Morchella SCLEROTIA PRODUCTION THROUGH GRAIN SUPPLEMENTATION

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SUMMARY

Edible fungi from the Morchella *genus are important at national and international level because of their high commercial value. Nevertheless, their artificial production still represents a challenge, even though patents for their cultivation do exist. A number of studies point out that obtaining sclerotia is a necessary part of the process for domestication of the genus and commercial production. This study consists of two experiments. In the first one, mycelial growth was assessed for five strains of* Morchella *using four different grains (maize, oats, wheat and rye), assuming that greater mycelial growth implies a more abundant production of sclerotia. For all the strains tested the highest response was obtained with rye and the most extensive growth was observed in CP508 (17.90cm^2). In the second experiment, the effect of rye supplementation on sclerotia production was evaluated using modifications to the jar method of Ower et al. (USPat. 4,594,809; June 1986). The treatment with rye supplemented with compost and gypsum (RCG) led to the best result, and the most productive strain in this experiment was CP506 (8.47g). Sclerotia were obtained between the third and fourth week following inoculation, in all treatments. Under the experimental conditions of this study, no barrier effect became evident and no effect of the nutrient-poor medium in sclerotia production was found. Therefore, it may be that the differentiation in the formation of sclerotia is due to factors such as the presence of nutritious elements and growth promoters contained in the compost.*

Introduction

The ascomycetes from the *Morchella* genus are edible mushrooms that are economically important and of high commercial value, both nationally and internationally. Although patents have been registered for commercial production (Ower, 1982; Ower *et al*., 1986), their 'production' is still dependent on manual picking in the field. Their production under controlled conditions still has difficulties, with low productivity and malformations in fruiting bodies (Molina *et al*., 1993; Barnes and Wilson, 1998; Stott and Mohammed, 2004).

Advances in the process of domestication of *Morchella* have promoted the view that sclerotia are resistant structures that permit the fungus to survive in adverse conditions and are essential for reproduction and the production of the fruiting body (Ower , 1982; Ower *et al*., 1986, 1988; Stott and Mohammed, 2004) under both natural and controlled conditions (Volk and Leonard, 1990; Pilz *et al*., 2007). The production of sclerotia has been established as a precursor for the domestication of this fungus (Stott and Mohammed, 2004).

The best known method for obtaining sclerotia was described by Ower *et al*. (1986), who patented the jar method. This method involves filling a jar half full of wheat grain as a substrate, covering it with a layer of perforated aluminum to act as a physical barrier, and then filling the remainder with a layer of soil divided into sections with nutritious and non-nutritious, or nutrient-poor, media. In 1989 and 1990 Volk and Leonard made adjustments to this method, obtaining sclerotia in an average time of four weeks. Similarly, Amir *et al*. (1993) obtained them in culture medium, and Buscot (1993) described two types of growth: lateral growth, produced by mycelial growth breaking through the glass walls of the Petri dishes, and terminal growth, produced in aging culture media. Formation in culture medium has also been documented (Philippoussis and Balis, 1995; Faris *et al*., 1996), as well as the role this plays in storing nutrients (Buscot, 1989; Buscot and Bernillon, 1991). All these studies point to the view that the mass production of sclerotia offers the possibility of it serving as 'seed' for domestication experiments (Singh *et al*., 1999).

Ower *et al*. (1986) and Buscot (1993) indicated that either a physical barrier, a non-nutrient (nutrient-poor medium) area or some adverse conditions are required, in such a way that the mycelium stops growing and transforms itself, to become a compact structure that will mature and transform into sclerotia. However, recent studies in

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OBTENCIÓN DE ESCLEROCIOS DE *Morchella* MEDIANTE LA SUPLEMENTACIÓN DE GRANOS

