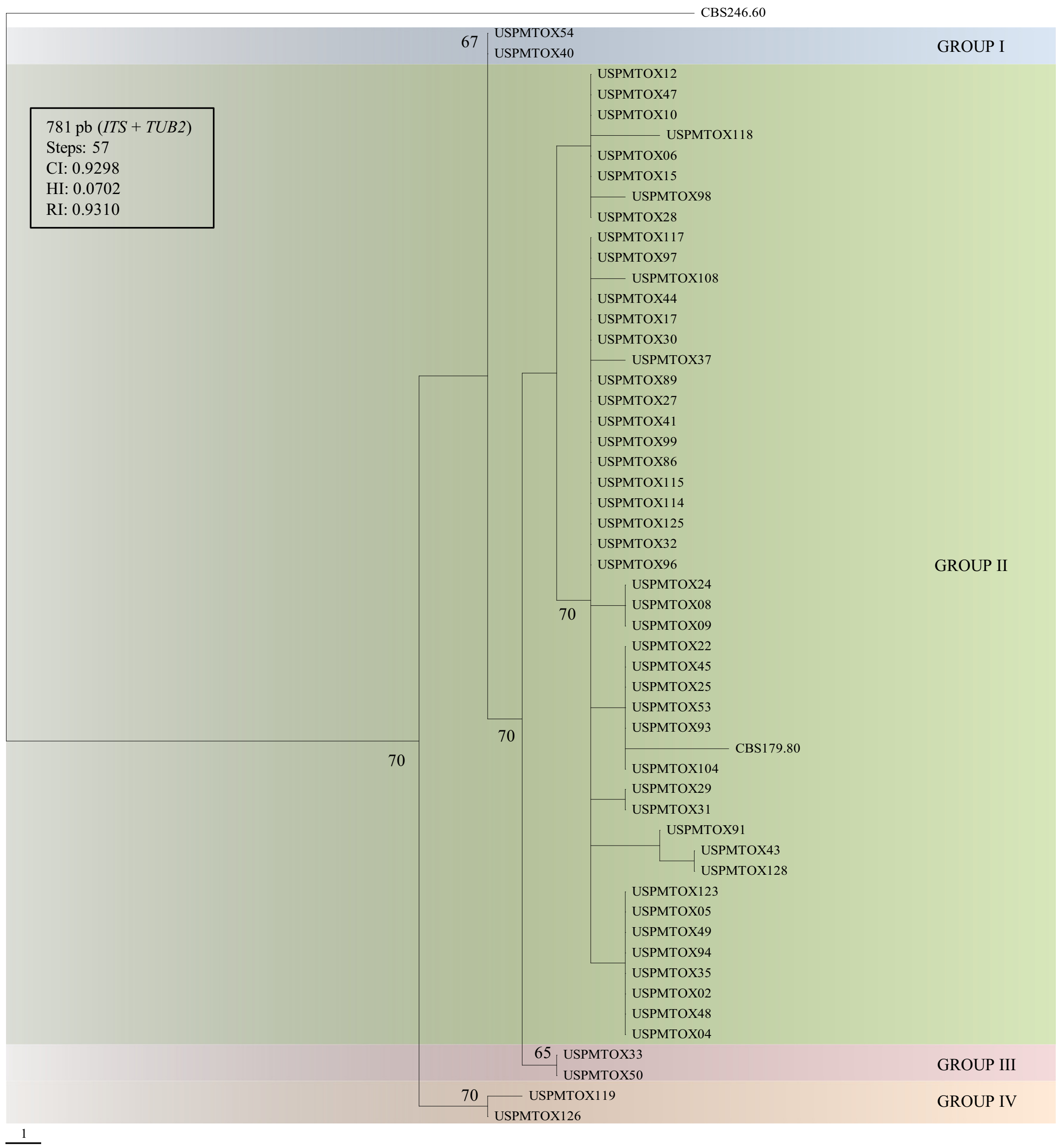

Fig. 1. Most-parsimonious tree obtained from the combined sequence data of two loci (*ITS* and *TUB2*) of 53 isolates of *Epicoccum sorghinum*, reference strains (*E. sorghinum* CBS 179.80), and *E. pimprinum* (CBS 246.60) as outgroup. Numbers above nodes represent the bootstrap values.

