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Ramophialophora chlamydospora, a new species from an alkaline lake of Wadi-El-Natron, Egypt

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Abstract

Ramophialophora chlamydospora, a dematiaceous hyphomycete, recovered from alkaline water of Lake Fasida in Wadi-El-Natron, Egypt, is described. It has phenotypic characterization of branched conidiophores ending with terminal phialides with collarettes, on which dacryoid, brown conidia are formed in slimy heads, and the formation of abundant chlamydospores, a characteristic morphological feature for this new species, that differentiates this new species from the other four species of *Ramophialophora*. Sequence analysis of ITS region revealed its relationship with members of order Sordariales. A culture of the new fungus was preserved as pure one and deposited in the culture collection of the Assiut University Mycological Centre as AUMC 11013. The ITS sequence data was uploaded to the GenBank as KX446768 and the morphological characterization was uploaded to MycoBank as MB828700.

Key words – Alkaline lakes – Phylogeny – *Ramophialophora* – Sordariales – Wadi-El-Natron

Introduction

Wadi-El-Natron depression in Egypt has twenty saline lakes which dry up in summer and become hypersaline and rich with natron, a mixture of sodium salts (Lucas and Harris 1962). Lake Fasida is a small and shallow basin which dries up completely during the summer resulting in the formation of thick deposits of salts on its bottom (Shortland et al. 2011). During surveys and isolation of extremophilic fungi from hypersaline, alkaline lakes of Wadi-El-Natron, Egypt (Al-Bedak 2017; Ismail et al. 2017), an interesting strain was recovered on 1 % glucose-Czapek's agar at 25 °C, from water of Lake Fasida and it showed a strong relationship to genus *Ramophialophora*.

The genus *Ramophialophora* is a dematiaceous hyphomycete which has phialides with collarettes and its conidia are formed in glutinous masses. In addition, branched conidiophore in some species of that genus, is a characteristic morphological feature that never described in *Phialophora* or any other similar genera (Calduch et al. 2004). Up to now, only four species of *Ramophialophora* were described; the type species *R. vesiculosa* (Calduch et al. 2004) isolated from forest soil sample in Asturias Province, Spain; *R. humicola* (Madrid et al. 2010) from a soil sample collected from Ronda, Spain and *R. globispora* from plant debris and *R. petraea* from rock sample in Karst caves in China (Zhang et al. 2017).

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Materials and methods

Sampling site

Wadi-El-Natron is situated between 30º17' and 30º38' N and 30º2' and 30º30' E in the Western Desert adjacent to the Nile Delta, Egypt, below the sea level by 23 m and below the water level of Rosetta branch of the Nile river by 38 m (Abd-el-Malek and Rizk 1963). Water samples were collected from Lake Fasida during Feb 2012 (Ismail et al. 2017) and their pH, total dissolve solids (TDS), carbonates and bicarbonates were determined.

Strain isolation

The fungus was recovered on 1 % glucose-Czapek's agar from water of Fasida lake using pour-plate technique (Sanders 2012). The medium has the following composition (g/L): glucose, 10; Na_2NO_3, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005, agar, 15; Rose Bengal, 0.05 and chloramphenicol, 0.25 and the pH was adjusted at 7.3. The dishes were incubated for 7-15 days at 25 °C. The developed colony of the new fungus was purified and preserved on PDA slants at 4 °C as pure culture.

Morphological studies

Cultural morphology and growth rates were studied on Czapek's Dox agar (Cz), potato dextrose agar (PDA), malt extract agar (MEA), oat agar (OA), corn meal agar (CMA), Cz supplemented with 1 %, 2 %, 3 %, 4 % and 5 % NaCl and Cz with pHs of 6, 7, 8, 9 and 10. The inoculated plates were incubated at 25 °C for 7 days in case of Cz + NaCl concentrations and Cz with different pHs, and 14 days in case of Cz, PDA, MEA, OA and CMA. Microscopic features on PDA were examined in lacto-phenol cotton blue.

