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A Study on Multiplication Response of "*Tricholoma matsutake*"(Pine Mushroom) Conidio to Cultural Media Environment[1]

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松茸菌(*Tricholoma matsutake*)의 培養環境에 대한 增殖反應에 관한 硏究[1]

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ABSTRACT

This study was conducted to examine the physiology of pine mushroom mycelia cultured with various media for artificial culture of pine mushroom. The results obtained were as follows: 1) Among the various media, the medium composed of honey, boiled pine mushroom and soil extract fluid, fibrous root extract fluid, dry yeast, KH_2PO_4 inositol, folic acid, and biotin was the best for the growth of pine mushroom mycelium. 2) The optimum temperature for germinating pine mushroom spore and for culturing pine mushroom mycelium, was 24°C and the optimum pH was 4.5. 3) There was no significant difference in growth between the mycelium separated from the tissue of pine mushroom sporophore and that separated from the spore. 4) No noticeable effect was found on the growth if such salts as $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CaCl_2$ and ferric citrate were added to the Hamada's medium. 5) The addition of fibrous root extract promoted the growth of pine mushroom mycelium. 6) As a carbon source of artificial media, honey was more effective than glucose. 7) The culture infiltration of Mortierlla growing often in Fairy Ring was good for the growth of mycelium compared with the control. 8) The addition of fibrous root extract, inositol, biotin, and folic acid to artificial culture media was greatly effective in growth. When the temperature was lowered 19°C after mycelium has appeared, the formation of primordium was observed.

Key words: Tricholoma matsutake; *cultural media environment; multiplication response.*

要 約

松茸는 赤松의 細根에 活物寄生한다고 하나 그 本質이 아직 究明되지 못하고 있음으로 人工栽培의 確立이 模索되고 있는 중이다. 本論文에서는 一次的으로 松茸에서 純粹分離한 菌을 培養하여 生理的 特性을 究明하고 人工原基를 만들어 人工的인 松茸栽培의 本質을 究明하여 松茸栽培 方法을 돕고자 松茸菌의 生理特性에 對하여 조사한 結果는 다음과 같다. 1) 各種 培養基上에서의 松茸菌系의 發育은 蜂蜜+松茸發生土壤煎汁+赤松鬚根抽出液+乾燥酵母+KH_2PO_4+Inositol+葉酸+Biotin의 培地가 가장 優秀하였다. 2) 松茸胞子의 發芽 및 菌系培養에서 最適溫度는 24℃, 最適 pH는 4.5였다. 3) 松茸菌系培養에 있어서 松茸子實體의 組織에서

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分離한 것과 胞子에서 分離한 것은 그 發育에 差異가 없었다. 4) Hamada 培地에 各種 微量重金屬鹽類 $ZnSo_4$, $MnSo_4$, $MgSo_4$, $CaCl_2$ 枸櫞酸鐵 等의 添加는 別效果가 없었다. 5) 赤松鬚根抽出物 添加는 菌系發育에 確實한 效果가 있었다. 赤松鬚에는 松茸菌系의 生長을 促進하는 因子가 存在한다는 것을 알 수 있었다. 6) 松茸菌系 培養用 人工培養基의 C源으로서 glucose보다 蜂蜜의 效果가 크다. 7) Fairy Ring에 가장 많이 存在하는 微生物인 Mortierlla spp의 培養濾液을 松茸菌系 人工培養基에 添加한 것이 對照値에 比하여 菌系의 發育이 良好하였다. 8) 松茸人工原基의 培地에 赤松鬚根抽出物 Insitol Biotin 葉酸의 添加는 效果가 크다. 菌系의 蔓延된 後 温度를 내려서 19℃로 維持할 때 primordium의 形成을 確認하였다.

INTRODUCTION

The scientific name of pine mushroom is *Tricholoma matsutake* (s. Its & Imai) Singer, Agaricales 227, 1949 (published in 1951) *Armillaria matsutake* S. Ito & Imai, Bot. Mag. Tokyo: 39. 327. 1925. Pine mushroom is geographically distributed mainly in the *Pinus* genus and occasionally in *Tsuga*, *Abies*, and *Picea* genus in some part of Manchuria, the Korean Peninsula, and the Japanese Islands whose altitude reaches ±0m-2,000m above the sea.

