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Microfungal flora of *Tricholoma matsutake* producing and nonproducing sites in the forest of *Pinus densiflora*

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적송 (*Pinus densiflora*) 림내 송이(*Tricholoma matsutake*) 발생지와 미발생지의 토양 균류의 수직 분포

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ABSTRACT: The vertical distribution of the fungal population for the soil samples from two sites of producing and nonproducing of *Tricholoma matsutake*, song-yi mushroom, were examined at Yangyang and Myung-joo, Gangweon province . By the dilution plate method, a total number of propagules of fungi per gram of soil was observed to be low at the song-yi producing sites but high at the song-yi nonproducing sites under the communities of *Pinus densiflora*. The tendency of the number of fungal propagules were decreased with the increasing vertical depth. In the incubation method at 42°C, six genera and nineteen species of the fungi were isolated from two sites; *Aspergillus fumigatus, Acremonium* sp., *Talaromyces stipitatus, Penicillium lilacinum, P. oxalicum* and *Westerdykella multispora*. The most dominant species by this method was *A. fumigatus*. From heat treatment method at 70°C, seven genera and nineteen species were isolated; *Aspergillus fumigatus, Alternaria alternata, Neurospora sitophila and Mucor* sp.. In the ethanol treatment method, one genera and one species was isolated *Mortierella* sp.. From the three isolation methods, it was found that the total number of the soil fungi and the frequency of species appeared were the highest at the soil of upper layer whereas the lowest at the soils of lower layer in its vertical distribution.

KEYWORDS: Microfungal flora, *Tricholoma matsutake, Pinus densiflora*.

Tricholoma matsutake, song-yi, was a wild product known as a delicate flavour of food. Recently, Korean song-yi mushroom was exported to Japan and foreign countries, so that it contributed to the Korean GNP and the rural economy.

Gradually the value of *T. matsutake* begins to increased, so the investigation for *T. matsutake* production was initiated early in the 1960's. There after, it was found that *T. matsutake* was ectomycorrhizae by the physiological and ecological investigation (Ito, 1981; Ogawa, 1981a, 1981b; Kang *et al.*, 1989).

However it is almost impossible to practice a series of experiments for the symbiosis on all of mycorrhizal fungi because of the difficults of isolation, pure culture and inoculation (Ogawa, 1985).

At the present, the condition of song-yi growth, physiological characteristics, growth of Shiro (mycelium mass of *T. matsutake*) and a process of formulation in *Pinus densiflora* forest, mycorrhizae of *T. matsutake* and its activation have been studied (Ogawa, 1981 a). Beside, acknowledge about competition between other filamentous fungi or soil microorganisms and Shiro, and ecological change were understood by Ogawa (1981b, 1985).

The morphological features of mycorrhizae are depending on the combination of both hosts. Moreover the ectomycorrhizae being formed on a plant species are different by each fungal partner. The estimation of mycorrhizal masses ('Shiro') and the identification of fungal partners are of important to study the ecological roles of mycor-

rhizal fungi in the forest ecosystem. The number of soil fungi was reduced in the mass of the Shiro, but the normal fungal flora was recovered according to the diappearance of desiccation of soil. The Song-yi Shiro as it was like arc could be formed at either wet site or dry site and probably its original form was circular forming fairy ring. So it was assumed that the frequncy of Shiro formation was high in the soil of song-yi production and the fruit body production also continued for long time. *T. matsutake* from fruiting body from *P. densiflora* forest from 15-20 years to 40-50 years.

The flora of higher fungi in forest are dependent on host plants, so soil properties and soil microbial flora are also continuously variable due to the development of forest ecosystem(Ogawa, 1981a). Hydrogen ion concentration of song-yi production soil was pH 4.2 - 4.5 in Japan(Ogawa, 1981b), pH 6.55 in Korea(Ryoo *et al.*, 1980), while that of song-yi nonproduction soil was pH 5.4-6.1 in Korea(Ryoo *et al.*, 1980). The song-yi producing sites in Korea was eastern part of Taebaek Mountains of Gangweon prefecture. Two sites of Yangyang and Myung-joo, are one of the typical production sites of *T. matsutake.*

This paper describes the population of the soil microfungi and vertical distribution in the production and nonproduction sites of *T. matsutake.* Isolation and vertical distribution of the microfungi in two sites was investigated by the incubation method, heat treatment method, and ethanol treatment method.

