ROLE OF COPPER ON GROWTH, DEVELOPMENT AND PHYSIOLOGICAL EFFECT OF *PLEUROTUS NEBRODENSIS*

HU QING-XIU*, WU ZHENG-WU, ZHANG RUI-YING, ZOU YA-JIE AND YANG XIAO-HONG
Institute of Agricultural Resources and Regional Planning, CAAS, Beijing 100081, China
hqx9758@126.com

ABSTRACT

Pleurotus nebrodensis is an important rare edible fungus with independent intellectual property rights in China, has commercialized and large scale culture firstly realized in 1990s in Beijing, is a widely popular product in the market, and has current yearly yield over 200,000 tonnes. This paper focuses on influence of copper on growth and development of *P. nebrodensis* and on activities of extracellular *laccase* and manganese peroxidase; and meantime, and change of flow rate of H^+ and Ca^{2+} on mycelia surface under the action of copper is determined, to make physiological effect of copper clear. It is shown by research result that, copper supplemented substrate can obviously promote growth rate (4.35 mm/d) of *P. nebrodensis* mycelia at a content of 50 mg/kg, significantly different with that of the control group. At copper content of 50 mg/kg, growth cycle and biological efficiency of *P. nebrodensis* are 166.22 days and 50.66%, prolonged by 16 d and improved by 4.61%, respectively. In growth period of *P. nebrodensis* mycelia, laccase has high activity, and even higher (significantly different with that of the control group) when the copper content in the substrate is 25-50 mg/kg, but has activity decreased when the copper content is over 50 mg/kg. In growth period of *P. nebrodensis* fruiting body, manganese peroxidase has activity higher than that in growth period of mycelia, and has highest activity at copper content of 50 mg/kg, indicating certain correlation with yield. The content of copper in the substrate can change flow direction of extracellular H^+ and Ca^{2+} on mycelia surface. All these results will provide scientific basis for culture techniques and theory of *Pleurotus nebrodensis*.

Keywords: *Pleurotus nebrodensis*, mycelial growth, yield, enzyme activity, ion flow

INTRODUCTION

Pleurotus nebrodensis belong to *Pleurotus*, Pleurotaceae, Agaricales, Basidiomyceta. It is a rare edible fungus, is called Bailinggu or Aweigu in China [1]. *P. Nebrodensis*was firstly described and recorded by Inzengae in 1863, and names as *Agaricus nebrodensis*, and was renamed as *P. nebrodensis* (Inzengae Quelet) in 1886. Recent researches indicate that, *P. nebrodensis* in China is an independently evolving branch of *Pleurotus eryngii* and should be named as *P. eryngii* var. *tuoliensis* [2-3].

P. nebrodensis was successfully cultivated in China in 1983 for the first time, and is popular in the market as the fruiting body is large, white, tender and delicious and rich in nutrition. Meanwhile, research on medicinal value of *P. nebrodensis* has attracted increasing attention [4]. At present, artificial culture techniques of *P. nebrodensis*are generalized in most area of northern China, and a good deal of culture experience has been obtained. Despite the great market value of *P. nebrodensis* products, low unit production and long culture period increases production risk and restricts the production and development of *P. nebrodensis.* In recent years, researches on *P. nebrodensis* mostly focus on strain identification [5-8], culture substrate selection and the like, and research reports on micronutrient element utilization and physiology are few. Microelement plays a very important role in substance and energy metabolism in growth and development process of edible fungi, not only is the constituent of cell structure and multiple enzymes and enzyme activator, but also participates in biochemical reaction in cells. Microelement can promote growth of fungi in *Pleurotus*. For example, manganese element supplemented in culture substrate can promote mycelial growth and increase yield and can also promote lignin degradation [9]. Beelman [10] found that accumulation of Cu can improve yield and quality of *A. bisporus*, and increase of Cu content in basidiomycete may increase activity of tyrosinase and cause browning of fruiting body. There is a significant difference in inherent levels of Cu in different strains of basidiomycete, but *P. nebrodensis* does not accumulate Cu and disease resistanceis not correlated with Cu content inbasidiomycete [11].

