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Microdochium bolleyi (Ascomycota: Xylariales): Physiological characterization and structural features of its association with wheat

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Summary: Plant roots can be colonized by asymptomatic fungal strains belonging to several taxa, among them, the group defined as Dark Septate Endophytes (DSE). *Microdochium bolleyi* commonly colonizes wheat roots and other crops. It is considered a weak pathogen or even a non-pathogenic fungal species, which has also been considered as a potential biocontrol agent against aggressive soil-borne pathogens in cereal crops. We isolated a strain of *M. bolleyi* from wheat roots sampled in a crop field in Argentina, and characterized its abilities to grow in different carbon and nitrogen sources, to produce indole and to solubilize phosphorus; also several enzymatic activities were evaluated. In addition, resynthesis was performed under controlled conditions in order to characterize root fungal colonization under both, optical and transmission microscopy. The strain 22-1 colonized wheat root parenchymal tissue, forming chlamysdospores inside parenchymal cells and root hairs, and poorly grew in carbon and nitrogen sources. This fungus also synthesized indoles in *in vitro* culture, but it cannot solubilize phosphorus. Only amylase activity was detected out of seven enzymatic activity measured. *Microdochium bolleyi* (strain 22-1) colonized the roots, it formed typical DSE fungal structures and behaved like a "true endophyte"; however further studies are necessary to elucidate its role in the association with wheat.

Key words: Dark septate endophytes, *Microdochium bolleyi*, root endophytes, wheat crop.

Resumen: *Microdochium bolleyi* (Ascomycota: Xylariales): Caracterización fisiológica y caracteres estructurales de su asociación con trigo. Las raíces de las plantas hospedan una gran diversidad de hongos, entre ellos, se encuentran los Endofitos Septados Oscuros (ESO). *Microdochium bolleyi* coloniza las raíces de trigo y otros cereales, aunque algunos autores lo han considerado un patógeno débil, otros han demostrado su acción biocontroladora contra patógenos agresivos del suelo. En el presente trabajo, se aisló una cepa de *M. bolleyi* (22-1) de raíces de trigo. Esta cepa fue metabólicamente caracterizada y se realizó un ensayo de resíntesis bajo condiciones controladas con el fin de caracterizar la colonización del hongo en la raíz bajo microscopía óptica y de transmisión. Su crecimiento fue escaso en las fuentes de carbono y nitrógeno evaluadas, sintetizó indoles en cultivo in vitro, pero no mostró habilidades para solubilizar el fósforo, por último, solo se detectó actividad amilasa. La cepa 22-1 coloniza la corteza radicular del trigo, formando clamidosporas melanizadas inter e intracelularmente y en el interior de los pelos radiculares. *Microdochium bolleyi* (cepa 22-1) coloniza la raíz de trigo formando las típicas estructuras de los ESO y comportándose como un "verdadero endófito", sin embargo, se necesitan más estudios para terminar de dilucidar su papel en la asociación con el trigo.

Palabras clave: Endofitos septados oscuros, hongos endofitos, *Microdochium bolleyi,* trigo.

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Introduction

Most plant species can associate with a wide diversity of root endophytic fungi, including the denominated Dark Septate Endophytes (DSE). These endophytes comprise a group of cosmopolitan fungi, mostly of ascomycetes species included in different orders as Helotiales, Pleosporales, Sordariales and Xylariales (Jumpponen, 2001; Jumpponen & Trappe, 1998). DSE are characterized by melanized septate hyphae and microesclerotia or chlamydospores that can colonize the parenchymal tissue of the roots either intra- or intercellularly (Addy *et al*., 2005; Muthukumar & Tamilselvi, 2010; Sieber & Grünig, 2013), without causing disease symptoms on the host plant (Jumpponen, 2001). Among DSEs, *Phialocephala fortinii* is one of the best studied taxa, recorded in different environments and plant species (Brenn *et al*., 2008; Jumpponen & Trappe, 1998). Isolates of this fungus inoculated in several experimental trials, using a range of host plant species and culture conditions (Fernández & Cagigal, 2017; Newsham, 2011; Wilcox & Wang, 1987), yielded positive, neutral or negative effects on plant growth. Similarly, it has been demonstrated that some asymptomatic strains of well-known dark pathogenic fungal taxa (*viz. Curvularia* spp*, Phoma* spp.) could be included within this group, as they colonize the root parenchymal tissue with melanized mycelium, behaving as typical DSE fungi (Loro *et al*., 2012; Priyadharsini & Muthukumar, 2017).