Materials and methods

Sampling sites

Soil samples were collected from two sites of song-yi production and nonproduction on Yangyang and Myung-joo area at July and August, 1989. Soils were taken vertically from each site of *P. densiflora* forest. Soil samples were divided into seven parts representing depths of fallen leaves layer, 1 cm (surface layer), 1-5 cm, 5-10 cm, 15-20 cm, 20-25 cm, and 25-30 cm from the soil surface.

Media

Media for isolation and identification of fungi were Malt-Yeast Agar(MYA), Malt Extract Agar (difco manual, MA), Czapek's solution Agar(CA), Malt Extract Agar(MEA), and Potato Dextrose Agar(PDA) (Min *et al.*, 1981).

Isolation and identification

Depth fluctuation of total fungal propagules at two areas including the several layers of soil, and incubation method at 42℃, heat treatmemt method at 70℃ for 15 minutes and ethanol treatmemt method were applied in this experiment.

The soil sample was suspended in 5 m*l* of sterilized water, and 0.2 m*l* of the suspension was spread onto MYA plate for the dilution plate method. For the incubation method at 42℃, these plates were incubated at 42℃ for two days and the isolates were transferred to malt agar (MA) slants and incubated at 37℃ for growth.

For the heat treatment, one part of the suspension was treated at 70℃ for 15min and 0.2 m*l* of the suspension was spread onto the MYA plate, and incubated at 24℃ for the isolation. For the ethanol treatment method, 0.3 m*l* of the soil suspension was resuspended in 0.3 m*l* ethanol absolute, and 0.2 m*l* of the resuspension was spread onto MYA plate. These plates were incubated at 24℃ for seven days and the isolates were transferred to malt agar (MA) slants and incubated at 24℃ for growth. The isolates were identified by the several papers (Gilman 1957; Ito and Yokoyama, 1981; Matsushima, 1975; Raper & Thom, 1986; Raper & Fennell, 1965; Ellis, 1971, 1976).

Dilution method

Each soil suspension prepared was different in its soil amounts; one gram of soil from fallen leaves layer-5 cm depth, one and half gram of soil from 5-10 cm depth, two grams of soil from 10-20 cm depth, two and half grams of soil from 25-30 cm depth and three grams of soil from 25-30 cm depth. Each soil sample was added to 10 m*l* of saline in test tube and shaked thoroughly. The aliquots were referred to as soil suspension. A part of soil suspension, 0.2 m*l*, was spreaded on MYA plate and incubated at 42℃ for two days followed by the incubation at 37℃ to isolate the

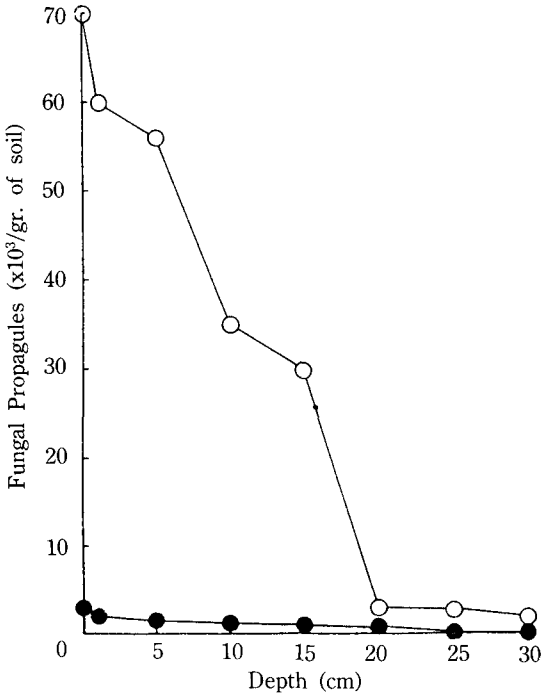

Fig. 1. Vertical distribution of total fungal population at Yang-yang. Song-yi producing site (●) and song-yi nonproducing site (○).