Interestingly, although pine mushroom belongs to Pinaceae, it doesn't appear in *Larix* forest. Generally, pine mushroom develops on the 20-90 year old red pine forest, with the optimum range of age between 40 and 60 years. It develops on such acid rocks as granite, geneiss, sand stone. Although its has two seasons of development, namely in spring and autumn, its development concentrates on September and October. Since Korea is geographically covered with forests occupying about 70% which almost consists of red pine forest, it seems that nature has endowed Korea with high potential of pine mushroom field. Accordingly, some effective economical policies along these lines will contribute to the increase in the incomes which exceed those from main forest products. However, current levels of technology associated with artificial multiplication of pine mushroom do not assure its success because there remains so many areas to be solved in regard to pine mushroom cultivation. Only continous long term research will solve the many problems involved. Although pine mushroom is reported to be parasitic on the fungus root of red pine, there is no sufficient research to identify its essence.

Therefore, some attempts have been made for artificial method of cultivation. The main objective lies in the artificial cultivation through inoculating the artificial rudiment which is produced by culturing fungi seperated from pine mushroom, into red pine forest appropriately treated to develop pine mushroom.

It requires the environmental survey to study the pine mushroom development in red pine forest, in parallel with the research for life environment of pine mushroom fungi. Especially it is, therefore, imperative to establish the theory and methodology on the development and the growth of pine mushroom fungi, based on the research concerning their biological nature.

Presently, the artificial cultivation of pine mushroom requires more than the research on pine mushroom fungi themselves. To facilitate it, more study should be made on the relationship between roots of red pine and pine mushroom fungi, and on the environment of the pine mushroom development. Additionally, it is necessary to establish the technology connected with artificial rudiment and inoculation mentioned above. Since these areas are to wide to be covered here, this paper will concentrate on both the selection of the artificial cultivation radical and the cultivation of pine mushroom mycelium.

LITERATURE REVIEW

Unlike oak mushroom and agaric mushroom, pine mushroom has no established cultivation technology which relates to the transplantation of species fungi. The reason lies in the fact that oak mushroom and agaric mushroom form sporophore

even in the cultivation mycelium on the artificial culture medium because they, saprophyte, are characterized by vigorous growth potential and easy cultivation of fungi.

By contrast, it is difficult for pine mushroom to form sporophore in the cultivated mycelium. It also grows slowly under pure cultivation. Hence, the establishment of artificial cultivation of pine mushroom fundamentally requires the explanation of its characteristics.

The germination of pine mushroom spore in the sector of spore and mycelium in pure culture was stated to be easy by Mitumura,[14] Nisikada,[22,23,24,25] however, suggested opposite results.

Masi[17] and Nisikada[23] reported that pine mushroom mycelium showed more rapid growth when cultivated in the sporophore tissue, particularly in the external tissue of sporophore's basic part. Subsequently, spore and sporophore tissue were reported to show identical slow growth.

Although there appeared a number of research on pine mushroom between 1910s and 1930s, most of them less contributed to the sound development of basic research.

Tominaga[27,28,29,30,31] provided detailed reports relative to the formation and germination of sporophore spore of pine mushroom, the formation and the division of the secondary mycelium fungus root of pine mushroom and red pine, and the formation and the development of pine mushroom sporophore.

In relation to the separation of pine mushroom fungi and the pure cultivation of mycelium Hamada,[12,16]. Nisikada,[22,23] and Mitu[14,15,16] reported their research results. As a parent rock for the development of pine mushroom, Wakymizu[34] reported that granite quartz, porphyry, liparite, sand stone, amphibole, and sillceous were most appropiate.

According to Kinugawa,[11] a close relationship exists between the growth and the humidity of mycelium and sporophore. He also stated that the moisture content of the soil where pine mushroom grew was as low as 10-30% and that it had a significant influence on the growth of the mycelium of the soil. Besides the discussion of the soil pH by Hamata,[6] Russel (1950) reported that the range of the soil pH in pine mushroom was between 3.0 and 6.5, with its optimal range of 4.5 to 5.0. According to Kotama[10], fairy ring pH was 4.2 to 4.4. Hamata's[5] results indicated that growth temperature reached from 5°C to 30°C, with its optimal around 24°C.

