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Physiological Study of the Wild Edible Mushroom *Leucocalocybe mongolica*

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Leucocalocybe mongolica (S. Imai) X.D. Yu & Y.J. Yao, a well–known edible and medicinal mushroom is endangered as its habitat is continually under threat from human activities, which has been lessening year after year. In this paper, the biological characteristics of the fungus of strain isolated from the wild fruit body were studied. For the screening of single optimal factor, carbon sources, nitrogen sources, inorganic salt and growth factor were tested for the mycelia growth rate. As a result, the fungus grew better on maltose, starch and cellobiose as the carbon sources; yeast extract, beef powder, beef extract as the nitrogen sources; K_2HPO_4, $Fe_2(SO_4)_3$ as the inorganic salt; some decoctions such as of *Pleurotus ostreatus*, carrot, soybean sprouts as the growth factor. The orthogonal experiments with the above selected three conditions for each of the four factors were then performed and the optimal cultivation conditions were determined. A descending order of the impact for the four factors was growth factor > inorganic salt > nitrogen source > carbon source and the F–test showed very significant difference among all the four factors. The optimal culture condition for *L. mongolica* was the combination of starch and beef extract, decoction of 2% carrot, no added inorganic salt. Based on this conclusion of the orthogonal experiment, the optimal medium in mycelium cultures was applied to temperature and pH experiments, anointed with pH=6.5 and at 25°C were suggested that good growth of mushrooms.

Key words: *Leucocalocybe mongolica*, biological characteristics, single factor, orthogonal test

INTRODUCTION

Leucocalocybe mongolica (S. Imai) X.D. Yu & Y.J. Yao, its former latin name *Tricholoma mongolicum* S. Imai (Imai, 1937), Chinese name is "Bai–mo"or "Kou–mo" (Fig. 1) *T. mongolicum* was proposed to erect a new genus, *Leucocalocybe*, based on morphological and molecular evidence. (Yu *et al*., 2011); That was verified by later experimenters (Dong *et al*., 2013). It is distributed in grassland of Inner Mongolia of China, Mongolia, far east of Russia. And always form the fairy ring.

Due to their highly desirable flavor and short fruiting season, *L. mongolica* are collected by indigenes and mycophiles. "Kou Mo" enjoy a widespread reputation dating back to ancient times in the history of China. The White Mushroom (*Tricholoma mongolicum* S. Imai) is regarded as the best quality of "Kou–mo" (Liu *et al*., 1980). Besides that, It benefits stomach, inhibits cancer, lowers blood pressure and cholesterol (Wu *et al*., 2007). In addition, it is source of natural medicines for local minorities. Evenks boiled it with mutton with the efficacy of fortify one's health and remission blood–heat. Mongolian think it can be applied in the treatment of botulism of wound and detoxify (Wunir *et al*., 2009). The mushroom belongs to the important taxa in economy of biodiversity from China (Liu *et al*., 2003).

Fig. 1. *Leucocalocybe mongolica* (S. Imai) X.D. Yu & Y.J. Yao

In recent years, *L. mongolic*a Imai has drawn more and more attention because of its bioactivities. The fruit bodies have anti– tumor activity (Bau *et al*., 2012). Fungal polysaccharide has always been one of the hot research topics. The extraction conditions, purification and activity screening of *L. mongolica* polysaccharide were taken (Wang *et al*., 2006; Hou., 2008; Wu *et al*., 2012; You., 2014). It has the anti–proliferative effect n on human cervical carcinoma cell and hepatoma cell (Ge *et al*., 2009). It has a high antioxidant activity and can decrease the risk of atherosclerosis (Bao *et al*., 2014).

Lectins from fruit bodies and cultured mycelia of *L. mongolica* have aroused great concern for scholars. Extraction experiments were completes (Wang *et al*., 1998; Yao *et al*., 2007). Physiological activities were found, such as immunomodulatory and antitumor activities (Wang *et al*., 1996; Liu *et al*., 2005) antiproliferative activity (Wang *et al*., 1995) actions on macrophages, splenocytes and life–span in sarcoma–bearing mice (Wang *et al*., 1997). Hypotensive and vasorelaxing activities (Wang *et al*., 1996) .