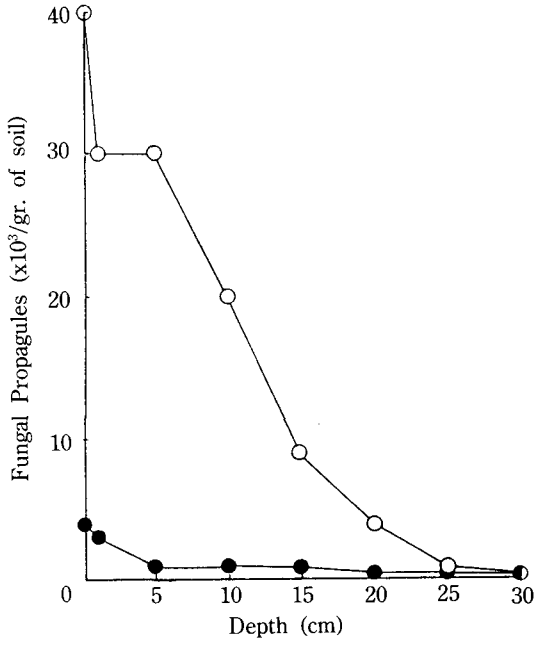

Fig. 2. Vertical distribution of total fungal population at Myung-joo. Song-yi producing site (●) and song-yi nonproducing site (○).

fungi. Some part of each soil suspension, 0.5 m*l* was treated at 70℃ for 15 minutes. Other part of each soil suspension 0.3 m*l*, added to 0.3 m*l* ethanol absolute. After this resuspension was treated at room temperature for 15 minutes. From this aliquot, each duplicate of 0.2 m*l* soil suspension was spread on MYA plate and incubated at 24℃ for the isolation of other groups of fungi.

Results

Soil samples were collected from two sites, Yang-yang and Myung- joo in Gangwon prefecture, from July to August, 1989.

All soil samples used in the present investigation were subjected the dilution plate method, the incubation method at 42℃, heat treatment method, and ethanol treatment method.

Vertical distribution of fungal propagules

The results of the dilution plate method obtained soils from song-yi producing and song-yi nonproducing in two areas were shown in Fig. 1 and 2.

Comparing with the vertical distribution of fungal population at two sites, the number of the fungal propagules showed on the dilution plates was the highist in the soil of the upper layer followed by the middle layer and the lower layer in soil samples at song-yi nonproducing of two areas, while the fungal flora from each soils of song-yi producing did not show the fluctuation with depth as the same results described above. At the upper layer in each field, more than 10^4 propagules per gram of soil were obtained comparing with that of the middle or the lower layers.

Generally, the decresing tendency of the number of fungal propagules per gram of soil with the increase of depth was proved in the soils of song-yi nonproducing at two areas.

Fungal isolation by the incubation method

1) Fungal isolation from two sites

From the soil samples collected from the song-yi producing soil at Yang-yang area, 7 species classified in 5 genera were isolated as shown in Table I, comprising 2 species of Ascomycotina and

Table I. List of microfungi from song-yi producing and song-yi nonproducing sites by the incubation method at 42℃ at Yang-yang (Fungal propagules/grams of soil).

Sites	Song-yi production								Song-yi nonproduction								Total
fungi \ Depth (cm)	S*	1	5	10	15	20	25	30	S*	1	5	10	15	20	25	30	
Ascomycotina																	
Talaromyces																	
emersonii												17					17
T. stipitatus		13	13	9						13							48
Thermoascus																	
aurantiacus												6	6				12
Westerdykella																	
multispora				8	6	5								15	8	7	49
Deuteromycotina																	
Acremonium																	
sp.							4	4									8
Alternaria																	
alternata									5	13							18
Aspergillus																	
clavatus											12				4		16
A. flavus										13							13
A. fumigatus	37	25	12								25						99
A. ochraceus	38	12															50
A. terreus						6											6
Penicillium																	
echinulatum															10		10
P. frequentans		13	12	8													33
P. lilacinum												17	12				29
P. oxalicum									38	25	13						76
P. ruburum														5			5
P. variable									50	25							75
P. viridicatum													*19*				*19*
Unidentification	13	13	12	9	6	5	4	3	38	13	25	6	6	10	13	17	193
Subtotal	88	76	49	34	18	10	8	7	131	102	75	46	43	30	35	24	
Total							290									486	776