Kodama[10] provided the report that normal sporophore could be developed when mycelium layer was cooled in the latter part of August, with soil temperature maintained between 18°C and 24°C.

As for artificial cultivation, a number of research has made pure cultivation of mycelium common practices since 1945, in addition to Misumura's report[14,15,16] on the artificial cultivation of pine mushroom. Much research results were also reported on the physiological ecology of pine mushroom and physical, chemical properties of mycelium.[1,3,4,5,7,8,13,26,33]

In Japan, Misumura[14] published that sporophore was made when pine mushroom mycelium made exogenous fungus root in the root of red pine in 1908.

Following Bernard in France, Burgeet in Germany also made active research on indigenous fungus root in 1909. Professor Melin[18,19,20,21] at Upsala university in Sweden was a leading scientist in the field of fungus root research. A wide range of research was conducted on the physiological ecology of the fungus forming fungus root, the method for the artificial cultivation of the fungus forming fungus root, physiology of fungus root, and environmental condition to form fungus root. It was in 1917 when Melin started making experimentation to synthesize mushroom mycelium including Boletus with young trees. Although Masui[17] began his fungus root research based on Melin's synthetic experiment, his conclusion on pine mushroom unfortunately failed to be supported by successive experiments.

His conclusion that pine mushroom mycelium was parasitic by absorbing sugar content from the

root of red pine can be accepted by the microscopic research on raw material.

Slankis in Canada wrote in detail on some conditions to which research on exogenous fungus root of forest trees was subject, in "Recent Advance in Botany" (1938-1942).

While Hiromoto[7] made detailed research on life relationship and pine mushroom fungus root, Misune did on the relationship between pine mushroom development and host, pine mushroom development, and red pine fungus root.

Following the Explanation of the conditions to form fungus root (conditions for settlement of mycelium by Bjorkman, fungus root research in the world entered into the stage where scholars including Harley studied the absorption and movement of form mycelium to root, using isotope.

MATERIALS AND METHODS

For the extraction of sporophore tissue and the separation of pine mushroom spore, fresh and complete sporophore was used, for other purposes usual existing radicals were used.

After putting sterilized Petri-dish into various agar cultivation radicals and dropping the spore onto the cultivation radical by the slurry method to fix a slice of pileus with vaseline, investigation was taken to see whether the spore kept at 24°C for germination.

In order to inoculate a variety of cultivation radical in relation to mycelium growth, the mycelium cultivated by test tube was used under the same condition. In regard to the separation from sporophore, the same size of mycelium cut by triangular needle within the stipe mycelium was inoculated.

General method of preparation was followed to obtain the barley extract and potato decoction. As a decoction to infiltrate the soil for pine mushroom development, a mixture composed of 200 g of the soil for pine mushroom development and 1,000 cc of well water were heated and filtered.

Besides, dry yeast selling at the market was added to red pine fibrous root extracting decoction made by red pine fibrous root into pieces, extracting by alcohol and finally eliminating the alcoholic and content from those filtered with germ. Filtered cultivation liquid of three weeks in the honey dry yeast KH_2PO_4 sterilized well water of Mortierlla spp which was separated from pine mushroom fairly ring.

RESULTS AND DISCUSSION

Although a wide range of research regarding pine mushroom is required for artificial cultivation of pine mushroom, immediate preconditions include

(1) a knowledge about the formation of culture medium where the growth of pine mushroom mycelium is faster and stronger than yeast glucose.

(2) an physiological investigation on the metabolism from mycelium to the formation of sporpphore.

Table 1. Relationship between the medium and germination of pine mushroom spore.

Media	Spore of Germination	Abstract
Boiled pine mushroom agar	–	Agar 2%
P.D.A.	+	"
Boiled barely decoction agar	+	Germination after 6 days in 3% boiled decoction poor growth of hypha.
Boiled red pine fibrous root decoction agar	–	No germination of spore until 20 days.
Boiled pine mushroom agar extracting from soil fluid	Germination of 7 days	Soil growing pine mushroom 200g + water 1,000cc.
Meyer's medium agar	–	Filtering after boiled.
Boiled pine mushroom extracting from soil fluid adding glucose agar	Germination of 6 days	Good hypha growth

The experimental results connected with (1) are shown in Table 1-11. Table 1. presents the liquid infiltrating the soil of pine mushroom development is effective for the germination of pine mushroom spore, while the artificial cultivation radical without natural substances such as Mr. Meyer agar gets no germination.