Molecular identification

DNA extraction

0.2 g of 7-day-old fungal mycelia of *Ramophialophora chlamydospora* grown on PDA, were grounded and transferred to 1.5-ml microfuge tubes. 800 µl CTAB buffer composed of 3 % CTAB, 1.4 M NaCl, 0.2 % Mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0 and 1 % PVP-40, were added to each tube. After incubation at 65 °C for 30 min, 800 µl of CI Mix with the composition of 24 ml chloroform and 1 ml isoamyl alcohol, were gently added and mixed with the tube contents. A clear supernatant was obtained by centrifugation at 10000 xg for 10 min. For DNA precipitation 2/3 volume of isopropanol (precooled at -20 °C) was added and mixed gently. The samples were incubated at 4 °C overnight, thereafter centrifugation at 13000 xg for 10 min. The supernatant was discarded and the pellet was pooled and washed with 200 µl washing buffer composed of 76 % ethanol and 10 mM ammonium acetate. The washing buffer was carefully decanted and the pellet was suspended in 200 µl TE buffer supplemented with 10 mg/ml RNase. After incubation at 37 °C for 30 min, 100 µl of 7.5 M ammonium acetate and 750 µl ethanol were added and mixed gently. Samples were centrifuged at 13000 xg for 10 min at room temperature. The supernatant was completely discarded and the pellet was suspended in 100 µl sterile distilled water.

PCR for rDNA and sequencing using ITS1 and ITS4 primers

The PCR reaction was performed using SolGent EF-Taq. The universal primers ITS1 and ITS4 (White et al. 1990) were used. In the PCR tubes 1µl of DNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10x complete buffer and 40 µl of sterile ddH2O, 10 pmol of ITS1 (5´ TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´ TCC TCC GCT TAT TGA TAT GC 3´) were added. Then the PCR amplification was carried out using the following sequence: one round of amplification consisting of denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 20 sec, annealing at 50 °C for 40 sec and extension at 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The PCR products were then purified with the SolGent PCR Purification

Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. The purified PCR products were confirmed on 1% agarose gel by electrophoresis using size marker. The bands were eluted and sequenced in the forward and reverse directions.

Phylogenetic analyses

Sequence data for the representative type strains of all published *Ramophialophora* species were downloaded from GenBank. Sequences produced in this study and those from GenBank were aligned using ClustalX (Thompson et al. 1997) and optimized manually. Sequence alignments for all data sets were uploaded to TreeBASE http://purl.org/phylo/treebase/phylows/study/TB2:S23783 (study no. 23783), and sequence for the novel species was deposited in GenBank. Maximum-parsimony (MP) and Maximum-likelihood (ML) phylogenetic analyses were performed using PAUP* 4.0 (Swofford 2001). Maximum-likelihood (ML) analysis (Felsenstein 1981) was carried out under these settings: heuristic searches with random stepwise addition of 100 replicates and TBR rearrangements. The best optimal model of nucleotide substitution for the ML analyses was determined using akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall 1998). K81 was the best fit for the ITS dataset. Phylogenetic trees were visualized using Njplot (Perriere and Gouy 1996) and edited using Adobe Illustrator CS6.

Results

The water sample collected from Lake Fasida registered alkaline pH of 9.53, TDS 7.12 % with high carbonates content of 40.8 g/L and bicarbonates of 23.5 g/L.