S*: surface, fallen leaves layer

5 species of Deuteromycotina. Fourteen species classified in six genera were obtained from the song-yi nonproducing soils as shown in Table I, consisting 4 species of Ascomycotina, and 10 species of Deuteromycotina. As shown in Table II, microfungi from the soil samples by the incubation method at 42℃ at Myung-joo area was investigated. It was found that 2 species of Ascomycotina and 5 species of Deuteromycotina were isolated from song-yi producing sites, while 4 species of Ascomycotina and 9 species of Deuteromycotina from song-yi nonproducing sites.

As shown in Table I and II, fungal species identified were *Acremonium* sp., *Aspergillus fumigatus,*

Table II. List of microfungi from song-yi producing and nonproducing sites by the incubation method at 42℃ at Myung-joo area (Fungal propagules/grams of soil).

Sites / Depth (cm) / Microfungi	Song-yi production								Song-yi nonproduction								Total
	S*	1	5	10	15	20	25	30	S*	1	5	10	15	20	25	30	
Ascomycotina																	
Talaromyces emersonii												25					25
T. stipitatus			15	8						13							34
Thermoascus aurantiacus													6				6
Westerdykella multispora					12	10	4							10	8	7	51
Deuteromycotina																	
Acremonium sp.						10	8	4									22
Alternaria alternata									25	12							37
Aspergillus clavatus											25						25
A. flavus										13							13
A. fumigatus	25	13	12	8	6	5	4	3	25	25	25	18	12	5	4	3	193
A. ochraceus	25	13															38
A. terreus				8	6												14
Penicillium echinulatum														5			5
P. frequentans			13	9													22
P. lilacinum											25	17	12				54
P. oxalicum									25	25							50
P. ruburum														10			10
P. variable									25	13							38
Unidentification	25	25		8					25	25	15	44					167
Subtotal	75	51	38	41	24	25	16	7	125	126	90	104	30	30	12	10	
Total								277								527	804

S*: surface, fallen leaves layer

A. ochraceous, A. terreus, Penicillium frequentans, Talaromyces stipitatus, and *Westerdykella multispora* from soil samples at song-yi producing site in Yang- yang and Myung-joo, on the other hand, *Alternaria alternata, Aspergillus clavatus, A. flavus, A. fumigatus, Penicillium echinulatum, P. lilacinum, P. oxalicum, P. ruburum, P. variable, P. viridicatum, Thermoascus aurantiacus, Talaromyces stipitatus, T. emersonii,* and *Westerdykella multispora* from soil samples at song-yi nonproducing site in Yang- yang and Myung- joo.

From these results, it was concluded that *A. fumigatus* was dominant from soil samples at song-yi producing and nonproducing sites in two areas.

2) Distribution of microfungi

Incubation of the plate culture at 42℃ greatly reduced total fungal population and number of co-

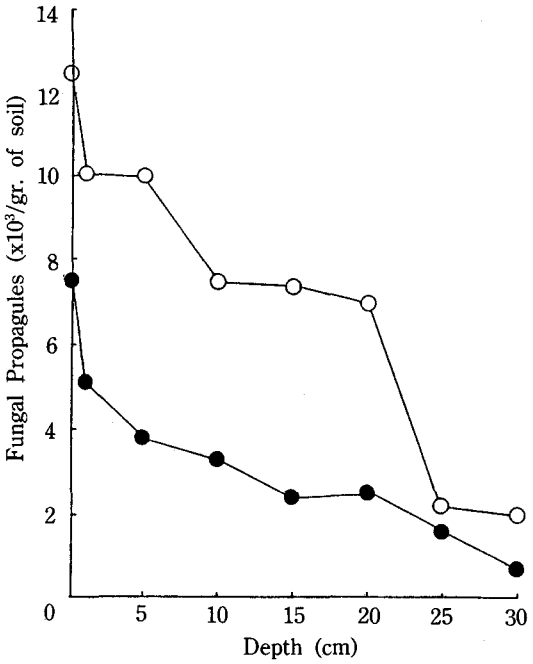

Fig. 3. Vertical distribution of fungal population at Yang-yang by the incubation method. Song-yi producing site (●) and song-yi nonproducing site (○).