In the present research, divalent Cu (in the form of copper salt) at different concentrations was added into conventional culture medium for *P. nebrodensis*, activities of manganese peroxidase and laccase in the whole growth cycle were determined, differences between enzyme activities and yields in different culture media were compared, and change of flow rate of H^+ and Ca^{2+} on mycelia surface was determined by NMT, to make effect of copper on growth and development of *P. nebrodensis*clear, to find optimal Cu content for growth and development, to learn physiological effect of Cu, and to provide scientific basis for improvement in culture medium, promotion in growth, increment in yield and establishment of culture techniques and theory of *P. nebrodensis*.

MATERIALS AND METHODS

Experiment strain

Pleurotus nebrodensis 00485 provided by China Center for Mushroom Spawn Standards and Control ((CCMSSC).

Main reagents and apparatuses

$CuSO_4 \cdot 5H_2O$ and H_2O_2 (analytically pure), HAC-NaAC buffer solutions (0.1 mol/l acetic acid-sodium acetate, self made) with pH 4.6, pH 5.0 and pH 5.8, ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma).

UV-2800H ultraviolet spectrophotometer (Unico (Shanghai,China) Instrument Co., Ltd) and LK-S3D thermostat water bath (Beijing Likang Company, China).

Experimental Methods

Spwan: Culture of stock strain was cultured on potato dextrose agar (PDA) in an incubator at 25±1°C for 12 days. Culture process of mother spawn included preparing culture medium from wheat 1000 g, gypsum 10 g and lime 3 g, sterilizing by conventional method, bagging, inoculating by aseptic operation, and culturing in an incubator at 25±1°C for 15 days. Liquid medium was made using the following ingredients: peeled potato (200g), dextrose(20g), peptone (3g) and warm tap water (1200ml), natural pH. The liquid spawn substrate was autoclaved for 45min at 121°C, cooled and inoculated with mycelial plugs from PDA (5 mm diameter; 5plugs/flask). Shake culture was performed at rotation speed 130/min, 22°C in dark for 13 days. The substrate standard formulation for production substrate (63% moisture) included cotton seed hulls (80% d.w.), bran(15%), corn flour (3%), calcium carbonate (1%) and calcium sulfate (1%).

Experiments of liquid culture at different copper contents: The liquid medium was added with copper ion (copper sulfate) at different concentrations, loaded in 1000 ml triangular flasks at an amount of 500 ml, sterilized and cooled, and inoculated with *P. nebrodensis* 00485. Addition of Cu^{2+} content is shown in Table 1. Mycelia were respectively collected by filtering the fermentation liquid, oven dried at 45°C and weighed. Dry weights of mycelia from different flasks were compared. The experiment was completely randomized with 5 replicates per treatment.

Table 1. Design of additive content of Cu in experiment of liquid culture

Treatments	CK	A1	A2	A3	A4	A5	A6	A7	A8
Cu^{2+}(mg/l)	0	50	100	150	200	250	300	350	400
CuSO4·5H2O (mg/l)	195.32	390.64	585.96	783.28	978.6	1173.92	1369.24	1566.56	195.32

Experiments of production at different copper contents: The materials of the culture substrate were mixed and added with Cu^{2+} solution with different Cu^{2+} contents according to Table 2, and bagged in 17 cm×33 cm×0.05 cm high pressure polypropylene plastic bags at a amount of 350 g, with 45 bags for each Cu^{2+} content. The bagged substrate was sterilized at high pressure at 121°C by conventional method, cooled, and inoculated with mother spawn by aseptic operation. The culture and fruiting management was performed according to the literature [12].