Phylogenetic analysis

The ITS dataset comprised 23 sequences, of which 11 are *Ramophialophora* and the remaining sequences for related taxa belong to the order Sordariales (Fig. 1). The maximum parsimony dataset consisted of 557 characters with 254 characters as constant information, 63 characters as variable characters which were parsimony-uninformative, and 240 characters were counted as parsimony-informative characters. Maximum Parsimony analyses resulted in two most parsimonious tree with a tree length of 793 steps, a consistency index of 0.6482, a retention index of 0.7267 and a rescaled consistency index of 0.4711. Maximum likelihood analysis yielded one tree (-ln likelihood = 4700.430) (Fig. 1). The new species *Ramophialophora chlamydospora* consistently grouped with the four species of *Ramophialophora* along with *Cercophora appalachianensis* and *Coronatomyces cubensis* but with low weak support. However, *Ramophialophora chlamydospora* formed a highly supported clade (92 % ML/84 % MP) with *R. globispora*, *R. petraea* and *Cercophora appalachianensis* (Fig. 1). Phylogenetic trees obtained from Maximum Parsimony yielded trees with similar overall topology with the one shown in Fig. 1.

Taxonomy

Ramophialophora chlamydospora AH Moubasher, MA Ismail, OA Al-Bedak & RA Mohamed, sp. nov. Fig. 2

GenBank number: KX446768; MycoBank number: MB828700

Typification: EGYPT. Wadi-El-Natron: Lake Fasida, from alkaline water sample, 2 Feb 2012, Osama A. Al-Bedak (holotype AUMC 11013).

Etymology – Referring to the formation of abundant chlamydospores.

Cultural characteristics

Colonies on PDA attaining 50 mm in 14 days at 25 °C, composed of immersed greyish-brown mycelium (9F3-4/10F3-4), and orange grey conidial tufts (5B2/6B2). Conidiophores erect, sinuous, cylindrical, septate, branched, brown to dark brown at the lower part, becoming paler towards the apex, smooth, thick-walled, (– 7) 12–20 (–35) × 2–3 µm (n=50). Conidiophore branches ending with phialides. Phialides monophialidic, smooth-walled, often waisted, sinuous, 15–20 × 2–4 µm (n=50)

with conspicuous, collarettes, mostly 3–5 µm wide. Sporulation abundant after 14 days of incubation. Conidia one-celled, subhyaline, brown, smooth-walled, dacryoid, mostly 2.5–3.5 (– 4.0) × 2–3 µm (n=50), often with a cylindrical hilum, aggregated in slimy masses. Chlamydospores abundant, produced from immersed mycelia, at first hyaline, later brownish, in chains, clustering with age (Fig. 2). Sexual state not observed within 60 days of culturing.