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People favor its taste and rich nutrition, as well as it is potential use value on pharmaceutical developments. Wild resources are not increasing to meet human demand. So domestication experiments of *L. mongolica* need to do. It was reported that successfully fruiting was trying with cultivation mode of "bed" (Tian *et al*., 1992). Despite these continued efforts, however, artificial cultivation has not been successful. It is still lack of an actual breakthrough on cultivation techniques. Wild resources of *L. mongolica* were in the state of predatory development. Inaddition, the habitat of *L. mongolica* faced with a severe challenge. Such as grassland degradation for overgrazing, desertification of grassland. There is a very necessary for physiology research of *L. mongolica*.

MATERIALS AND METHODS

Fungal strain isolation and identification

The strain was originally isolated from the fruit body which was collected in New Barag Left Banner, Nei Monggol Autonomous Region, China (GPS information N48.0865, E118.8809444) at an altitude of 808 m in 2015. The strain was preserved in strains library of fungi diversity laboratory which are attached to engineering research center of Chinese ministry of education for edible and medicinal fungi in Jilin agricultural university. The voucher specimen was preserved in Herbarium Mycology of Jilin Agriculture University (HMJAU). The strain preservation number MCCJLAU2015C1. It was maintained on potato dextrose agar at 4°C. The Internal Transcribed Spacer (ITS1–5.8S–ITS2 of nuclear ribosomal DNA) was amplified from the culture and 625 bp of the fragment were obtained from DNA sequ–encing. (Sangon Co., Ltd., Shanghai, China). Through sequence alignment, the strain and the voucher specimen are with the sequence similarity of 100%. Sequences generated in this study were submitted to GenBank and accession numbers is KX641897.

The basal medium and culture conditions

The strain was transferred into Petri dishes by aseptic operation. It was propagated on potato dextrose agar at 25°C. When mycelia had grown all over Petri dishes, it was punched into 5 mm homogeneous pieces around concentric circles by the puncher. These pieces of mycelia were used to transfer into culture mediums of single factor test and the orthogonal test.

The basal medium contained glucose 2%, beef extract 0.2%, KH_2PO_4 0.1%, VB_1 0.01%, Agar 2%, distilled water. The medium was autoclaved at 121°C for 30 min.

Culture dishes were inoculated which then were put to incubator with 25°C. All the experiments were replicated in six Petri dishes.

Colonies diameter was measured at 24 hours intervals, mycelial growth vigor were observed simultaneously. The experiment was continued for 20days.

Single factor test of carbon and nitrogen source

Carbon sources of monosaccharides (glucose, galactose, mannose, fructose, xylose), disaccharides (sugar, maltose, lactose, cellobiose), polysaccharides (starch, pectin), ethyl alcohol, glycerinum were tested. Apart from outside ethyl alcohol and glycerinum, 2% addition level was added individually to the basal medium to replace the glucose. Ethyl alcohol and glycerinum was added individually to the basal medium to replace the glucose with volume fraction of 0.5%. A medium free from any carbon source served as a control.

Nitrogen compounds of inorganic nitrogen (NH_4NO_3, $NaNO_2$, $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, urea), and complex organic nitrogen (beef extract, beef powder,casein, peptone, yeast extract, yeast powder,soybean meal) were studied after the tests for carbon source. For each nitrogen source, 2% addition level was added individually to the basal medium to replace the beef extract. The medium lacking nitrogen served as control.

Single factor test of inorganic salt and growth factor

Macro–elements and trace elements were tested. With regard to macro–elements, 0.1% addition level was added individually [K_2HPO_4, KH_2PO_4, $Ca(H_2PO_4)_2 \cdot H_2O$, $CaCl_2$, NaCl, $MgSO_4$] to the basal medium to replace the KH_2PO_4. Relatively, $Fe_2(SO_4)_3$, $FeCl_3$, $Zncl_2$ were added individually into culture medium to obtain a final concentration of 0.001% to the basal medium to replace the KH_2PO_4. The medium lacking inorganic salt served as control.