Microdochium bolleyi (syn: *Idriella bolleyi*) is another well-known species of DSE. It has been extensively isolated from roots and stem bases of several cereals and grasses (Domsch *et al*., 1980; Salt, 1977). Although it has been reported to cause minor damage under particular conditions, it is mainly considered as non-pathogenic (Kirk & Deacon, 1987; Punithalingam *et al*., 1979), even *M. bolleyi* has been patented as a take-all biocontrol agent in cereals and grasses (Fox-Roberts & Deacon, 1988). Mandyam *et al*. (2010) characterized strains of dark septate endophytes identified as *Microdochium* sp. isolated from sampled roots of C4 grasses in Kansas (USA). These authors have demonstrated that two *Microdochium* sp. isolates used a variety of complex nutrient sources and produced different extracellular enzymes, suggesting the potential role of these fungi in organic matter mineralization and plant nutrition.

Although, DSE studies have focused on descriptive analyses of the colonization and fungal strains, mostly isolated from natural ecosystems (Jumpponen & Trappe, 1998; Newsham, 2011), while, when considering crop species, the information related to their association with DSEs is scarce and limited (Wang *et al*., 2016; Yuan *et al*., 2010; Rothen *et al*., 2017; Fernandes *et al*., 2015; Muthukumar & Tamilselvi, 2010). Argentina produces approximately 2% of the global wheat production, with an average planting area of 6 million hectares in the last 5 years (Andrade & Satorre, 2015), thus becoming one of the most important crops in the country.

Despite *M. bolleyi* is commonly found associated with cereals and grasses, forming typical DSE structures, few studies have focused on its physiological abilities and none of them have considered its interaction with wheat from an ultra-anatomical point of view. The aims of this work were: *i)* to isolate and identify a strain of *M. bolleyi* from wheat roots and to evaluate physiologically this endophytic fungus and *ii)* to describe the anatomy of the interaction at two levels, optical and ultra-anatomical, based on resynthesis tests 30 days after inoculation.

Materials and Methods

Isolation and culture

The fungal strain was isolated from wheat roots, sampled in a productive field in the location of Ferré (Buenos Aires Province, Argentina 34° 06' S-61° 09' W). This area, known as rolling or central sub-humid Pampas, is one of the most productive agricultural regions in the country. The soil is Argiudoll type (pH 7), the annual rainfall, near 900 mm, is concentrated during spring and summer periods and the mean annual temperature is 16°C. Isolation and culture of endophytic fungi from roots were carried out following the methodology described by Silvani *et al*. (2008). Whole wheat plants (n=15) were sampled in June 2013 and transported to the laboratory within 24hs. The roots were excised and washed prior surface sterilization with 3% NaOCl (v/v) and antibiotics (0.05% w/v Penicillin, 0.05% w/v Ampicillin, 0.05% w/v Streptomycin, 0.05% w/v Tetracycline). The roots were washed with sterile water and cut

into segments of 2-3 mm. Then, each segment was transferred to drops of water-agar medium (Gel-Gro®) and incubated at 25°C in the dark. Emerging hyphae from root ends were checked periodically under a binocular microscope and carefully plated onto malt extract agar (MEA) for further growth and fungal characterization. From 24 fungal isolates, one strain (22-1) corresponding morphologically to the genus *Microdochium* was selected due to observable typical DSE structures. The selection was done through preliminary resynthesis assay with wheat plants.

Morphological and molecular identification

The isolate of *Microdochium* morphotype (strain 22-1) was cultured on MEA medium to characterize vegetative growth and sporulation. In order to approach the species level identification of the strain, descriptions provided by Ellis (1971) and Hernández-Restrepo *et al.* (2016) were considered. For molecular identification, isolate 22-1 was cultured in liquid malt extract 20% (w/v) at 25°C for one week. Genomic DNA was extracted using UltraClean™ Microbial Isolation Kit (MO BIO). The internally transcribed spacer (ITS) region of the fungal rDNA was amplified using the primers ITS1 and ITS4. PCR was performed using iProofTM High Fidelity DNA Polymerase (BIO RAD) in a 50 µl reaction volume containing 5–10 ng DNA under conditions described by Triebel *et al.* (2005). The PCR product was purified with UltraClean™ PCR Clean-up DNA Purification Kit (MO BIO) and sequenced with primers ITS1 and ITS4 on genotyping service provide by Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (FCEN-UBA). Sequences obtained from pair primers were aligned to obtain a consensus sequence using Bioedit (Hall, 1999) and compared with others present in the 'nr' database with Blastn (Altschul *et al*., 1990). All sequences were aligned with MAFFT program version 6 (Katoh & Toh 2008) available online (http: //mafft.cbrc.jp/ alignment/ server/), assigning a cost to the opening 15 and 6 to the extent of the gaps. To infer the phylogenetic tree, the alignments were subjected to a neighbor-joining analysis using the heuristic search option in MEGA v. 5 (Tamura *et al*., 2013). The support nodes obtained were calculated by the Bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Indole production