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RESUMEN

Los hongos comestibles del género Morchella *son importantes a nivel nacional e internacional por su alto valor comercial. Sin embargo, su producción artificial es aún un reto, a pesar de que existen patentes para su cultivo. Diversos estudios coinciden en que la obtención de esclerocios es uno de los aspectos necesarios a desarrollar para lograr su domesticación y producción comercial. Este trabajo consta de dos experimentos, en el primero se evaluó el crecimiento micelial de cinco cepas de* Morchella *empleando cuatro tipos de granos (maíz, avena, trigo y centeno), bajo el supuesto que a un mayor crecimiento micelial se tiene una mayor producción de esclerocios. Se encontró que el centeno tuvo mayor respuesta en todas las cepas y de éstas la más productiva fue la CP508 (17,90cm²). En el segundo experimento se evaluó el efecto de la suplementación del centeno para la producción de esclerocios, usando modificaciones al método del frasco de Ower et al. (USPat. 4.594.809; Junio 1986), observando que el tratamiento de centeno suplementado con composta y yeso (CCY) fue el mejor. La cepa más productiva fue la CP506 (8,47g), obteniendo esclerocios entre la tercera y cuarta semana de inoculación en todos los tratamientos. Bajo las condiciones del experimento no se manifestó una respuesta del efecto barrera y el medio pobre-rico en la producción de esclerocios, por lo que la diferenciación y formación de estas estructuras puede deberse a factores como elementos nutritivos y promotores del crecimiento contenidos en la composta.*

OBTENÇÃO DE ESCLERÓCIOS DE *Morchella* PELA SUPLEMENTAÇÃO DE GRÃOS

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RESUMO

Os cogumelos comestíveis do gênero Morchella *são importantes a nível nacional e internacional por seu alto valor comercial. No entanto, sua produção artificial é ainda um desafio, mesmo existindo patentes para seu cultivo. Diversos estudos coincidem em que a obtenção de esclerócios é um dos aspectos necessários a desenvolver para conseguir sua domesticação e produção comercial. Este trabalho consta de dois experimentos, no primeiro foi avaliado o crescimento micelial de cinco cepas de* Morchella *empregando quatro tipos de grãos (milho, aveia, trigo e centeio), considerando que, com maior crescimento micelial há uma maior produção de esclerócios. Encontrou-se que o centeio teve maior resposta em todas as cepas e destas a mais produtiva foi a CP508 (17,90cm²). No segundo experimento foi avaliado o efeito da suplementação do centeio para a produção de esclerócios, usando modificações ao método do frasco de Ower et al. (USPat. 4.594.809; Junho 1986), observando que o tratamento de centeio suplementado com compostagem e gesso (CCY) foi o melhor. A cepa mais produtiva foi a CP506 (8,47g), obtendo esclerócios entre a terceira e quarta semana de inoculação em todos os tratamentos. Sob as condições do experimento não se manifestou uma resposta do efeito barreira e o meio pobre-rico na produção de esclerócios, portanto a diferenciação e formação destas estruturas podem dever-se a fatores como elementos nutritivos e promotores do crescimento contidos na compostagem.*

various culture media indicate that the barrier effect and a nutrient-poor medium do not necessarily promote the transformation of mycelia, and that this happens possibly because of nutrients or other substances in the substrate where it grows (Alvarado *et al*., 2008).

To sum up, the production of *Morchella* depends on the formation of sclerotia. However, obtaining these structures continues to present scientific and technological problems that must be overcome if it is to be domesticated and intensively produced under controlled conditions (Stott and Mohammed, 2004). The aim of the present study is to evaluate the most suitable grain for the mycelial growth of *Morchella* and to determine the effect of supplementing the type of grain used for the production of sclerotia, modifying the jar method proposed by Ower *et al*. (1986). Two experiments were conducted. The first one intended to evaluate mycelial growth of several strains employing different grains, assuming that alternative grains to wheat, the conventional grain used, exist and lead to better responses in terms of sclerotia production, and that a greater mycelia production causes more abundant sclerotia formation. Thus, the most suitable grain for sclerotia production can be determined. In the second experiment grain supplementation was evaluated by modifying the method devised by Ower *et al*. (1986), assuming that the addition of nutrients to the grain will have positive effects in terms of the production of sclerotia and that adverse conditions are not necessary for obtaining these structures.