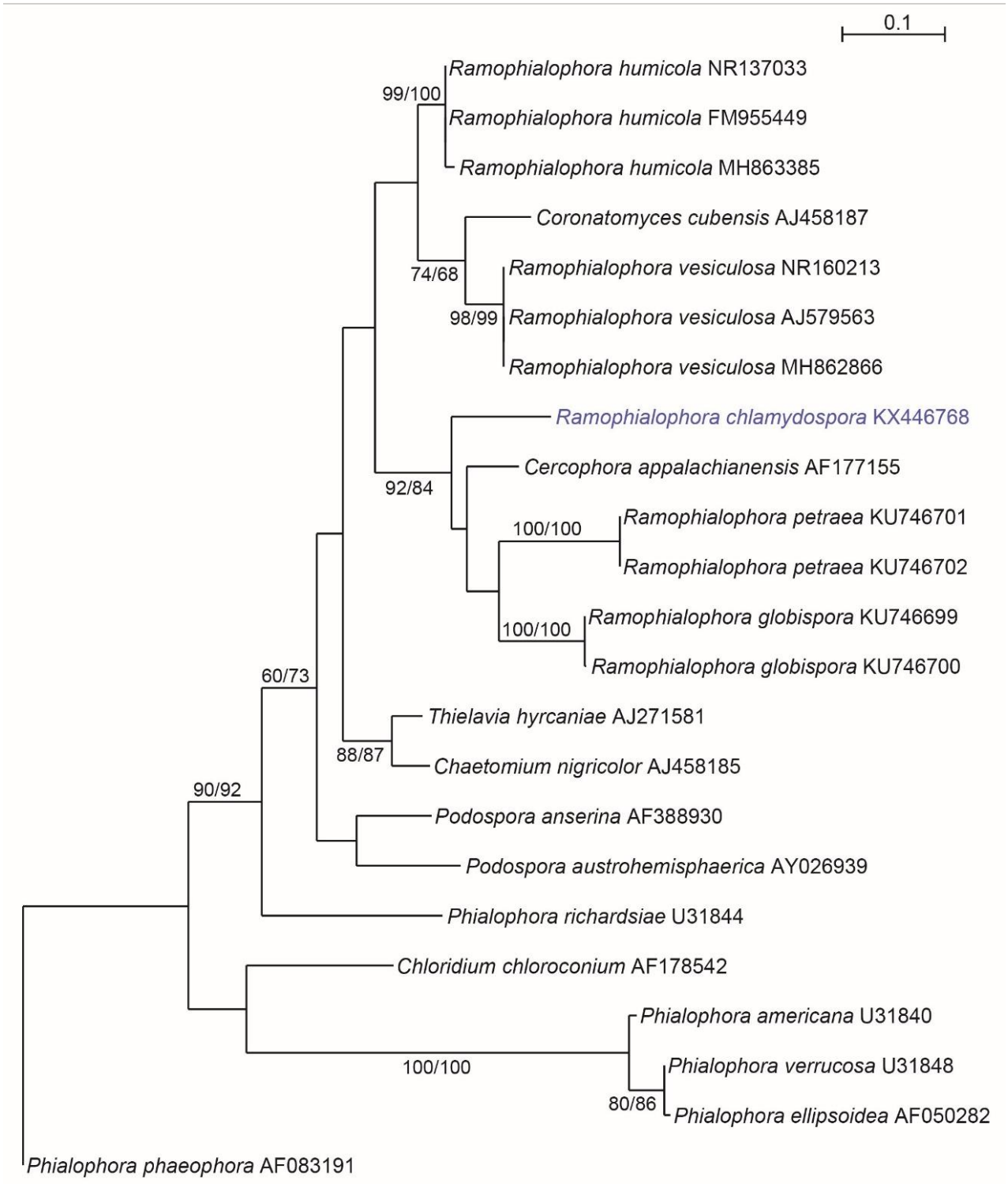

Fig. 1 – Maximum likelihood tree based on sequencing of the ITS regions of *Ramophialophora chlamydospora* AUMC 11013 with the most similar members of order Sordariales in GenBank.

Colonies on Cz, OA, CMA and MEA attaining 40 mm, 60 mm, 60 mm and 30 mm in 14 days at 25 °C. Sporulation sparse to rare on MEA, OA and CMA, moderate on Cz after 14 days of incubation. On Cz with different pH values of 6, 7, 8, 9 and 10 after 7 days of incubation at 25 °C, colonies reaching 55 mm, 45 mm, 65 mm, 80 mm and 75 mm in diameter, respectively (Fig. 3) illustrating that the fungus is alkaliphilic. On Cz supplemented with NaCl with concentrations of 1 %, 2 %, 3 %, 4 % and 5 %, colonies attaining 60 mm, 30 mm, 7 mm and 5 mm, respectively, and it completely failed to grow on 5 % NaCl concentration (Fig. 3).