For indole production assessment, the fungal isolate was grown in three replicates 250-ml flasks with broth containing (L^{-1}): glucose 2.5 g; sodium succinate 2.5 g; K_2HPO_4 6 g; KH_2PO_4 4 g; KOH 2.1 g; NH_4Cl 1 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; NaCl 0.1 g; $CaCl_2 \cdot 2H_2O$ 0.02 g; $FeCl_3$ 0.01 g; and $Na_2MoO_4 \cdot 2H_2O$ 0.002 g (Fuentes-Ramirez *et al*. ,1993). Liquid medium was supplemented with tryptophan (100µg/L), and incubated at 25°C on a rotary shaker in darkness for 7 days. To detect the presence of indole, 1 mL of the medium was centrifuged (10,000 rpm, 10 minutes) and the supernatant was mixed with an equal volume of the Salkowski chromogenic reagent and incubated for 30 min (Ehmann, 1977). The test was considered positive when a pink-red color change of the supernatant was observed.

Phosphorous biosolubilization

To test the ability of *M. bolleyi* for phosphorous solubilization, the fungus was inoculated on Petri dishes containing solid NBRIP (National Botanical Research Institute's phosphate growth medium), a medium developed for screening phosphate solubilizing microorganisms (Nautiyal, 1999). This medium contains insoluble calcium phosphate ($Ca_3(PO_4)_2$) as the only source of phosphorus. Five replicate Petri dishes were incubated in the dark at 25°C for 7 days. Positive solubilizing capacity was determined when a visible halo was evident surrounding the fungal colony on agar plates. The pH of the media was adjusted to 7.0 before autoclaving.

Enzymatic activities

Microdochium bolleyi (strain 22-1) was tested for seven hydrolytic capabilities. These were determined on a basal medium (Caldwell *et al*., 1991) supplemented with the corresponding substrate for each enzyme and agar 1.5%. All tests were performed in triplicate in Petri dishes of 4.5 cm diameter, containing 10 ml of each medium adjusted to pH 6. Plates were incubated for 1-2 weeks at 25°C. Inoculated basal media plates without test substrate and non-inoculated reaction plates were run as controls. Polysaccharide hydrolysis was determined with 1% starch (SIGMA), 1% carboxymethylcellulose (CMC-SIGMA), 1% xylan (SIGMA), 1% apple pectin and 2.4% chitin

(SIGMA) as the sole carbon source. The chitin solution was prepared following the protocols described by Hankin & Anagnostakis (1975).

Iodine solution 1% was added to the medium to reveal enzymatic activity for starch, and congo red for CMC and xylan (Teather & Wood, 1982). The reaction was considered positive when a translucent halo formed. Chitin utilization was detected when clear zones were observed around colonies in the opaque agar. Hydrolysis of fatty acid esters was determined by the formation of an opaque halo of calcium palmitate crystals in the basal medium supplemented with 1% Tween 40 (Caldwell *et al*., 1991). Finally, protein hydrolysis was determined by the formation of a clear halo in basal medium with gelatin 3% as the sole nitrogen source (Gerhardt, 1981).

Metabolic profile analysis

Substrate utilization by *M. bolleyi* was tested using pre-made FF microplates (Biolog® catalog #1006) containing 95 different carbon and nitrogen sources. For inoculum preparation, *M. bolleyi* was cultivated in MEA at 25°C. After 1 week the conidia were collected and suspended in a tube containing 5 ml of sterile FF-IF broth (0.25% Phytagel® and 0.03% Tween 40 in DI water). Fungal growth was estimated by measuring turbidity at 750 nm and 490 nm and using the formula: Mycelial Growth = [(abs x 490 nm- abs A1 490 nm) - (abs x 750 nm- abs A1 750 nm)] * 1000. Measurements were made at day 7. Results were graphically presented through a histogram.

Resynthesis and colonization

The resynthesis assay was performed with the identified strain of *M. bolleyi* (strain 22-1). Five pots of 200ml were filled with an autoclaved mixture of soil: vermiculite: perlite in 1: 1: 1 (v/v/v) proportion. Hypochlorite (5%) surface-sterilized wheat seeds were sown in the substrate and inoculated with three 1x1-cm plugs with fungal mycelium, taken from the hyphal edge of a 10-day-old fungal colony culturing on MEA. In order to visualize the fungal root colonization, the plants were harvested after 30 days and the roots were processed following the technique developed by Phillips & Hayman (1970) and observed with a light microscope (LM) at 40x magnification.