Table 2. Design of additive content of Cu in experiment of production

Treatments	CK	C1	C2	C3	C4
Cu^{2+}(mg/kg)	0	25	50	100	150
CuSO4·5H2O (mg/l)	0	97.66	195.32	390.64	585.96

Mycelia growth rate, bagful time, low temperature stimulation time and harvesting date were recorded during the experiment. Average growth rate was calculated by randomly selecting 5 substrate bags from each formulation, marking mycelia growing height by drawing a line on the bag 10 days after inoculation, and marking once every 5 days for 3 times in total. Bud appearing time was recorded when the first fruiting bud appeared. Harvesting time was recorded as the number of days from the beginning of harvesting and terminal of harvesting. The weight of each fruiting body was recorded during the harvesting. The biological efficiency was calculated based on fresh weight of fruiting body from the first harvesting.

Biological efficiency = (fresh weight of fruiting body/dry weight of culture substrate)×100%.

Enzyme activity determination: Sample was collected 20 days after inoculation, and once every 15 days. Sample was collected 2 cm below inoculation surface in elevation direction. Extraction of extracellular enzyme was performed by weighing frozen sample 20.0 g with an analytical balance, placing in a 200 ml beaker, adding distilled water 100 ml, extracting in thermostat water bath at 30 °C for 1 h, filtering with two layers of filter paper, collecting filtrate as crude enzyme solution, and setting the volume at 100 ml in a volumetric flask.

Determination of laccase (Lac) activity was performed according to Robert Bourbonnais method, which was modified according to the speed of the laccase oxidizing ABTS. The reaction was performed at 25 °C in a 5 ml system containing 2 mL of 0.l mol/l sodium acetate buffer solution (pH 5.0) containing 0.5 mmol/l ABTS, 2.9 ml of distilled water, and 0.1 ml of crude enzyme solution for 4 min. Absorbance at 420 nm was detected. The enzyme unit is defined as the amount of the enzyme that increases absorbance per minute by 0.1. Determination of manganese peroxidase (MnP) activity was performed in a 5 mL system containing 0.11 mol/l pH4.5 sodium lactate buffer solution 4 ml, 10 mmol/l manganese sulfate 0.25 ml, 10 mmol/l guaiacol 0.25 ml and crude enzyme solution 0.25 ml at 30 °C for 30 min with reaction started by dropwise addition of H_2O_2 (6 mmol/l) 0.25 ml. Absorbance at 240 nm was detected. The enzyme unit is defined as the amount of the enzyme that increases absorbance per minute by 0.1.

Determination of ion flow on mycelia surface by NMT: The determination was performed 15 d after inoculation for the first time, and performed in the whole growth period of *Pleurotus nebrodensis* at the same frequency as that of enzyme activity determination. The flow of two ions (Ca^{2+} and H^+) was determined each time, with the same sampling location as that of enzyme activity determination. Five parallel tests were performed at each sampling location.

Statistical analysis

Experimental data was analyzed by statistical software Microsoft Excel and SPSS. Multiple comparison between average of each group was analyzed by least significant difference method (LSD), and variance analysis was performed for analysis of growth potential of *P. nebrodensis* mycelia and yield of fruiting body.

RESULTS AND ANALYSIS

Determination on biomass of liquid culture under different copper ion contents

Copper ion content in culture medium has significant influence on biomass of *P. nebrodensis* under liquid culture condition (result shown in Fig. 1). When copper ion content in the culture medium is less than 100 mg/l, biomass of *P. nebrodensis* increases with the increase of copper ion content

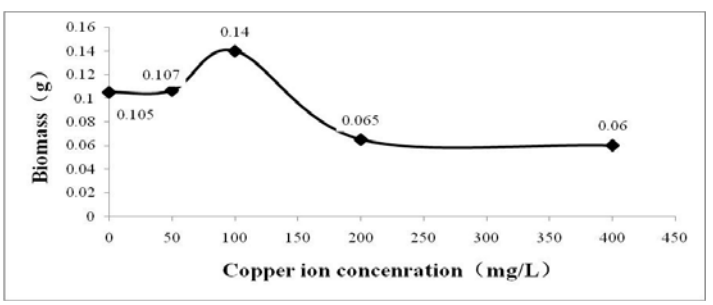

Figure 1. Comparison of biomass of *P. nebrodensis* at different Cu contents in liquid media

and reaches maximal value of 0.14 g/l at copper ion content of 100 mg/l; When copper ion content in the culture medium is greater than 100 mg/l, biomass of *P. nebrodensis* gradually declines, and the biomass has no significant change when the copper ion content is 250-400 mg/l.