Materials and Methods

Biological material

The following strains of *Morchella* were employed: CP509, CP507, CP508, CP499, and CP506. The first strain (*M. esculenta*) was imported from the USA and the remaining four (*M. conica*) were collected from a pine forest in the State of Mexico (100°01'46.30''N, 19°18'08.73''W). These strains were identified by gene amplification analysis of the ITS (*Internal Transcript Spacer*) region of ribosomal DNA, and are safeguarded in the strain bank of the Colegio de Postgraduados, Campus Puebla, Mexico.

Culture medium

These strains were sown in culture medium made with compost extract obtained from mushroom compost from the Riojal production plant (Las Vigas, Veracruz, Mexico). For medium preparation, 1.2kg of compost was placed in 4 liters of distilled water and the mixture heated over a weak flame for 15min, stirring constantly. This compost was then combined in the following proportions: 800ml of mixture, 20g of malt (Bioxon[R]) and 20g of agar (Bioxon[R]), completing with distilled water up to 1 liter. The culture medium was sterilized in a conventional way; inoculated with each strain and incubated in darkness at 26°C. Sclerotia appeared *in vitro* within a period of three weeks.

TABLE I
ASSESSMENT OF TREATMENTS FOR PRODUCTION OF *Morchella* SCLEROTIA

Treatment	Characteristics
RHMC	90g rye + 10g horse manure + 1g micronutrients + 10g compost + 0.2g $CaCO_3$
RSMA	80g rye + 10g sand + 1g micronutrients + 4.5g ash
RCG	100g rye + 10g compost + 1g gypsum
RC	100g rye

Treatments and experimental design

A first experiment consisted in assessing the mycelial growth for five *Morchella* strains (CP509, CP507, CP508, CP499, and CP506) employing four types of grain (maize, oats, wheat, and rye) to determine the most appropriate one for sclerotia production. A completely randomized experimental design was used with 10 replicates per treatment. Wheat represented the control, as this is the conventional grain used for the production of *Morchella* sclerotia. The different grains were soaked until 60% humidity was reached and subsequently sterilized for 30min at 120°C under 15lb of pressure. Ten grams of grain were put in each Petri dish with a circular implant of 0.5mm diameter for each strain, incubating at 27°C in darkness for 9 days. Mycelial growth (cm^2) was determined by tracing the outline of the surface growth every third day, for each replicate; this was done based on the 3.12 Arc View GIS program.

A second experiment consisted in evaluating the effect of the grain supplementation on sclerotia production, using modifications to the jar method devised by Ower *et al.* (1986). This consisted in adding to the grain that in the first experiment showed the best results when compared to wheat, which is the grain usually employed for this process, different elements that theoretically promote *Morchella* growth, such as manure (Volk and Leonard, 1990), compost (Stamets, 1993; Faris *et al.*, 1996; Alvarado *et al.*, 2008), ash (Pilz *et al.*, 2007) and micronutrients (Alvarado *et al.*, 2008). The different treatments (Table I) were carried out with and without a physical barrier (perforated aluminum sheet), in order to determine the influence of the barrier effect on sclerotia formation. In this case, the modification consisted in using polypropylene bags, which were placed in transparent plastic containers of 250ml, so that they took on a cylindrical shape (instead of jars), where a layer of 100g of soil (pH 6.5 and 21% humidity) was added, sterilized for 30min under conventional conditions, without any division differentiating the nutrient-poor medium (Figure 1). The control (RC) was rye grain alone, without any supplement. The treatments were established in a completely randomized experimental design with 10 replicates and incubated in darkness at 26°C during four weeks.