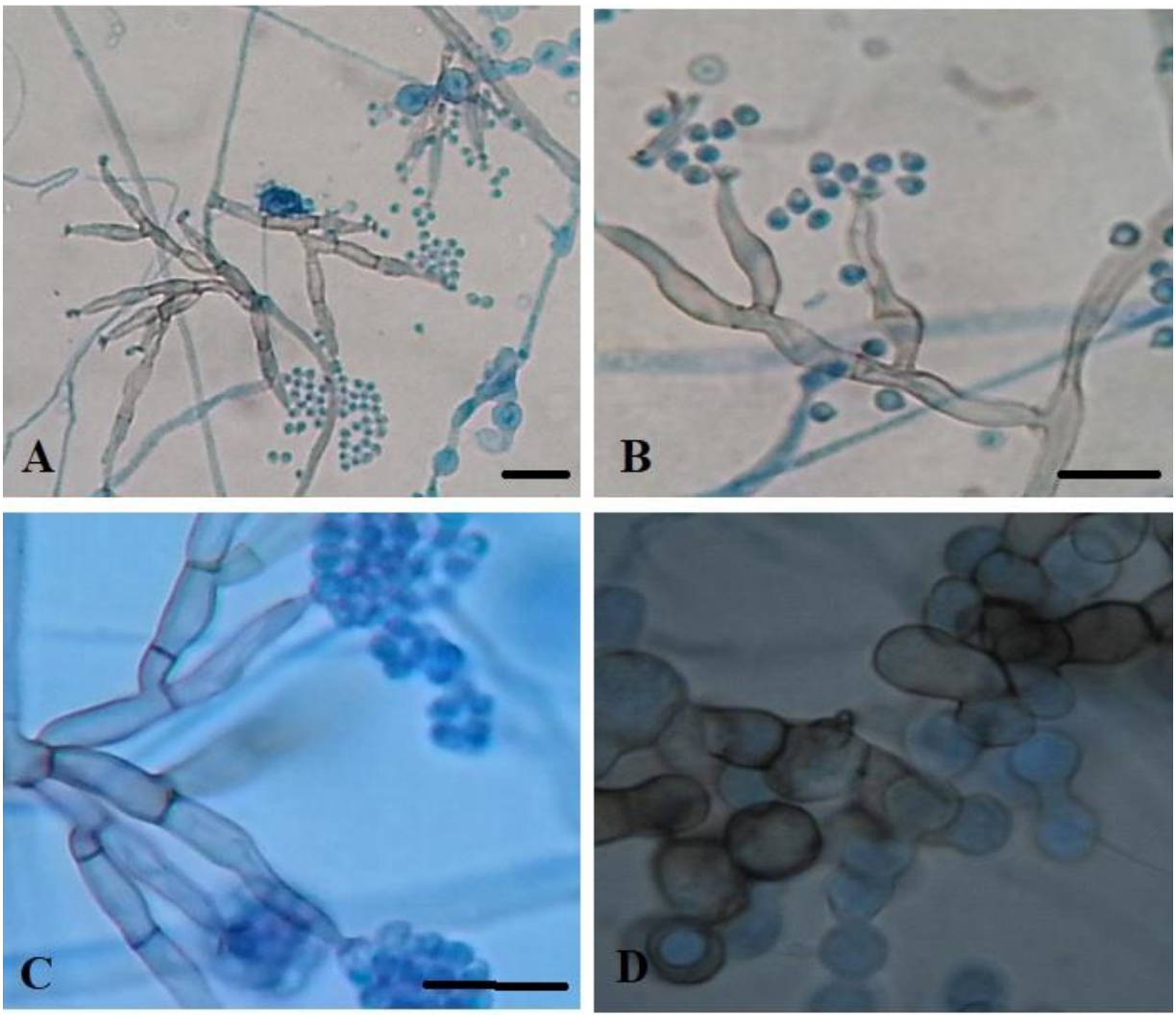

Fig. 2 – Morphological characters of *Ramophialophora chlamydospora* showing. A branched conidiophores. B phialides with flaring collarettes bearing dacryoid conidia. C branched conidiophores and slimy conidial heads on phialides. D chlamydospores. Scale Bars = 10 µm.

Molecular study

Sequence analysis of ITS region confirmed a closest relationship of the new species with the other members of Sordariales. The type strain was deposited in the culture collection of the Assiut University Mycological Centre (AUMC) as AUMC 11013 and the ITS sequence was uploaded to GenBank with accession number KX 446768. The percentage of sequence similarity to the closest matching taxa of *Ramophialophora* and other closer species of Sordariales did not exceed 92 % revealing that the current species is a new taxon (Fig. 1).

