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ORIGINAL ARTICLE

The effect of carbon and nitrogen sources on the formation of sclerotia in *Morchella* spp.

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Abstract Cultural assays were used to compare the effect of various carbon (C) and nitrogen (N) sources on in vitro sclerotial formation and development, specifically in mycelial cultures of black and yellow morels (*Morchella elata* and *Morchella crassipes*, respectively). While different C and N sources supported abundant mycelia growth, these nutritional parameters also influenced sclerotial formation. Carbon sources such as ribose, cellobiose, galactose, xylose, sucrose and mannitol produced many (18–125) large-sized (diameter 0.16–0.43 cm) and cream-colored sclerotia in *M. crassipes*; in *M. elata*, small-sized (diameter 0.16–0.28 cm) and brown-pigmented sclerotia were formed in media containing ribose, galactose, sorbose and mannitol. Among the nitrogen sources, sodium nitrate and yeast extract caused both morel species to produce significantly fewer sclerotia (6–24) of significantly smaller size (diameter 0.11–0.27 cm). Carbon sources such as mannitol and ribose and N sources, sodium nitrate and yeast extract produced numerous large-sized sclerotia in morels.

Keywords Morels · Sclerotia · *Morchella crassipes* · *Morchella elata* · Carbon and nitrogen sources · Nutritional parameters

Introduction

Morels are wild, edible ascomycetous mushrooms belonging to the family Helvellaceae. Fruiting bodies of morel fungi (*Morchella* spp.) are highly prized for their medicinal and nutritional qualities (Nitha et al. 2007). Black-fleshed fruiting bodies are produced by *M. angusticeps*, *M. elata* and *M. conica*, while *M. esculenta*, *M. crassipes* and *M. deliciosa* produce yellow or white mushrooms (Wipf et al. 1996, 1999). Morels have long been considered saprobes (Ower 1982), although some studies indicate that they might be facultatively mycorrhizal (Lakhanpal et al. 1991; Buscot 1993a; Harbin and Volk 1999). *Morchella* spp. mostly grow in soil that is rich in organic matter, usually in dense deciduous forest litter (Weber 1995). The epigeal fructifications (ascocarps) often appear in clusters during spring in cool temperate zones, when soil temperature warms to about 10°C.

Investigations have shown that there is a stage in the life cycle of morels called the sclerotium. Ower et al. (1986) showed the importance of sclerotia in the life cycle of *Morchella* spp., and their role in the production of fruiting bodies under controlled conditions. This important finding underlines the need to determine the optimal conditions for the formation, growth and maturation of sclerotia. Sclerotia are hard-surfaced resting bodies composed of fungal cells that act as a nutrient reservoir; they are resistant to unfavorable environmental conditions. Nutrients, mainly neutral lipids in the form of triglycerides, are stored in the sclerotia and during the sexual cycle; substantially, all the nutrients for fruiting body development are drawn from sclerotia (Ower et al. 1986). Sclerotia remain dormant for long periods of time and resume growth on the return of favorable environmental conditions (Volk and Leonard 1990). Two types of sclerotia have been found in morels: a relatively small ‘early encrusting sclerotium’ (EES) and a larger ‘late isolated sclerotium’ (LIS) (Buscot 1993a). LIS have temperature requirements, cold resistance and biochemical properties (quality and type of mycosporins) that

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parallel the subterranean mycelial structure connected with ascocarps in nature. Recently, Masaphy (2010) reported the successful initiation and development of fruiting bodies through different morphological developmental stages, such as sclerotium formation, scelerotium germination, asexual spore formation, formation of initial knots, and development of the fruiting body in *Morchella rufobrunnea* under soil-less conditions.

Carbon (C) sources, nitrogen (N) sources, and agro-wastes play an important role in the production of lignolytic enzymes in morels, and these in turn affect their behavior in cultivation (Kanwal and Reddy 2011). In the present investigation, variations in sclerotial formation by *M. crassipes* and *M. elata* were evaluated versus different C and N sources. For commercial cultivation of morels, large-sized and abundant sclerotia are needed that can act as spawn cultures for fruiting body formation. The present study deals with the selection of carbon and nitrogen sources to produce sclerotia that can be used for cultivation purposes.

