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Full Length Research Paper

The effect of cultivation conditions on the mycelial growth of a dark-septate endophytic isolate

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The EF-37 isolate, one of DSE fungi, is beneficial to the growth and development of its host plant, *Saussurea involucrata* Kar. et Kir. The cultivation requirements including basic culture medium, temperature, light, pH, carbon source and nitrogen compounds were studied for their effects on mycelial growth of a dark-septate endophytic (DSE) fungus EF-37 by using one-factor-at-a-time method. Potato dextrose agar (PDA) was the best medium for the growth of endophyte EF-37. Our studies showed that 20°C, 24 h dark cultivation and pH 7 significantly influenced the growth of endophyte EF-37 on PDA medium. Moreover, glucose and calcium nitrate were found to be the best nutrients for EF-37 growth. Under the optimal cultivation conditions, DSE fungus EF-37 isolate could grow actively. This is the first study about the effect of cultivation conditions on the growth of this strain, which provides the preparatory knowledge for the biological characteristics of DSE fungus EF-37.

Key words: Cultural conditions, dark-septate endophytic (DSE) fungus EF-37, mycelial growth, optimization, *Saussurea involucrata* Kar. et Kir.

INTRODUCTION

Dark-septate endophytes (DSE) comprise a miscellaneous group of root-inhabiting fungi (Jumpponen, 2001). DSE fungi are distributed all over the world and infect all kinds of plants, especially under harsh climatic conditions such as alpine environments (Jumpponen and Trappe, 1998; Addy et al., 2005). DSE fungi have been found to have a mutualistic, mycorrhiza-like relationship with their host plants (Jumpponen and Trappe, 1998). Despite many studies around DSE have been worked out, very little is known of their biological characteristics. *Saussurea involucrata* Kar. et Kir. which grows in the alpine zone of 2,600 m or higher, is an endangered medicinal plant and is listed as a second-grade national protected wild plant in China (Fu, 1992). In our previous work, we reported that two DSE fungi, EF-M and EF-37 were isolated from the root of *S. involucrata.* And the two DSE fungi, especially EF-37 had the promoting role on its host plant, *S. involucrata* (Wu and Guo, 2008; Wu et al., 2009a). Therefore, we chose EF-37 for more study. In order to further investigate the interaction between DSE fungus EF-37 and *S. involucrata* as well as exploiting in larger ecological or physiological studies with the fungus in the future, it is necessary to find out the biological characteristics of EF-37. The objective of this paper was to quantify the effect of basic culture medium, temperature, light, pH, carbon source and nitrogen compounds on the mycelial growth of DSE fungus EF-37.

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MATERIALS AND METHODS

Microorganism

DSE fungus EF-37 was isolated from the roots of *S. involucrata* Kar. et Kir. and deposited at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. For long-term storage, agar plugs of the strain were kept in 10% glycerol at -80°C. For short-term storage, the strain was generally maintained on PDA at 4°C in the dark. After we had researched the interaction studies between DSE fungus EF-37 and *S. involucrata*, we isolated the strain from the culture bottles where it was interacted with *S. involucrata.* The activation, colony morphology and growth of the EF-37 isolate were studied on PDA. The molecular identification of DSE fungus EF-37 was similar to the previous work (Wu et al., 2009a).

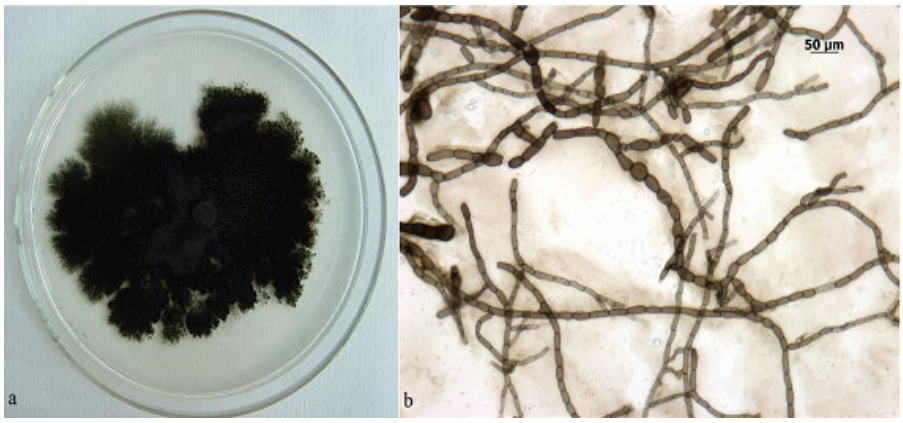

Figure 1. The morphological characteristics of DSE fungus EF-37. a, the colonies of EF-37 on PDA with calcium nitrate; b, Microscopic characteristics of EF-37 in light micrograph (×400).