were transferred to flasks containing potato dextrose broth (Difco, Detroit, Michigan) and were incubated at 22 °C for 10 days (Oliveira et al., 2013). The culture broths were sterilized by 0.22 µm Millex filters (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Ireland) and used to determine the production of 19 extracellular enzymes by the API-ZYM® system (Bio-Merieux, Marcy l'Etoile, France). Briefly, 65 µl of each fungal culture broth were deposited in the 20 cupules, and the galleries were maintained at 30 °C for 4 h in the dark. Thereafter, a drop of ZYM A reagent (25 g of Tris–hydroxymethyl aminomethane + 11 ml of hydrochloric acid 37% + 10 g of sodium lauryl sulphate + 100 ml of H_2O) and ZYM B reagent (0.12 g ‘Fast Blue BB’ + 40 ml of methanol + 60 ml dimethyl sulphoxide) was added in each cupule. The results were determined in nanomoles (nmol) of the hydrolyzed substrate according to the intensity of the color reaction on a scale of 1–5, i.e., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 = > 40 nmol. All of the measurements were taken in duplicate, and potato dextrose broth was used as the negative control.

2.4.2. *TeA production*

Eleven *E. sorghinum* isolates were selected for TeA-producing ability tests based on their phylogenetic position. At least one isolate from each of the four phylogenetic groups was tested. Centrifuge tubes (50 ml) containing 5 g of rice and 3 ml of distilled water were autoclaved for 20 min at 121 °C. Grains were inoculated with 3 mycelial plugs (5 mm) obtained from OA culture of each isolate and incubated in the dark at 25 °C for 21 days. For TeA extraction and detection the protocols of Oliveira et al. (2017) were followed, according to which 5 g of ground rice sample was homogenized for 90 min with 15 ml of acetonitrile:water (1:1, v/v), acidified with 110 µl of formic acid. After centrifugation, an aliquot of 100 µl of acetonitrile extracts was taken and diluted with 900 µl of the mobile phase.

TeA was determined by API 5000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an Ion Electrospray Ionization (ESI) source in the negative ionization mode. The column used was a 50 mm × 4.6 mm × 1.8 µm SB C-18 (Agilent). Multiple-reaction monitoring (MRM) was used for TeA determination. The precursor pick of TA (m/z 196.0) and two products peaks m/z 139 (DP - 75, CE -26 V, CXP -19 V) m/z 112.0 (DP – 75, CE -34 V, CXP -15 V) were monitored to accomplish both quantitation and qualification criteria. A binary gradient at a flow rate of 0.4 ml/min was performed with solvent A (water) and solvent B (methanol), following the conditions described by Oliveira et al. (2017). A calibration curve was obtained using six TeA standard concentrations (20, 200, 400, 600, 800, and 1000 μg/kg). Good linearity with a correlation coefficient higher than 0.999 was obtained for the calibration range. The LOD (S/N = 3) and LOQ (S/N = 10) for TeA were 4 and 10 μg/kg, respectively.

3. Results

3.1. Species identification and phylogenetic analysis

All of the 25 sorghum grain samples were contaminated with *E. sorghinum*, in a frequency of isolation ranging from 69.7 to 77.2%. Based on the morphological description reported in the *Phoma* Identification Manual (Boerema et al., 2004), the 53 isolates were identified as *E. sorghinum*. However, due to a high variability found in macroscopic features, especially in colony colors, analysis of DNA sequences (*ITS* and *TUB2*) were essential to confirm the identity of the isolates. With the primers used, the lengths of the *ITS* and *TUB2* fragments obtained were 445 bp and 336 bp, respectively. The combined *ITS* + *TUB2* alignment was 781 bp in length. Both loci were congruent and were therefore concatenated (P = 0.6667). There were 730 constant characters, 37 parsimony-uninformative variable characters and 14 parsimony-informative characters. A most-parsimonious tree of 57 steps in length with a CI of 0.9298, HI of 0.0702 and RI of 0.9310 showed four main groups with well-supported clades as outlined below: Group I, with 67% of bootstrap, included two *E. sorghinum* isolates; Group II (70% bs), contained the majority of *E. sorghinum* isolates (n = 47), with multiple subclades, including the reference strain (*E. sorghinum* CBS 179.80). Group III (65%) and Group IV (70% bs) included two *E. sorghinum* isolates each (Fig. 1).