Ultra-structural studies

To complement the interaction studies, colonized roots were studied with a transmission electron microscope (TEM). Wheat seedlings cultivated on Murashige-Skoog (MS-Sigma) medium (pH 5.8 at 25°C) were inoculated with *M. bolleyi*. Thirty days later, segments of roots were cut and washed in phosphate buffer, then fixed in 0.25% glutaraldehyde and 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4) for 18h at 4°C, rinsed in phosphate buffer under light vacuum and post-fixed in buffered 2% osmium tetroxide for 2h at 4°C, dehydrated in an ethanol-acetone series and included in Durcupan epoxy resin. Thin sections (0.5 µm) were cut with a manual ultramicrotome (Sorvall MT1); these sections were stained with toluidine blue (0.05% [w/v] in benzoate buffer, pH 4.4, for 45 s) and observed with a Zeiss EM 109T transmission electron microscope (service of the Faculty of Medicine (UBA).

Results

Identity

The morphology of strain 22-1 agrees well with the description of *M. bolleyi* (phylum Ascomycota, Pezizomycotina, Sordariomycetes, Xylariomycetidae, Xylariales, Microdochiaceae) provided by Hernández-Restrepo *et al*. (2016). In pure culture, young colonies were smooth and white-pink in color (Fig. 1A) but later, a melanin-pigmented zone gradually expanded outwards from the colony center. This pigmentation was correlated with the formation of chlamydospores that covered the entire Petri dish after 2-3 weeks. *M. bolleyi* (strain 22-1) produced crescent-shaped conidia arising from denticulate loci on hyaline septate hyphae (Fig. 1B). The globose or sub-globose conidiogenous cells described by de Hoog & Hermanides-Nijhof (1977) were occasionally observed. In the phylogenetic analysis, strain 22-1 was grouped with members of *M. bolleyi* (100% of bootstrap support) (Fig. 2). The ITS sequence was submitted to NCBI GenBank with the accession number KF600798.

Fungal physiological characterization

Indole production was evident due to the turning of the Salkowski's chromogenic reagent from

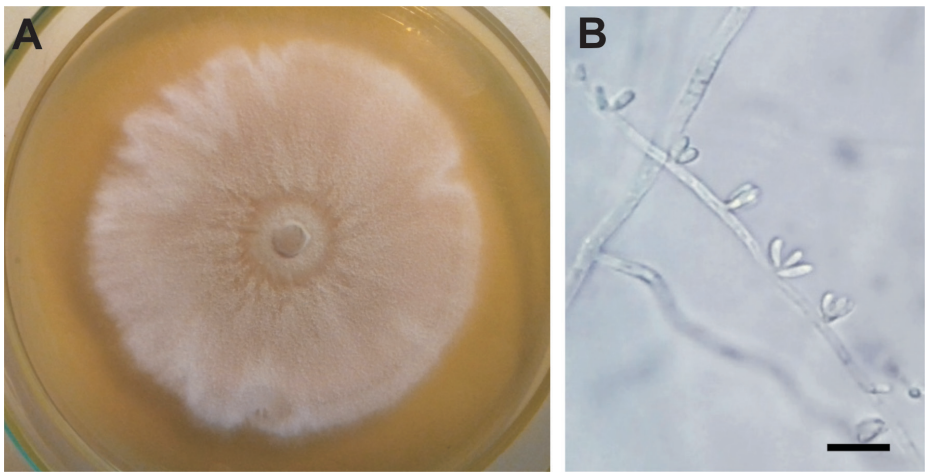

Fig.1. *Microdochium bolleyi.* **A**: Colony general appearance of strain 22-1 grown on MEA at 25ºC for 7 days. **B**: Hyaline unicellular conidia on cylindrical conidiogenous cells. The scale bar represents 10µm.

colorless to intense pink color after seven days of fungal incubation. Regarding its P-solubilization ability, the *M. bolleyi* strain isolated from wheat roots did not produce a translucent halo in the tested NBRIP medium. On the other hand, enzyme activity assays indicated that strain 22-1 produced only amylases, out of the seven enzymatic capacities evaluated.