Experimental set-up

The plates used in the experiments were 90 mm Petri dishes containing 20 ml agar medium. In the middle of the plates, there were inoculated with 7 mm agar plugs cut with a sterile cork borer from the edge of actively growing EF-37 isolate colony on PDA.

Culture medium experiments

The cultural medium for tests were respectively, PDA medium, cornmeal agar (CA) medium, Sabouraud's medium, oatmeal agar (OA) medium, wheat bran agar (WBA) medium and Czapek's medium (Fang, 1998). All culture plates were incubated in a growth chamber at 25℃ in the dark.

Light experiments

The effect of light on the mycelial growth of the EF-37 isolate was studied by growing the fungal cultures on PDA under different photoperiods of 24 h light, 12 h light/12 h dark and 24 h dark. All plates were incubated at 25℃.

Temperature experiments

The effect of temperature on the mycelial growth of the EF-37 isolate was studied by growing the fungal cultures on PDA at 5, 10, 15, 20, 25 and 30℃. All plates were incubated in the dark.

pH experiments

The effect of pH on the mycelial growth of the EF-37 isolate was studied by growing the fungal cultures on PDA with a pH range of 3, 4, 5, 6, 7, 8, 9 and 10 adjusted by 1 mol·L^{-1} hydrochloric acid or 1 mol·L^{-1} sodium hydroxide solution. All plates were carried out in a growth chamber at 20℃ in the dark.

Carbon source experiments

PA (containing only potato and agar) was used as the basic medium. It contained 2% of one of the following carbon sources: D(+)-glucose, D(-)-fructose, D(+)-sucrose, D(+)-lactose, maltose, starch, D-mannitol, glycerin, phaseomannite and D-sorbitol. A medium free of any carbon source served as a control (Satchuthananthavale and Cooke, 1967). All solid cultures inoculated EF-37 isolate were placed in a growth chamber at 20℃ in the dark.

Nitrogen source experiments

PDA was used as the basic medium and contained respectively, 0.4% of the following nitrogen compounds: ammonium sulphate, ammonium nitrate, calcium nitrate, sodium nitrate, potassium nitrate, L-phenylalanine, L-methionine (Met), L-arginine (Arg), beef extract and peptone. The medium lacking nitrogen served as control. All solid cultures were inoculated with the EF-37 isolate and placed in a growth chamber at 20℃ in the dark.

Statistical analysis

All experiments were carried out in a complete randomized block design and each treatment consisted of 10 culture plates. The mycelial growth of EF-37 isolate was observed after seven days. The diameter of the colonies was measured with a caliper gauge along two diameters at right angles to one another and the average diameter for each plate was calculated. All of the experiments were repeated three times and data were statistically analyzed using one-way ANOVA. Differences among treatments were determined using Duncan's multiple range tests at a significant level of $p=0.05$. Data are presented as means ± standard errors (SE).

RESULTS

Microorganism

The strain with swollen, dark and septated hyphae produced no conidia or other reproductive structures (Figure 1) and the molecular analysis of the ITS nuclear rDNA region showed that it closely resembles DSE

Table 1. The effect of different cultivation conditions on the mycelial growth of the EF-37.

Culture medium	The colony diameter (cm)	Light	The colony diameter (cm)	Temperature (℃)	The colony diameter (cm)	pH	The colony diameter (cm)
PDA	3.06±0.11a	24 h light	1.94±0.11c	5	0.96±0.08d	3	1.03±0.05e
CA	2.94±0.10a	12 h light/12 h dark	2.76±0.12b	10	2.32±0.08c	4	1.65±0.08d
Sabouraud's	0.70±0.02d	24 h dark	3.15±0.15a	15	2.76±0.10b	5	2.78±0.18b
OA	2.14±0.12b			20	3.18±0.09a	6	3.05±0.05ab
WBA	1.62±0.13c			25	3.06±0.10ab	7	3.27±0.14a
Czapek's	1.76±0.11c			30	2.80±0.11b	8	2.81±0.12b
						9	2.76±0.13b
						10	2.51±0.08c

Values are presented as the mean ± SE.
Values designated with different letters are significantly different at P<0.05.

fungus EF-37 (100% similarity to the GenBank sequence from DSE fungus EF-37 FJ843591). Therefore, we could confirm that the strain we isolated again was DSE fungus EF-37.