3.2. Phenotypic characterization

Significant variations were observed on morphological features of the 53 *E. sorghinum* isolates. Colonies on OA and MEA reached a diameter between 40 and 75 and 56–80 mm, respectively (Table 2). Colonies were cottony or velvety with a wide range of colors, especially greyish green or whitish/salmon pink (Fig. 2). No clear-cut morphological difference between phylogenetic groups was observed; however, most of the isolates of group II (62%) produced a reddish diffusible pigment on MEA. No pigment production was observed in any isolates of group I and III.

All the isolates studied displayed the typical micromorphological features of *E. sorghinum* (Fig. 3). We did not observe any associations between micromorphological variation and the phylogenetic results. In general, the isolates produced globose, glabrous, brown pycnidia 62–162 μm wide with straight neck, hyaline (immature) or sometimes brown (mature) (Fig. 2). Chlamydospores were uni- or multicellular, botryoid-alternarioid, hyaline to brown, intercalary or sometimes terminal, 5–70 μm wide (Fig. 3). Conidia were variable in shape, mostly ovoid-ellipsoidal (4–5 × 2–2.3 μm).

3.3. Physiological characterization

There were no qualitative and quantitative differences between the 53 *E. sorghinum* isolates in enzymatic production patterns. The following 5 extracellular enzymes were produced in the same concentration by all isolates: esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-b-glucosaminidase. On the other hand, a considerable variability in mycotoxin production was observed. All the *E. sorghinum* isolates (n = 11) were able to produce TeA at levels ranging from 98.6 to 148,000 μg/kg (mean: 38462.05 μg/kg) (Table 3).

4. Discussion

A detailed understanding of fungal genetic, phenotypic and physiological diversity has proved to provide new insights for both taxonomy and mycotoxin management. In this context, a polyphasic approach was undertaken to characterize one of the most important species involved in the sorghum-grain mold complex.

Epicoccum sorghinum has been isolated in a range of crops, especially in cereals grown in tropical and subtropical areas (de Oliveira et al., 2018; Oliveira et al., 2018). However, due to difficulties in morphological identification, its distribution has been underestimated. Currently, studies that use molecular tools to identify species within the *Phoma* complex have achieved satisfactory results and have also contributed to indicate a potential risk for mycotoxin contamination in food (Oliveira et al., 2017; Stokholm et al., 2016). In our study, both *ITS* and *TUB2* were able to discriminate the *E. sorghinum* isolates at species level and are indispensable for a correct identification due to the high phenotypic variability found in this species.

Our results revealed that *E. sorghinum* isolates from Brazil showed a considerable genetic and phenotypic intraspecific variability. Based on phylogenetic analyses, the isolates were clustered into four well supported groups. A high phenotypic variation was detected, particularly in colony color and in the production of a reddish pigment in isolates from group II, but no other morphological group marker was found. In general, the macro and micromorphology features are in agreement with the description for this species in the "*Phoma* Identification Manual", the main reference of *Phoma* taxonomy. However, the broad range of colony color observed in our study (e.g. grey, white, salmon, olivaceous grey, whitish-orange and sometimes salmon or greyish in the same colony) has not yet completely described in the literature and agrees with the view of previous authors who suggested that *E. sorghinum* probably includes cryptic species (Fig. 2). With regard to physiological characterization, there are no differences in extracellular enzymatic production between the isolates; however, it's interesting to note that the lowest TeA production was observed in isolates that did not produce pigments (group I and III). Further studies using a large number of isolates are necessary to confirm if pigment and mycotoxin production could be useful taxonomic markers in *E. sorghinum*.

Our findings are in agreement with previous studies that also reported genetic and morphological variability in *E. sorghinum* strains (*Phoma sorghina*). Pažoutová (2009) studied *P. sorghina* strains from Southern Africa and Texas (USA) and found a considerable intraspecific variability. Isolates from Texas produced intense red pigments while dark hyphae and intercalary chlamydospores were found in African isolates. However, no evidence for the presence of specific lineages or cryptic speciation has been found (Pažoutová, 2009). Aveskamp et al. (2009), based on multigene analyses (ITS, actin, and β-tubulin) of 13 *E. sorghinum* strains, have found a high level of intraspecific genetic variation and suggested that a high recombination rate plays a critical role in the diversification of this species.