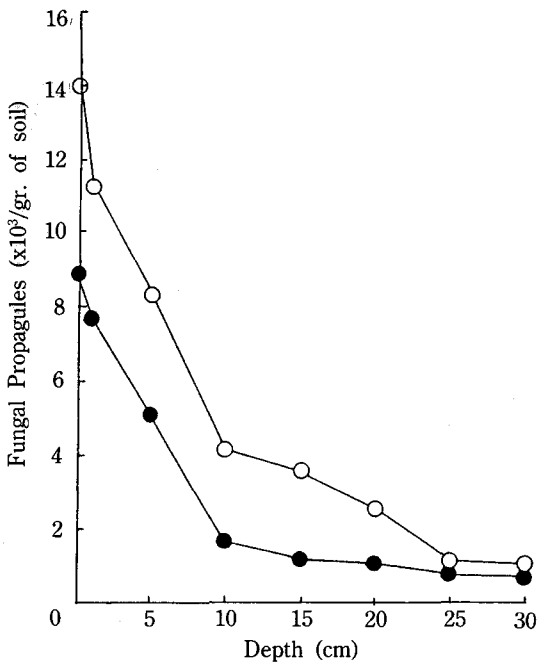

Fig. 4. Vertical distribution of fungal population at Myung-joo by the incubation method. Song-yi producing site (●) and song-yi nonproducing site (○).

lonies from song-yi producing soil samples showed small number of propagules than those of song-yi nonproducing soil samples.

The fungal population per gram of soil was decreased with the increased depth from the soil surface at any sampling sites. The number of fungal propagules per gram of soil by the selection method of incubation at 42℃, were particularly of dominant species with *Aspergillus fumigatus*. Vertical distribution of dominant species was the highest at the upper layers, decreased at the middle and lower layers. These results were in good agreement with the tendency of fungal distribution at four sampling sites.

Fungal selection with heat treatment method

1) Fungal species isolates at two sites

From the heat treatment at 70℃, 9 species in 4 genera were isolated from song-yi producing sites and fifteen species in seven genera from the song-yi nonproducing sites in Yang- yang, as shown in Table III.

From the soil samples of Myung-joo, five species in three genera and twelve species in seven genera were isolated from the soil samples of song-yi producing and song-yi nonproducing sites in Myung-joo, respectively. Microfungi were composed of four species in Deuteromycotina and one species of Zygomycotina in song-yi producing sites, while eight species in Deuteromycotina, three species in Ascomycotina and one species Zygomycotina in song-yi nonproducing sites at Myung-joo area (Table IV).

Fungal species isolated from two sample sites was *Aspergillus fumigatus, A. ochraceus, A. niger, P. anthinellum, P. nigricans, Mucor* sp., *Alternaria alternata, Aspergillus fumigatus, A. versicola, Penicillium challisii, P. cyclopium, P. echinulatum, P. lilacinum, P. puberulum, P. variable, Neurospora sitophila, Talaromyces stipitatus, Westerdykella multispora,* and *Mucor* sp. In the heat treatment method at 70℃, it was found that most microfungi isolated were upper(surface-10 cm) and middle layer(10-20 cm) showing that they are thermotolerant fungi. From these results, it was found that *A. fumigatus* was dominant species at two sites.

2) Vertical distribution of soil fungal popula-

Table III. List of microfungi from soil samples of song-yi producing and song-yi nonproducing by heat treatment method at Yang-yang (Fungal propagules/grams of soil).

