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Analyses in Biogeographic Distribution and Colonization Strategy of Ectomycorrhizal Ammonia Fungi in *Hebeloma* Subgenus *Porphyrospora*

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Porphyrospora 亜属菌の生物地理的分布と増殖戦略の解析

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ABSTRACT

Occurrences of ectomycorrhizal ammonia fungi belonging to *Hebeloma* subgenus *Porphyrospora* have been recorded from Japan, New Zealand, and Australia. The aligned sequences were analyzed based on their β-tubulin genes (long and short sequences) by neighbor-joining method. The phylogenetic trees indicate that specimens examined can be segregated into two groups, one comprising *Hebeloma* sp. from New Zealand and *H. aminophilum,* and another containing *H. vinosophyllum.* To clarify the taxonomic rank of the two groups, mating tests were undertaken among *H. vinosophyllum* from Japan, *Hebeloma* sp. from New Zealand and *H. aminophilum* from Australia. The results are consistent with the groupings that *Hebeloma* sp. from New Zealand is the same species to *H. aminophilum.*

Thereafter, studies based on physiological mechanism were carried out to elucidate the biogeographic distribution and the colonization strategy of these ammonia fungi. The results show isolates grew in ammonium-nitrogen concentrations from 0.1 mM to 300 mM where 3 mM was the optimum ammonium-nitrogen concentration and adapted to pH 4.0 to pH 8.0 where pH 6.0 and pH 7.0 were the optimum values for these isolates to grow under 25°C incubation.

Basidiospore germination in *Hebeloma vinosophyllum* was stimulated by 10 mM to 500 mM NH_4Cl aqueous solution at pH 4.5 to 9.0. The basidiospore germinated at 10°C to 35°C with an optimum at 25°C to 30°C. The percent germination value decreased with the increased duration of storage both under dry and wet conditions. There was 19.3% germination ability left

when stored at 15°C under dry condition for 150 days. The humidity and temperature affected the longevity of *H. vinosophyllum* basidiospores.

Monokaryon of *Hebeloma vinosophyllum* was also isolated and cultured to fruit in *vitro*. Though fruiting characteristics were different from dikaryon, the monokaryotic fruiting was able to produce basidiospores which germinated when suspended into NH_4Cl aqueous solution. This indicates ammonia fungi as pioneer one in forests or grass lands should have propagation strategies for colonization following a large amount of ammonium-nitrogen disturbance.

GENERAL INTRODUCTION

"Ammonia fungi" are defined as a chemoecological group of fungi that have been recognized by experimental observation by Sagara & Hamada (1965) and Sagara (1973, 1975). These fungi appear on the disturbed habitats by a high concentration of ammonium-nitrogen with sequentially developing reproductive structures (Sagara 1975). The sequential appearance of reproductive structures (successive occurrence) of these fungi generally proceeds as follows: anamorphic fungi → ascomycetes → smaller basidiomycetes → larger basidiomycetes (Sagara 1975). Ammonia fungi are divided into two groups, one comprising species that can be induced to fruit under laboratory conditions and another comprising species that appear only in the field when nitrogenous materials such as urea is applied (Sagara 1975). The appearance of reproductive structures of ammonia fungi results either from mycelial growth that prefers or tolerates high concentrations of ammonium-nitrogen under alkaline to neutral conditions, and/or from spore germination that is stimulated by ammonium-nitrogen under the same conditions (Suzuki 1989, 2006). The nutrient modes of ammonia fungi can be divided into two types: saprobic and biotrophic (ectomycorrhizal), based on the observation of mycorrhiza formation of the fungal species in the field (Sagara 1975, 1995; Fukiharu & Hongo 1995; Sagara et al. 2000), and based on the measurement of cellulolytic and ligninolytic enzyme activities of the fungi (Enokibara et al. 1993; Yamanaka 1995a; Soponsathien 1998a, b). In the field, the saprobic fungi occur earlier, and fruit for shorter period, than ectomycorrhizal species (Sagara 1975; Yamanaka 1995b). Reproductive

structures of ammonia fungi have generally disappeared by four years after the initial stimulus (Sagara 1975). Under natural conditions, ammonia fungi fruit on the forest floor or in grassland after decomposition of the animal body or excrement, and are included in the broader ecological group of "postputrefaction fungi" (Sagara 1992, 1995).

Saprobic ammonia fungi are likely to be the main microbes that decompose litter amended with a large amount of nitrogen (Fukiharu et al. 1997a; Sato & Suzuki 1997; Suzuki 2006). For ectomycorrhizal ammonia fungi, Sagara (1995) proposed "a tripartite cleaning symbiosis" as their roles in the forest. In other words, sacrobic and ectomycorrhizal ammonia fungi are expected to have important roles in the cycling of nutrients, especially of carbon and nitrogen, in forests and grasslands under conditions of high concentrations of nitrogen. Mycorrhizal fungi can extract carbon from litter and transport it to plants (Falck & Falck, 1954). Frank (1888) first suggested that mycorrhizae were important in the uptake of nitrogen from forest floors. As they are a large biomass component, both the fungus and fine roots will immobilize substantial quantities of nitrogen in producing their own growth. Mycorrhizal fungi can also increase the uptake of phosphate. Phosphate is taken up by the hyphae and transported to the host. Herrera et al. (1978) found mycorrhizal hyphae on a decomposing leaf and rapid transport of phosphate from that leaf to a host plant via mycorrhizal hyphae. In addition to carbon, nitrogen and phosphate, mycorrhizal fungi take up and translocate almost any element required for plant growth and mycorrhizal also enhance the transport of water from soil to plant (Allen 1991).

These roles suggest the possibility that biogeographic distribution of

ammonia fungi, particularly ectomycorrhizal ammonia fungi, may be primarily based on relationship with vegetation in each area. Mycorrhizal plants and fungi would be dramatically affected by changing world climate as would all other organisms. Mycorrhizal fungi, as other organisms, have evolved and adapted to specific habitats. Studies on the biogeography of ectomycorrhizal fungi have been integrated well into surveys on the distribution of these fungi (Horak 1983). Mycorrhizal fungi were demonstrated to move rapidly and widely across disturbed areas by both wind and animals (Allen 1987, 1988; Warner et al. 1987). Animals and wind both respond to topographic barriers. The pattern of dispersal of the fungi and their host plants is a function of the responses of the vector to the topographic features and the extent to which the vector migrates. However, few similar attempts have been made. Nevertheless, even these fungi have important dispersion patterns. For example, simply surveying the locations where species have been described reveals some interesting pattern. Many fungi probably have restricted habitats just as do many plants. As climates change, the ability of the mycorrhizal fungi to change or disperse as well as the responses of the host plants would be a major determinant in the ability to maintain productive ecosystems.

The community of ammonia fungi is considered to be a basic component of the normal fungus community in forest and grassland ecosystems. It is important to survey the species composition of ammonia fungi in different vegetation types in different regions since this will aid understanding of the roles of ammonia fungi in the global ecosystem (Suzuki 2006).

Most research on ammonia fungi following application of urea in the field

and/or laboratory has been undertaken in four areas: Japan (Sagara 1975, 1992, 1995; Fukiharu & Hongo 1995; Yamanaka 1995a,b; Fukiharu et al. 1997a; Sato & Suzuki 1997; Suzuki & Toyokawa 1999; Fukiharu et al. 2000a, b; Sagara et al. 2000; Imamura & Yamoto 2001; Suzuki et al. 2002b; He & Suzuki 2004b), Taiwan (Fukiharu et al. 1997b), New Zealand (Sagara et al. 1993; Suzuki et al. 2002a) and Australia (Suzuki et al 1998, 2002a; Nagao et al. 2003). In other areas, ammonia fungi have been studied in a few urea-treated sites in Europe and North America (Sagara 1992; Sagara unpubl. data; Suzuki et al. unpubl. data).

Patterns of biogeographic distribution of ammonia fungi can be categorized into six regional distribution types: ubiquitous, Northern Hemisphere, East Asia, East Asia and Oceania, Australia and New Zealand, and Australia endemic (Suzuki et al. 2003).

The species composition of ammonia fungi in each area might be more strongly related to the flora than fauna, since ammonia fungi fruit on the forest floor and in grassland after decomposition of the animal body or excrement, not on animal bodies themselves. New Zealand and Australia have very different native flora and fauna from those of Northern Hemisphere (Alexander 1996; Taylor & Smith 1997). These features make the study of species composition of ammonia fungi in New Zealand and Australia as well as in Japan, especially relevant to knowledge of biogeographic distribution of these fungi.

Sagara and Hamada (1965), and Sagara (1973, 1975) applied a wide variety of chemicals and substances to forest soils in Japan to study possible effects on fungi and, as a result, found a group of fungi which fruit

sequentially on soil after treatment with urea, aqueous ammonia, or any nitrogenous materials which release ammonia on decomposition and cause an alkaline condition on soil. The number of species belonging to this group totals approximately 35 in Japan including *Hebeloma radicosoides* Sagara, Hongo & Murak, *Hebeloma spoliatum* (Fr.) Karst, *Hebeloma vinosophyllum* Hongo (*Pinus-Chamaecyparis* forest in Kyoto).

In the Northern Hemisphere, *Hebeloma luchuense* Fukiharu & Hongo, *H. radicosoides* and *Alnicola lactariense* Clémencon & Hongo have been collected from *Castanopsis* and *Quercus* forests following application of urea (Sagara 1975, 1992, 1995; Suzuki 1992; Clémencon & Hongo 1994; Fukiharu & Hongo 1995; Fukiharu & Horigome 1996; Fukiharu et al. 1997b; Shimabukuro 2000). Other Northern Hemisphere ammonia fungi are *H. vinosophyllum* from *Castanopsis,* and *Pinus* forests (Sagara 1975, 1992, 1995; Fukiharu & Hongo 1995; Yamanaka 1995b; Fukiharu & Horigome 1996; Fukiharu et al. 2000a,b), and *H. spoliatum* from *Quercus* and *Fagus* forests (Sagara 1975; Fukiharu & Horigome 1996). These two species have also been collected from the forest floor when a large amount of ammonia was present (Sagara 1975, 1992, 1995; Suzuki 1992; Yamanaka 1995b; Fukiharu & Horigome 1996; Fukiharu et al. 1997b, 2000a, b). *Laccaria amethystine* Cook and *L. bicolor* (Maire) Orton sensu Imazeki & Hongo (1987) occurred at higher frequency in urea-treated plots than in surrounding areas, from *Fagus, Quercus*, coniferous, and mixed deciduous-coniferous forests (Sagara 1975, 1992, 1995; Yamanaka 1995b). In contrast, *H. luchuense, H. radicosoides, H. spoliatum, H. vinosophyllum,* and *A. lactariolens* were not recorded in any urea plots or in the vicinity of

decaying animal bodies in New Zealand or Australia (Bougher et al. unpubl. data; Sagara et al. unpubl. data).

Studies of ammonia fungi in the Southern Hemisphere over the past decade have recorded the following species. In *Nothofagus* forest in New Zealand, application of urea yielded *Amblyosporium* sp., *Doratomyces* sp., *Ascobolus* spp., *Humaria* sp., *Peziza* spp., *Coprinopsis* spp., *Tephrocybe* sp., *Hebeloma* spp., *and Laccaria* spp.(Sagara et al. 1993; Sagara et al unpubl. data). In a *Eucalyptus* forest in Western Australia, *Ascobolus* sp., *Peziza* sp., *Thecotheus urinamans*, *Coprinopsis* sp., *Tephrocybe* sp., and *Hebeloma* sp. were recorded following application of urea (Nagao et al. 2003; Bougher et al. unpubl. data).

The number of ammonia fungi recorded from the Southern Hemisphere (Pegler 1977; Horak 1980; May & Wood 1997; Suzuki et al. 1998; Segedin & Pennycook 2001; Nagao et al. 2003) is still much less than from the Northern Hemisphere (e.g., Lange 1935 - 1940; Smith 1938, 1984; Van Brummelen 1967, 1995; Moser 1983; Pirozynski 1969; Sagara 1975, 1992, 1995; Orton & Watling 1979; Fukiharu & Hongo 1995; Wang & Sagara 1997; Sagara et al. 2000; Imamura 2001). This needs for further study in the Southern Hemisphere in order to elucidate the role of the fungal community in each habitat.

The ammonia fungi present in different forest vegetations in both Hemispheres will always comprise saprobic and ectomycorrhizal species. Some ammonia fungi, both saprobic and ectomycorrhizal, from the Southern Hemisphere, have closely similar counterpart species belonging to the same genus in the Northern Hemisphere, e.g. *Coprinopsis phlyctidospora* (Oceania

type) versus *C. phlyctidospora* (Eurasia type) and *Hebeloma aminophilum* R. N. Hilton & O. K. Miller versus *H. vinosophyllum*, both in subgenus *Porphyrospora*. Members of these pairs may be separated from each other by substrate or host specificities, and/or by topographic of other physical barrier (s) (Suzuki et al. 2003).

In this paper, ectomycorrhizal ammonia fungi in *Hebeloma* subgenus *Porphyrospora* collected from different geographical areas, particularly for globally distributed species were used as typical species of Northern and Southern Hemisphere for further study. This study comprises four parts. Firstly, a species collected from New Zealand was identified through both sequence analysis of β-tubulin genes and test of inter-compatibility. Secondly, examination of vegetative growth for these ammonia fungi under different ammonium-nitrogen concentrations and pHs was carried out. Basing on the results, it may elucidate the biogeographic distribution of ammonia fungi in the two Hemispheres. Thirdly, an ammonia fungus, *Hebeloma vinosophyllum* from Japan on physiological response of basidiospore germination to different environmental factors was inspected. At the last chapter, reproductive ability of this fungus was observed. Data from the results may reveal the colonization strategy of ammonia fungi. Furthermore, this study may help us to understand the dispersal and speciation of these ammonia fungi in the field.

CHAPTER I

Phylogenetic analysis and identification of ammonia fungi in *Hebeloma* subgenus *Porphyrospora* based on β-tubulin gene and mating test

Introduction

An ectomycorrhizal species, *Hebeloma aminophilum* R.N. Hilton & O.K. Miller was first collected near a decaying large kangaroo body in a mixed *Eucalyptus* (*Eucalyptus marginata* and *E. calophylla*) forest near Manjimup, Western Australia, and assumed to be sarcophilous (Hilton 1978; Miller & Hilton 1987). The occurrence of *H. aminophilum* was later reported from a urea-treated *Eucalyptus* forest (Suzuki et al. 1998). A *Hebeloma* species [subgenus *Porphyrospora* (Singer 1986)] was obtained from *Nothofagus* and *Leptospermum* - *Kunzea* - *Agathis* forests in New Zealand and from a *Eucalyptus* forest in Western Australia. The collections from both countries showed similar macro- and micro-morphological characters, broadly fitting the species concept of *H. aminophilum* and differing from the species concept of *H. vinosophyllum* Hongo (Suzuki et al. unpubl. data). *H. aminophilum* has not been recorded from the Northern Hemisphere in spite of many observations of urea-treated plots in various habitats and many observations at sites with decayed animal bodies. It appears that *Hebeloma* subgenus *Porphyrospora* contains at least two species of ammonia fungi, *H.*

vinosophyllum in the warm, northern, temperate zone of East Asia, and *H. aminophilum* in the warm, southern, temperate zone of New Zealand and Australia (Suzuki et al. 2003).