It is clear that a wide genetic and phenotypic variability has been found in *E. sorghinum* isolated from different geographical locations. To our knowledge, this is first study to provide a description of *E. sorghinum* present in Brazil. However, our knowledge of the genetics and diversity of this species remains limited to specific regions of the globe (e.g. South Africa, USA and Brazil), which makes the proposal of segregate species a premature decision. Further studies should be conducted to clarify the mechanisms involved in the process of speciation of this species, such as reproductive mode, host specificity, vegetative compatibility and genetic structure of populations worldwide.

Regarding to the mycotoxigenic ability of *E. sorghinum*, our results confirmed that this fungus can be considered an important source of TeA contamination in sorghum grains. All 11 isolates tested were

Table 2
Macromorphological characters of 53 *E. sorghinum* isolates grown on oatmeal (AO) and malt extract agar (MEA).

Isolate	Diameter (mm)		Diffusible pigment		Texture		Border		Exudate	
	OA	MEA	OA	MEA	OA	MEA	OA	MEA	OA	MEA
USPMTOX2	72	76	+	+	V	V	R	R	−	+
USPMTOX4	52	56	−	−	V	V	R	R	−	+
USPMTOX5	62	70	−	−	V	V	R	R	+	−
USPMTOX6	68	80	−	+	C	V	R	R	−	−
USPMTOX8	65	72	−	−	V	C	R	R	+	−
USPMTOX9	60	72	−	−	V	C	R	R	−	−
USPMTOX10	70	78	−	−	C	V	R	R	−	−
USPMTOX12	63	78	−	+	V	C	R	R	−	−
USPMTOX15	59	72	−	−	C	C	R	R	−	−
USPMTOX17	60	65	−	+	V	C	R	R	+	−
USPMTOX22	60	65	−	−	V	V	R	R	+	−
USPMTOX24	56	60	−	−	V	V	R	R	+	−
USPMTOX25	61	71	−	−	V	C	R	R	−	−
USPMTOX27	68	70	+	+	C	C	R	R	−	−
USPMTOX28	48	69	−	+	C	C	R	R	+	−
USPMTOX29	66	79	−	−	C	C	R	R	+	+
USPMTOX30	75	80	−	−	C	V	R	R	−	−
USPMTOX31	62	66	−	−	V	V	R	R	−	−
USPMTOX32	65	70	−	+	V	C	R	R	+	−
USPMTOX33	64	70	−	−	V	C	R	R	+	+
USPMTOX35	65	70	−	+	V	V	R	R	−	−
USPMTOX37	66	73	−	+	V	V	R	R	+	+
USPMTOX40	60	72	−	−	C	V	R	R	+	+
USPMTOX41	60	68	−	−	V	V	R	R	−	−
USPMTOX43	70	75	−	+	V	V	R	R	+	−
USPMTOX44	64	80	−	+	V	V	R	R	+	−
USPMTOX45	65	77	−	+	V	V	R	R	−	−
USPMTOX47	50	80	−	+	V	V	R	R	−	+
USPMTOX48	65	75	−	+	V	V	R	R	+	+
USPMTOX49	63	68	−	−	V	V	R	R	+	+
USPMTOX50	55	65	−	−	C	C	R	R	−	−
USPMTOX53	50	60	−	+	V	C	R	R	−	−
USPMTOX54	40	60	−	−	C	C	R	R	+	−
USPMTOX86	65	75	−	+	V	C	R	R	+	+
USPMTOX89	66	74	+	+	V	C	R	R	+	−
USPMTOX91	65	63	−	+	V	V	R	R	−	−
USPMTOX93	70	77	−	−	V	V	R	R	+	−
USPMTOX94	50	60	−	+	V	V	R	R	−	−
USPMTOX96	50	65	−	+	C	V	R	R	+	−
USPMTOX97	70	75	−	+	V	V	R	R	+	−
USPMTOX98	66	79	−	−	V	V	R	R	−	−
USPMTOX99	66	75	−	+	V	C	R	R	−	−
USPMTOX104	60	68	+	+	V	V	R	R	−	+
USPMTOX108	65	72	−	+	V	V	R	R	+	−
USPMTOX114	67	78	−	−	V	C	R	R	+	−
USPMTOX115	65	75	−	−	V	V	R	R	−	−
USPMTOX117	65	80	−	+	V	C	R	R	−	−
USPMTOX118	70	80	+	+	V	C	R	R	+	+
USPMTOX119	74	80	−	−	V	C	R	R	−	−
USPMTOX123	60	70	−	+	V	V	R	R	−	−
USPMTOX125	70	75	−	−	V	V	R	R	+	+
USPMTOX126	70	75	−	+	V	V	R	R	+	−
USPMTOX128	68	75	−	+	V	V	R	R	+	+
CBS 179.80	65	69	−	−	V	V	R	R	−	−