Mycelial growth rate and bagful time

Experimental results (shown in Table 3) indicate that, copper ion has little effect on mycelial vigor but can promote mycelial growth. The copper ion can promote mycelial growth at the content of 25 mg/kg, showing no significant difference with that of control group. At copper ion content of 50 mg/kg, mycelial growth rate (4.35 mm/d) is obviously increased, significantly different with that of control group at $p<0.05$ and having no significant difference with that of control group at $P<0.01$, and the bagful time is shortened by 7 days. The copper ion at content of 100mg/ kg has certain promoting effect on growth of *P. nebrodensis* mycelia, showing significant difference with that of control group at $p<0.05$, but the growth of mycelia becomes slow. Hence, copper ion can promote mycelial growth at low content, but suppress mycelial growth at high content.

Table 3. Comparison of mycelial growth rate, bagful time and mycelialvigor in different copper concentration

Treatments	Bagful time/d	Mycelial growth rate Average(mm/d)	Mycelial vigor Significance of difference		
			0.05	0.01	
CK	71.29	3.49±0.38	a	A	+++
C1	68.61	3.89±0.24	ab	A	+++
C2	64.74	4.35±0.38	b	A	++++
C3	66.34	4.22±1.13	b	A	+++
C4	65.48	4.02±2.00	ab	A	++++

Note: The data is test average ± SD (n=30). The alphabet shows significance of difference at $p<0.05$ or $p<0.01$. + indicates low mycelial vigor, ++ indicates general mycelial vigor, +++ indicates good mycelial vigor, and ++++ indicates vigorous mycelial growth.

Yield, culture cycle and biological efficiency

It is shown by result analysis that, addition of copper ion at certain content can shorten culture cycle of *P. nebrodensis* and improve biological efficiency. At copper content of 50 mg/kg, growth cycle and biological efficiency of *P. nebrodensis* are 166.22 days and 50.66%, prolonged by 16 d and improved by 4.61%, respectively, when compared with those of CK. Analytic result indicates no statistically significant difference between the test formulations and the CK in average unit yield at ($p<0.01$). (Table 4).

Table 4. Comparison of yield, culture cycle and biological efficiency among different formulas

Treatments	Culture cycle	Average yield/g	Yield Significance of difference		Biological efficiency / %	Ratiowith biological efficiency of CK
			0.05	0.01		
CK	175.86	161.18	a	A	46.05%	/
C1	174.22	161.87	a	A	46.25%	0.2%
C2	166.22	177.30	a	A	50.66%	4.61%
C3	169.47	175.88	a	A	50.25%	4.2%
C4	170.17	169.88	a	A	48.54%	2.49%

Note: The data is test average ±SD (n=30). The alphabet shows significance of difference at $p<0.05$ or $p<0.01$.

Manganese dependent peroxidase and laccase

Research result shows that (Fig. 2) in the whole growth and development process of *P. nebrodensis*, *laccase* has an activity trend of high in mycelia growing period, sequential slow decline in post-ripening period, temperature difference stimulation period, bud appearing period and fruiting body growing period, and sharp drop after harvest. Manganese dependent peroxidase has small activity fluctuation in mycelia growing period and post-ripening period, evident activity increase in temperature difference stimulation period, high activity level at early stage of bud appearing period, gradual activity decrease in late stage of fruiting body growing period, and the same activity as that in mycelia growing period (Fig. 2). Change trend of laccase and manganese peroxidase in activity is in coincidence with that in research of role of manganese on *P. nebrodensis*.

It is found by analysis combining Table 4 and Fig 2 that, experimental group with high activity of manganese dependent peroxidase has high yield. The difference of peroxidase activity between different experimental groups can be considered as the difference in yield. The bigger the difference in enzyme activity is, the bigger the difference in yield, and vice versa.