The recorded variable consisted of the fresh weight of sclerotia and these were harvested one by one, removing soil residues with a soft, pig bristle brush. Biological efficiency (BE) was calculated for each treatment, dividing the fresh weight of sclerotia for each sample by the dry weight of substrate (34.5g) and multiplying by 100 (Chang and Miles, 1989; Volk and Leonard, 1989). This fieldwork method is commonly used in the edible mushroom industry.

Figure 1. Production of *Morchella* sclerotia by modification of the jar method of Ower *et al.* (1986). a: physical barrier (sheet of perforated aluminum) separating the grain from the layer of ground; b: sclerotia growing through the layer of ground, obtained after the third week of incubation; c: sclerotia 28 days after inoculation

Statistical analyses consisted of variance analyses and multiple comparison of means, using Tukey test ($p<0.05$), employing the STATISTICA® software (2000). Both experiments were analyzed by applying variance analysis in a factorial arrangement, especially designed to determine the response of each variable, as well as their interactions.

Results

Mycelial growth

In the first experiment, mycelial growth of the different strains of *Morchella* for the different types of grain showed significant differences (Tukey $p<0.05$) among treatments, with rye (28.6cm^2) manifesting the most prolific growth of any of the strains analyzed, followed by oats (15.2cm^2), wheat (6.5cm^2) and maize (4.5cm^2). Therefore, rye grain was chosen for assessment in the second experiment (evaluation of grain supplementation). The strain with the highest response among treatments was CP508 (17.90cm^2), as compared to other strains tested: CP507 (16.44cm^2), CP499 (14.95), CP506 (10.5) and CP509 (8.45 cm^2).

In terms of the interaction between grains and strains, different statistical groups were observed. The most prolific growth in any treatment occurred with rye (strains CP499; CP507; CP506 and CP508) while maize produced the poorest results. The strain showing the most prolific growth was CP508, in the case of all grain types. Although the remaining strains manifested different amounts of growth, this varied according to the type of grain. However, strains CP509, CP499, and CP506 manifested a less prolific growth (Figure 2).

Sclerotia production

In the second experiment significant differences were found in terms of the effect of grain supplementation comparing treatments, barrier effect and strains. The treatment with the highest value was RCG (10.93g) compared to RSMA (5.97), RHMC (4.55) and RC (3.06g). The treatments without a barrier (7.91g) manifested more prolific sclerotia production than those with a barrier (5.35g), and the best strain for the production of these structures was CP506 (8.47g), followed by a second, statistically similar group which included the strains CP507 (5.82), CP499 (7.21), and CP508 (8.30g), with strain CP509 (4.90g) presenting the lowest yield.

Interaction between treatments and barrier effect showed significant differences, with RCG being the best, both without (13.05g) as well as with a barrier (8.85g); whereas contrarily, RC was the treatment

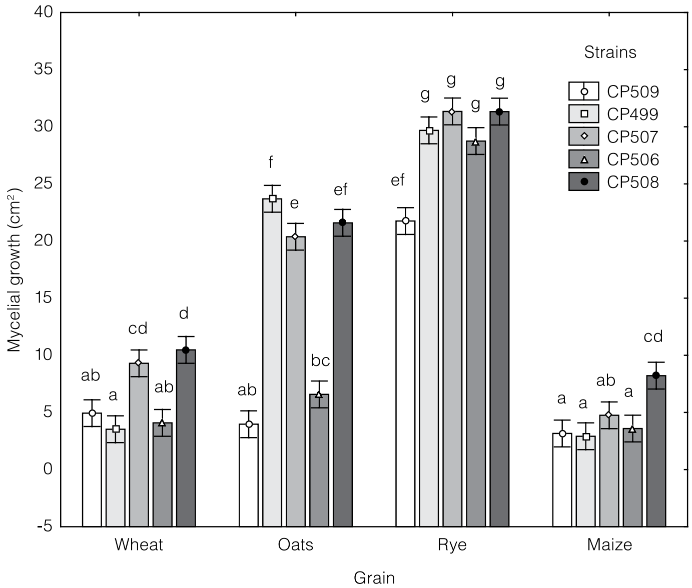

Figure 2. Mycelial growth (cm^2) of *Morchella* in different types of grain for five strains studied. Treatments with a different letter are statistically different (Tukey p<0.05).