Table 2. indicates that the optimal temperature for the pine mushroom spore development is 24°C.

Table 3. reveals that pH 4.0-4.5 is the most appropiriate for the growth of developed mycelium of pine mushroom spore.

Table 4. also shows that 24°C is the optimal temperature for the growth of pine mushroom mycelium.

It can be seen from Table 5 that the optimal cultivation radical, the main objective of this experiment, of mycelium growth includes honey,

Table 2. Relationship between the temperature of culture and growth of pine mushroom mycelium

Temperature	After 4 days	After 6 days	After 10 days	Length of hyphe growth after 14 days
10°C	–	–	–	–
15°C	–	–	–	30 ~ 150μ
20°C	–	–	+	30 ~ 300μ
24°C	–	+	++	50 ~ 400μ
26°C	–	+	++	40 ~ 400μ
29°C	–	–	–	–
32°C	–	–	–	–

Inoculation of pine mushroom spore on Hamada's media adding 3% of honey instead of glucose

Table 3. Relationship between the H-ion concentration of medium and germination of pine mushroom spore.

pH	After 4 days	After 20 days	Abstract
8.5	–	–	Media; honey, boiled red pine mushroom extracting from soil fluid, dry yeast, pH of agar : 2.5 – 8.5. Filtering after 3% of honey, 200g of boiled pine mushroom extracting from soil fluid and 1 l of water
7.5	–	–	
6.5	–	–	
5.5	+	++	
5.0	+	+++	
4.5	+	+++	
4.0	+	+++	
3.5	–	–	
2.5	–	–	

Table 4. Relationship between the temperature of culture and growth of pine mushroom mycelium

Temperature	After 14 days	After 28 days	After 42 days	After 56 days
0°C	– mm	– mm	– mm	– mm
5	–	+	6.0	6.5
10	+	6.0	9.0	12.0
15	5.5	9.5	17.0	23.0
20	7.0	12.0	22.5	29.0
24	7.0	15.0	26.0	30.5
27	7.0	12.0	15.0	18.0
30	4.5	4.5	5.5	6.5
32	–	–	–	–
35	–	–	–	–

Media ; dry yeast: Boiled pine mushroom extracting from soil fluid; honey.
Red pine fibrous root extracting decoction: KH_2PO_4 · Inositol, Biotin.

Table 5. Relationship between the medium and growth of pine mushroom mycelium.

Media	Degree of growth	Diameter of mycelium cluster	Abstract
Boiled pine mushroom agar extracting soil fluid	+	5.0mm	Cultering for 3 days, 24°C
Boiled red pine fibrous root decoction · Boiled pine mushroom agar extracting from soil fluid	+	5.5	
Glucose · Boiled pine mushroom agar extracting from soil fluid	+++	10.0	glucose 2%
Raulin's decoction agar	–		
Peffer's decoction agar	–		
Honey · Boiled pine mushroom extracting from soil fluid · Dry yeast agar	++++	15.0	Honey 40g, water 1l, Dry yeast 5g
Hamada's medium	+++	11.0	
Honey · Boiled pine mushroom extracting from soil fluid · Red pine fibrous root extracting decoction · Dry yeast agar	++++	15.5	Filtering sterilized alcohol extract from pine root
Narkrans synthetic medium	+++	10.0	
Honey · Boiled pine mushroom extracting from soil fluid · Red pine fibrous root extracting decoction · Dry yeast · KH_2PO_4 · Inositol · Folic acid · Biotin	++++	16.5	Maximum growth of hypha

pine mushroom, boiled pine mushroom extracting soil fluid, red pine fibrous root extracting decoction, dry yeast, KH_2PO_4, Inositol, Biotin, the cultivation radical of folic acid, and some other similar cultivation radicals, and that no mycelium was multiplicated in the pure artificial cultivation radical such as Raulin's liquid agar and Peffer's liquid one. This represents the fact that there exists the possibility to search better cultivation radical.

Table 6 shows that when a part of sporophore was transplanted to multiply mycelium the specific part of sporophore itself has no significant effect on the growth of mycelium.

According to table 7, although micro heavy metal salts constitutes of an essential element for the cultivation of fungi, there appears no significant difference between a mixture of Hamada's media with micro heavy metal salts and control.