In the metabolic profile analysis, of the 95 different carbon and nitrogen sources evaluated

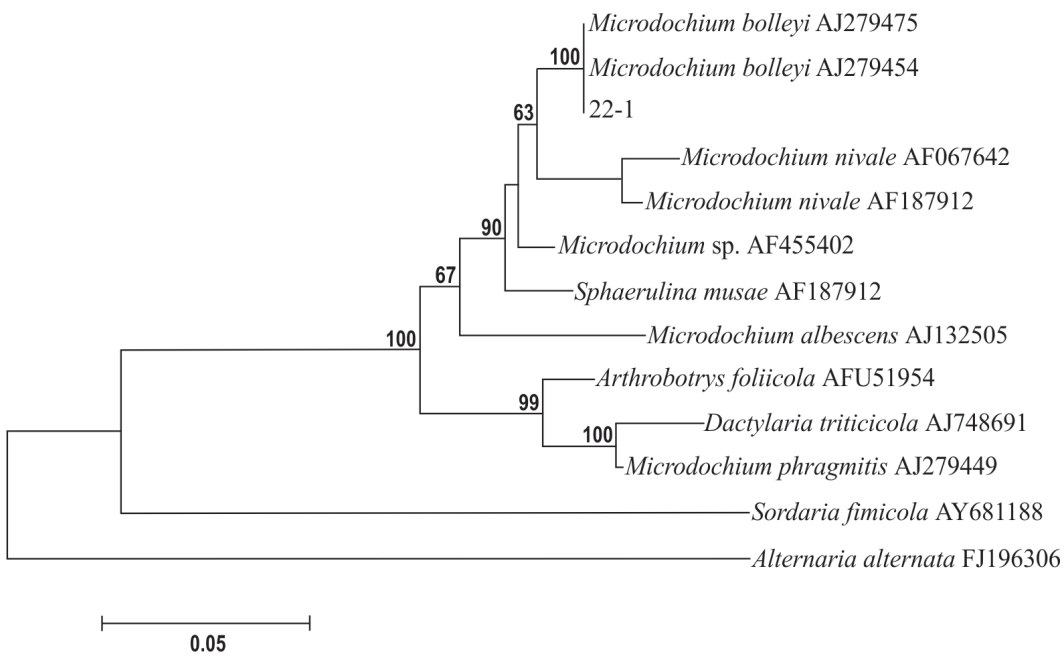

Fig. 2. Strict consensus of the most parsimonious trees resulting from the ITS data matrix analysis. Numbers above branches refer to bootstrap values.

in the BIOLOG® microplates, the histogram displayed five mycelial growth ranges, from R1 (less used substrates) to R5 (more used substrates). According to the histogram, only a few substrates (9, R5) were well used by strain 22-1 (Fig. 3). The most used ones were Adenosine-5-Monophosphate, Putrescine, Glucose-1-Phosphate, 2-Aminoethanol, Glycyl-L-Glutamic Acid, D-Saccharic Acid, L-Serine, L-Ornithine, Adenosine; and the least used ones were B-Cyclodextrin, D-Melibiose, a-D-Lactose, Maltitol, i-Erythriol, m-Inositol, Lactulose, a-Cyclodextrin and Glucoronamide (see attached information for further data). (Table).

Resynthesis and ultra-structural studies

In LM observations, the isolated strain 22-1 showed typical structures of DSE fungi, colonizing the intercellular spaces of wheat root cortical tissue with hyaline hyphae that did not stain with trypan blue. Melanized and compacted chlamydospores formed inter- and intracellularly in cortical cells and also were frequently found inside the root hairs, occupying the entire space thereof (Fig. 4A-B), but no colonization of the vascular cylinder or signs of tissue necrosis or disorganization were observed. TEM observations revealed that the hyphal penetration occurred directly at the junction of the epidermal cells, without formation of appressoria. Abundant hyphae in epidermal cells and intercellular space were not associated with the development of papillae or degradation of the plant cell wall (Fig. 4C-D); electronically dense material was observed around these intercellular hyphae.

Discussion

In Argentina, DSE of crops are not well-known yet, their identities are poorly understood, and even less their interaction with cereal species (Rothen *et al*., 2017). In this work, a strain of a DSE fungus, morphologically identified as *M. bolleyi*, was isolated from wheat roots in agricultural soils in the province of Buenos Aires. Through a phylogenetic analysis, we unequivocally confirmed its identification. *Microdochium bolleyi* is frequently isolated from cereals and grasses in diverse environments (Mandyam *et al*., 2010;