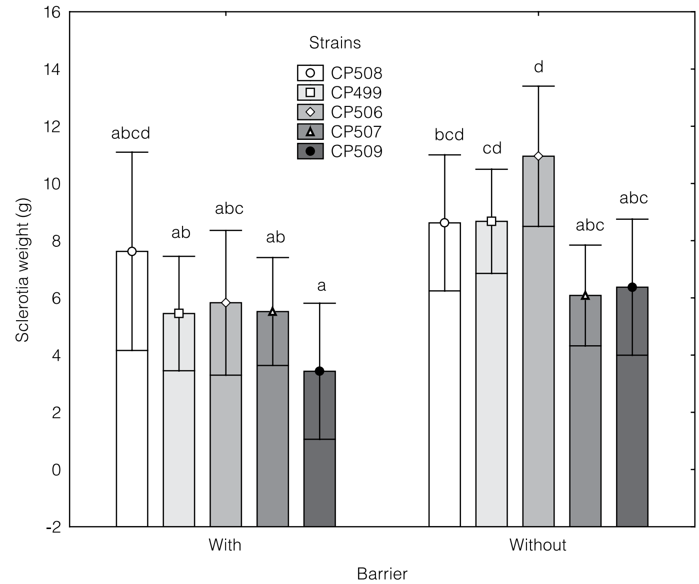

Figure 4. Production of sclerotia (g) of *Morchella* in different strains with and without a physical barrier. Treatments with a different letter are statistically different (Tukey, p<0.05).

with the lowest sclerotia production (Figure 3).

Interaction between strains and barrier effect showed statistical differences (Tukey, p<0.05) and statistically similar treatments were apparent, with a greater response being evident for all the strains in treatments without a barrier (Figure 4); with CP506 (10.95g), without a barrier, representing the one with highest sclerotia production.

It was apparent that sclerotia production is related to the interaction between treatments and strains (Figure 5); for example, treatments RHMC and RSMA did not produce sclerotia in the case of strains CP506 and CP509. It was shown that RCG (100g rye + 10g compost + 1g gypsum) represented the best treatment and that it manifested the highest sclerotia production out of any of the strains.

Biological efficiency (BE)

It was found that RCG (31.70%) is the treatment with the greatest BE, followed by that of RSMA (17.32%), RHMC (13.19%) and RC (8.88%). As for the barrier effect, BE was highest in the treatments without a barrier (22.93%), compared to those with a barrier (15.50%). Regarding the strains studied, there were statistically different groups; the one with the highest BE was made up of CP506 (24.55%), CP508 (24.07%) and CP499 (20.92%) strains, and the one with the lowest BE included CP507 (16.87%) and CP509 (14.21%). Finally, interactions (Table II) demonstrated that the RCG treatment with or without a barrier manifested the greatest efficiency for producing sclerotia with the greatest size and weight (Figure 6). Treatment RC, however, presented the lowest results. In terms of the interaction between strains and barrier, it was found that the most efficient was the CP506 without a barrier, and the least efficient was CP509 with a barrier.