C: cottony; V: velvety; R: regular; +: present; −: absent.

confirmed as TeA producers. In the last years, the emerging mycotoxin TeA has gained great attention in the scientific community, especially after the Bavarian Health and Food Safety decided, as the first official decision, to limit the TeA content in sorghum/millet-based infant food (500 μg/kg) (Rychlik et al., 2016). However, most of the studies of TeA contamination in food link it only to the presence of *Alternaria* species, considered the most important source of TeA occurrence. *Epicoccum sorghinum* has been reported in a wide range of cereals, especially in sorghum grains. In one of the few studies that reported the risk of *E. sorghinum* for food security, the authors confirmed that this species was the responsible for a human hemorrhagic disorder known as 'onyalay'. In a recent paper (Oliveira et al., 2017), a high level of TeA was detected in Brazilian sorghum grains (> 500 μg/kg) and confirmed that *E. sorghinum* was responsible to produce TeA during the pre-harvest period. In this study, high levels of TeA were reported from *E. sorghinum* (> 120,000 μg/kg) and are in agreement with the results observed in our study (mean of 38,462.05 μg/kg). Therefore, special attention should be paid to the presence of this species in food and its by-products.

5. Conclusion

Results from this study revealed that *E. sorghinum* isolated from Brazil shown a wide genetic, phenotypic and physiological variability and confirm the high intraspecific diversity that has also been reported of this species from other countries. The isolates were cluster in four