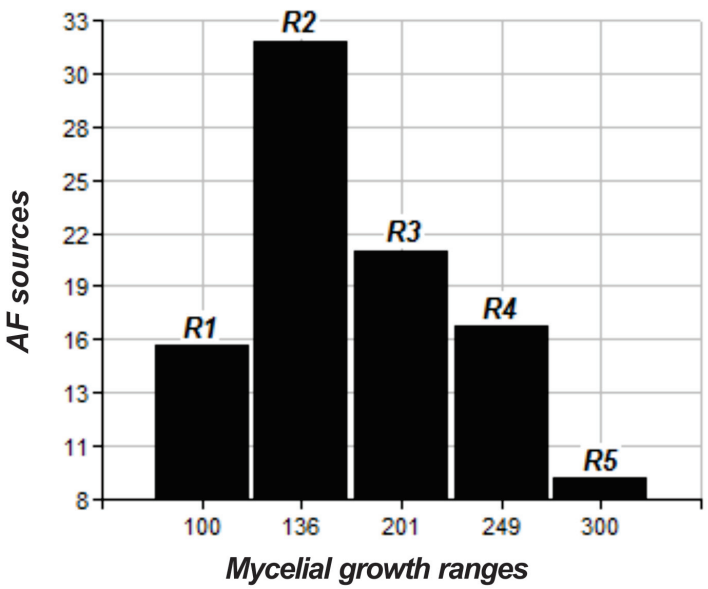

Fig. 3. Histogram representing the absolute frequencies (AF) of substrates used by *M. bolleyi* for five mycelial growth ranges (R1 to R5). The values of mycelial growth were calculated using the following formula: Mycelial Growth = [(abs x 490 nm- abs A1 490 nm) - (abs x 750 nm- abs A1 750 nm)] * 1000.

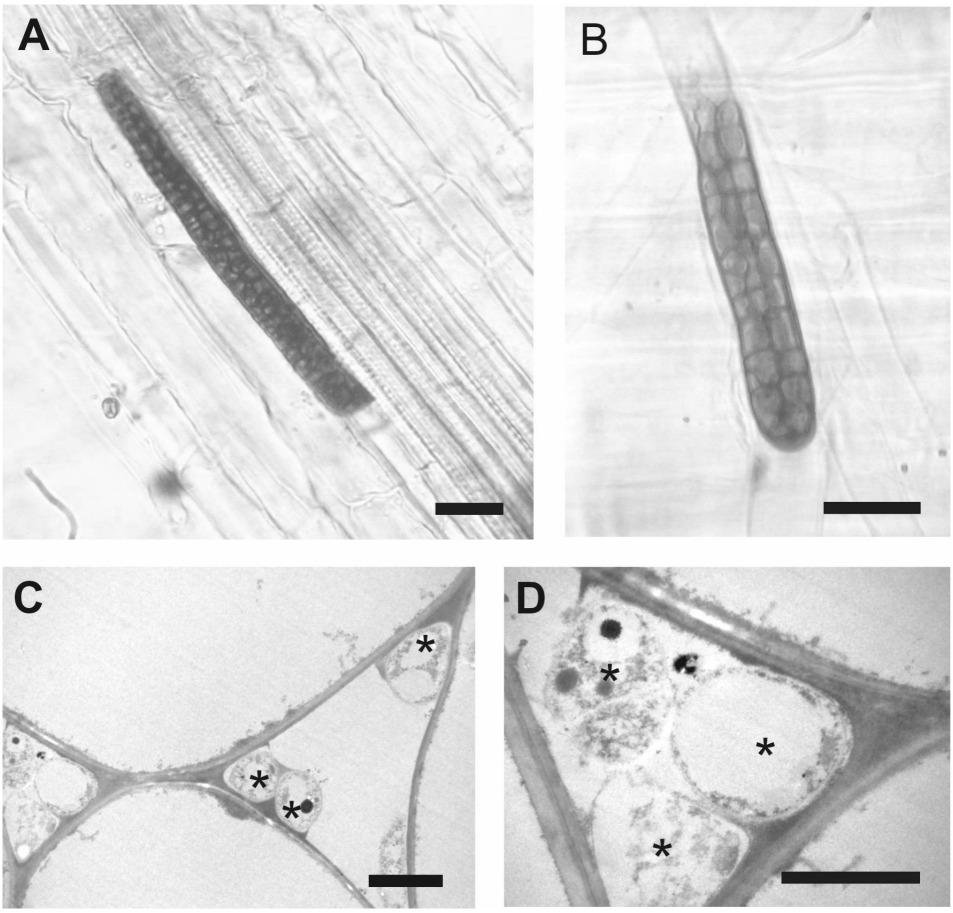

Fig. 4. *Microdochium bolleyi* colonization of wheat roots observed by optical microscope (**A-B**) and TEM (**C-D**). **A**: Chlamydospore inside a cortical root cell, scale bar 10µm. **B**: Chlamydospore inside a root hair, scale bar 10µm. **C-D**: Hyphae colonizing the intercellular space in the cortical tissue (*) with surrounding electrically dense material, scale bars 1 µm.