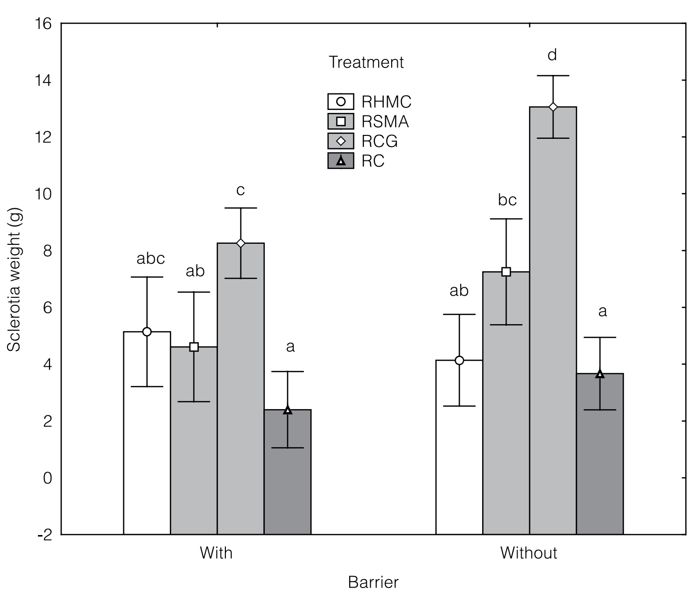

Figure 3. Sclerotia production (g) of *Morchella* in different treatments with and without a physical barrier. Treatments with a different letter are statistically different (Tukey, p<0.05).

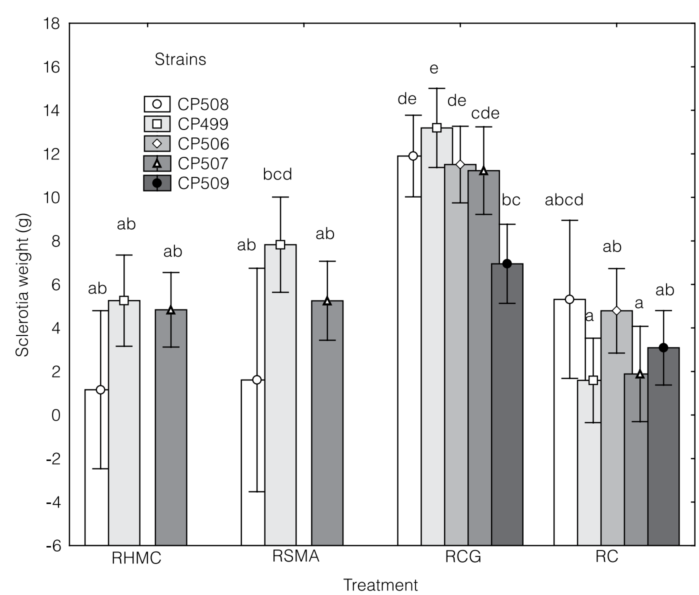

Figure 5. Production of sclerotia (g) of *Morchella* with different treatments and different strains. Treatments with a different letter are statistically different (Tukey, p<0.05).

TABLE II
PRODUCTION OF *Morchella* SCLEROTIA

Treatment	Barrier	Average fresh weight (g)*	Biological efficiency (%)
RCG	Without	13.06	37.8 a
	With	8.26	23.9 b
RSMA	Without	7.25	21.0 bc
	With	4.61	13.4 cd
RHMC	Without	4.14	12.0 cd
	With	5.14	14.9 bcd
RC	Without	3.67	10.6 d
	With	2.40	7.0 d
Strain			
CP508	Without	8.62	25.3 abc
	With	7.62	22.4 abcd
CP499	Without	8.67	25.5 ab
	With	5.45	16.0 cd
CP506	Without	10.95	32.2 a
	With	5.83	17.1 bcd
CP507	Without	6.08	17.9 bcd
	With	5.52	16.2 cd
CP509	Without	6.37	18.7 bcd
	With	3.43	10.1 d

*Each quantity is the average of 10 replicates. Treatments marked with a different letter are statistically different (Tukey, $p<0.05$).

Discussion

The importance of sclerotia in the life cycle of *Morchella* is discussed herein, as these structures represent an essential stage for subsequent fruiting (Volk and Leonard, 1990). Under natural conditions they allow the fungi to survive in adverse conditions, which may include limited nutrients, poor humidity, extreme temperatures (Volk and Leonard, 1990), intense rainfall, prolonged winters (Ower, 1982) as well as floods and snow (Stamets, 1993). Besides, they facilitate the relationship with secondary tree roots and grasses, in response to natural disasters, such as fires (Pilz *et al*., 2007).