Culture medium experiments

The growth experiments in six different culture media showed that PDA was the most suitable medium for the EF-37 isolate. After seven days of culture, the colony diameter of EF-37 was the following: PDA medium 3.06 ± 0.11 cm > CA 2.94 ± 0.10 cm > OA 2.14 ± 0.12 cm > Czapek's medium 1.76 ± 0.11 cm > WBA 1.62 ± 0.13 cm, but there was almost no mycelial growth on Sabouraud's medium (Table 1). Therefore, PDA was selected as the culture medium in the following experiments.

Light experiments

After seven days of culture, darkness was found to be the best condition for the growth of the EF-37 isolate. The colony diameter of EF-37 under 24 h dark condition was 3.15 ± 0.15 cm which was 1.14 times that of the colonies grown in 12 h light/12 h dark condition, and 1.62 times that of the colonies grown in 24 h light condition (Table 1). Therefore, the EF-37 isolate was cultured under dark condition in the following experiments.

Temperature experiments

After seven days of culture, the EF-37 isolate was found to grow in a large temperature range from 5 to 30℃, but it grew more rapidly and actively at 20℃ temperature (Table 1). Therefore, 20℃ was selected as growth temperature in the following experiments.

pH experiments

After seven days of culture, the EF-37 isolate was observed to adapt strongly to the range of pH, but it grew better at pH 5 to 8 (Table 1). The colonies diameter of EF-37 was the largest at pH 7, achieving 3.23 cm.

Carbon source

After seven days of culture, significant effects of carbon source on the growth of the EF-37 were observed. Glucose and lactose were the most suitable carbon source for the growth of the EF-37, followed by D-mannitol, D-sorbitol and maltose (Table 2). The EF-37 grew slowly in the medium containing D(-)-fructose, glycerin and phaseomannite as carbon source. From the practical point of view, glucose was chosen as the carbon source in the following tests.

Nitrogen source

After seven days of culture, many nitrogen sources were found to be suitable for the growth of the EF-37, but calcium nitrate was better than any other nitrogen source. The colony diameter on the PDA with calcium nitrate as the nitrogen source was 5.72 cm, 1.97 times that of the control lacking any nitrogen source. It was followed by peptone and L-phenylalanine (Table 2). Ammonium sulphate, ammonium nitrate, sodium nitrate, Met and Arg were not suited for the growth of the EF-37 because the colony diameter of EF-37 with these compounds was smaller than that of the control. Considering the low cost, easy availability and use, calcium nitrate was used as the nitrogen source in culturing the EF-37.

From the above results, the optimal culture conditions of DSE fungus EF-37 were PDA with calcium nitrate as

Table 2. Effects of carbon and nitrogen sources on the mycelial growth of the EF-37.

Carbon source	The colony diameter (cm)	Nitrogen source	The colony diameter (cm)
Control (culture medium lacking carbon source)	0.78±0.08f	Control (PDA containing no nitrogen source)	2.90±0.15de
Glucose	3.56±0.21a	Ammonium sulphate	1.76±0.23g
Fructose	0.76±0.09f	Ammonium nitrate	2.40±0.18ef
Sucrose	0.98±0.13e	Calcium nitrate	5.72±0.25a
Lactose	3.56±0.21a	Sodium nitrate	2.68±0.08e
Maltose	2.56±0.09c	Potassium nitrate	3.28±0.25d
Starch	2.30±0.12d	L-phenylalanine	4.12±0.27c
D-mannitol	2.76±0.18b	Met	1.98±0.14f
Glycerin	0.74±0.06f	Arg	1.34±0.11g
Phaseomannite	0.7±0.010f	Beef extract	2.94±0.19de
D-sorbitol	2.64±0.11bc	Peptone	4.84±0.32b

Values are presented as the mean ± SE.
Values designated with different letters are significantly different at $P<0.05$.

the nitrogen source, in a growth chamber at 20°C, at pH 7 and in the dark. And in the optimal culture conditions, the colony diameter of DSE fungus EF-37 was 1.97 times that on the PDA.

DISCUSSION

It was reported that DSE were mycorrhiza (Treu et al., 1996; Jumpponen, 2001) and had positive effects on the host growth (Shivanna et al., 1994; Fernando and Currah, 1996; Jumpponen and Trappe, 1998; Wu and Guo, 2008; Wu et al., 2009). The DSE fungus EF-37 isolate had promoting effects on the growth and active components of *S. involucrata* (Wu et al., 2010), and could therefore play a positive role in the artificial cultivation and sustainable utilization of *S. involucrata*. Considering all the above, we have studied some basic biological characteristics of this DSE fungus. This is the first report on the environmental and nutritional requirements for the mycelial growth of this species.