Species delimitations and relationships between species traditionally have been based on morphological characters (Bruchet 1970, Boekhout 1982, Singer 1986, Vesterholt 1989). In general, fungi are classified according to morphology, methods of reproduction, and modes of spore production, etc. For morphological evidence, it mainly based on colors and shapes of spore, or related to that of fruit-body. The morphological approach has its distinct advantages. But in some cases, taxonomy based on morphology is of limited value, because of the lack of suitable diagnostic features (Mikawa & Ohara 1998). It is sometimes difficult to distinguish a fungal species by its morphology under the level of genus, for species of fungi in a genus level are very close in their morphologies. In other cases, special conditions are needed to allow the development of the morphological feature on which the system is based. But many fungal species, especially some mycorrhizal fungi are not easy to fruit in *vitro,* but pileus or spore as the most important morphological features is necessary.

In the past decade, the molecular systematics for studying phylogenetic relationship and systematical evolution of organisms at the molecular level came into existence owing to the progress of the technique in molecular biology, especially the gene cloning technique, the PCR (Polymerase Chain Reaction) methods, the development of automatic sequencers, and so on. Nowadays, the phylogenetic relationship between organisms can be estimated by comparing information - storing macromolecules such as DNA,

RNA and proteins of organisms. In the field of mycology, the technique for analyzing nucleotide sequences of ribosomal DNA (rDNA) and β-tubulin genes has been adopted.

As we know, microtubules are involved in several basic cellular processes as segregation of genetic material, intracellular transport, maintenance of cell shape, positioning of cell organelles, extracellular transport by means of cilia, and movement of cells by means of flagella and cilia. The main constituent of microtubules is the tubulin, which is a globular protein. The most common members of the tubulin family are α-tubulin and β-tubulin, the proteins which makes up microtubules. These two proteins are encoded by separate gene, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom. The terminal coding sequence of β-tubulin gene is also widely used in taxonomy and molecular phylogeny. (Mages et al. 1995; Keeling et al. 1998; Schutze et al. 1999; Ayliffe et al. 2001; Edgcomb et al. 2001).

In bioinformatics, phylogenies, or evolutionary trees, are the basic structures necessary to reveal the differences among species, and to analyze those differences statistically. Generally speaking, there are three major methods of constructing phylogenetic trees: distance based methods, ex., least squares, neighbor joining; maximum parsimony; maximum likelihood. In this paper, neighbor joining method was used. Neighbor-joining (Saitou & Nei, 1987) is a method that is related to the cluster method but does not require the data to be ultra-metric. In other words it does not require that all lineages have diverged by equal amounts. The method is especially suited for data sets comprising lineages with largely varying rates of evolution. It can

be used in combination with methods that allow correction for superimposed substitutions.

Simultaneously, mating test, the biological species concept, based on sexual inter-compatibility as the criterion to delimit species this study is also used as well as the methods of phylogeny.

Study background

The fungi in this study, *Hebeloma* (Fr.) Kumm, a genus in the *Cortinariaceae* (*Agaricales*), occur worldwide in the temperate zone. Species of *Hebeloma* form ectomycorrhizae (Hacskaylo & Bruchet 1972), but a few species have been reported as ammonia fungi (Sagara 1995). Many species are associated with a number of tree species, and some are pioneer species, among the first ectomycorrhizal fungi to appear during succession (Gryta et al. 1997).

The classification of *Hebeloma* is as follows:
Mycota: *Eeukaryomycota*: *Eumycota*: *Basidiomycota*: *Eubasidiomycetes*: *Hymenomycetidae*: *Agaricales*: *Cortinariaceae*: *Hebeloma* (Imai & Hongo 1989). According to Singer in 1986, *Hebeloma* contains three subgenus as *Porphyrospora, Myxocybe* and *Hebeloma,* and subgenus *Hebeloma* is divided into two sections: *Hebeloma* (*Indusiata*) and *Denudata.*

Basidiospores in genus *Hebeloma* are usually warty rough, boat-shaped, and in deep brownish. But in subgenus *Porphyrospora,* spores seem to be a little fresh brown with a purple or reddish shade (Singer 1975). Species identification in this subgenus is difficult by their morphological features. Species like *H. vinosophyllum* isolated from Japan, *H. aminophilum* isolated

from Australia, and *H. sacrophyllum* and *H. porphyrospora* isolated from Europe, are belonging to *Porphyrospora*. It was suggested they should be the same morphological species for their very closely features in morphology. Isolates isolated from New Zealand in an application of urea - treated plots are suggested to be *Hebeloma* sp. which contained spores in reddish still needs to classify (Fig. I -1).

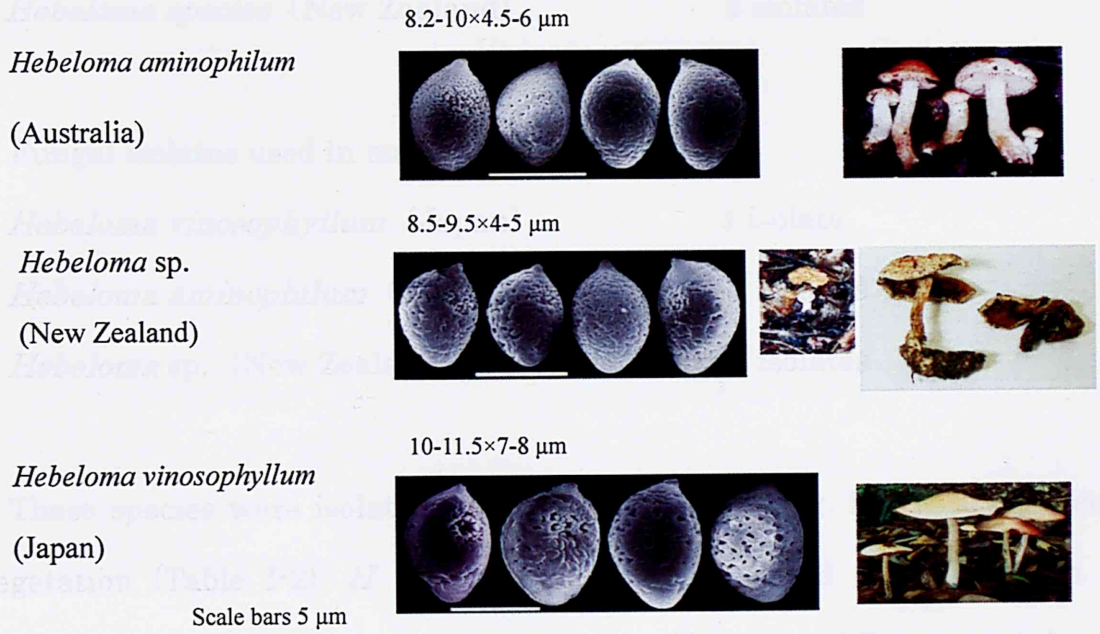


Figure I -1 Basidiomata and basidiospores of *Hebeloma* spp.

(from Fukiharu unpublished data)

Materials and Methods

Fungal specimens

1. Fungal isolates used in the phylogenetic analysis of sequence of ß-tubulin gene (Table I-1)

Hebeloma vinosophyllum (Japan)	7 isolates
Hebeloma aminophilum (Australia)	6 isolates
Hebeloma species (New Zealand)	3 isolates

2. Fungal isolates used in mating test (Table I-2)

Hebeloma vinosophyllum (Japan)	1 isolate
Hebeloma aminophilum (Australia)	2 isolates
Hebeloma sp. (New Zealand)	2 isolates

These species were isolated from different areas (Fig. I-2) with different vegetation (Table I-2). *H. vinosophyllum* was isolated from Japan in a *Quercus* and *Castanopsis* dominated forest. *H. aminophilum* was isolated from Dwellingup, southwestern Australia in *Eucalyptus marginata* and *E. calophylla* mixed forest. *Hebeloma* sp. was isolated from *Nothofagus menziesii* and *N. fusca* mixed forest near Taupo in the North Island of New Zealand.

Quercus is a plant growing in the northern temperate zone in the family *Fagaceae*, oaks. Some are evergreen or deciduous trees and vary from shrubs to great trees. Evergreen oaks flower in spring and fruit in autumn, deciduous ones with wind-pollinated flower before new leaves fully grown

and shed leaves when acorns have fallen. Most species are cultivated for ornamental and timber tree. Acorns are used for swine-food, bark for dye. Leaves and acorns are poisonous because of their content of gallotannins (Mabberley 1997).

Table I -1 Fungal specimens for the phylogenetic analysis of sequence of β-tubulin gene

Species	Strain No.	Location
Hebeloma vinosophyllum	CHU Aoki	Musashino, Saitama, Japan
Hebeloma vinosophyllum	FB-14216	Kouchi, Japan
Hebeloma vinosophyllum	CHU Kiyosumi	Chiba, Japan
Hebeloma vinosophyllum	FB-32636	Kyoto, Japan
Hebeloma vinosophyllum	FB-14520	Shizuoka, Japan
Hebeloma vinosophyllum	CHU Hasumi	Tochigi, Japan
Hebeloma vinosophyllum	FB-14502	Shizuoka, Japan
Hebeloma aminophilum	E 480	Shake Gully, Southern Quinninup, Australia
Hebeloma aminophilum	CHU 2-4	Dwellingup, Australia
Hebeloma aminophilum	CHU 6034-1	Dwellingup, Australia
Hebeloma aminophilum	CHU 6032-1	Dwellingup, Australia
Hebeloma aminophilum	CHU 6-2	Dwellingup, Australia
Hebeloma aminophilum	CHU 3-4B	Dwellingup, Australia
Hebeloma sp.	CHU 2101	Taupo, North Island, New Zealand
Hebeloma sp.	CHU 931	Taupo, North Island, New Zealand
Hebeloma sp.	CHU 932	Taupo, North Island, New Zealand

Table I -2. Fungal specimens for mating test

Species	Strain No.	Vegetation	Location
H. vinosophyllum	CHU Kiyosumi	*Quercus* and *Castanopsis* dominated forest	Japan
H. aminophilum	CHU 3	*Eucalyptus marginata and*	Dwellingup, Australia
H. aminophilum	CHU 3-4B	*E. calophylla* mixed forest	Dwellingup, Australia
Hebeloma sp.	CHU 931	*Nothofagus menziesii and*	Kaimanara State Forest Park, Taupo, North Island, NZ
Hebeloma sp.	CHU 932	*N. fusca* mixed forest	Kaimanara State Forest Park, Taupo, North Island, NZ

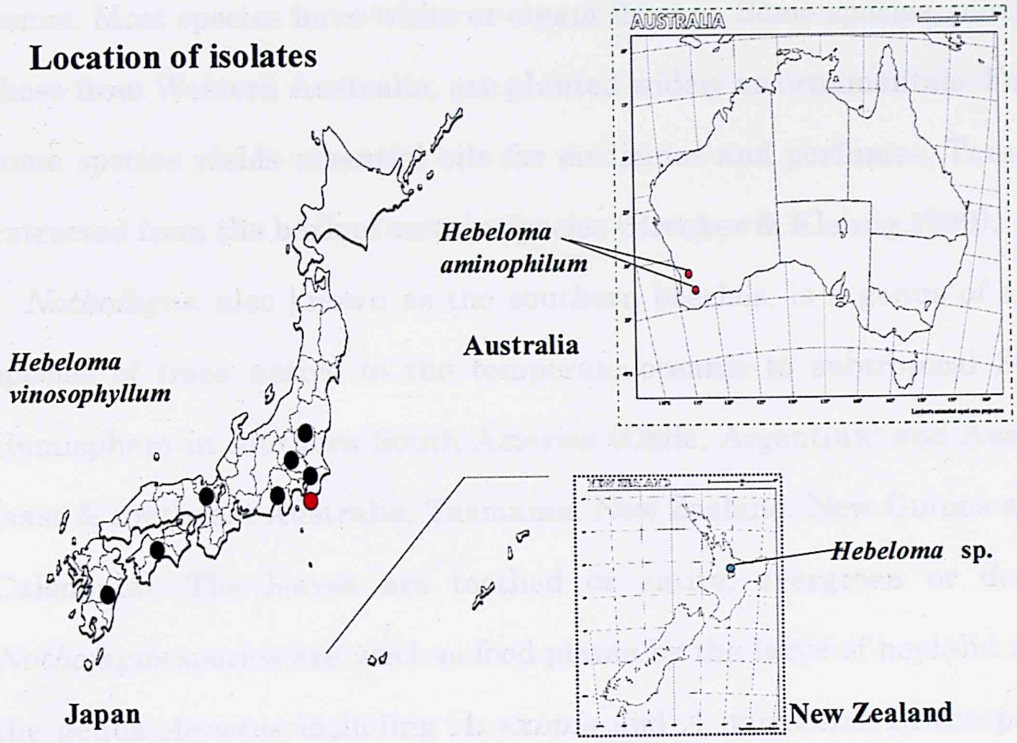


Figure I -2 Location of specimens isolated in mating test

Castanopsis is a genus of evergreen trees belonging to the beech family, *Fagaceae*. The genus contains about 120 species, which are native to tropical and subtropical eastern Asia. A total of 58 species are native to China, with 30 endemic; the other species occur further south, through Indochina to Indonesia, and also in Japan. Most species are used for timber, and the nuts of many are edible. Its dead wood serves as host to many mushroom types, including the eponymous shiitake (Mabberley 1997).

Eucalyptus is a large and important genus of Australian forest trees includes about 500 species in the family *Myrtaceae*. The tree grows best in

areas with an average temperature of 15°C. *Eucalyptus* is an evergreen genus. Most species have white or cream flowers. Some species, particularly those from Western Australia, are planted widely as ornamentals. Foliage of some species yields essential oils for medicines and perfumes. Tannins are extracted from the bark of certain species (Brooker & Kleinig 1999).

Nothofagus, also known as the southern beeches, is a genus of about 35 species of trees native to the temperate oceanic to subtropical Southern Hemisphere in southern South America (Chile, Argentina) and Australasia (east & southeast Australia, Tasmania, New Zealand, New Guinea and New Caledonia). The leaves are toothed or entire, evergreen or deciduous. *Nothofagus* species are used as food plants by the larva of hepialid moths of the genus *Aenetus* including *A. eximia* and *A. virescens*. In the past they were commonly included in the family *Fagaceae*, but genetic tests by the Angiosperm Phylogeny Group revealed them to be genetically distinct, and they are now included in a family their own, the *Nothofagaceae* (Wardle 1984).