Discussion

The morphological examination of *R. chlamydospora* showed some of the typical phenotypic features of *Phialophora* such as phialides with flaring collarettes on which conidia are formed in

slimy heads, however, it distinguished from *Phialophora* species by its branching conidiophores. The novel species was isolated from alkaline water sample (pH = 9.53) from Lake Fasida which contained high carbonates (40.8 g/L) and bicarbonates (23.5 g/L) contents. The novel species showed better growth on alkaline media with pH values of 9 and 10, indicating that the fungus is alkaliphile. Four species of *Ramophialophora* have been described up to date. These species are ecologically different from the new species since the habitat of *R. chlamydospora* is an alkaline water while *R. vesiculosa* (Calduch et al. 2004) and *R. humicola* (Madrid et al. 2010) were isolated from a soil sample in Spain, and *R. globispora* from plant debris and *R. petraea* from rock sample in Karst caves in China (Zhang et al. 2017). Our strain is the only species of that genus which isolated from an alkaline water sample. It resembles *R. vesiculosa* and *R. humicola* in the presence of branched conidiophores and dacryoid conidia and differs from them in the absence of vesicle at the end of conidiophores, but conidiophores of the new species terminated directly with phialides bearing conidia in slimy head. *Ramophialophora chlamydospora* is distinguished from *R. globispora* by its branched conidiophores and forming conidia in slimy heads whereas *R. globispora* is characterized by its unbranched conidiophores bearing tufty and terminally penicillate phialides and long macroscopic conidial beam formed with the aggregation of the conidial chains and its conidia are globose. Also, *R. petraea* differs from our isolate in the presence of reduced conidiophores and its phialides not abundant. The presence of chlamydospores in the new species is a characteristic feature, which differentiate it from all the known species of that genus. The differences between *Ramophialophora* species are illustrated in Table 1.