Some natural conponents and vitamins were tested as growth factors. Vitamin E, vitamin B1 were selected, 0.01% addition level was added individually to the basal medium to replace the VB_1. Half treatment of natural conponents milk powder addition by 2%, corn flour addition by 3% malt extract powder addition by 1% was individually insteaded of VB_1 in the basal medium. 8 natural conponents *Pleurotus ostreatus*, carrot, soybean sprouts, broad leaf, potato, needle leaf, *Leymus chinensis*,wheat bran which was severally weighed 20 g/L, boiled the juice and filtered was added individually to the basal medium to replace the VB_1. The medium lacking growth factor served as control.

Optimization by orthogonal matrix method

The orthogonal L_9 (3^4) was used to obtain the optimal medium in mycelium cultures. After the test by Single factor test, the best three levels from carbon sources, nitrogen sources, norganic salts and growth factors which were selected as the four factors of exercise at three levels orthogonal design. (The best three levels were selected in accordance with a comprehensive conclusion of mycelial growth rate and mycelial growth vigor).

Single factor test of temperature and pH

Based on this conclusion of the orthogonal experiment, the optimal medium in mycelicum cultures was obtained. The optimal medium were inoculated which then were put individually to incubator with 13, 15, 20, 25 and 27°C.

With regard to single factor test of pH, the optimal medium was selected. ThepH test will be divided into two parts: pH (1) test and pH (2) test. pH (1) test :The initial pH was adjusted to 4, 5, 5.5, 6 and 77 using 1 M HCl or 1 M NaOH, which then were put to incubator with 25°C. The pH (2) test :The initial pH was adjusted to 6, 6.5, 7, 7.5 and 8 using 1 M HCl or 1 M NaOH, which then were put to incubator with 25°C.

Analysis of data

All the data obtained from this investigation were analysed by analysis of variance and the tests of significance were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Effect of carbon source on mycelia growth

There were significant effects of carbon source on the mycelial growth of *L. mongolicum* (Table 1). The wild mushroom was isolated from grassland can grow on 14 kinds of culture medium. Colonies have shown mycelial growth vigor semblable. From perspective of biochemical composition on carbon source, monosaccharide, disaccharide, polysaccharide, small molecule alcohol and pectin can be utilized by *L. mongolica* in order to meet the nutritional needs. Mmaltose, soluble starch, cellobiose were more effective than control. Among the carbon sources tested, the highest mycelial growth rate was obtained with maltose, followed by starch and cellobiose. These carbon sources are superior to control. The rest of carbon sources are in order, glycerinum, fructose, mannose, ethyl alcohol, lactose, sucrose, glucose. The worst are galactose, xylose, pectin. Carbon sources are essential cytoskeletal components, supply simultaneously mycelium with energy. The composition of 0.2% beef extract are present in the basal medium in the test. Constituents of beef extract are complicated. Beef extract as an organic nutrients was utilized by fungus as a nitrogen source. It possesses the property of the carbon source concurrently. 10 carbon sources were relatively superior to control. From the significant effects point of view, maltose, starch and cellobiose are good candidates for the carbon sources, compared with other carbon sources. Therefore, the three carbon sources were selected as the carbon source in the following experiments.

Effect of nitrogen source on mycelia growth

Among 13 nitrogen sources and control examined, there were significant effects of nitrogen source on the mycelial growth of *L. mongolica* (Table 2). Mycelium can grow on 14 mediums. From perspective of biochemical composition on nitrogen sources, organic nitrogen and inorganic nitrogen can be utilized by *L. mongolica* in order to meet the nutritional needs. From the point of mycelial growth vigor, organic nitrogen sources were more effective than inorganic nitrogen sources. From a other perspective of mycelial growth rate, organic nitrogen sources were more effective apart from casein and soybean meal. Some components has a negative effect on growth. Nitrogen sources play a key role in the cycle of n–containing compounds synthesis and mycelium growth. Accroding to mycelial growth rate, mycelium on 12 mediums grow faster than control in addition to Urea. the possible reason is that biuret, three urea and cyanuric acid have been producted from urea through condensation reaction under high pressure at high temperature.