DNA preparation

A dikaryotic isolate, was inoculated on MY agar plate [malt extract (Bacto, Becton, Dickinson and Co., USA), 10 g; yeast extract (Bacto, Becton, Dickinson and Co., USA), 2 g; agar (Nakalai, Japan), 15 g; pure water, 1,000 ml; pH 6.5] and incubated at 25.0±0.5°C for about 10 days. Then a piece of mycelium was cut by a 8 mm· diameter cork borer and transplanted to a 200 ml conical flask where containing 100 ml of MY medium. The MY culture was incubated at 25.0±0.5°C in the dark for 2 weeks with reciprocal shaking

in 60 reciprocations per minute (TAITEC BioShaker BR-300LF). Obtained mycelia then were put to freezer under −80°C for 24 hours. Thereafter, frozen mycelia were dried by freeing dryer (EYELA Freez Dryer FOU-830) for 24 hours. DNA extraction method was as follows:

1. Weigh out 10 - 20 mg of freezing dried mycelium and grind with blue pestles;
2. Add 500 µl of CTAB buffer and grind samples a bit more;
3. Incubate samples at 60°C for 30 minutes;
4. Add 500 µl of 24 : 1 Chloroform : Iso amyl alcohol and mix well by shaking tubes;
5. Centrifuge at 4°C for 15 minutes at the speed of 15,000 rpm;
   Following centrifugation, there were three layers: top: aqueous phase, middle: debris and proteins, bottom: chloroform.
6. Pipette off the aqueous phase taking care not to suck up any of the middle or chloroform phases. Pipetting slowly helps with this;

7 Place the aqueous phase into a new labeled eppendorf tube;

8. Add 300 ml isopropanol (two third volumes of the aqueous phase part) and mix well;
9. Let sit in freezer for 15 min;
10. Centrifuge for 3 min at maximum speed;
11. Pour or pipette off the liquid, being careful not to lose DNA;
12. Add 300 µl of cold 70% ethanol and mix;
13. Centrifuge for 1 min at maximum speed;
14. Pour or pipette off the liquid, being careful not to lose DNA;
15. Add 300 µl of cold 95% ethanol and mix;

16. Centrifuge for 1 min at maximum speed;

17. Pour or pipette off the liquid, being careful not to lose DNA;

18. Dry the pellet: Place samples in the speed vacuum for 20 min until dry;

19. Re-suspend samples with 200 µl of TE buffer. Allow to re-suspend for overnight in refrigerator before running a test gel using 5 µl of the DNA.

PCR amplification and the sequence of ß-tubulin gene

Primers used in polymerase chain reaction (PCR) amplification were F-ßtub1, F-ßtub2r, F-ßtub3, F-ßtub4r (Voigt et al. 2003). The 20 µl reaction mixture contained 0.8 µl each primer, 2 µl template DNA, 10 mM dntp 1.6 µl, 10 × reaction buffer 2.0 µl, Taq DNA Polymerase (Bioneer) 0.1 µl with 12.7 µl pure water. PCR (Cycler: GeneAmp9700) process was as follows:

1. 95°C, 2 min (1cycle)

2. 95°C, 40 s+50°C、45 s+72°C、50 s (30 cycles)

3. 72°C, 5 min (1 cycle)

4. 15°C ∞

After electrophoresis in 1% low melting point agarose gel (Takara), the amplified products were excised from the gel.

Phylogenetic analysis

Sequences were aligned using the CLUSTAL X multiple aligment program (Thompson et al. 1997). The aligned sequences were analyzed by the neighbor-joining method (Saitou & Nei 1987) using NEIGHBOR in MEGA version 2. The distance matrix was calculated using Kimura's two-parameter method, and the topology was tested with 1000 bootstrap trials (Felsenstein

1993).

Monokaryotic isolates

A dikaryotic isolate, *H. vinosophyllum* CHU Kiyosumi (from Japan) was inoculated on MY agar slant [malt extract (Bacto, Becton, Dickinson and Co., USA), 10 g; yeast extract (Bacto, Becton, Dickinson and Co., USA), 2 g; agar (Nakalai, Japan), 15 g; pure water, 1,000 ml; pH 6.5] and incubated at 25.0±0.5°C until a basidioma formed (about two weeks). A piece of sterilized filter paper (No. 1, Advantec) was placed into the test tube against the inside surface facing the pilei (Fig. I-3). The slant was then placed horizontally to induce negative geotropism of the stipes. Basidiospores were dispersed onto the surface of the filter paper placed just beneath the pilei. Thereafter, the basidiospores were collected aseptically and suspended in sterile 100 mM $(NH_4)_2HPO_4$ aqueous solution (pH 8.1) to induce germination (Suzuki et al., 1982). The spore suspensions were diluted several times by the same chemical solution and spread over the MY agar plate medium separately each with only 1 ml. After 4 - 7days of incubation at 25.0±0.5°C in the dark, a mycelium derived from each basidiospore was isolated. The monokaryotic isolates were confirmed by the absence of clamp connections after about 10 days of their growth. The monokaryons used for the experiments in this paper were assigned temporary numbers, Hvk 001 ~ Hvk 031.

Figure I-3 Spore prints made by the dikayotic basidiomata

Since *H. aminophilum* (from Australia) and *Hebeloma* species (from New Zealand) have lowered fruiting abilities during subcultures, the dikaryotic isolates of *H. aminophilum* CHU 3, *H. aminophilum* CHU 3-4B and *Hebeloma* sp. CHU 931, *Hebeloma* sp. CHU 932 were inoculated separately on both MY agar slide culture and MY liquid medium, and incubated at 25.0±0.5°C in the dark. The slide cultures were incubated for 7 - 10 days and the liquid one was incubated for 20 days with reciprocal shaking (60 reciprocations per minute). The hyphal suspensions in the liquid cultures were diluted by sterile pure water into different times and spread over MY

agar plate media separately with 1 ml for each concentration, and then incubated for 5 - 7days at 25.0±0.5°C in the dark. Both the mycelia grown on the slide glass and agar plate were cut by the hyphal tips under a microscope and then inoculated separately on the agar slant. The monokaryons obtained were used for the mating tests. They were assigned temporary numbers, Ha 3-001 ~ 025, Ha 3-4B-001 ~ 008 and Hsp 931-001 ~ 017, Hsp 932-001 ~ 010.

Mon-mon mating tests

The mating inoculation was conducted by plating plugs of two different monokaryotic stocks 5 mm apart in the center of a MY agar plate. After incubating for about 4 weeks at 25.0±0.5°C in the dark, a piece of mycelium was collected from both the contact zone of two colonies and the outer edge of each colony on the line joining the center of the two inoculum plugs. Thereafter, compatible crossings as those having clamp connections formed throughout the paired colony were identified (Fig. I-4). Individual pairings were performed three times. Monokaryotic isolates having different kinds of incompatibility factors were selected as testers for the following di-mon mating tests.

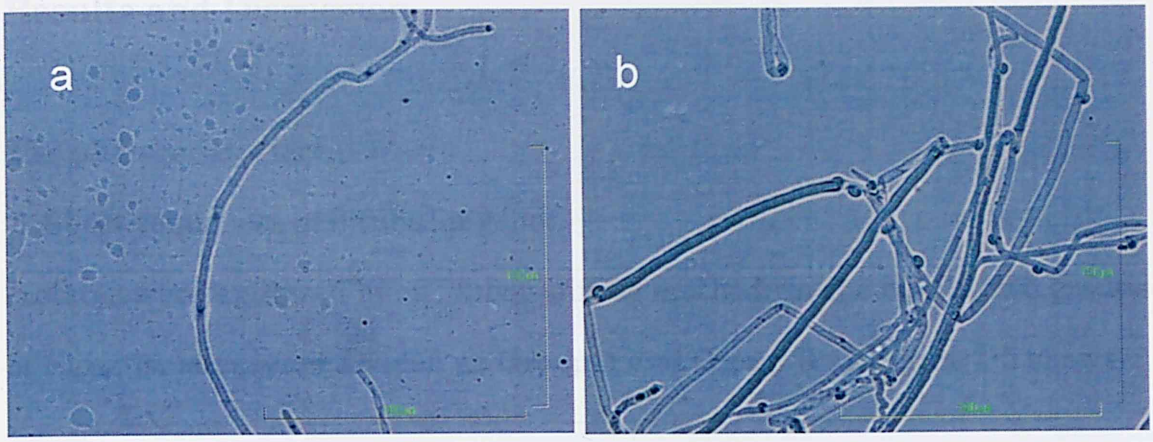


Figure I -4 Mycelia have (or have no) clamp connections formed throughout the paired colony. a: clamp connections did not formed; b: clamp connections formed.

Di-mon mating tests

The mating inoculation was conducted by plating a monokaryotic tester and a dikaryotic isolate 5 mm apart in the center of a MY agar plate. After the incubating for about 4 weeks at 25.0±0.5°C in the dark, a piece of mycelium was removed from the outer edge of the monokaryotic colony. Thereafter, crossings as those having clamp connections at an outer edge of the tester colony or incompatibility were determined. Individual pairings were performed three times.

Results and Discussion

The phylogenetic trees

1. Short sequences of ß-tubulin gene

Isolates were analyzed by neighbor joining method. In the result, two groups of these isolates were divided as Group A and Group B as Figure I-5 shows:

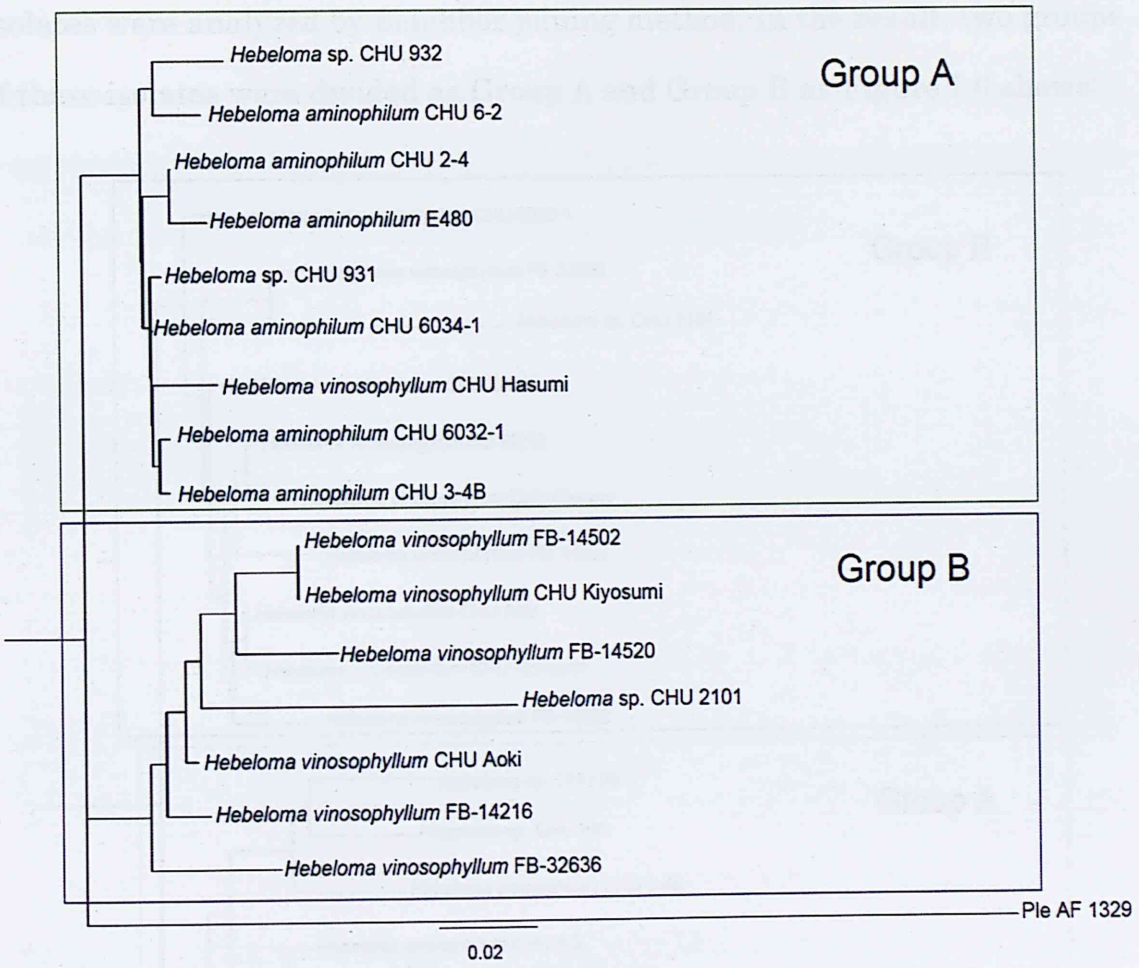


Figure I-5 The phylogenetic tree based on short sequence of β-tubulin genes by neighbor joining method

In Group A, isolates of *H. aminophilum* from Australia and isolates of *Hebeloma* sp. from New Zealand were put into this group. In Group B,

isolates of *H. vinosophyllum* appeared in Japan were put into this group. The question in this phylogenetic tree is that an isolate of *H. vinosophyllum* CHU Hasumi was put into Group A and, another isolate of *Hebeloma* sp. CHU 2101 was put into Group B. It is necessary to confirm the two isolates again in further experiment.

2. Long sequence of ß-tubulin gene

Isolates were analyzed by neighbor joining method. In the result, two groups of these isolates were divided as Group A and Group B as Figure I-6 shows:

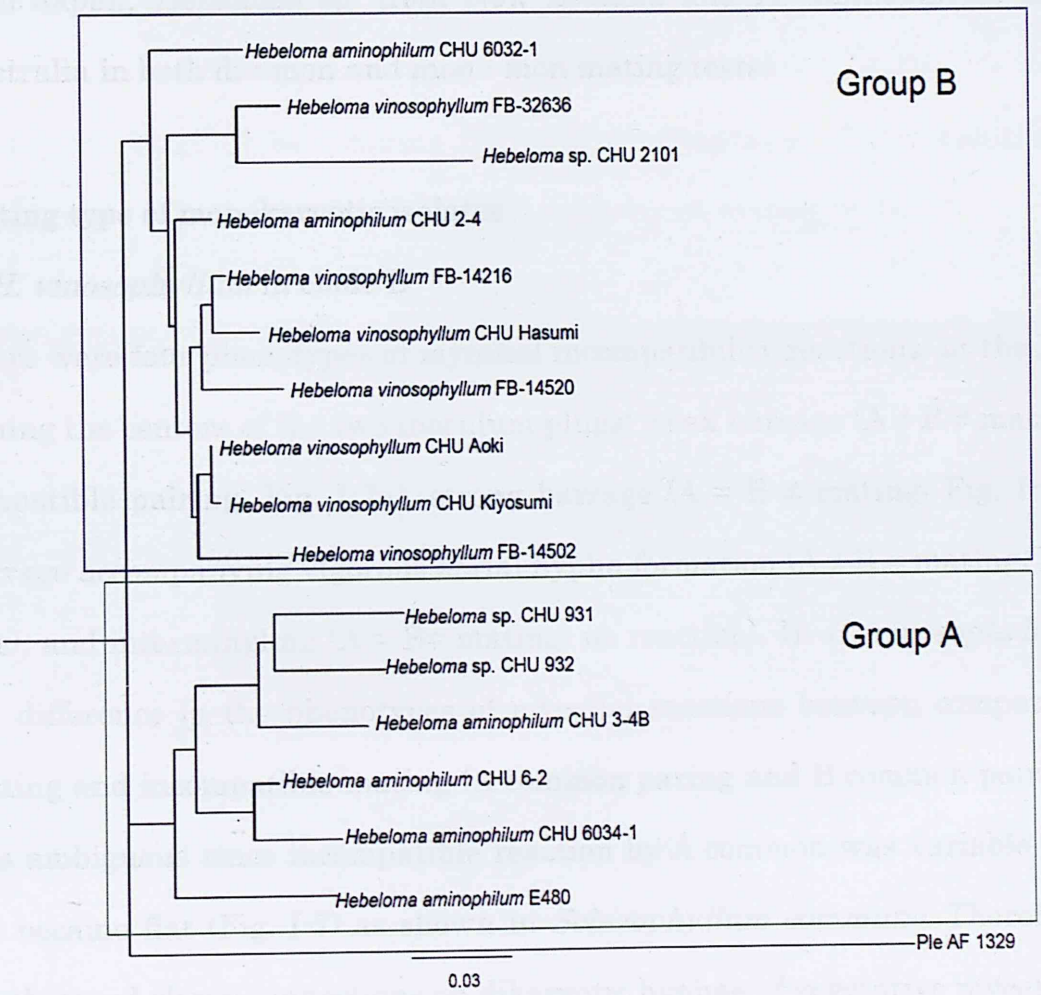


Figure I-6 The phylogenetic tree based on long sequence of ß-tubulin genes by neighbor joining method

In Group A, most isolates of *H. aminophilum* from Australia and isolates of *Hebeloma* sp. from New Zealand were put into this group. In Group B, isolates appeared in Japan were put into this group. As a result, two isolates of *H. aminophilum* and one isolate of *Hebeloma sp.* were put into Group B. The result still needs to be confirmed later in experiment.