In the cultivation of fungi, different culture media such as PDA medium, WBA medium, CA medium, OA medium and others have often been used for mycelial growth and spores production of fungi. In the research here described, among six culture media tested, PDA was the best for the mycelial growth of the EF-37 isolate. That was similar to the work of Winder (1999) on *Fusarium avenaceum* who reported that PDA rich in starch and glucose was a much better growth substrate for this fungus.

Although it was suggested that light effects on fungal growth should be interpreted cautiously (Griffin, 1994), it has been shown that light influences the growth rate of fungi (Tan, 1978). In our present work, light had a significant effect on mycelial growth and the 24 h dark treatment was optimum, in agreement with the work of Weitz et al. (2001) on *Omphalotus olearius* and *Panellus stipticus*. Townsend (1954) also found that rhizomorphs of *Armillaria mellea* grew faster at 25°C in the dark than in the light. These observations are in contradiction with those reported by Berliner (1961) which indicated that *A. mellea* and *P. stipticus* were not affected under light experiment. Weitz et al. (2001) believed that the different effects of the light on the fungi could be due to different light intensities and quality used. Another reason may be that the EF-37 was isolated from the roots of *S. involucrata* and was acclimated to living in the dark conditions.

Temperature affects the growth of all organisms because it controls the rates of metabolic reactions. The temperature range at which most organisms function is between 0 and 40°C, the lower limit being associated with the transition phase at freezing and the upper limit being associated with the increasing influence of catabolism (Gillooly et al., 2001). Some authors demonstrated that *Penicillium digitatum* and *Penicillium italicum* could germinate and grow at temperatures ranging from 4 to 30°C; they detected no growth or germination of either species at 37°C (Lacey, 1989; Staddon, 2002; Plaza et al., 2003). Other studies have also found an optimum temperature for growth of basidiomycetes (wood-rotting) between 20 and 30°C (Cartwright and Findlay, 1934; Boddy, 1983). The temperature optima for mycelial growth of the EF-37 that were found in this study had almost the same changes as those described in previous studies.

Townsend (1954) found that the pH range over which rhizomorphs developed was 3.6 - 8.0, with an optimum at pH 5.6. Weitz et al. (2001) reported that the optimum pH for growth was pH 3.5 for *A. mellea* and *O. olearius*, pH 3.5 - 4 for *P. stipticus* and pH 4 for *Mycena citricolor* and they had a limited pH range. In our studies, the EF-37 isolate grew in large pH range, which could make it adapt

easily to the surroundings.

Glucose, maltose and sucrose have been reported to be best carbon sources for maximum mycelial growth (Asiegbu, 2000; Dong and Yao, 2005). The highest mycelial growth of *Psathyrella atroumbonata* Pegler, a Nigerian edible mushroom, was obtained in glucose medium (Jonathan and Fasidi, 2001). Other authors have shown that *Tuber borchii* mycelia readily utilize glucose and fructose as carbon sources but grow poorly on sucrose (Saltarelli et al., 1998). In the present work, among the various carbon sources tested, glucose and lactose had obvious effects on the growth of the EF-37 isolate, which was in agreement with the previous studies (Dong and Yao, 2005). These authors reported that disaccharides had stronger effects on mycelial growth than the monosaccharides tested, except D(+)-glucose. While the mycelial growth of the EF-37 on medium containing D(-)-fructose, glycerin and phaseomannite was relatively weak compared to that on control medium, growth may have been inhibited by breakdown products.

The most widely used nitrogen sources for mycelial growth are ammonia, ammonium salts, amino acids and complex organic nitrogen (Garraway and Evans, 1984). Our study showed that the EF-37 isolate could grow better in different nitrogen sources especially calcium nitrate. In addition to nitrate nitrogen, it was likely that calcium ions play an important role in the mycelial growth of the EF-37. The importance of calcium was reported by Chandra and Purkayastha (1977) for *Calocybe indica* Purkayastha and A. Chandra. Dong and Yao (2005) also believed that calcium had some effects on the mycelial growth of *Cordyceps sinensis* in submerged culture. Therefore, the effect of mineral elements on the mycelial growth of the EF-37 isolate deserved further studies.

In our study, the EF-37 isolate was not highly dependent on environment and nutrition conditions, and could easily adapt to basic culture medium, temperature, illumination, pH, carbon source and nitrogen compounds. This was probably the reason why the EF-37 isolate could promote growth and development of its host plant, *S. involucrata* in extreme conditions.

The effects found in this study have determined the culture conditions required to optimize growth that will facilitate further studies to gain a better understanding of DSE fungus. Such an understanding is necessary for further investigating the factors affecting the growth of the EF-37 isolate and its relationship with its host plant, *S. involucrata*.

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