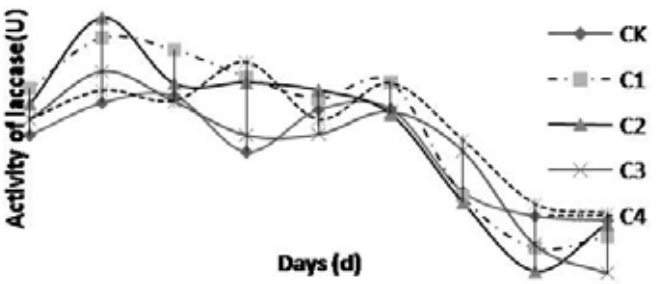

Figure 2. Comparison of the Lac in different periods among different experimental groups

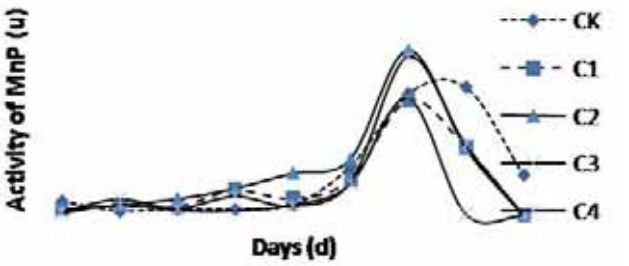

Figure 3. Comparison of the MnP in different periods among different experimental groups

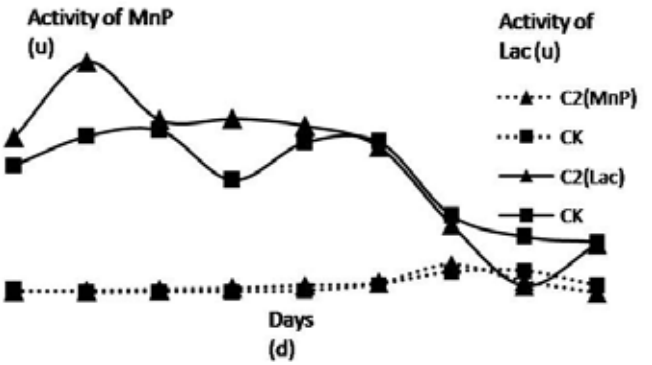

Figure 4. Comparison of activity change of the two enzymes in different period both C2 and CK

Change of H^+ and Ca^{2+} flux

Copper ion has maximal influence on growth rate, culture cycle and biological efficiency at concentration of 50 mg/kg, thus treatment C2 is selected for determining flux of H^+ and Ca^{2+} on mycelia surface (Fig. 5 and 6). Flux change of H^+ and Ca^{2+} on mycelia surface has no correlation with content of added copper ion. It is estimated that outward flow rate of H^+ may has no direct influence on change of Ca^{2+} flow rate, but the addition of copper ion can directly changed ion flow rate and direction in *P. nebrodensis* mycelia. The mechanism and path of these influences needs further research.

CONCLUSION AND DISCUSSION

It is shown by research result that, addition of copper at certain content can promote growth of *P. nebrodensis*. Copper can obviously promote growth rate (4.35 mm/d) of *P. nebrodensis*, shorten culture cycle (166.22 days) and yield (177.30 g). Whereas, result of research on *P. eryngii* by Rodriguez Estrada and Royse DJ [11] showed that copper at content of