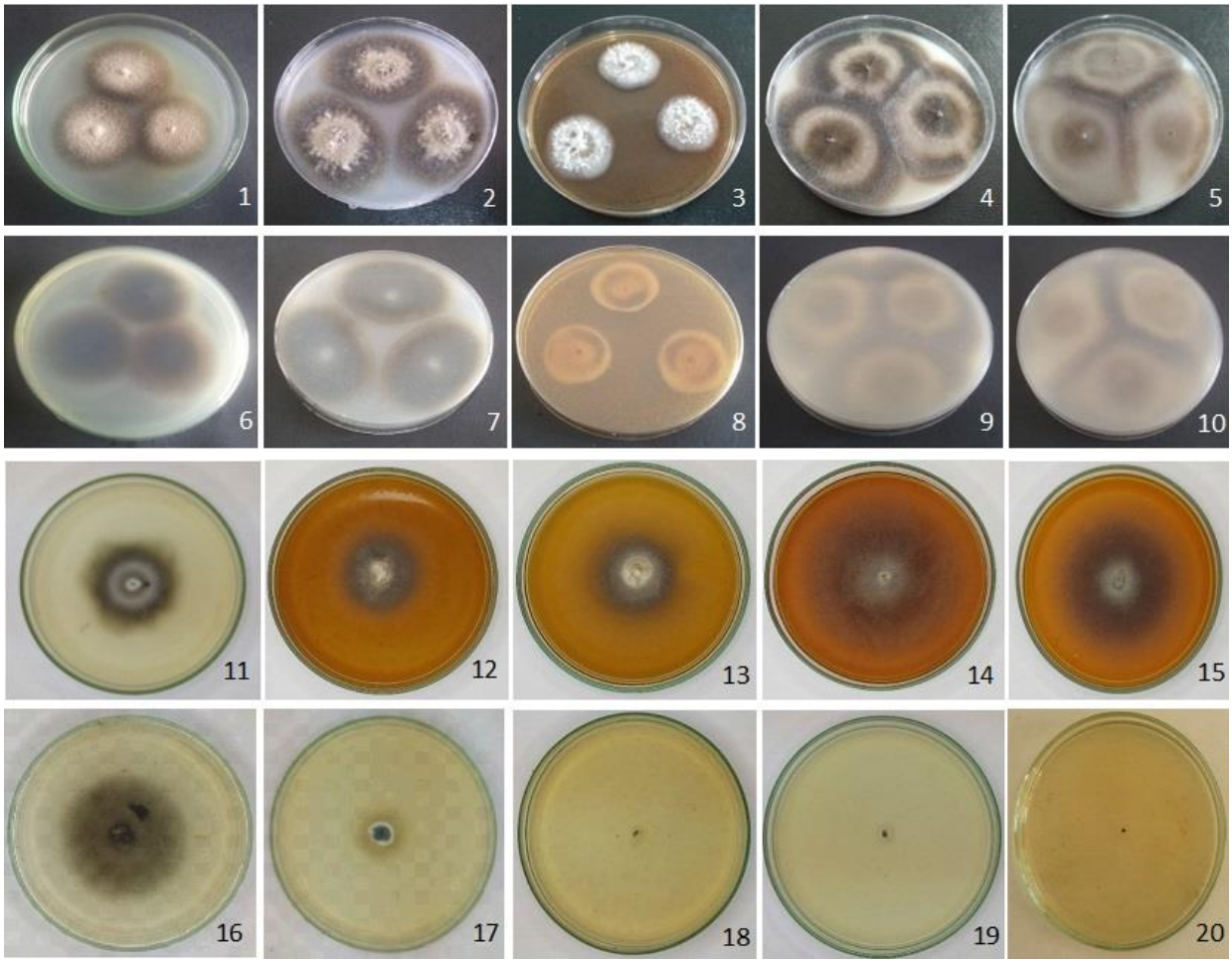

Fig. 3 – Growth of *Ramophialophora chlamydospora* at 25 °C on: 1 Czapek's Dox agar (Cz). 2 Potato dextrose agar (PDA). 3 Malt extract agar (MEA). 4 Oat agar (OA). 5 Corn meal agar (CMA). 6-10 reverse on the same media. 11-15 Cz (pH 6, 7, 8, 9 &10). 16-20 Cz + NaCl (1 %, 2 %, 3 %, 4 % & 5 %).

Table 1 Distinguishing characters of *Ramophialophora* species.

***Ramophialophora* spp.**	**Conidiophores**	**Vesicles**	**Phialides**	**Conidia**	**Chlamydospores**
R. vesiculosa	Branched	Present	Simple	Dacryoid, in slimy heads	Absent
R. humicola	Branched	Present	Simple	Dacryoid, in slimy heads	Absent
R. globispora	Unbranched	Absent	Penicillate	Globose, in chains	Absent
R. petraea	Reduced	Absent	Simple	Globose, in slimy heads	Absent
R. chlamydospora	Branched	Absent	Simple	Dacryoid, in slimy heads	Abundant

Key to known species of Ramophialophora

1. Conidiophores unbranched, conidia globose ……..2
- Conidiophores branched, conidia dacryoid ……..3
2. Phialides penicillate, inconspicuous collarette, conidia in chains ……..*R. globispora*
- Phialides not abundant, ampulliform or sometimes irregular, conidia in slimy heads ……...*R. petraea*
3. Chlamydospores abundant ……..***R. chlamydospora***
- Chlamydospores absent ……4
4. Vesicles, 3.5–6.5 µm at the broadest part, conidia 2.5–3 µm ……..*R. vesiculosa*
- Vesicles, 1.5–4.0 µm at the broadest part, conidia 2.5–4.0 µm ……..*R. humicola*

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