Basing on results of the phylogenetic trees of both long and short sequence β-tubulin gene for subgenus *Porphyrospora*, further study to clarify the taxonomic rank of the two groups, was undertaken among *H. vinosophyllum* from Japan, *Hebeloma* sp. from New Zealand and *H. aminophilum* from Australia in both di - mon and mon - mon mating tests.

Mating type of monokaryotic isolates

1. *H. vinosophyllum* in clade B

There were four phenotypes of mycelial incompatibility reactions: at the line joining the centers of the two inoculum plugs: weak barrage (A ≠ B ≠ mating: compatible pairing; Fig. I-7a), strong barrage (A = B ≠ mating; Fig. I-7b), barrage accompanying vigorous aerial hypha formation (A ≠ B = mating; Fig. I-7c), and intermingling (A = B= mating: no reaction). In *H. vinosophyllum*, the difference in the phenotypes of mycelial reactions between compatible mating and incompatible mating (A common paring and B common pairing) was ambiguous since incompatible reaction by A common was variable and not became flat (Fig. I-7) as shown in *Schizophyllum commune*. Therefore, we observed clamp connections on dikaryotic hyphae of vegetative mycelium derived from pairings. Pairings were designated as compatible when clamp connections formed on three parts of the parent mycelia as described above.

By the mating between monokaryotic isolates, Hvk 004, Hvk 008, or Hvk 012 and those, Hvk 002, Hvk 003, or Hvk 029, a pseudoclamp, i. e. a clamp cell does fully fuse with hyphal cell, was formed only in the contacted parts of the colonies whereas, by the mating between monokaryotic isolates, Hvk 001, Hvk 010, or Hvk 018 and those, Hvk 002, Hvk 003, or Hvk 029, clamp connections were observed at the contacted parts and both opposed sides of the paring colonies. This indicates that the offspring derived from the mating was AB heterokaryon. The results indicate that the mating type of *H. vinosophyllum* based on CHU Kiyosumi was tetrapolar (Table I-3). Therefore, four monokaryotic isolates, i. e., Hv k001 (A_1B_1), Hv k003 (A_2B_2), Hv k008 (A_1B_2), Hv k011 (A_2B_1), having different combinations of incompatibility factors were selected, as the testers for subsequent mating tests.

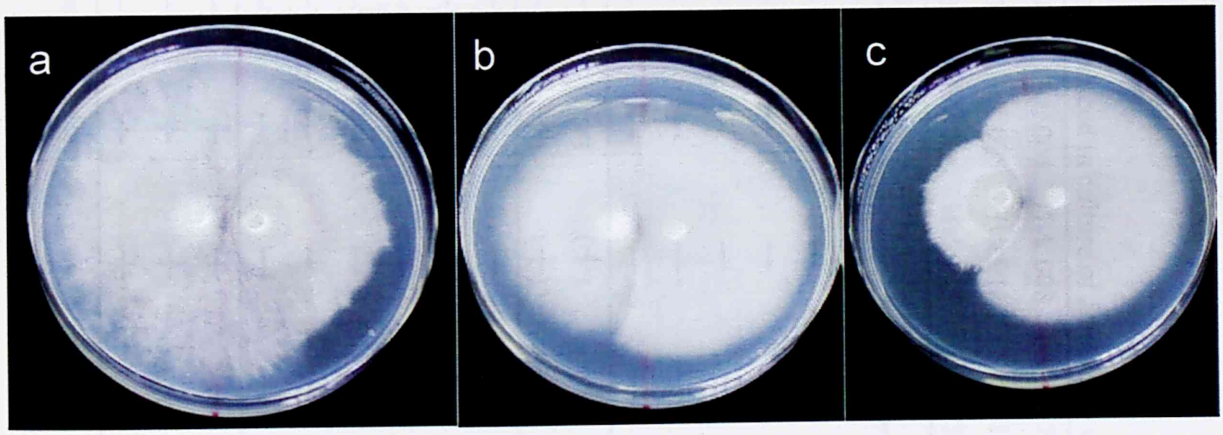


Figure I-7 Phenotype of three possible combination of monokaryons in *Hebeloma vinosophyllum* a: $A_1B_1 \times A_2B_2$ (Hvk 001 × Hvk 003), b: $A_1B_1 \times A_1B_2$ (Hvk 001 × Hvk 008), c: $A_2B_2 \times A_1B_2$ (Hvk 003 × Hvk 008)

Table I-3. Mating pattern among 31 monokaryotic isolates obtained from basdiospores produced in culture of *Hebeloma vinosophyllum* CHU Kiyosumi.

			Hvk 001 Hvk 007 Hvk009	Hvk010 Hvk 014 Hvk015	Hvk016 Hvk018 Hvk 019	Hvk020 Hvk030 Hvk031	Hvk 002 Hvk 003 Hvk 029	Hvk 004 Hvk 005 Hvk 006	Hvk 008 Hvk 012 Hvk 013	Hvk 017 Hvk 021 Hvk 023	Hvk 024 Hvk 026	Hvk 011 Hvk 022 Hvk025	Hvk027 Hvk 028
			A_1B_1				A_2B_2	A_1B_2				A_2B_1	
Hvk 001	Hvk016		—	—	—	—	+	—	—	—	—	(+)	(+)
Hvk 007	Hvk018		—	—	—	—	+	—	—	—	—	(+)	(+)
Hvk009	Hvk 019	A_1B_1	—	—	—	—	+	—	—	—	—	(+)	(+)
Hv k010	Hvk020		—	—	—	—	+	—	—	—	—	(+)	(+)
Hvk 014	Hvk030		—	—	—	—	+	—	—	—	—	(+)	(+)
Hvk015	Hvk031		—	—	—	—	+	—	—	—	—	(+)	(+)
Hvk 002	Hvk 029	A_2B_2	+	+	+	+	—	(+)	(+)	(+)	(+)	—	—
Hvk 003			+	+	+	+	—	(+)	(+)	(+)	(+)	—	—
Hvk 004	Hvk 017		—	—	—	—	(+)	—	—	—	—	+	+
Hvk 005	Hvk 021		—	—	—	—	(+)	—	—	—	—	+	+
Hvk 006	Hvk 023	A_1B_2	—	—	—	—	(+)	—	—	—	—	+	+
Hvk 008	Hvk 024		—	—	—	—	(+)	—	—	—	—	+	+
Hvk 012	Hvk 026		—	—	—	—	(+)	—	—	—	—	+	+
Hvk 013			—	—	—	—	(+)	—	—	—	—	+	+
Hvk 011	Hvk027		(+)	(+)	(+)	(+)	—	+	+	+	+	—	—
Hvk 022	Hvk 028	A_2B_1	(+)	(+)	(+)	(+)	—	+	+	+	+	—	—
Hvk025			(+)	(+)	(+)	(+)	—	+	+	+	+	—	—

+, clamp connections formed; (+), pseudoclamps formed; —, clamp connections did not form

2. *H. aminophilum* in clade A

Monokaryotic isolates were obtained from the dikaryotic isolates of *H. aminophilum* CHU 3 and *H. aminophilum* CHU 3-4B. Twenty five monokaryotic isolates of *H. aminophilum* CHU 3 were paired off for mating test. The results indicate two types of monokaryotic isolates having different incompatibility. Therefore the monokaryotic isolates, i. e., Ha 3-003, Ha 3-007, having different incompatibility, were selected as the testers for subsequent mating tests. Whereas, only one type of monokaryotic isolate having an incompatibility were obtained from the dikaryotic isolates of *H. aminophilum* CHU 3-4B. Therefore two monokaryotic isolates, i. e., Ha 3-4B-001, Ha 3-4B-002, having the same incompatibility factors, were selected as the testers for subsequent mating tests.

3. *Hebeloma* species in clade A

Monokaryotic isolates were separately obtained from the *Hebeloma* sp. CHU 931 and *Hebeloma* sp. CHU 932. Seventeen monokaryotic isolates of *Hebeloma* sp. CHU 931 were paired off for mating test. The results indicate two types of monokaryotic isolates having different incompatibility. Therefore, the monokaryotic isolates, i. e., Hsp 931-006, Hsp 931-010, having different incompatibility, were selected as the testers for subsequent mating tests. Whereas, only one type of monokaryotic isolate having an incompatibility was obtained from the dikaryotic isolates of *Hebeloma* sp. CHU 932, therefore two monokaryotic isolates, i. e., Hsp 932-001, Hsp 932-002, having the same incompatibility factors were selected as the testers for subsequent mating tests.

Compatibility of intra - and inter - clade matings

Mating between isolates in clade A and those in clade B by di-mon and mon-mon showed no clamp connection formation in all tested vegetative mycelia (Table I-4). Therefore, the isolates of *H. aminophilum* collected in Australia and *Hebeloma* sp. collected in New Zealand, and those of *H. vinosophyllum* collected in Japan were incompatible and behaved as different biological species.

Mating between monokaryotic isolates of *H. aminophilum* CHU 3 and those of *Hebeloma* sp. CHU 931 were compatible. Mating between Ha 3-003 and Ha 3-004 and, between Hsp 932-001 and Hsp 932-002 formed pesudoclamp whereas mating between Ha 3-007 and Ha 3-010, and between Hsp 932-001 and Hsp 932-002 formed clamp connections. These results indicate that *H. aminophilum* in Australia and *Hebeloma* sp. in New Zealand are the same biological species and samples collected from Australia and New Zealand are also tetrapolar (Table I-5).

For the biogeographical distribution of *H. vinosophyllum* and *H. aminophilum,* the latter was first collected near a decaying large kangaroo body in a mixed *Eucalyptus* (*Eucalyptus marginata* and *E. calophylla*) forest near Manjimup, Western Australia, and assumed to be sarcophilous (Hilton 1978; Miller & Hilton 1987). The occurrence of *H. aminophilum* was later reported from a urea-treated *Eucalyptus* forest (Suzuki et al. 1998). *H. aminophilum* has not been recorded from the Northern Hemisphere in spite of many observations of urea-treated plots in various habitats and many observations at sites with decayed animal bodies. A *Hebeloma* sp. was also recorded in New Zealand in *Nothofagus* forest (Sagara 1993; Suzuki 2003)

following application of urea. *H. vinosophyllum* has been collected from Japan in *Castanopsis* and *Pinus* forests (Sagara 1975, 1992, 1995; Fukiharu & Hongo 1995; Yamanaka 1995b; Fukiharu & Horigome 1996; Fukiharu et al. 2001a, b). Though these isolates were recorded in different geographical areas and with different vegetations, morphologically, they are very close and very hard to identify by their basidiomata or basidiospores.

Table I - 4. Mating tests between isolates of *Hebeloma aminophilum* from Australia, isolates of *Hebeloma* sp. from New Zealand and isolates of *Hebeloma vinosophyllum* from Japan

	Monokaryotic tester isolates of *Hebeloma vinosophyllum* CHU Kiyosumi							
	A_1B_1		A_2B_2		A_1B_2		A_2B_1	
	Hvk 001	Hvk 018	Hvk 002	Hvk 003	Hvk 008	Hvk 012	Hvk 011	Hvk 022
Dikaryotic isolate								
H. aminophilum CHU 3	—	—	—	—	—	—	—	—
H. aminophilum CHU 3-4B	—	—	—	—	—	—	—	—
H. aminophilum E480	—	—	—	—	—	—	—	—
Hebeloma sp. CHU 931	—	—	—	—	—	—	—	—
Hebeloma sp. CHU 932	—	—	—	—	—	—	—	—
*H*ebeloma sp.. CHU 2101	—	—	—	—	—	—	—	—
Monokaryotic isolates								
H. aminophilum CHU 3								
Ha 3-003	—	—	—	—	—	—	—	—
Ha 3-004	—	—	—	—	—	—	—	—
Ha 3-007	—	—	—	—	—	—	—	—
Ha 3-010	—	—	—	—	—	—	—	—
H. aminophilum CHU 3-4B	—	—	—	—	—	—	—	—
Ha3-4B 001	—	—	—	—	—	—	—	—
Ha3-4B 002	—	—	—	—	—	—	—	—
Hebeloma sp. CHU 931								
Hsp 931-003	—	—	—	—	—	—	—	—
Hsp 931-006	—	—	—	—	—	—	—	—
Hsp 931-001	—	—	—	—	—	—	—	—
Hsp 931-010	—	—	—	—	—	—	—	—
*H*ebeloma sp. CHU 932	—	—	—	—	—	—	—	—
Hsp 932-001	—	—	—	—	—	—	—	—
Hsp 932-002	—	—	—	—	—	—	—	—

—,clamp connections did not formed

Table I -5. Mating tests between *Hebeloma aminophilum* from Australia and *Hebeloma* sp. from New Zealand

		Hebeloma sp. CHU 931[a]				*Hebeloma* sp. CHU 932		*H. aminophilum* CHU 3-4B[b]	
		Hsp 931-003	Hsp 931-006	Hsp 931-001	Hsp 931-010	Hsp 932-001	Hsp 932-002	Ha 3-4B 001	Ha 3-4B 002
		A_1B_1	A_1B_1	A_2B_2	A_2B_2	A_3B_1	A_3B_1	A_2B_2	A_2B_2
H.aminophilum CHU 3[a]									
Ha 3-003	A_1B_1	—	—	+	+	(+)	(+)	+	+
Ha 3-004	A_1B_1	—	—	+	+	(+)	(+)	+	+
Ha 3-007	A_2B_2	+	+	—	—	+	+	—	—
Ha 3-010	A_2B_2	+	+	—	—	+	+	—	—
H. aminophilum CHU 3-4B[b]									
Ha 3-4B 001	A_2B_2	+	+	—	—	c	c		
Ha 3-4B 002	A_2B_2	+	+	—	—	c	c		
Hebeloma sp. CHU 932[b]									
Hsp 932- 001	A_3B_1	(+)	(+)	+	+			c	c
Hsp 932-002	A_3B_1	(+)	(+)	+	+			c	c

+, clamp connections formed; (+), pseudoclamps formed; —, clamp connections did not form

a, two mating types of monokaryotic isolates were obtained.

b, only one mating type of monokaryotic isolate was obtained.

c, mating test was not done for only one mating type of each isolate gotten.

Conclusion

The results showed *H. aminophilum* isolated from Australia and *H. vinosophyllum* isolated from Japan were divided into separate group, and *Hebeloma* sp., the target isolates from New Zealand was also divided into a same group with the isolates obtained from Australia.