Materials and methods

Strain and its maintenance

Fruiting bodies of *Morchella crassipes* (yellow morel) and *M. elata* (black morel) were collected from an area under bamboo plantations at Banuri, Palampur, Himachal Pradesh, India. These fungi were identified based on their morphological features, including shape, size and color of fruiting bodies and ascospores. Moreover, the fruiting bodies were designated to the species level by sequence analysis of the internal transcribed spacer (ITS) regions of the rRNA gene (Kanwal et al. 2010). The sequences have been submitted to the GenBank database of NCBI under the accession numbers GQ228466 (*M. crassipes*) and GQ228470 (*M. elata*). Pure cultures were isolated from internal parts of fresh ascocarps and maintained at 25°C on Potato Dextrose Agar medium in Petri dishes. The cultures have been deposited at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, under the accession numbers MTCC 10162 (*M. elata*) and MTCC 10166 (*M. crassipes*).

Media for production of sclerotia

Both *M. crassipes* and *M. elata* were cultured on modified Buscot media (Buscot 1993b) supplemented with glucose (10 g/L) and sodium nitrate (250 mg/L) along with 1.5% agar. All experiments were conducted in 300-mL glass culture jars (Kasablanka, Mumbai, India) containing 50 mL of medium. The jars were constricted with plastic enclosures. Mycelial discs (5 mm diam.) cut from the edge of actively-growing 6-day-old cultures were placed at the center of the jars so that the hyphae were in contact with the upper surface of the agar. The jars were then incubated at 25°C in the dark.

Effect of C and N sources on production of sclerotia

Sclerotial formation was evaluated on agar media containing different carbon sources (10 g/L): ribose, arabinose, xylose, mannose, galactose, fructose, sorbose, rhamnose, sucrose, cellobiose, soluble starch (amylase) and mannitol in place of dextrose. There were also different nitrogen sources: casein, peptone, yeast extract, tryptone, sodium nitrite ($NaNO_2$), ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3) in place of sodium nitrate ($NaNO_3$) (250 mg/L). Three replicates were maintained for each treatment.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were compared with Tukey's test using the GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA).

Results

Effect of different carbon and nitrogen sources on production of sclerotia

For *M. crassipes*, dense and circular growth of brown-colored mycelium was observed. Ribose, cellobiose, galactose, glucose, xylose, sucrose and mannitol served as the best C sources for sclerotia formation (Table 1; Fig. 1a, b). Rhamnose, mannose, fructose, soluble starch and sorbose did not promote sclerotial formation. Large-sized sclerotia were observed in the presence of mannitol. Younger sclerotia were light yellow in color and turned to brown with maturity. Sclerotia formed in xylose (mean size: 0.2± 0.075 cm) and sucrose (mean size: 0.16±0.073 cm) were much smaller in size compared to mannitol (mean size: 0.43±0.1 cm) and formed on the agar surface rather than the sides of glass jars. Sclerotial formation was not observed in most of the nitrogen sources tested except in sodium nitrate, ammonium nitrate, peptone and yeast extract, where less numerous and often smaller sclerotia were observed (Table 1).

In *M. elata* cultures, dense profusely branched, light brown mycelium was observed with all the C sources tested. Sclerotia were smaller in comparison to the sclerotia formed by *M. crassipes* (Table 1). Ribose, galactose, glucose, sorbose and mannitol promoted the formation of

Table 1 Average number and size of sclerotia formed by *Morchella crassipes* and *M. elata* under different carbon and nitrogen sources after 18 days

Nutritional sources	*M. crassipes*		*M. elata*	
	Number of sclerotia/jar	Average size of sclerotia (cm)	Number of sclerotia/jar	Average size of sclerotia (cm)
Carbon sources				
Ribose	125.0 a	0.33 b	105.0 a	0.28 a
Rhamnose	–	–	–	–
Cellobiose	73.0 b	0.30 b	–	–
Galactose	18.0 d	0.21 c	82.0 b	0.20 c
Mannose	–	–	–	–
Xylose	47.0 c	0.20 c	–	–
Fructose	–	–	–	–
Sucrose	19.0 d	0.16 d	–	–
Soluble starch	–	–	–	–
Glucose	23.0 d	0.24 c	97.0 a	0.23 b
Sorbose	–	–	20.0 c	0.16 d
Mannitol	74.0 b	0.43 a	65.0 a	0.23 b
Nitrogen sources				
Casein	–	–	–	–
Peptone	14.0 b	0.14 c	–	–
Yeast extract	24.0 a	0.26 a	5.4 b	0.11 b
Tryptone	–	–	–	–
Sodium nitrate	12.0 b	0.27 a	9.0 a	0.12 a
Sodium nitrite	–	–	–	–
Ammonium chloride	–	–	–	–
Ammonium nitrate	6.0 c	0.18 b	–	–