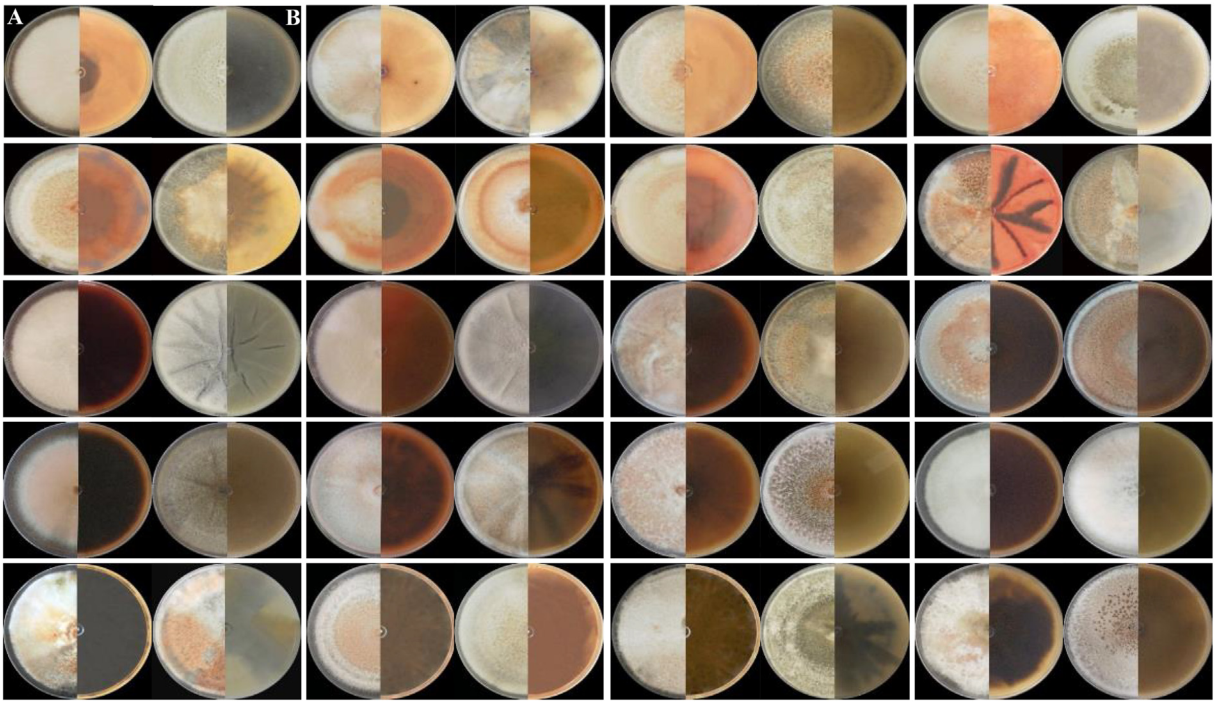

Fig. 2. Variation of colony colors (front) and pigment production (reverse) of *Epicoccum sorghinum* isolates on malt extract agar (A) and oatmeal agar (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

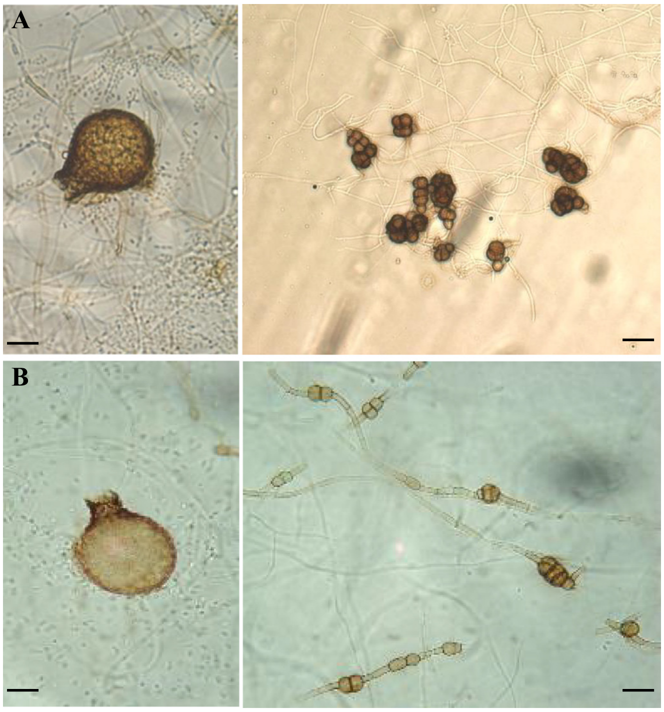

Fig. 3. Micromorphological structures of *Epicoccum sorghinum*. (A) Hyaline/brown glabrous pycnidia; (B) hyaline/brown uni- or multicellular chlamydospores. Scale bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phylogenetic groups, but we have not found any consistent morphological and or physiological marker that able us to delimitate new species. The broad range of colony color found in this study can be useful to better describe the macromorphology feature of *E. sorghinum*. Furthermore, all selected *E. sorghinum* isolates (n = 11) were confirmed as TeA producer and should be considered an important source of this mycotoxin in sorghum grains. These findings contribute to the knowledge of this widespread species and assert that the presence of *E. sorghinum* in cereal, especially in sorghum producing-countries, may play a role in TeA contamination.

Table 3
Tenuazonic acid (TeA) production by representative *Epicoccum sorghinum* isolates from each phylogenetic group.

Isolates	Phylogenetic group	TeA (μg/kg)	GenBank accession	
			ITS	*TUB2*
USPMTOX40	G I	98.6	MG969865	MH249626
USPMTOX27	G II	17,300		
USPMTOX29	G II	23,400		
USPMTOX31	G II	12,100		
USPMTOX43	G II	15,390		
USPMTOX48	G II	148,000	MG969871	MH249627
USPMTOX49	G II	72,700		
USPMTOX91	G II	44,800		
USPMTOX94	G II	121,000		
USPMTOX33	G III	926	MG969862	MH249625
USPMTOX126	G IV	1700	MG969894	MH249624

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