Results of mating experiment are consistent with the groupings based on their ß-tubulin gene sequences. Therefore, the results of the phylogenetic and hybridization studies as well as morphological studies presented in this paper revealed that the *Hebeloma* sp. from New Zealand is the same species to *H. aminophilum,* but different species from *H. vinosophyllum.*

CHAPTER Ⅱ

Effects of pH and ammonia-nitrogen concentration on vegetative growth of ectomycorrhizal ammonia fungi

Introduction

Patterns of biogeographic distribution of ammonia fungi can be categorized into six regional distribution types: ubiquitious, Northern Hemisphere, East Asia, East Asia and Oceania, Australia and New Zealand, and Australia endemic. Among the ammonia fungi, closely related counterpart species in each hemisphere are recognized (Suzuki et al. 2003).

In ubiquitous (global) distribution type, several species of saprobic and ectomycorrhizal ammonia fungi have been collected from various sites in temperate regions in both Northern and Southern Hemispheres; In the Northern Hemisphere distribution type, four saprobic ammonia fungi have been collected only from temperate areas of the Northern Hemisphere; In East Asia distribution type, the saprobic and ectomycorrhizal ammonia fungi were collected from Japan and Taiwan. Among ectomycorrhizal ammonia fungi, *Alnicola lactariolens, Hebeloma radicosoides, H. luchuense* and *H. vinosophyllum* were recorded (Sagara 1975, 1992, 1995; Suzuki 1992; Yamanaka 1995a,b,c; Fukiharu & Hongo 1995; Fukiharu & Horigome 1996; Fukiharu et al. 2000a, b); In Australia and New Zealand distribution type, the ectomycorrhizal ammonia fungus *Hebeloma aminophilum* has been

collected from temperate regions of New Zealand and Australia, namely in the North Island, New Zealand, near Perth, Western Australia, and in south-eastern Australia (May & Wood 1997; Suzuki et al. 1998; Fungimap data T. May pers. comm.). Recently, Young (2002) recorded this fungus from two other Australian regions, Tasmania (cold temperate zone) and northern Queensland (tropical region). It is not known whether both fungi are distributed widely in the Southern Hemisphere or are confined to New Zealand and Australia.

Yamanaka (2003) and Licyayo & Suzuki (2006) had reported the effect of pH or ammonium - nitrogen concentration on the growth of ammonia fungi. Their studies included many saprobic ammonia fungi, but less in ectomycorrhizal ammonia fungi. Since ectomycorrhizal fungi concern closely with the vegetation in each area on the distribution of biogeography, in this study, ectomycorrhizal species occurred in urea-treated plots in both Northern and Southern Hemispheres were selected, that included *H. vinosophyllum*, *H. radicosoides*, *H. spoliatum*, *A. lactariense*, *H. aminophilum* and *Hebeloma* sp. Among these isolates, *H. aminophilum* and *Hebeloma* sp. were separately collected from Australia and New Zealand. *Hebeloma* sp. from New Zealand was also considered to be the species of *H. aminophilum* (see Chapter I). They are belonging to genus *Hebeloma* subgenus *Porphyrospora* as well as the species of *H. vinosophyllum* which collected from Japan. The main purpose for this study is to elucidate the biogeographic distribution of these *Porphyrospora* ammonia fungi based on their physiological characteristics, namely their vegetative growth responses to different pHs and ammonium-nitrogen concentrations. Other species

selected because those species were occurred in the urea-treated plots with appearance of *H. vinosophyllum* and, they are belonging to genus *Hebeloma* except *A. lactariense* which considered as a species of *Hebeloma* before.

In this study, the effects of pH and ammonia-nitrogen concentration on the vegetative growth were discussed.

Materials and Methods

Fungal species

1. *Hebeloma* species from *Nothofagus* forest in New Zealand

   Isolate No.: *Hebeloma* sp. CHU 931, *Hebeloma* sp. CHU 932, *Hebeloma* sp. CHU 2101;

2. *Hebeloma aminophilum*, R. N. Hilton & O. K. Miller from *Eucalyptus* (*Eucalyptus marginata* and *E. calophylla*) forest in Australia

   Isolate No.: *Hebeloma aminophilum* CHU 3, *Hebeloma aminophilum* CHU 3-4B, *Hebeloma aminophilum* CHU 6032;

3. *Hebeloma vinosophyllum* Hongo from *Castanopsis* and *Pinus* forests in Japan

   Isolate No.: *Hebeloma vinosophyllum* FB-32636, *Hebeloma vinosophyllum* CHU fast, *Hebeloma vinosophyllum* CHU Hasumi;

4. *Hebeloma radicosoides* Sagara, Hongo & Murak from *Castanopsis* and *Quercus* forests in Japan

   Isolate No.: *Hebeloma radicosoides* NAO 659, *Hebeloma radicosoides* NAO 679, *Hebeloma radicosoides* NAO 680;

5. *Hebeloma spoliatum* (Fr.) Karst from *Quercus* and *Fagus* forests in Japan

   Isolate No.: *Hebeloma. spoliatum* NAO 664, *Hebeloma. spoliatum* NAO 674, *Hebeloma. spoliatum* FB-12395;

5. *Alnicola lactariense* Clémencon & Hongo from *Castanopsis* and *Quercus* forests in Japan

   Isolate No.: *Alnicola lactariense* 202

Culture conditions

1. Different pHs

MY medium [malt extract (Difco, Becton, Dickinson and Co., USA) 10 g; yeast extract (Difco, Becton, Dickinson and Co., USA) 2 g; distilled water, 1,000 ml] was adjusted to different pH values by HCl (from pH 3.0 to pH 5.0) and KOH (from pH 6.0 to pH 9.0): pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0 and pH 9.0. Then the culture solutions were autoclaved to sterilize for 15 minutes under 121°C.

2. Different ammonium-nitrogen concentrations

The basal medium was composed of glucose, 22.22 g; KH_2PO_4, 0.33 g; $MgSO_4 \cdot 7H_2O$, 0.33 g; $CaCl_2 \cdot 2H_2O$, 0.11 g; $ZnSO_4 \cdot 7H_2O$, 0.33 mg; $FeSO_4 \cdot 7H_2O$, 0.15 mg; $CuSO_4 \cdot 5H_2O$, 0.10 mg; $MnSO_4 \cdot 5H_2O$, 0.10 mg; $NaMoO_4 \cdot 2H_2O$, 0.02 mg; thiamine hydrochloride, 0.50 mg; nicotinic acid, 0.10 mg, and distilled water, 1,000 ml. Ammonium chloride (NH_4Cl) was used as a nitrogen source added to the culture solution above at different concentrations: 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, 30 mM, 100 mM, 300 mM, and 1,000 mM. The media were adjusted to pH 7.0 by KOH (Licyayo & Suzuki 2006). Distilled water was used as control. Media were sterilized by filtration (acetate cellulose, 0.2 μm pore size, Advantec).

Inoculum culture

Strains stocked at 5°C were used to inoculate on MY agar plates for 2-week growing. In order to obtain actively inocula, growing strains were transplanted once more on MY agar plates for another 2-week growing. Then inoculum disks were cut with a 4 mm cork borer in diameter and inoculated

into a 50 ml-conical flask where hold 20 ml of the liquid medium. Inocula were taken all from the peripheral region of the colonies. Three replicates were prepared for each treatment. Incubation was at 25.0±0.5°C in the dark for 20 days.

Mycelium harvesting, final pH and final ammonium-nitrogen concentration

The well-developed mycelia were taken out and wrapped with aluminum foil then put into an oven dried at 60°C for 48 hours. Dried samples were weighed using an electron weighting scales.

The final pH values of both NH_4Cl culture media and MY media were measured using a glass electrode pH meter (Horiba pH/Ion meter F-23).

The final ammonium-nitrogen concentration of NH_4Cl culture media was measured using High Performance Liquid Chromatography (HPLC) (Hitachi LaChrom HPLC System Pump L-7000, Column Oven L-7300, Conductivity Detector L-7400, Autosampler L7200, Integrator D-7500 for data processing and Gelpack GL-IC-C65 (611-5M12) column), Flow rate: 1.00 ml/min, oven temperature: 40°C, Injection volume: 15 µl, and the eluting solvent used was 2.0 mM H_2SO_4.

Statistical analysis

All statistical analyses were performed using Statce12 software (OSM Publishing Co, Japan) and the analysis was by Tukey-Kramer method.

Results and Discussion

Effects of pH on biomass production

Table Ⅱ-1 shows 16 isolates grew from pH 4.0 to pH 8.0. Among them, pH 6.0 and pH 7.0 were the optimum values for most of these 16 isolates to grow. Some isolates got their high biomasses production too at pH 5.0 or pH 8.0.

Biogeographically, isolates from both New Zealand and Australia produced the highest biomasses at either pH 6.0 or pH 7.0. Other isolates from Japan yielded their highest biomasses mostly at pH 6.0 or pH 7.0, but some higher production were also obtained at pH 5.0 or pH 8.0. Figure Ⅱ-1 to Figure Ⅱ-6 show more clear for these 16 isolates in growth responses at different pHs.

Hebeloma sp. CHU 931: Optimum mycelial growth was at pH 6.0. There were significant differences at every interval pH scale from 4.0 to 8.0 for vegetative growth;

Hebeloma sp. CHU 932: Optimum mycelial growth was at pH 6.0. The yield showed there were non significant differences in between pH 5.0 and 7.0, pH 5.0 and 8.0, pH 7.0 and 8.0 for vegetative growth;

Hebeloma sp. CHU 2101: Optimum mycelial growth was at pH 7.0. The yield showed there were non significant differences between pH 5.0 and 6.0, pH 6.0 and 8.0, pH 7.0 and 8.0 for vegetative growth;

Table Ⅱ-1. Biomass of 16 isolates of ammonia fungi cultivated in MY medium under different pHs

fungal species	isolate	biomass (mg dry wt mycelium) under different initial pHs						
		3	4	5	6	7	8	9
Hebeloma species	CHU 931	0a	32.0±1.7b	71.0±1.5d	88.3±0.9f	79.0±1.2e	56.7±2.7c	0a
	CHU 932	0a	11.7±0.9b	22.3±0.9c	37.0±1.2d	25.0±0.6c	22.7±1.3c	0a
	CHU 2101	0a	30.0±0.6b	41.7±1.2c	44.7±1.5cd	53.7±1.8e	48.7±0.9de	0a
Hebeloma aminophilum	CHU 3	0a	34.0±2.1b	74.0±2.1d	95.0±1.2f	81.7±1.2e	55.7±2.4c	0a
	CHU 3-4B	0a	27.7±1.5b	45.3±1.5c	49.3±1.2cd	54.7±2.0d	22.3±1.2b	0a
	CHU 6032	0a	23.3±1.9b	42.3±1.9d	45.3±1.7de	50.7±0.9e	31.7±1.2c	0a
Hebeloma vinosophyllu	FB-32636	0a	32.0±1.2b	82.7±2.2c	87.0±1.2c	102.7±1.5d	81.7±2.2c	0a
	CHU Fast	0a	30.3±0.7b	82.7±1.5c	90.0±0.6d	100.7±1.5e	79.7±0.7c	0a
	CHU Hasumi	0a	9.0±0.6b	53.3±1.5c	74.7±0.3de	78.3±0.7e	74.0±1.5d	0a
Hebeloma radicosoides	NAO 659	0a	29.0±1.2b	53.0±1.5c	74.3±2.0e	93.3±1.5f	64.7±1.5d	0a
	NAO 679	0a	8.3±1.2b	58.0±1.2e	84.3±2.0f	51.3±0.9d	21.3±1.5c	0a
	NAO 680	0a	25.7±0.9b	51.0±1.2c	89.7±1.8d	85.0±1.5d	54.7±2.2c	0a
Hebeloma spoliatum	NAO 664	0a	37.0±1.2b	92.3±2.4d	99.7±1.8e	96.7±0.9de	51.3±0.7c	0a
	NAO 674	0a	42.3±1.9b	95.3±2.4d	101.3±1.2d	98.0±1.5d	58.0±1.2c	0a
	FB-12395	0a	44.0±2.1b	99.7±2.2d	109.0±0.6e	96.7±2.2d	53.0±2.3c	0a
Alnicola lactariense	202	0a	43.0±1.5b	76.3±0.3c	78.3±1.2cd	91.3±1.5e	82.0±1.2d	0a

Means and SE calculated from three replicates of each treatment; yields followed by different letters in the same rows are significantly different at $P<0.05$ according to the Tukey-Kramer test.

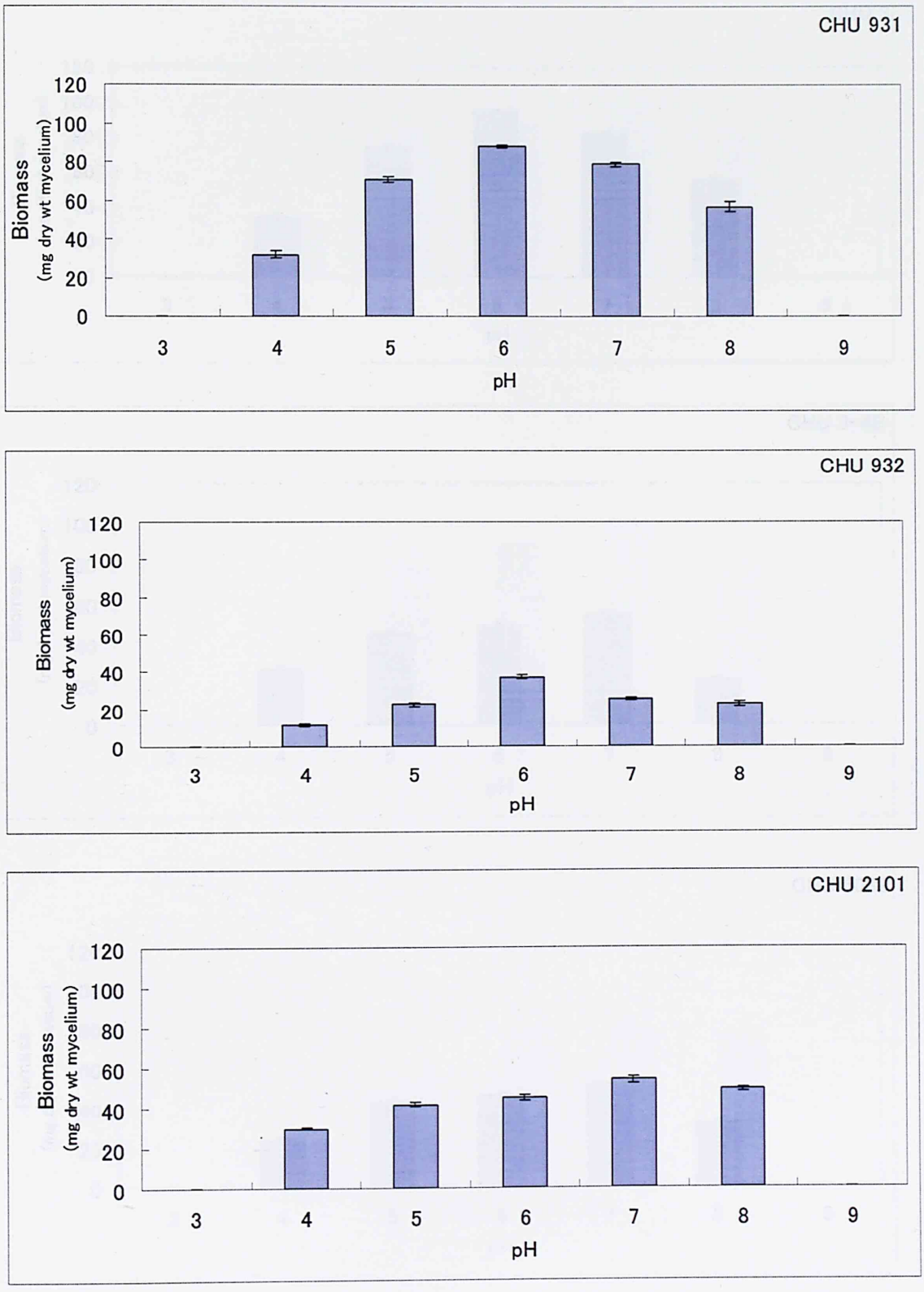


Figure Ⅱ-1 Mycelial yields of isolates of *Hebeloma* sp. at different pHs; Bar indicates SE of the means.