Wirsel *et al*., 2001). Although some studies have shown that this fungus produces weak pathogenic effects (Waller, 1979; Kirk & Deacon, 1987; Hong *et al*., 2008), it is not considered a pathogenic species and it is even able to control take-all diseases (Fox-Roberts & Deacon, 1988), behaving like a commensalist or as a fungus promoting plant growth (Dawson & Bateman, 2001; Liljeroth & Bryngelsson, 2002; Mandyam *et al*., 2010; Zhang *et al*., 2008).

Physiological characterization

The isolated strain of *M. bolleyi* did not solubilize P when calcium phosphate was used as an insoluble phosphorus source. The capacity to solubilize P, though, has been shown in others DSE species, using solid and liquid media with three phosphorus sources: calcium, aluminum and iron phosphate (Priyadharsini & Muthukumar, 2017; Spagnoletti *et al*., 2017). For instance, *Aspergillus ustus,* considered as a DSE fungi (Barrow & Osuna, 2002), and *Curvularia geniculata* (Priyadharsini & Muthukumar, 2017) promote plant growth due to their ability to solubilize P from unavailable phosphate. Further research is needed on this aspect of strain 22-1, using more sensitive methods and different sources of phosphorus.

When considering the enzymatic activities, Cadwell *et al*. (2000) and Mandyam & Jumpponen

(2005) observed a large battery of hydrolytic enzymes in some strains of DSE fungi. In particular, for other *Microdochium* species, Mandyam *et al*. (2010) detected the presence of amylase, cellulase, tyrosinase and gelatinase. The strain of *M. bolleyi* studied here showed positive results just for the amylase test and for a few of the carbon and nitrogen sources evaluated. Some authors have suggested that the capability of DSEs to allow hosts roots to access mineralized nutrients is related to the saprophytic ability (Upson *et al*., 2010; Usuki & Narisawa, 2007). The few enzymatic abilities and the low proportion of organic compounds used would show a certain limitation by this fungus to mineralize organic sources.

The strain studied produced indole in liquid medium, as has been described for other DSE fungi by Lahlali *et al*. (2014) and Berthelot *et al*. (2016), among others. Some authors suggest that the production of microbial IAA would be of great importance for the establishment of symbiosis (Hilbert *et al*., 2012; Tranvan *et al*., 2000), while others propose that microbial IAA may induce the growth of plants and could confer improved stress tolerance (Kazan & Manners, 2009; Shi & Chan, 2014). Further tests are required, under different nutritional and stress conditions, to determine how the production of IAA by *M. bolleyi* may affect its interaction with the host.

Interaction with wheat

By means of the re-synthesis, we confirmed that the strain studied produced typical DSE structures (Sieber & Grünig 2013). Unlike many of these endophytes, its hyphae showed little or no melanization at all, with melanin being only found in chlamydospores. This characteristic of the intraradical mycelium has been observed in one strain of *Microdochium* sp. (Kageyama *et al*., 2008), *Phialophora graminícola* (Newsham, 1999), *Phialocephala fortinii*, and some other DSE as well. Barrow & Aaltonen (2001) consider that the poor melanization of the hyphae in DSE fungi can be attributed to a more active metabolic state in relation to the possible exchange of nutrients with the host, since they observed that melanized hyphae were more extensive in roots of dormant or inactive *Atriplex canescens* plants, while hyaline hyphae were most abundant in roots of physiologically active plants. Murray & Gadd (1981) examined further the morphology of an isolate of *M. bolleyi* and observed the same pattern of colonization in barley, where the roots remained healthy despite being highly colonized. These authors hypothesize that the asymptomatic colonization is due to the prevalence of chlamydospores chains, which are structures of resistance with low metabolic activity. Moreover, intercellular hyphae colonized mainly the cortical parenchyma and root hairs without reaching the vascular cylinder, an aspect that has often been related to non-phytopathogenic fungi (Mandyam & Jumpponen, 2005; Peterson *et al*., 2008). The pattern of colonization and the lack of typical defense response of host tissues (Morita *et al*., 2003; Shimizu *et al*., 2005) together with the absence of any visible disease symptoms make strain 22-1 a "true endophyte" (Mostert *et al*., 2000).

There are very few studies that analyze the DSE plant-fungus interaction at the ultrastructural level (Peterson *et al*., 2008, Tsuneda *et al*., 2009). Here, no defense responses were found when the interaction between *T. aestivum* and *M. bolleyi* was studied with TEM, only an electronically dense material was observed around the intercellular hyphae. This is similar to fibrillar material observed surrounding the hyphae in Chinese cabbage in the presence of *Heteroconium chaetospira* (Yonezawa *et al*., 2004) and in *Asparagus officinalis* inoculated with *P. fortinii* (Yu *et al*., 2001). Although in the current study the fibers were not observed, it could be due to an inefficient fixation of the material.