Sites	Song-yi production								Song-yi nonproduction								Total
Microfungi \ Depth (cm)	S*	1	5	10	15	20	25	30	S*	1	5	10	15	20	25	30	
Ascomycotina																	
Neurospora																	
sitophila									13	13							26
Talaromyces																	
stipitatus											17	12					29
Westerdykella																	
multispora					12	10	4			13	8	6					53
Deuteromycotina																	
Alternaria																	
alternata									38	25	12	8					83
Aspergillus																	
fumigatus	38	25	25	8	6	5	2		25	25	13	8	6	5	4	3	198
A. ochraceus	25																25
A. niger							8										8
A. versicolor										13							13
A. terreus				8	6												14
Penicillium																	
charlesii											13						13
P. cyclopium									25	12							37
P. echinulatum													12	10	4	3	29
P. funiculosum									12	12							24
P. granulatum	25										13						38
P. janthinelum								4									4
P. lilacinum															4	3	7
P. nigricans								3									3
P. puberulum											13						13
P. variable									13	12							25
Zygomycotina																	
Mucor sp.	13	13	13						13	13	13	8	6	5	2	3	102
Unidentification	13	13	12	17	12	10	4	4	13	13	13	9	6	5			144
Subtotal	114	51	50	33	36	25	18	11	152	151	115	51	30	25	14	12	
Total							338								550		888

S*: surface, fallen leaves layer

tion

In heat treatment of soil suspension at 70℃, total fungal population reduced at two sampling sites as shown in Fig. 5 and 6, as compared with direct dilution plate method(Min *et al.*, 1981, 1982, 1987).

The results in Fig. 5 and 6 showed that the fungal population isolated by the heat treatment

Table IV. List of microfungi from soil samples of song-yi producing and song-yi nonproducing by heat treatment method at 70℃ at Myung-joo (Fungal propagules/grams of soil).

Sites	Song-yi production								Song-yi nonproduction								Total
Microfungi \ Depth (cm)	S*	1	5	10	15	20	25	30	S*	1	5	10	15	20	25	30	
Ascomycotina																	
Neurospora																	
sitophila									13	12	9						34
Talaromyces																	
stipitatus												9	6				15
Westerdykella																	
multispora											12						12
Deuteromycotina																	
Alternaria																	
alternata									25	25	12	8					70
Aspergillus																	
fumigatus	25	25	13	9	6	6	4	3	25	13	13	8	6	6	4	4	170
A. niger			25														25
A. ochraceus	38	13							13	13							77
Penicillium																	
charlesii												13	8				21
P. cyclopium									25	13							38
P. echinulatum														12	10		22
P. funiculosum									13	12							25
P. granulatum		13															13
P. lilacinum																4	4
Zygomycotina																	
Mucor sp.	13	13	13						13	13	12	9	6	5	4	4	105
Unidentification	13	13	13	8	6	5	4	4	13	13	13	8	6	5	4	3	131
Subtotal	89	77	64	17	12	11	8	7	140	114	71	55	32	28	22	15	
Total							285								477		762

S*: Surface, fallen leaves layer

method at 70℃ were very low, but it was dependent on the soil depth and producing or nonproducing sites of *T. matsutake.*

From these results, it was concluded that the distribution of the fungi at each soil depth was found to be the same tendency as those obtained by the incubation method at 42℃. Total number of microfungi from soil samples of song-yi nonproducing sites were higher than those from soil sample of song-yi producing sites. And total number of colonies were higher than the results of incubation method at 42℃.

Dominant species by heat treatment method at 70℃ was *A. fumigatus,* and vertical distribution of dominant species was related with soil depth, although the dominant fungi were remained more or less constant at sites of song-yi producing and song-yi nonproducing in two areas.

Fungal isolation by the ethanol treatment method

For the ethanol treatment method, 0.3 m*l* of soil suspension was added to 0.3 m*l* of absolute