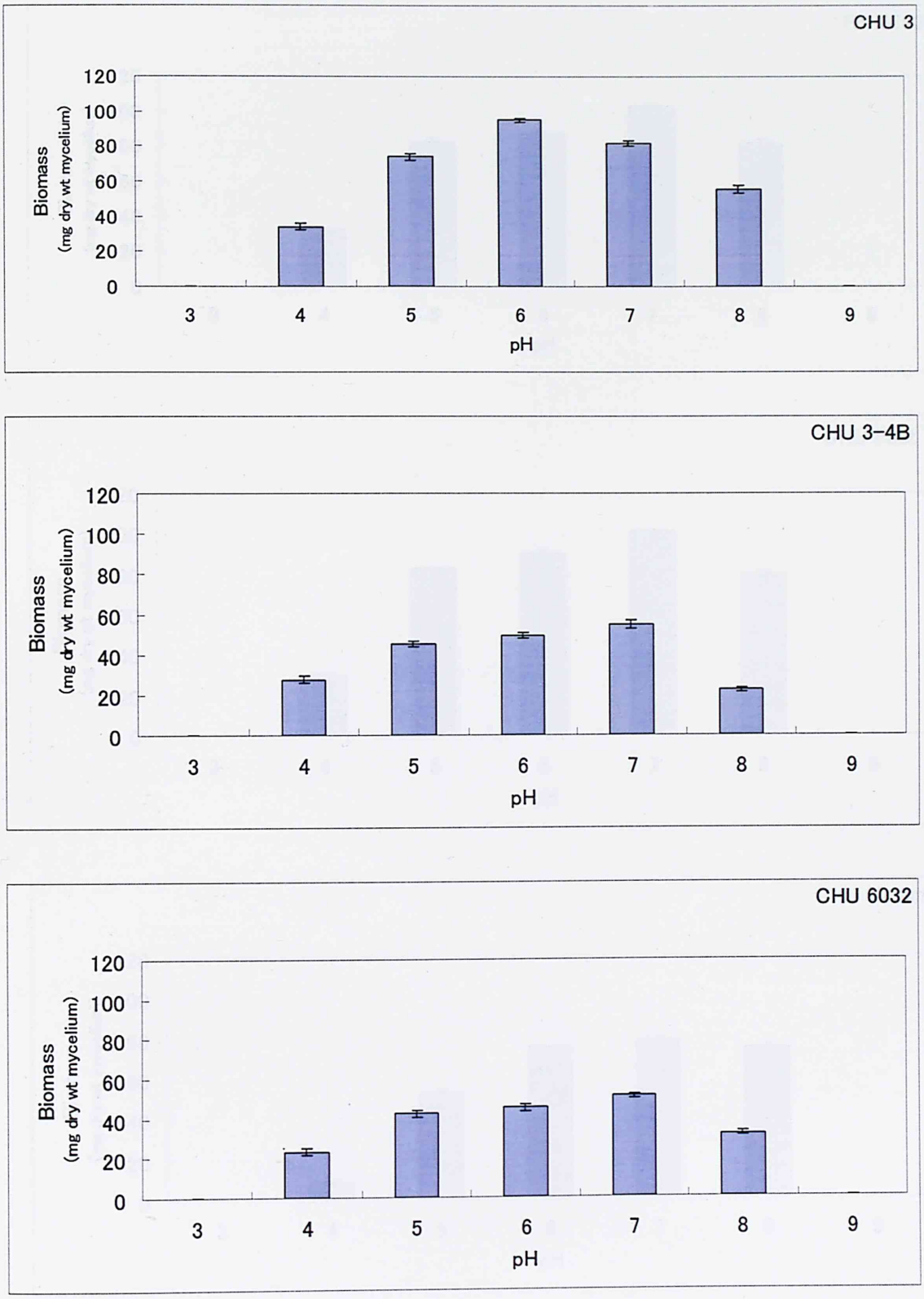


Figure Ⅱ-2 Mycelial yields of isolates of *Hebeloma aminophilum* at different pHs; Bar indicates SE of the means.

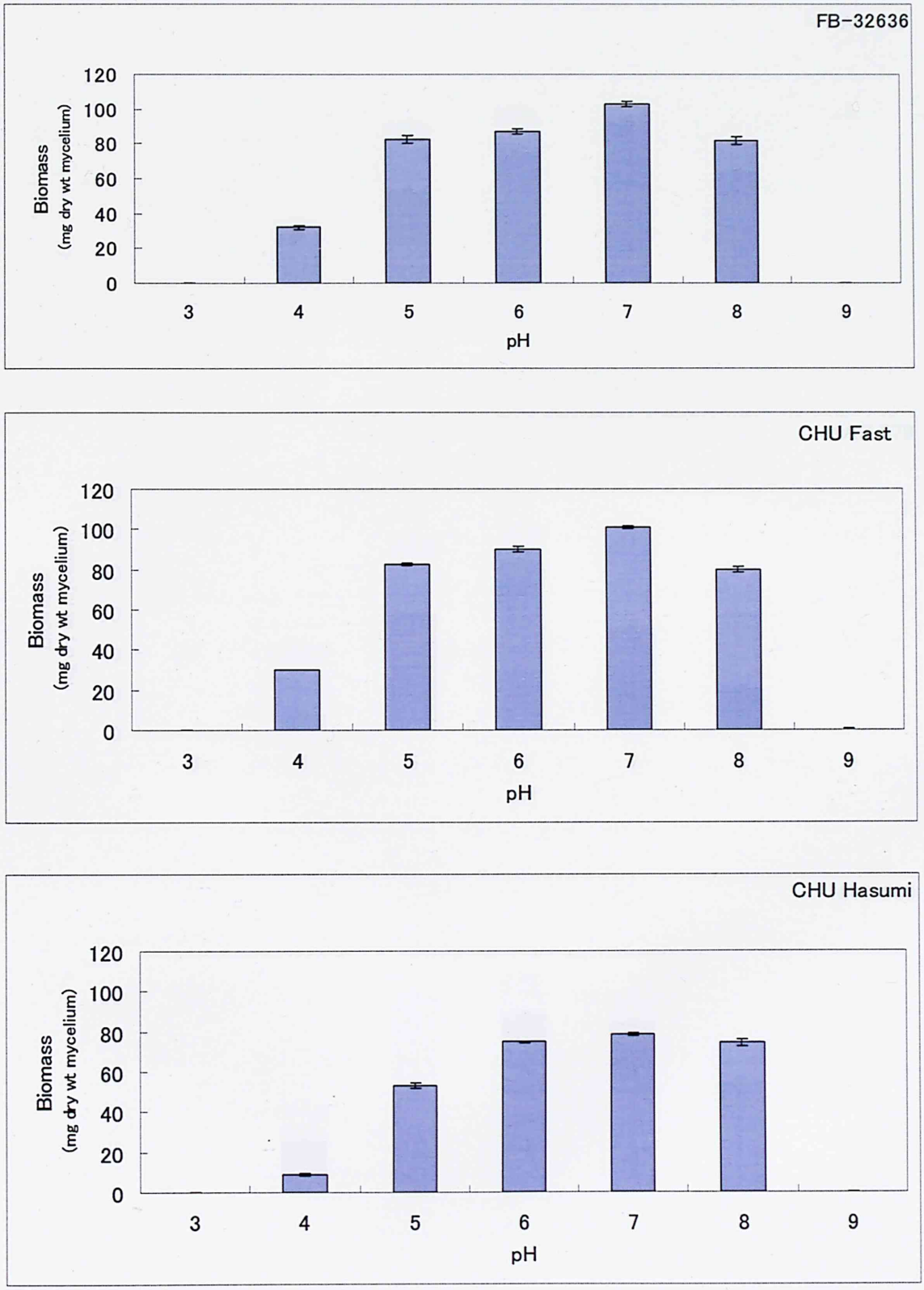
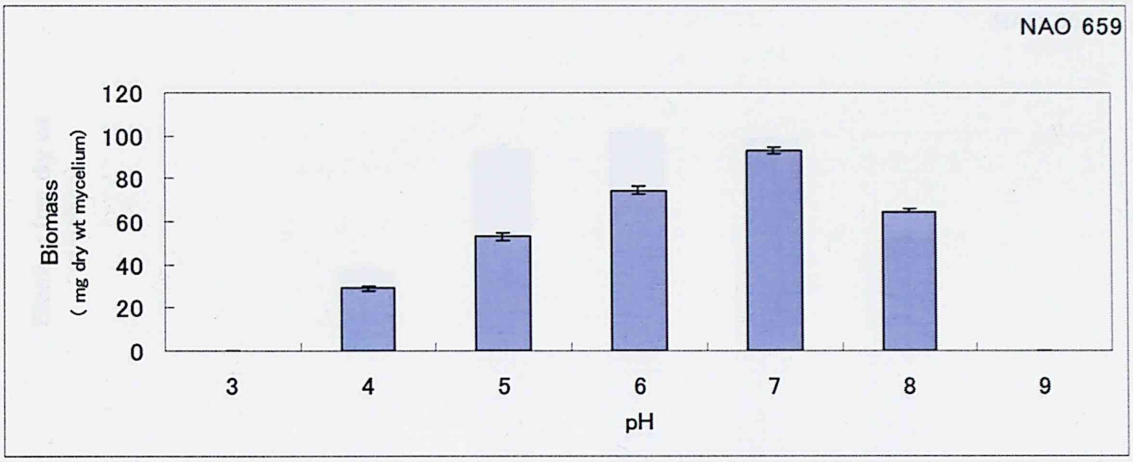
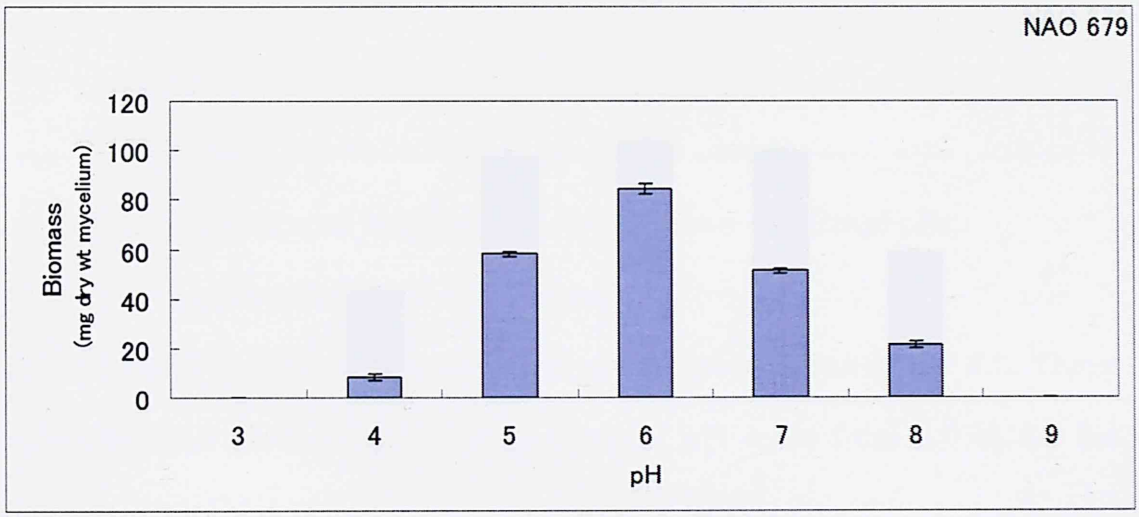


Figure Ⅱ-3 Mycelial yields of isolates of *Hebeloma vinosophyllum* at different pHs; Bar indicates SE of the means.





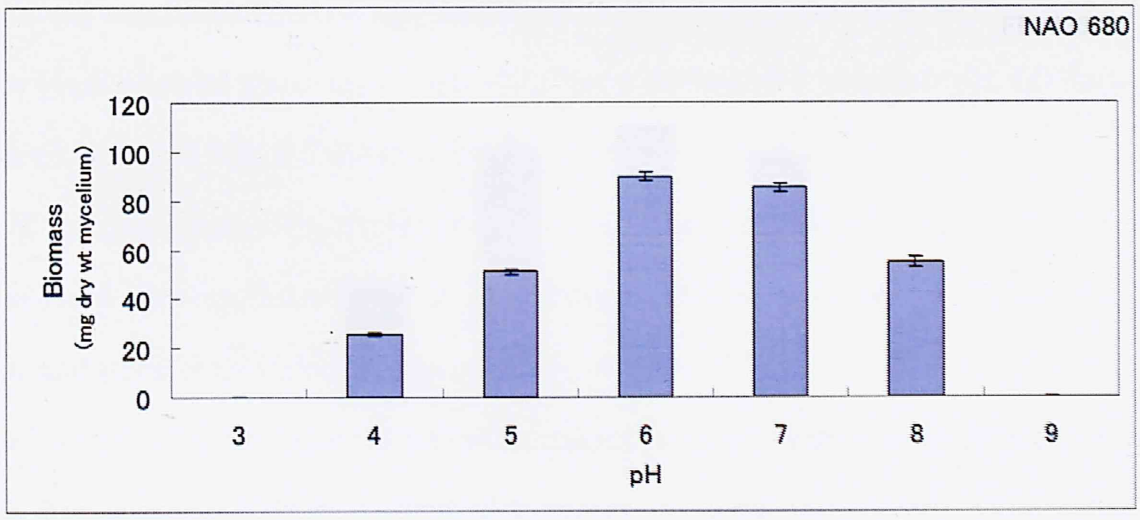


Figure II-4 Mycelial yields of isolates of *Hebeloma radicosoides* at different pHs; Bar indicates SE of the means.