Table 1. Effect of carbon sources on mycelial growth of *Leucocalocybe mongolica*

Carbon source	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
maltose	3.48	a	A	++
starch	3.24	ab	AB	++
cellobiose	3.18	b	AB	++
control	3.06	bc	BC	++
glycerinum	2.91	cd	BCD	++
fructose	2.83	cd	CD	++
mannose	2.82	cd	CD	++
ethyl alcohol	2.77	d	CDE	++
lactose	2.71	de	DEF	++
sucrose	2.68	de	DEF	++
glucose	2.45	ef	EF	++
galactose	2.37	f	F	++
xylose	1.99	g	G	++
pectin	0.28	h	H	++

* Date represent the average values of six replicates.

** "+++" vigorous my celial growth, "++" intermediate mycelial growth, "+" weak mycelial growth.

Table 2. Effect of nitrogen sources on mycelial growth of *Leucocalocybe mongolica*

Nitrogen source	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
yeast extract	2.57	a	A	+++
beef powder	2.46	ab	A	+++
beef extract	2.41	b	A	+++
yeast powder	1.94	c	B	+++
peptone	1.74	d	C	++
NH_4NO_3	1.72	d	C	+
$NaNO_2$	1.71	d	C	+
$(NH_4)_2SO_4$	1.67	d	C	+
Casein	1.45	e	D	+++
$NaNO_3$	1.29	f	DE	+
$(NH_4)_2HPO_4$	1.28	f	DE	+++
soybean meal	1.14	f	EF	+++
control	0.99	g	F	+
urea	0.40	h	G	+

*, ** As in Table 1

Table 3. Effect of Inorganic salts on mycelial growth of *Leucocalocybe mongolica*

Inorganic salt	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
K_2HPO_4	3.09	a	A	+++
control	2.83	b	B	+++
$Fe_2(SO_4)_3$	2.42	c	C	++
$MgSO_4$	2.30	cd	CD	++
KH_2PO_4	2.28	cd	CD	++
$Ca(H_2PO_4)_2 \cdot H_2O$	2.25	cd	CD	++
$CaCl_2$	2.23	d	CD	++
NaCl	2.22	d	CD	++
$FeCl_3$	2.22	d	CD	++
$Zncl_2$	2.12	d	D	++

*, ** As in Table 1

Comprehensive analysis which combine mycelial growth rate with mycelial growth vigor, yeast extract, beef powder, beef extract are good candidates for the nitrogen sources, compared with other nitrogen sources. Therefore, the three nitrogen sources were selected as the nitrogen source in the following experiments.

Effect of inorganic salt on mycelia growth

It is clearly shown from the study that effect of inorganic salt on mycelial growth of *L. mongolica* (Table 3). The K_2HPO_4 and no added inorganic salt were more effective on mycelial growth vigor, other inorganic salt added weakened the mycelial growth. Mushrooms need some inorganic salts and trace elements as its composition and regulation of physiological active substancesin the process of growth to reproduction. The experiment shows that mycelial growth of *L. mongolica* about supplements severely treated. Comprehensive analysis which combine mycelial growth rate with mycelial growth vigor, K_2HPO_4 are good candidates for the inorganic salt, compared with other nitrogen sources. No added nitrogen sources, $Fe_2(SO_4)_3$ were selected to do in–depth study. Therefore, the three nitrogen sources were selected as the nitrogen source in the following experiments.