The production of sclerotia has been successfully used for obtaining fruit bodies under controlled conditions of cultivation (Ower, 1982; Volk and Leonard, 1990; Pilz *et al*., 2007), so that a number of studies agree that it is necessary to obtain sclerotia in order to subsequently achieve its domestication and commercial production (Ower, 1982; Volk and Leonard, 1990; Stott and Mohammed, 2004).

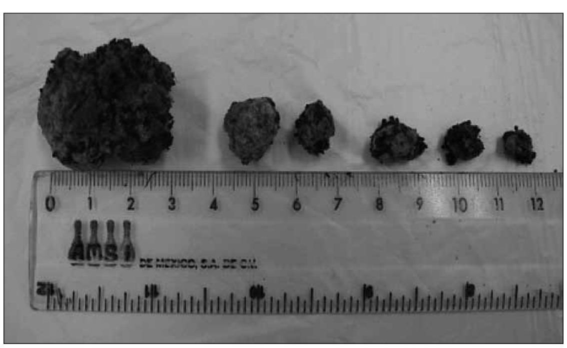

Figure 6. *Morchella* sclerotia obtained 28 days after inoculation

In spite of the scientific and commercial applications requiring precise knowledge of the life cycle of *Morchella*, only a general description has been forthcoming (Volk and Leonard, 1990), defining no differences between species and not distinguishing between natural or artificial conditions. Besides this, confusion exists when referring to the various taxonomic groups of this mushroom (Pilz *et al*., 2007; Masaphy, 2010), as well as concerning its ecological interactions like mycorrhizal, saprophytes or facultatives (Dahlstrom *et al*., 2000),

A number of different grains have been employed for *Morchella* sclerotia production. However, the most adequate has not yet been clearly determined, in spite of being an important factor in sclerotia production. It should be practical, available, cheap, and effective, for it to be employed on a widespread industrial scale (Volk and Leonard, 1989). The grain most often employed until now has been wheat (Ower *et al*., 1986; Singh *et al*., 1999), although experiments with maize and oats have been carried out (Papinutti and Lechner, 2008) without outstanding results. In the present study, rye was found to be the best option, concurring with results from Volk and Leonard (1989) and also fulfilling the aforementioned requirements. Besides it conserves its structure, does not cause contamination and enables the rapid growth of all the strains being studied.

As for mycelial growth, strain CP508 showed the most prolific development (17.90cm^2) and was therefore expected to manifest the greatest production of sclerotia, although in reality this turned out to be one of the least productive strains (8.30g). Likewise, the strain which produced the greatest quantity of sclerotia (CP506) performed poorly in terms of mycelial growth. This suggests that mycelial development is not necessarily related to sclerotia production, and that each of them depend on different conditions and interactions.

Regarding the modifications made to the jar method of Ower *et al*. (1986), it was found that the use of polyethylene bags is a feasible option for the production of sclerotia, as suggested by Singh *et al*. (1999). The use of this type of container proved easier and its price is more affordable. The material had no negative effect on the production of the strains in this study, in contrast to that observed by Volk and Leonard (1989) who indicated that sclerotia did not develop in polyethylene bags whereas it did in the glass jars. These authors suggest that the walls of the jars contribute to sclerotia formation; a not very likely option, as inert materials such as glass could not possibly have any effect on sclerotia production.

When soil was handled as one single homogenous layer and not as a nutrient-poor substrate (Ower *et al***.**, 1986, 1988) this facilitated its handling in the laboratory, making it clear that its division into media which are either nutrient poor or rich divided into different layers is not necessary for the formation and production of sclerotia.