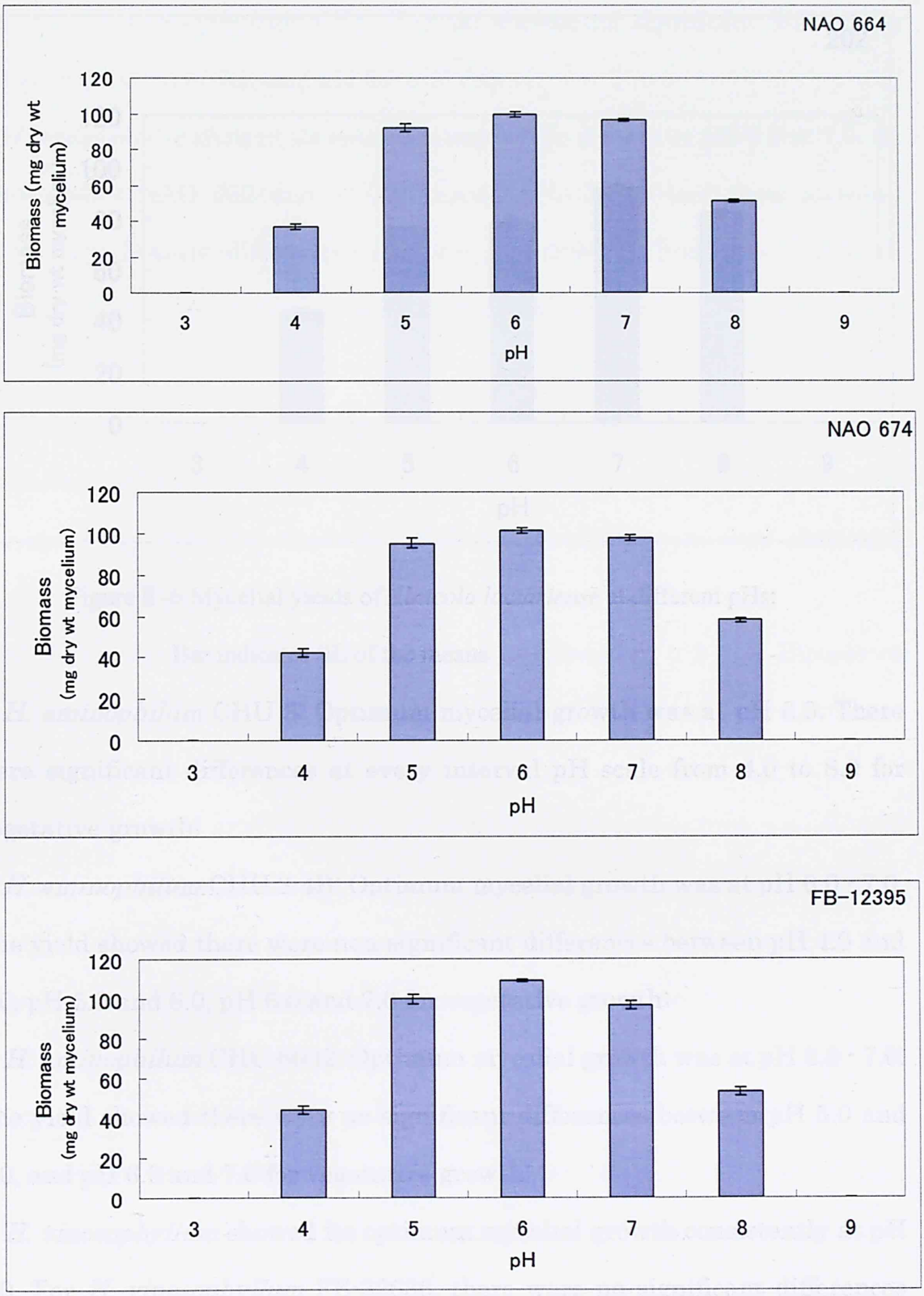


Figure Ⅱ-5 Mycelial yields of isolates of *Hebeloma spoliatum* at different pHs; Bar indicates SE of the means.

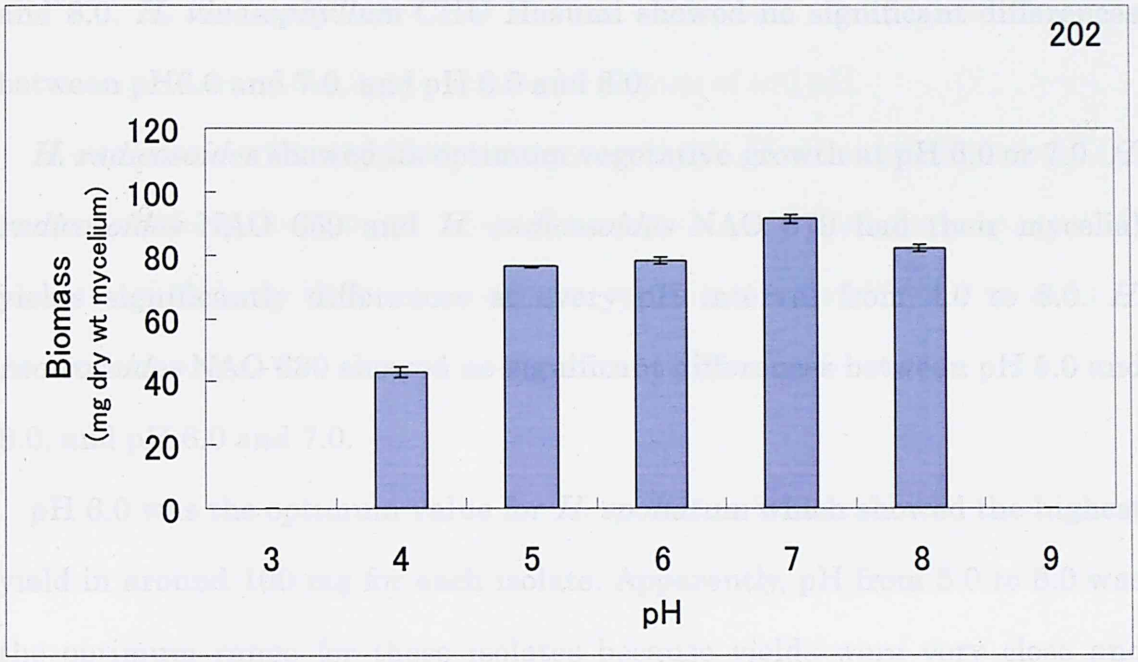


Figure Ⅱ-6 Mycelial yields of *Alnicola lactariense* at different pHs;

Bar indicates SE of the means.

H. aminophilum CHU 3: Optimum mycelial growth was at pH 6.0. There were significant differences at every interval pH scale from 4.0 to 8.0 for vegetative growth;

H. aminophilum CHU 3-4B: Optimum mycelial growth was at pH 6.0 - 7.0. The yield showed there were non significant differences between pH 4.0 and 8.0, pH 5.0 and 6.0, pH 6.0 and 7.0 for vegetative growth;

H. aminophilum CHU 6032: Optimum mycelial growth was at pH 6.0 - 7.0. The yield showed there were no significant differences between pH 5.0 and 6.0, and pH 6.0 and 7.0 for vegetative growth;

H. vinosophyllum showed its optimum mycelial growth consistently at pH 7.0. For *H. vinosophyllum* FB-32636, there were no significant differences between pH 5.0 and 6.0, pH 5.0 and 8.0, and pH 6.0 and 8.0. *H. vinosophyllum* CHU Fast showed no significant difference between pH 5.0

and 8.0. *H. vinosophyllum* CHU Hasumi showed no significant differences between pH6.0 and 7.0, and pH 6.0 and 8.0;

H. radicosoides showed its optimum vegetative growth at pH 6.0 or 7.0. *H. radicosoides* NAO 659 and *H. radicosoides* NAO 679 had their mycelial yields significantly differences at every pH interval from 4.0 to 8.0. *H. radicosoides* NAO 680 showed no significant differences between pH 5.0 and 8.0, and pH 6.0 and 7.0.

pH 6.0 was the optimum value for *H. spoliatum* which showed the highest yield in around 100 mg for each isolate. Apparently, pH from 5.0 to 8.0 was the optimum range for these isolates because yields were very close and higher than those at pH 4.0 or 8.0.

A. lactariense 202: Highest yield was obtained at pH 7.0. Biomasses between pH 5.0 and 6.0, and pH 6.0 and 8.0 showed no significant differences.

Relatively, these ammonia fungi grew well at pH ranges from a weak acid to a weak alkaline conditions with pH 6.0 to 7.0 as their optimum. Fries (1956) reported that *Coprinopsis* species grew well at above pH 8.0. *Coprinopsis* species are belonging to saprobic/ EP ammonia fungi. Yamanaka (2003) reported these ammonia fungi had their optimal pH range from pH 7.0 to 8.0. Data in this study and Chapter Ⅲ show that pH of the growing environment in neutral or alkaline condition always declined a little following the spore germination or mycelial growth. Therefore, the optimum pH for these fungi is correlated with the succession of saprobic/ EP → ectomycorrhizal/ LP fungi in the field where they sporulate. Sagara (1992) and Yamanaka (1995) also proved that EP species sporulated in neutral to

slightly alkaline conditions and LP on acidic soil. It suggests that the succession of ammonia fungi causes the change of soil pH.

Among these ectomycorrhizal ammonia fungi, *H. vinosophyllum* from the warm, northern, temperate zone of East Asia, and *Hebeloma* sp. and *H. aminophilum* from the warm, southern, temperate zone of New Zealand and Australia showed the similar responses to different pHs for their vegetative growth.

Changes of pH in the culturing media were also measured after harvesting. Table Ⅱ-2 shows the final pH of all treatments changed regularly from its initial values. Acid media changed by increasing slightly and those from pH

Table Ⅱ-2. Final pH of the MY media after cultivation

Fungal species	Isolate	Initial pH						
		3	4	5	6	7	8	9
Hebeloma sp.	CHU 931	3.4±0.0	4.2±0.0	5.6±0.0	5.7±0.2	6.7±0.0	7.2±0.0	8.3±0.0
	CHU 932	3.4±0.0	4.4±0.2	5.7±0.0	6.0±0.0	6.8±0.0	7.0±0.0	8.3±0.1
	CHU 2101	3.3±0.1	4.3±0.0	5.4±0.1	5.3±0.1	6.4±0.0	6.7±0.0	8.4±0.1
Hebeloma aminophilum	CHU 3	3.4±0.1	4.6±0.1	5.8±0.1	5.6±0.0	6.7±0.1	7.1±0.0	8.5±0.0
	CHU 3-4B	3.4±0.0	4.5±0.0	5.6±0.0	5.4±0.1	6.9±0.1	7.0±0.2	8.5±0.3
	CHU 6032	3.3±0.0	4.4±0.0	5.4±0.1	5.4±0.1	6.7±0.0	7.1±0.1	8.4±0.1
Hebeloma vinosophyllum	FB-32636	3.3±0.0	4.2±0.0	5.2±0.1	5.9±0.1	6.8±0.1	7.2±0.0	8.3±0.2
	CHU Fast	3.3±0.0	4.3±0.1	5.2±0.1	5.9±0.0	6.9±0.1	7.3±0.1	8.2±0.2
	CHU Hasumi	3.3±0.0	4.5±0.0	5.9±0.1	5.7±0.0	6.9±0.1	7.2±0.1	8.3±0.1
Hebeloma radicosoides	NAO 659	3.4±0.1	4.8±0.1	5.1±0.0	5.5±0.1	6.9±0.0	7.2±0.0	8.5±0.0
	NAO 679	3.4±0.1	4.5±0.0	5.4±0.0	5.8±0.0	6.8±0.0	7.0±0.1	8.4±0.0
	NAO 680	3.3±0.1	4.3±0.1	5.3±0.0	5.8±0.0	6.8±0.1	7.2±0.1	8.4±0.0
Hebeloma spoliatum	NAO 664	3.4±0.1	4.3±0.0	5.3±0.1	5.8±0.1	6.8±0.0	7.2±0.1	8.3±0.0
	NAO 674	3.3±0.1	4.2±0.2	5.2±0.1	5.7±0.0	6.8±0.1	7.3±0.0	8.3±0.1
	FB-12395	3.3±0.1	4.3±0.0	5.1±0.0	5.8±0.1	6.8±0.0	7.1±0.1	8.4±0.0
Alnicola lactariense	202	3.3±0.0	4.1±0.1	5.0±0.1	5.1±0.0	6.1±0.1	6.8±0.0	8.5±0.1

Means and SE calculated from three replicates of each treatment

6.0 to 9.0 decreased a little. It may be considered the actual optimum pH for

vegetative growth of these species was a little lower than the data showed. Because there were no great changes in pH by using MY liquid culture, it suggests that the stability of MY medium makes results in this experiment relatively reliable.

Effects of ammonium-nitrogen concentration on biomass production

Table Ⅱ-3 shows 16 isolates grew from 0.1 mM to 300 mM. Among them, 3 mM was the optimum ammonium-nitrogen concentration, irrespective of species or isolates. Within the growing range of ammonium-nitrogen concentrations, biomasses showed concentrations those higher than 3 mM from 10 mM to 300 mM were more suitable for the vegetative growth of these species than concentrations those lower than 3 mM from 0.1 mM to 1 mM. It means the mycelial growth of these isolates needs enough nitrogenous sources to support. Distilled water as a control showed no growth for all isolates as well as 1,000 mM solution did. Biomasses also showed species from the warm, northern, temperate zone of East Asia, and species from the warm, southern, temperate zone of New Zealand and Australia appeared the similar responses to different ammonium-nitrogen concentrations for their vegetative growth. Figure Ⅱ-7 to Figure Ⅱ-12 show more clear for these 16 isolates in growth responses on different ammonium-nitrogen concentrations.

Table Ⅱ-3 Biomass of 16 isolates of *Hebeloma* spp. cultivated in NH_4Cl synthetic medium under different ammonium-nitrogen concentrations

Fungal species	Isolates	Biomass (mg dry wt mycelium) under different ammonium-nitrogen concentrations (mM)									
		H_2O	0.1	0.3	1	3	10	30	100	300	1000
Hebeloma sp.	CHU 931	0a	4.3±0.9b	5.7±0.7b	15.6±1.2c	30.0±0.6f	27.0±0.6ef	25.3±0.7e	20.7±0.9d	14.3±0.3c	0a
	CHU 932	0a	1.3±0.3a	3.3±0.3ab	10.0±0.6c	24.7±0.7f	18.3±0.3e	15.0±0.6d	11.7±0.3c	3.6±0.3b	0a
	CHU 2101	0a	2.7±0.3ab	5.0±0.6b	14.7±0.9c	27.3±0.3e	26.0±0.6e	20.3±0.7d	19.0±0.6d	13.7±0.7c	0a
Hebeloma aminophilum	CHU 3	0a	4.7±0.3b	5.7±0.3b	14.3±0.9d	27.7±0.3f	22.3±0.7e	22.3±0.9e	16.3±0.3d	9.3±0.3c	0a
	CHU 3-4B	0a	2.3±0.3b	4.3±0.3b	10.7±0.3d	19.7±0.7f	15.7±0.6e	15.3±0.3e	12.3±0.3d	7.0±0.6c	0a
	CHU 6032	0a	1.7±0.3ab	4.7±0.7bc	10.3±0.9d	20.7±1.5g	17.3±0.9fg	16.3±0.7ef	12.3±0.9de	6.3±0.7c	0a
Hebeloma vinosophyllu	FB-32636	0a	3.3±1.3ab	6.7±0.3b	15.3±1.5c	33.3±1.2f	29.0±1.2ef	27.3±1.5e	21.7±1.3d	14.7±0.9c	0a
	CHU Fast	0a	2.0±0.6ab	5.3±0.3b	11.0±1.2c	30.3±0.9f	26.7±0.9ef	24.3±0.9de	22.0±1.0d	10.3±0.9c	0a
	CHU Hasum	0a	1.3±0.3a	2.0±0.6a	8.7±0.7bc	24.3±0.9e	18.3±0.9d	17.0±1.0d	11.3±0.9c	7.3±0.9b	0a
Hebeloma radicosoides	NAO 659	0a	3.7±1.2ab	6.0±0.6bc	13.0±0.6d	30.7±0.9f	18.3±1.3e	16.3±0.3de	14.0±0.6d	9.0±0.6c	0a
	NAO 679	0a	2.3±0.3ab	5.0±0.6b	9.7±0.3cd	27.0±1.7f	14.3±0.9e	13.7±1.2de	11.7±0.7de	6.3±0.3bc	0a
	NAO 680	0a	2.0±0.6ab	4.0±0.6b	8.7±0.3c	27.0±1.2e	14.7±1.5d	13.0±0.6d	11.0±1.0cd	5.3±0.3bc	0a
Hebeloma spoliatum	NAO 664	0a	3.0±0.6ab	6.7±0.3b	11.3±1.5c	22.3±0.9e	18.7±0.9de	18.7±1.3de	16.3±0.3d	8.7±1.2bc	0a
	NAO 674	0a	2.3±0.3ab	6.3±0.3bc	11.7±0.9d	24.0±1.5f	19.0±1.2e	18.7±0.9e	17.7±0.7e	8.7±0.9cd	0a
	FB-12395	0a	5.0±0.6b	7.0±0.6bc	10.7±0.3c	22.0±1.2de	18.0±0.6e	18.0±1.2de	16.0±1.2d	8.3±0.3bc	0a
Alnicola lactariense	202	0a	3.0±0.6b	6.3±0.3c	13.0±0.6d	18.0±0.6f	17.3±0.3ef	16.6±0.3ef	15.3±0.6de	13.0±0.6d	0a

Means and SE calculated from three replicates of each treatment; Yields followed by different letters in the same row are significantly different at $P<0.05$ according to the Tukey-Kramer test.