Conclusion

In Argentina, few studies have focused on the relationships between plants and DSE. In this work, a strain of *M. bolleyi* isolated from wheat roots was characterized. This fungus produced IAA, it did not grow in most of the sources of P and N, and only amylase activity was detected, suggesting it has low capacity to mineralize organic sources. When *M. bolleyi* colonized the roots, it formed typical DSE fungal structures and behaved like a "true endophyte" under the conditions evaluated. Further studies are necessary to broaden the knowledge of its role in association with wheat.

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Table. Fungal growth of *M. bolleyi*, in 95 different carbon and nitrogen sources, grouped in five mycelial growth ranges, from R1 (less used substrates) to R5 (more used substrates).

Range of utilization	Source	Mycelial growth
R5	Adenosin-5-Monophosphate	340.67
	Glycyl-L-Glutamic Acid	312.00
	Putrescine	310.33
	Glucose-1-Phosphate	298.67
	L-Serine	294.33
	Quinic Acid	290.67
	D-Saccharic Acid	289.33
	2-Aminoethanol	286.67
	Adenosine	285.33
R4	L-Glutamic Acid	283.33
	L-Ornithine	280.33
	g-Hydroxybutyric acid	271.33
	L-Malic Acid	262.00
	L-Proline	262.00
	Uridine	261.00
	Succinic Acid Mono methyl Ester	249.33
	L-Phenylalanine	249.33
	L-Threonine	249.33
	D-Xylose	243.33
	p-Hydroxy Phenyacetic acid	240.33
	L-Lactic acid	234.33
	a-Ketoglutaric Acid	233.00
	L-Pyroglutamic Acid	232.33
	Succinic Acid	230.33
	b-Hydroxybutyric acid	228.33
	D-Malic Acid	228.33
R3	L-Alaninamide	222.33
	L-Alanyl-Glycine	221.67
	g-Amino Butyric Acid	220.00
	D-Lactic acid Methyl ester	219.00
	L-Alanine	217.00
	N-Acetyl-L-Glutamic Acid	216.67
	Bromo Succinic Acid	213.33
	L-Aspartic Acid	213.33
	2-Keto-D-Gluconic Acid	206.67
	L-Asparagine	204.33
	Xylitol	203.33

Range of utilization	Source	Mycelial growth
R3	D-Gluconic Acid	203.00
	D-Trehalose	200.00
	N-Acetyl-D-Glucosamine	197.00
	D-Sorbitol	195.67
	Fumaric Acid	193.33
	Salicin	189.33
	D-Glucuronic Acid	184.00
	N-Acetyl-D-Mannosamine	181.67
	L-Sorbose	180.00
	Arbutin	176.00
	N-Acetyl-D-Galactosamine	171.33
R2	D-Tagatose	169.00
	Tween 80	161.67
	Sucrose	161.00
	D-Psicose	159.00
	D-Raffinose	152.00
	D-Ribose	147.00
	Sedoheptulosan	146.00
	D-Galacturonic Acid	144.67
	Succinamic Acid	144.00
	Stachyose	143.33
	D-Fructose	142.00
	Gentobiose	140.00
	Maltotriose	139.67
	L-Rhamnose	139.00
	Palatinose	138.67
	b-Methyl-D-Glucoside	135.00
	Glycerol	134.67
	a-Methyl-D-Galactoside	134.00
	Glycogen	131.33
	b-Methyl-D-Galactoside	130.33
	Turanose	128.67
	L-Arabinose	128.00
	L-Fucose	126.67
	D-Galactose	123.67
	a-D-Glucose	122.67
	D-Arabinose	121.67
	D-Cellobiose	120.67
	Dextrin	119.67

Range of utilization	Source	Mycelial growth
R2	D-Arabitol	118.67
	Amygdalin	118.33
	Maltose	118.00
R1	Adonitol	115.67
	D-Glucosamine	114.67
	a-Methyl-D-Glucoside	114.67
	D-Melezitose	111.00
	D-Mannitol	110.67
	Sebacic Acid	109.00
	D-Mannose	108.67
	B-Cyclodextrin	108.00
	D-Melibiose	106.67
	a-D-Lactose	106.33
	Maltitol	102.00
	i-Erythriol	101.33
	m-Inositol	100.00
	Lactulose	86.33
	Glucoronamide	59.33
	a-Cyclodextrin	58.33