Means in the same column followed by a common letter are not significantly different ($P<0.05$) according to Tukey's multiple range test. Mean values are from three jars

– denotes no sclerotia

numerous small-sized sclerotia. The sclerotia were brown in color when young and turned to dark brown on maturity. Sclerotia formed on the agar surface. No sclerotia formed in media containing rhamnose, cellobiose, mannose, xylose, fructose, sucrose or soluble starch, although media containing these substances supported the vegetative growth of the mycelium. Among the different N sources, sclerotia formed only in $NaNO_3$ and yeast extract (Table 1). For the latter, small-sized, brown-pigmented, irregular sclerotia formed on the perimeter of the culture.

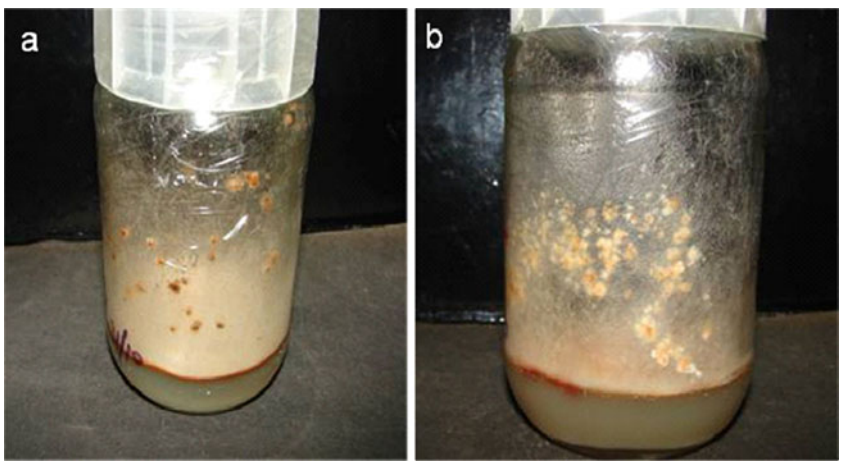

Fig. 1 Production of sclerotia by **a** *Morchella crassipes* with mannitol, and **b** *M. crassipes* in media with cellobiose as carbon source

Discussion

The substrate selected for the production of sclerotia should have an optimum carbon and nitrogen source that can yield a large number of sclerotia in the shortest time-span (Volk and Leonard 1989). In this study, both species produced dense vegetative growth in all the C sources. Carbon sources such as starch, maltose, fructose, glucose, glycogen and sucrose have been reported to be good carbon sources for all the *Morchella* spp., whereas ribose, sorbose, rhamnose, tartaric acid and malic acid are considered to be poor sources (Kaul 1978; Winder 2006).

Both *M. elata* and *M. crassipes* showed different patterns of sclerotial formation with different carbon sources. In many C sources, mycelia of *M. crassipes* produced large-sized sclerotia that were significantly more abundant versus the smaller sclerotia of *M. elata.* Sugars have been found to produce many sclerotia due to their influence on water potential (Amir et al. 1992, 1995). Previous reports indicated that C sources such as glucose, sucrose, maltose and starch are better sources for sclerotia formation in *Morchella* spp. (Volk and Leonard 1989; Guler and Ozkaya 2009). In the present study, mannitol produced large-sized sclerotia in both the cultures. Many sclerotia-forming fungi excrete water and soluble carbohydrates during the early stages of sclerotium development. This leads to a decrease in endogenous mannitol, causing a change in internal physiology that allows development of large-sized sclerotia (Cooke 1969; Winder 2006).

Among the different nitrogen sources, sodium nitrate and yeast extract served as good sources for sclerotial formation in both species. Casamino acids and yeast extract in combination with rye have been reported to enhance sclerotial formation in *M. crassipes* (Volk and Leonard 1989). Sclerotia formed in media were smaller in comparison to sclerotia formed in the combination of soil and agrowastes in this study.

In conclusion, considerable variations in morphology, location, size and pigmentation were observed for the production of sclerotia in different C and N nutritional sources. Carbon sources such as ribose, mannitol, and glucose, and N sources such as sodium nitrate served best for the formation of numerous large-sized sclerotia. Further, studies are required to delineate the optimal use of carbon and nitrogen sources to form a suitable medium for the production of large-sized and abundant sclerotia that can be used as spawn for fructification of morels under different environmental conditions.

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