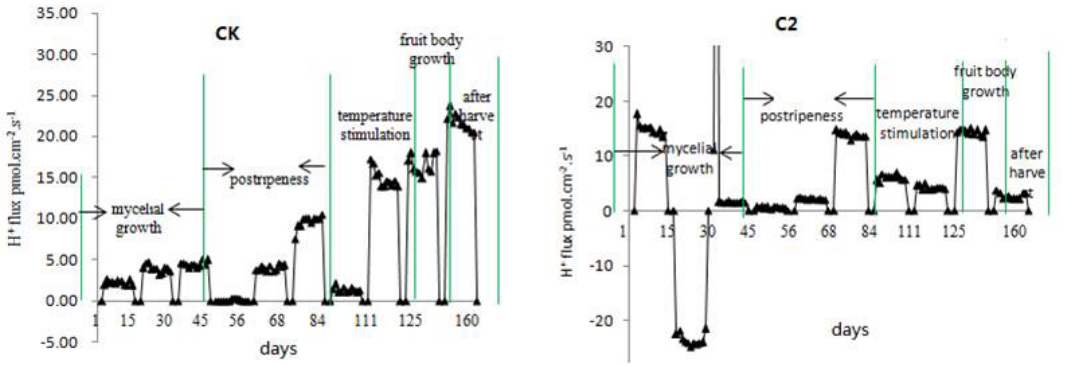

Figure 5. Change curve of H^+ flux on surface of mycelia of C2 group and CK group in different periods

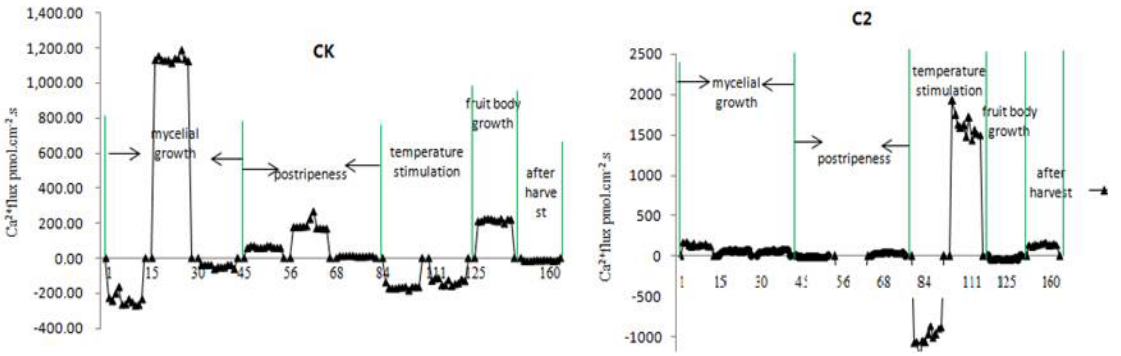

Figure 6. Change curve of Ca^{2+} flux on surface of mycelia of C2 group and CK group in different periods

150-250 μg/g (high content) can significantly reduce yield of *P. eryngii*, causes severe pathogenicity rate, but has low influence on biological efficiency, and average size and amount of *P. eryngii* fruiting body. It is also found that different strains have different copper tolerances, which is correlated with copper content in culture substrate [11]. Ling Yafei [13] found in the research on effect of mineral element, vitamins and hormone on growth of *Agrocybe aegirita* mycelia that, addition of copper can promote growth of *A. aegirita* mycelia but growth of *A. aegirita* mycelia has little demand on copper. Preliminary study on nutritive and physiological property of *Sparassis crispa* shows that addition of copper can obviously promote growth of *S. crispa* mycelia [14]. It is supposed that different edible fungi or different strains may have different tolerance on copper, which might be associated with gene of strains and copper content in culture substrate.

In enzyme activity experiment, laccase has highest activity in mycelia growing period, and sequential slow decline in post-ripening period, temperature difference stimulation period, bud appearing period, fruiting body growing period, and harvest period, which is concordant with the findings of Feng Zuoshan [15]. But in the research on change law of extracellular enzyme activity in growth and development process of *P. nebrodensis* by Zhou Changqing, *laccase* has low activity in mycelia growing period and post-ripening period, and significant activity increase in temperature difference stimulation period, which is completely different with the results of the research, and needs further confirmation. Manganese dependent peroxidase has low activity in mycelia growing period and post-ripening period, gradually increasing activity in temperature difference stimulation period, highest activity in bud appearing period, and gradually decreasing activity with the growth of *P. nebrodensis* fruiting body, which is in agreement with the results of research on *L. edodes* by Pan Yingjie [16]. Manganese dependent peroxidase has identical activity change trend in different strains, and has activity increase in early stage of fruiting body formation and activity decrease after harvesting of fruiting body. Relation between the manganese dependent peroxidase and formation of fruiting body deserves further study and discussion.

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