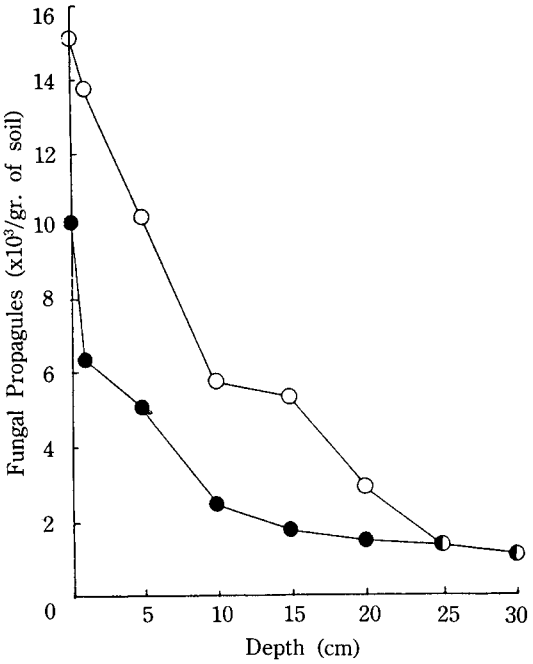

Fig. 5. Vertical distribution of fungal population at Yang-yang by the heat-treatment method. Song-yi producing site (●) and song-yi nonproducing site (○).

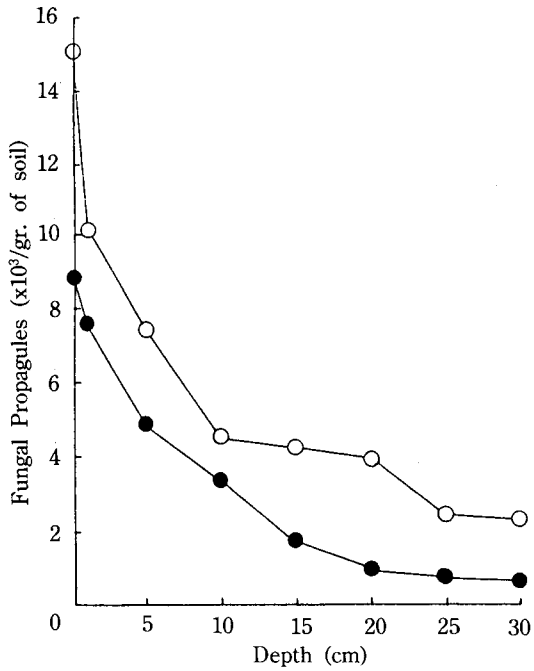

Fig. 6. Vertical distribution of fungal population at Myung-joo by the heat-treatment method. Song-yi producing site (●) and song-yi nonproducing site (○).

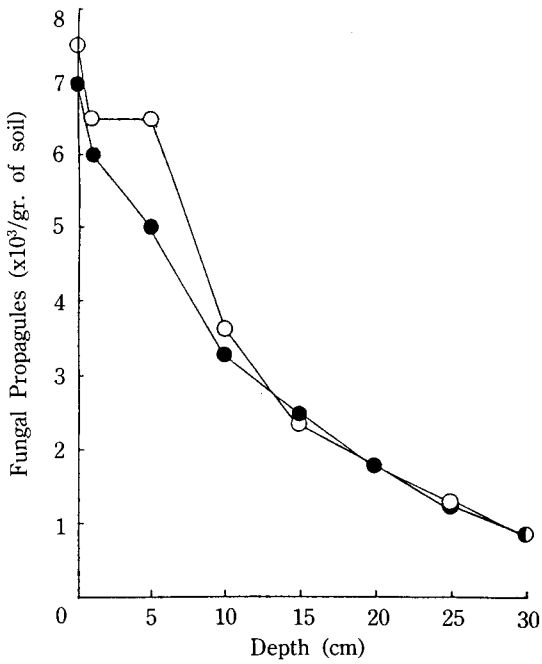

Fig. 7. Vertical distribution of fungal population at Yang- yang by the ethanol-treatment method. Song-yi producing site (●) and song-yi nonproducing site (○).