It indicates that the addition of micro heavy metal salts into cultivation radical has no effects.

The effect due to the addition of micro heavy metal salts can be expected when the composition of either Peffer or Meyer cultivation radical is purely refined and removed from micro heavy metal salts. However, since generally used composition

Table 6. Comparison of growth of mycelium isolated from spore and body of pine mushroom.

Part of gathering	After 14 days	After 28 days	Abstract
Base part of stipe	7.0mm	14.5mm	Diameter of mycelium cluster at 24°C
Lower part of stipe	6.5	14.5	
Inside part of stipe	7.0	15.0	
Upper part of stipe	7.5	14.5	
Inside part of pileus	6.8	14.5	

Media: Honey · Boiled pine mushroom extracting from soil fluid.
Red pine fibrous root extracting decoction · Dry yeast · Inositol · Agar.

Table 7. Effect of the growth of mycelium adding micro havey metal salts.

Division	After 15 days	After 30 days	Abstract
Hamada's medium	5.0mm	11mm	24°C Culture
Hamada's medium	5.5mm	11mm	
+ Micro havey metal salts	$ZnSO_4$ $7H_2O$ 4.5mg	$MnSO_4$ $4H_2O$ 5mg	Ferric citrate 5mg
	$MgSO_4$ $7H_2O$ 500mg	$CaCl_2$ $2H_2O$ 55mg	
	H_2O 1,000cc		

Table 8. Effect of growth of pine mushroom mycelium using promoters containing in fibrous root of pine

Division	After 5 days	After 15 days	After 25 days	After 35 days	Abstract
Honey · yeast · Agar		6.5mm	12mm	12.5mm	24°C Culture
Honey · yeast · Red pine fibrous root extracting decoction · Agar	+	7.5	14	15	
Glucose · Boiled pine mushroom extracting from soil fluid · Agar		6.0	11	12	
Glucose · Boiled pine mushroom extracting from soil fluid · Red pine fibrous root extracting decoction	+	7.0	13	14.5	

Filtering alcohol extract after pulverization red pine fibrous root.

of cultivation radical itself contains appropriate amount of micro heavy metal salts required for the growth of mycelium, the more addition of it gets no effect.

Table 8 presents the evidence that red pine fibrous root extracting decoction includes the matter which increases the growth of pine mushroom mycelium.

It has an implication relating to the fact that pine mushroom is parasitic on red pine fungus root.

According to table 9, although the relationship between *Mortierlla* spp. which has its densest distribution in Fairy Ring of the pine mushroom development forest and pine mushroom mycelium is unknown, it is clear that the addition of cultivation filter decoction into Hamada's media resulted in greater effect than the mycelium breeding of control.

Table 10 shows that the growth of mycelium in case of pine mushroom inoculation is not significantly differant from that in case of inoculation after the separation of sporophore tissue.

Table 9. Effect of the growth of pine mushroom mycelium using culture solution of Mortierlla SPP.

Division	After 14 days	After 28 days	Abstract
Hamada's medium	6.0mm	11.5mm	24°C Culture
Hamada's medium · Culture solution of Mortierlla SPP	7.0mm	13.5mm	

Mortierlla SPP separated from Faily ring of red pine mushroom was cultural on media composed of honey · yeast · KH_2PO_4 · water

Table 10. Comparison of growth of mycelium which are isolated from spore and body of pine mushroom (24°C)

Division	After 14 days	After 28 days	Abstract
Separation of pine mushroom spore	7.0mm	15.0mm	Spore was collected and transplant transplantation by triangle needle
Separation of sporophore	7.5	15.0	

Media : Honey · Dry yeast · Boiled pine mushroom extracting from soil fluid · Red pine fibrous root extracting decoction · Inositol · Folic acid · KH_2PO_4.

Table 11. Comparison of the growth of pine mushroom mycelium using glucose and honey as source of Carbon (24°C)

Division	After 14 days	After 28 days	Abstract
Glucose · yeast · Agar	6.0mm	12.0mm	Diameter of spore cluster.
Honey · yeast · Agar	7.0mm	13.0mm	Glucose, honey 2%, respectively.

In Table 11, it can be seen that adding honey has the dominating influence of glucose and honey as a C source which is essential for artificial cultivation radical.

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