Effect of growth factor on mycelia growth

There were significant effects of growth factor on the mycelial growth of *L. mongolica* (Table 4). Mycelium can grow on 14 mediums. From the point of mycelial

Table 4. Effect of growth factors on mycelial growth of *Leucocalocybe mongolica*

Growth factor	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
Pleurotus ostreatus	2.67	a	A	+++
VE	2.66	a	A	++
VB_1	2.53	a	AB	++
carrot	2.42	ab	AB	+++
control	2.41	ab	AB	++
milk powder	2.39	ab	AB	++
soybean sprouts	2.38	ab	AB	+++
broad leaf	2.34	ab	AB	+++
potato	2.16	b	BC	+++
corn flour	2.14	b	BC	+++
Malt Extract Powder	1.71	c	CD	+++
needle leaf	1.66	c	D	+++
leymus chinensis	1.63	c	D	+++
wheat bran	1.46	c	D	+++

*, ** As in Table 1

growth vigor, VE, VB_1, control, milk powder were more inferior than other growth factors. From a perspective of mycelial growth rate, *Pleurotus ostreatus*, VE, VB_1, carrot were more effective than control, milk powder, soybean sprouts, broad leaf were no significant difference from control, Followed by potato, corn flour, malt extract powder, needle leaf, *Leymus chinensis*, wheat bran. From the significant effects point of view, *Pleurotus ostreatus*, carrot and soybean sprouts are good candidates for the growth factors compared with other growth factors. Therefore, the three growth factors were selected as the carbon source in the following experiments.

Optimization by orthogonal matrix method

To investigate the relationships between variables of medium components for mycelial growth, the orthogonal layout L9 was employed. Based on the design of four factors and three levels , the experimental conditions for each experimental group were listed in Table 5. The orthogonal experiments with the above selected three conditions for each of the four factors were then performed and the optimal cultivation conditions were determined. The experimental conditions for each experimental group were listed in Table 5 with the experimental results concluded in the last column. Colonies have shown mycelial growth vigor semblable. Mycelial growth rate is only considering the option. To obtain a significant effect on mycelial growth, the optimum composition should be carbon source X_2, nitrogen source X_3, inorganic salt X_2, growth factor X_2. Namely, soluble starch, beef extract, mineral salt does not add, 2% carrot boiled the juice.

According to the magnitude order of R (maximum difference), the order of effect of all factors on mycelial growth could be determined nitrogen source growth factor (0.82) > nitrogen source (0.32) > inorganic salt 0.31) = carbon source (0.31). The results indicated that the effect of growth factor e was more important than that of the other three nutrients. To test the effects of the three factors, ANOVA was used and shown in Table 6. All factors, including growth factor, nitrogen source, inorganic salt, arbon source had significant effects on the mycelial growth of *L. mongolica*.

Effect of temperature and pH on mycelia growth

Based on this conclusion of the orthogonal experiment, the optimal medium in mycelium cultures was applied to temperature and pH experiments. The results obtained when *L. mongolica* was grown in a different temperature range reveal that best mycelial growth were attained at 25°C (Table 7). Therefore, this temperature was the optimum for the vegetative growth of this fungus. Colonies have shown mycelial growth vigor semblable about the temperatures of the test. With regard to mycelial growth rate, effect of mycelia growth on 25°C and 20°C haven't obvious differences. Besides 27°C and 15°C have obvious distinctness and slower growth rate compared with 20–25°C. Temperature range 20–25°C were beneficial to its growth. Tables 8 and 9 show that *L. mongolic*a grew in acidic, neutral and alkaline environments (pH 4.0–8.0). Comprehensive analysis of table 8 and table 9. It was observed that the best growth was obtained in slightly acidity medium of 6.5. Followed by that, which was the second best, was also recorded in neutral environment (pH 7.0). It could therefore be deduced that *L. mongolica* preferred an neutral medium to acid or alkaline pH 4.0, 5.0, 8.0 weaken mycelial growth that suggests that very strong acidic or alkaline environments were inhibitory to growth.