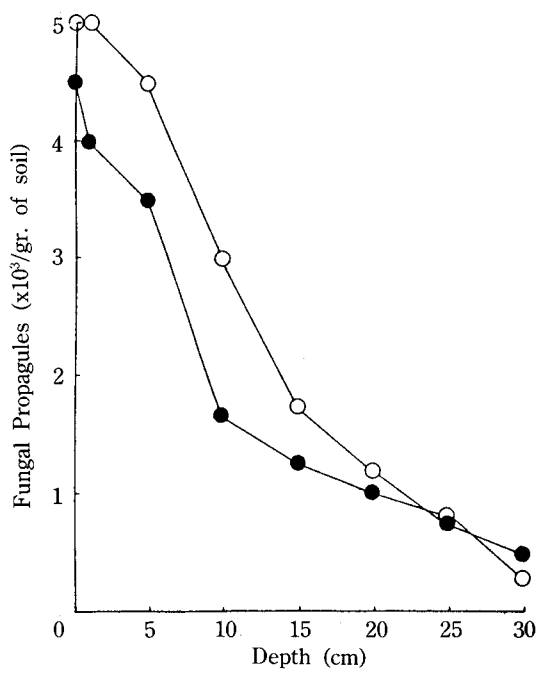

Fig. 8. Vertical distribution of fungal population at Myung-joo by the ethanol-treatment method. Song-yi producing site (●) and song-yi nonproducing site (○).

ethanol, after 15 min. incubation, 0.2 m*l* of the suspension was spread onto MYA plates, these plates were incubated at 24℃ for seven days and isolates were transferred to malt agar(MA) slants and incubated at 37℃ for growth.

The results were shown in Fig. 7 and 8. From these results, the number of fungal propagules per gram of soil and vertical distribution of soil fungal population were decreasing with the increasing depth the same as the incubation method and heat treatment method. But there was not a difference between two sites. In the ethanol treatment method at two sites, it was found that the dominant species was *Mortierella* sp.

Discussion

The present study was carried out to investigated the soil microfungal population and vertical distribution in song-yi producing sites and song-yi nonproducing sites . In this experiment, sampling and isolation method were used for the method of Min *et. al.* (1981 , 1987).

In comparision of the soil microfungi, the number of colonies from samples of nonproducing sites of song-yi was two times higher than that of song-yi producing sites. Also in vertical distribution study, the number of colonies were greatly reduced in proportion to the increasing depth in the dilution method. It was investigated that the decrease of microfungal population in song-yi producing site was agreed with the result of investigation by Ito(1981) and Ogawa(1981a).

The results of the vertical distribution showed almost the same temdency according to the three different experiments of incubation method(Warcup, 1955), heat treatment method(Awao & Mitsugi, 1973; Awao & Otsuka, 1973, 1974; Minoura *et al.*, 1973) and ethanol treatment method. Among isolated filamentous fungi from two sites, it is shown that dominant genera were *Aspergillus* and *Penicillium.*

摘 要

송이 (*Tricholoma matsutake*) 생산의 대표적인 지역으로 알려진 강원도 양양 지역과 명주 지역을 선택하여 송이 발생지와 미발생지의 미세균류를 분리 동정하였으며 수직분포를 조사하였다. 진균의 선별방법으로 희석법, 42℃에서 48시간 배양 후 37℃로 배양하는 방법, 70℃에서 15분간 열처리 방법, 그리고 ethanol처리 방법으로 토양의 수직적 분포를 파악하였다. 42℃배양법에서는 두 장소에서 분리된 토양균은 7속 18종이 분리되었으며, 송이 발생지에서는 *Aspergillus fumigatus, A. ochraceus, A. terreus, Acremonium sp., Penicillium frequentans, Talaromyces stipitatus*가 분리되었으며, 송이 미발생지에서는 *Aspergillus fumigatus, Penicillium lilacinum, P. oxalicum, Westerdykella multispora*가 우점종으로 나타났다. 70℃ 열처리 방법으로 분리한 경우 7속 20종이 분리되었으며, 송이발생지에서는 *Aspergillus fumigatus, Mucor* sp. 가 우점적으로 나타났으며, 송이 미발생지에서는 *A. fumigatus, Alternaria alternata, Mucor* sp., *Neurospora sitophila*가 우점종으로 나타났다. Ethanol처리 방법으로 분리한 경우는 1속 1종이 분리되었으며, 송이 발생지와 미발생지에 관계없이 우점종은 *Mortierella* sp.로 분류되었다. 이들 분리된 토양균은 전체균의 수와 우점종균수 그리고 나타나는 종의 빈도수는 그 수직적 분포에 있어서 상층에서 하층으로 내려갈수록 감소하였다.

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