As for sclerotia production, the results indicate that the grain supplemented with different nutritious elements contributes to the formation of all the strains studied, concurring with studies by Volk and Leonard (1989), who supplemented with different elements, such as peptone, malt extract and trace elements. It is considered that under the conditions of the present experiment good results were obtained with the RCG treatment, whose main component is compost, a cheap and easily accessible product. Besides possibly containing nutritious elements and certain growth promoters that contribute to induce sclerotia formation, a barrier effect or exogenous adverse conditions are not required (Alvarado *et al.,* 2008). These characteristics may promote its use on a larger scale, given its easy availability compared with the pure nutrients that are generally employed in *in vitro* culture media.

Treatments without barrier manifested the most prolific sclerotia production for the different combinations, as well as for all the strains studied. This result contrasts with previous research, which led to conclude that the physical barrier and nutrient-poor medium are necessary for inducing sclerotia formation (Ower *et al*., 1986; Volk and Leonard, 1989, 1990; Buscot, 1993).

Related to this, Buscot (1993) indicates that sclerotia production occurs in a culture medium where mycelial growth physically breaks through the wall of the Petri dish, or through the glass walls of a jar (Volk and Leonard, 1989, 1990). A similar effect may have occurred in the layer between the supplemented grain and the soil, causing the transformation of mycelia to become sclerotia. However, this did not necessarily constitute an adverse condition, and it could equally have been due to other factors, such as nutrients or other compounds contained in the supplemented grain.

In other species, such as *Sclerotia roofsii* Sacc**,** sclerotia can also be artificially induced when oxidative stress occurs as a result of the addition of hydrogen peroxide to the culture medium (Hadar *et al*., 1981**)** or by applying high temperatures (Georgiou *et al*., 2006), by mechanical damage to the mycelium (Carrol, 1991; Hadar *et al*., 1981), or by nutritional factors which cause cellular aggregation in the mycelium and a subsequent formation of sclerotia. Each one of these agents or a combination of them may go some way towards explaining the biogenesis of sclerotia (Georgiou *et al*., 2006). Thus, strains of *Morchella* may produce sclerotia as a response to a particular adverse condition.

It should be emphasized that the conditions described here are not the only ones which can cause the formation of sclerotia, as in nature they may appear as a result of particular stimulus, for example fires, soil disruption (Vogel, 1988; Stamets, 1993), intensive rainfall or prolonged winters (Ower, 1982). Likewise, the production of sclerotia is related to the capacity of fungi to reproduce in adverse natural conditions, which has been observed as much in the case of Ascomycetes as in Basidiomycetes (Georgiou *et al*., 2006).

This suggests that an adverse condition in the form of a barrier is not necessarily a precursor for sclerotia formation, as sclerotia were obtained in the case of all five strains being studied. Also, the modifications proposed and described here may help produce sclerotia at a low operational cost, as the emergence and growth of sclerotia occurred three to four weeks after inoculation, concurring with reports from Volk and Leonard (1989). This time lapse is much shorter than that reported in other studies, where up to 70 days were required for sclerotia formation (Singh *et al.,* 1999). Finally, the results concerning biological efficiency agree with those obtained in other studies of sclerotia production, and the highest BE was observed in treatments with no barrier. Also, *M. conica* manifested greater sclerotia production than *M. esculenta.*

Changes in the methods for obtaining sclerotia offer the possibility of defining the ideal conditions for domesticating *Morchella,* either under controlled conditions or by introducing them into the natural habitat. The latter may constitute either a conservation strategy or offer a means for repopulating natural environments. Future research should provide the needed knowledge for this technological development, proposing economic and efficient methods for *Morchella* production, similar to those which have been introduced for the production of other species of edible mushrooms.

Further research is necessary in order to clarify doubts concerning the morphogenesis of sclerotia and their role in the life cycle of various species of *Morchella*. In order to understand the dynamics of reproduction in these fungi, it is necessary to understand their adaptations, life cycle, mode of nutrition and reproductive strategies. In the future these aspects may offer other potential lines of research to discover practical methods for producing these fungi on a large commercial scale.

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