Table 5. The results of direct-viewing analysis of mycelial growth

Test Number	Carbon source	Nitrogen source	Inorganic salt	Growth factor	Mycelial growth rate (mm/d)*	Mycelial growth vigor (mm/d)**
1	1maltose	1yeast extract	1K_2HPO_4	1 *Pleurotus ostreatus*	2.66	++
2	1maltose	2beef powder	2No added	2 carrot	3.94	++
3	1maltose	3beef extract	3$Fe_2(SO_4)_3$	3 soybean sprouts	3.89	+++
4	2starch	1yeast extract	2No added	3 soybean sprouts	3.90	+++
5	2starch	2beef powder	3$Fe_2(SO_4)_3$	1 *Pleurotus ostreatus*	3.14	+++
6	2starch	3beef extract	1K_2HPO_4	2 carrot	3.96	++
7	3cellobiose	1yeast extract	3$Fe_2(SO_4)_3$	2 carrot	3.5	+++
8	3cellobiose	2beef powder	1K_2HPO_4	3 soybean sprouts	3.43	++
9	3cellobiose	3beef extract	2No added	1 *Pleurotus ostreatus*	3.15	++
T_1	52.45	50.31	50.22	44.76		
T_2	55	52.56	54.97	56.96	T=31.57	
T_3	50.42	55	52.68	56.15		
X_1	3.50	3.35	3.35	2.98		
X_2	3.67	3.50	3.66	3.80		
X_3	3.36	3.67	3.51	3.74		
R	0.31	0.32	0.31	0.82		

*, ** As in Table 1

Table 6. The results of F-test of mycelial growth

Source	Type Ⅲ Sum of Squares	df	MS	F	Sig.
Carbon source	0.7022	2	0.3511	15.6365	0.0001
Nitrogen source	0.7336	2	0.3668	16.3354	0.0001
Inorganic salt	0.7524	2	0.3762	16.754	0.0001
Growth factor	6.2051	2	3.1025	138.1702	0.0001
Error	0.8084	36	0.0225		
Total	9.2017	44			

Table 7. Effect of temperature on mycelial growth of *Leucocalocybe mongolica*

Temperature	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
25°C	4.87	a	A	+++
20°C	4.8	a	A	+++
27°C	3.4	b	B	+++
15°C	2.93	c	BC	+++
13°C	2.5	d	C	+++

*, ** As in Table 1

The optimal culture condition for *L. mongolica* was the combination of Starch plus beef extract, decoction of 2% carrot, no added inorganic salt. Based on this conclusion of the orthogonal experiment, the optimal medium in mycelium cultures was applied to temperature and pH experiments anointed with pH=6.5 and at 25°C were suggested that good growth of mushrooms.

The best media component obtained which are contribute to vegetative growth of *L. mongolica*. The mushrooms are rather more selective than other fungi in the size of the fruiting body requires the availability of more nutrients than are required by micro fungi. Mycelia

Table 8. Effect of pH (1) on mycelial growth of *Leucocalocybe mongolica*

pH	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
7	4.66	a	A	+++
6	4.49	a	AB	+++
5.5	3.89	b	B	+++
5	2.64	c	C	+++
4	2.15	d	C	+++

*, ** As in Table 1

Table 9. Effect of pH (2) on mycelial growth of *Leucocalocybe mongolica*

pH	Mycelial growth rate (mm/d)*	Significance levels		Mycelial growth vigor (mm/d)**
		0.05	0.01	
6.5	4.58	a	A	+++
7	4.18	ab	A	+++
6	4.10	ab	A	+++
7.5	4.00	ab	A	+++
8	3.80	b	A	+++

*, ** As in Table 1

growth is an important component part of mushroom cultivation.

Mushroom described as being ubiquitous, they are found just about everywhere. But *L. mongolica* has a restricted global distribution. Because of the special habitat, the correlative researches of physiology are necessary to take. Enzymes are activated to break down complex substrate components (e.g. cellulose, hemicelluloses and lignin) into simpler molecules which can be absorbed by mycelia as nutrients for growth and propagation. Different enzymes have tissue–specificity, correlating with the different develop phase and the difference between physiological and pathological condition. Base on the nutritional requirement of growth of *L. mongolica* mycelia, further work about enzyme which urgently needs to be done.

Effect of inorganic salt on mycelia growth on L. mongolica is remarkable. Most of the inorganic salt inhibits mycelium growth. There have been reports that five metal ions including Fe^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+} and Cu^{2+} could inhibit mycelium growth (Li, 2001). We get similar conclusion. So strictly control the metal ions content addition level is necessary.

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