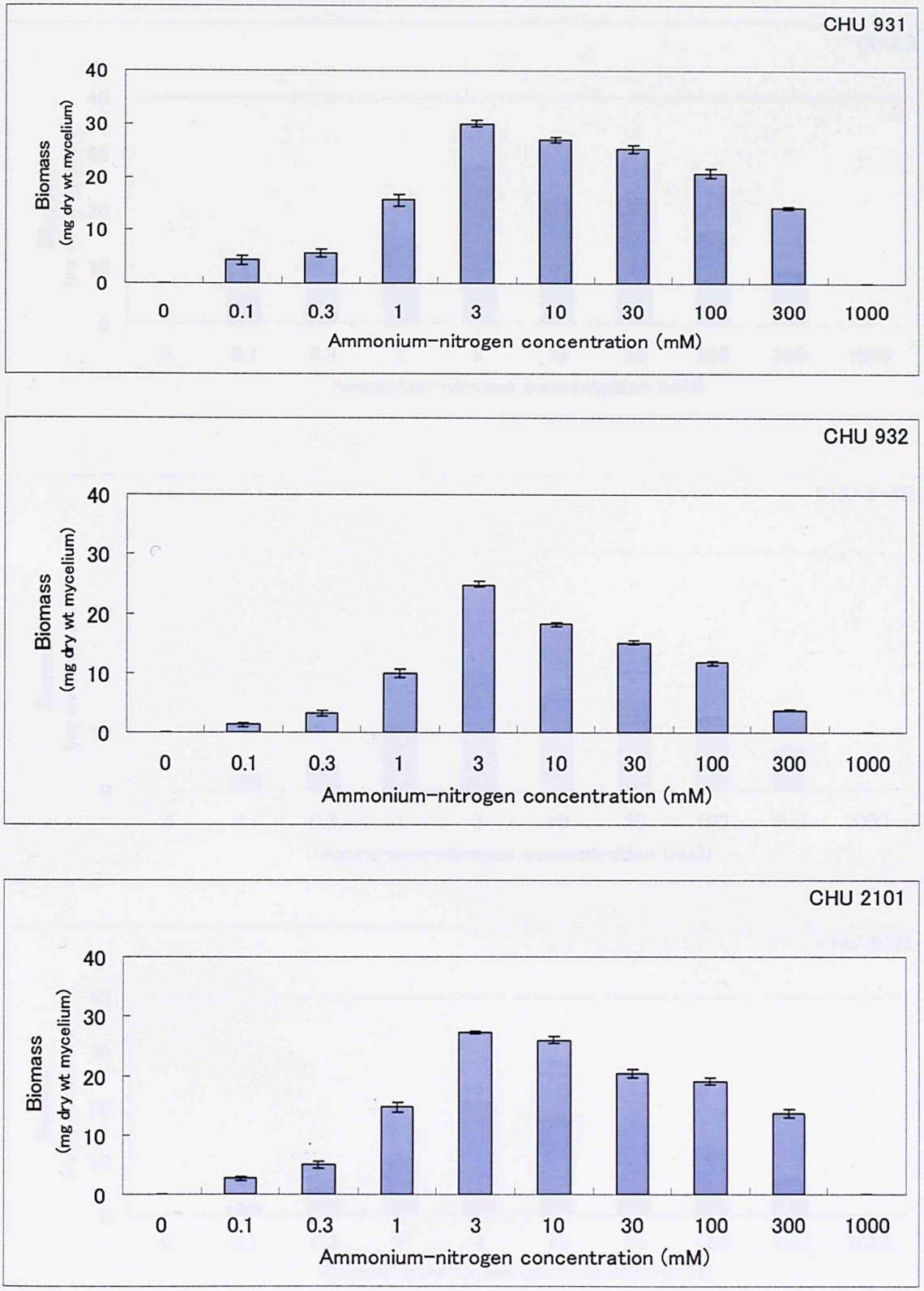


Figure Ⅱ-7 Mycelial yields of *Hebeloma* sp. in different ammonium-nitrogen concentrations; Bar indicates SE of the means.

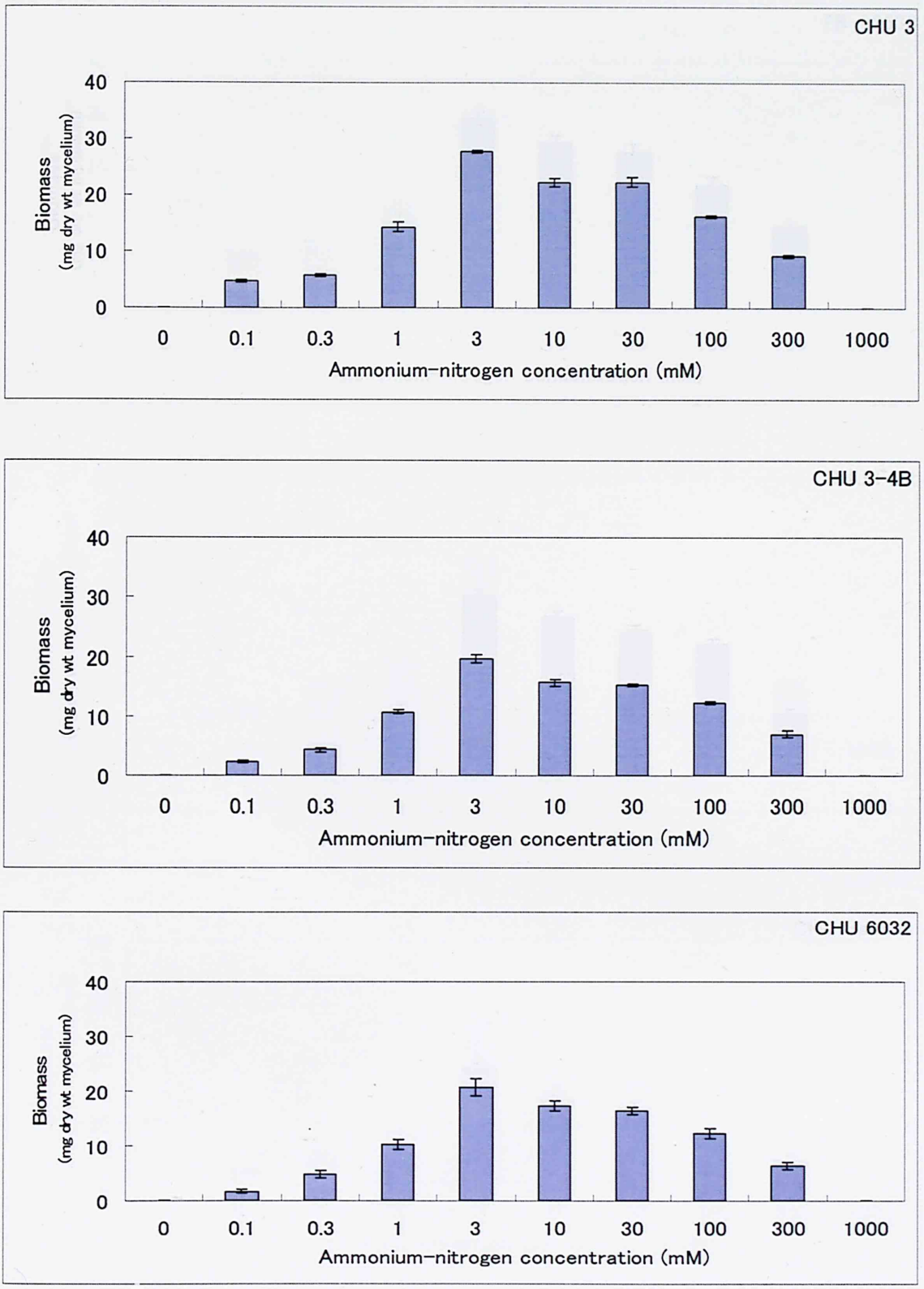


Figure Ⅱ -8 Mycelial yields of *Hebeloma aminophilum* in different ammonium-nitrogen concentrations; Bar indicates SE of the means.

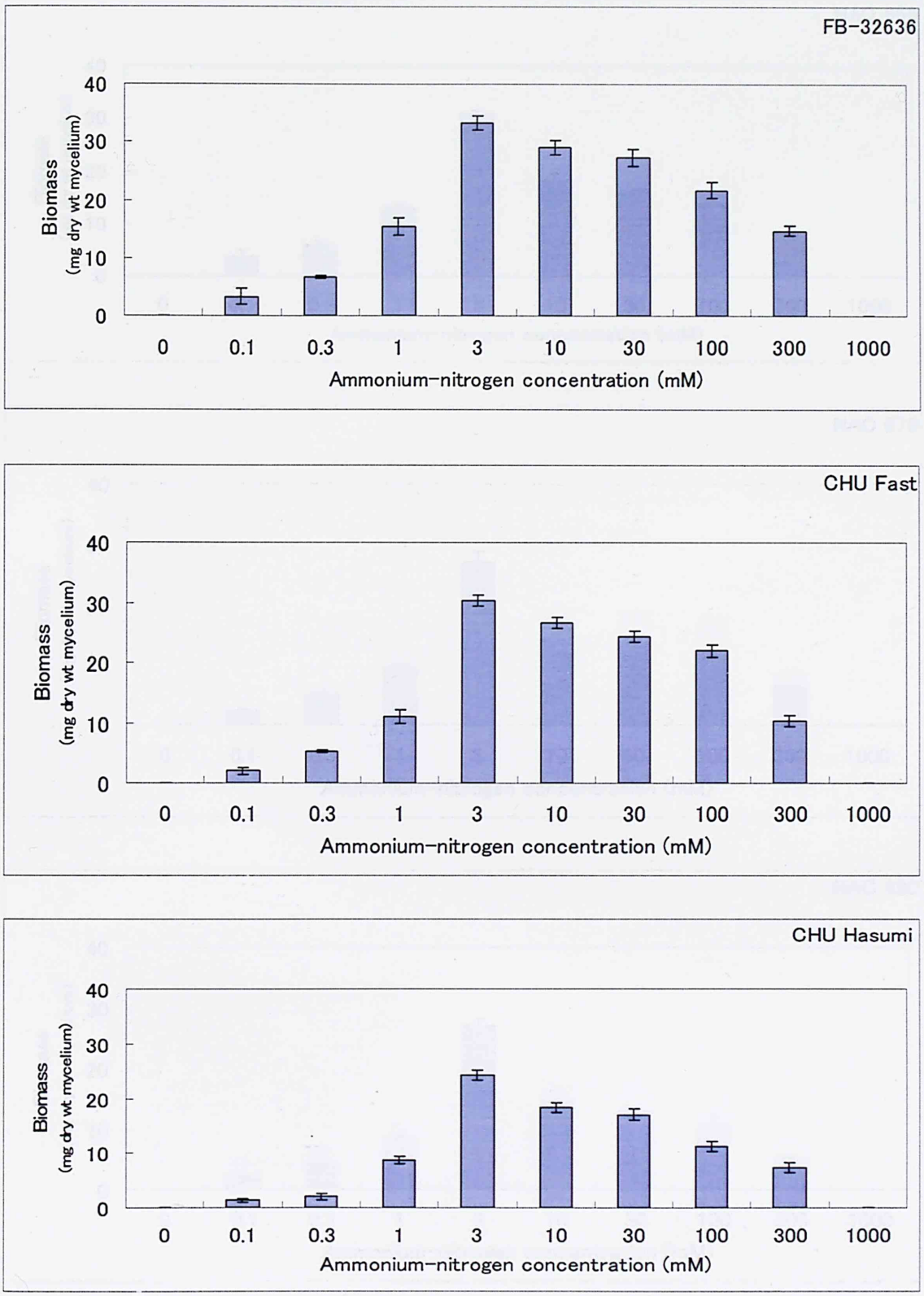
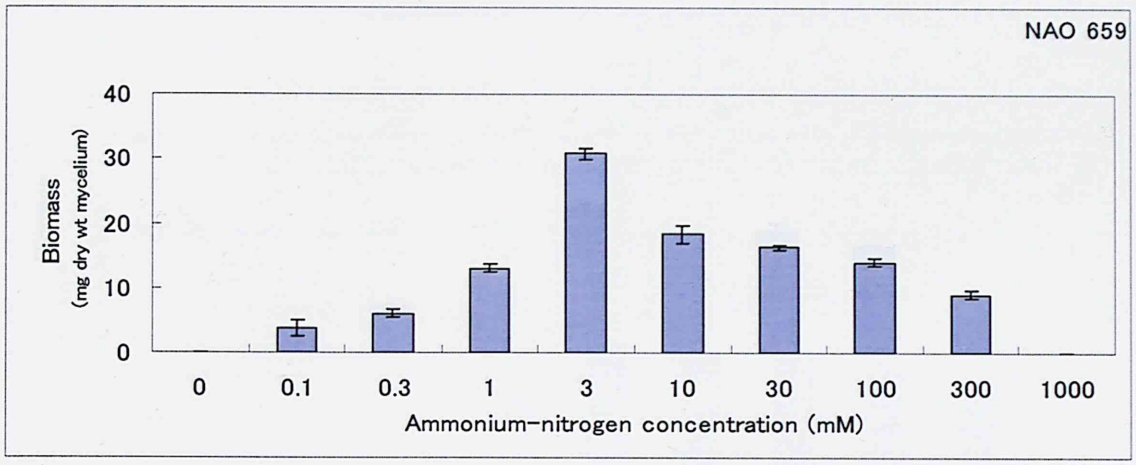
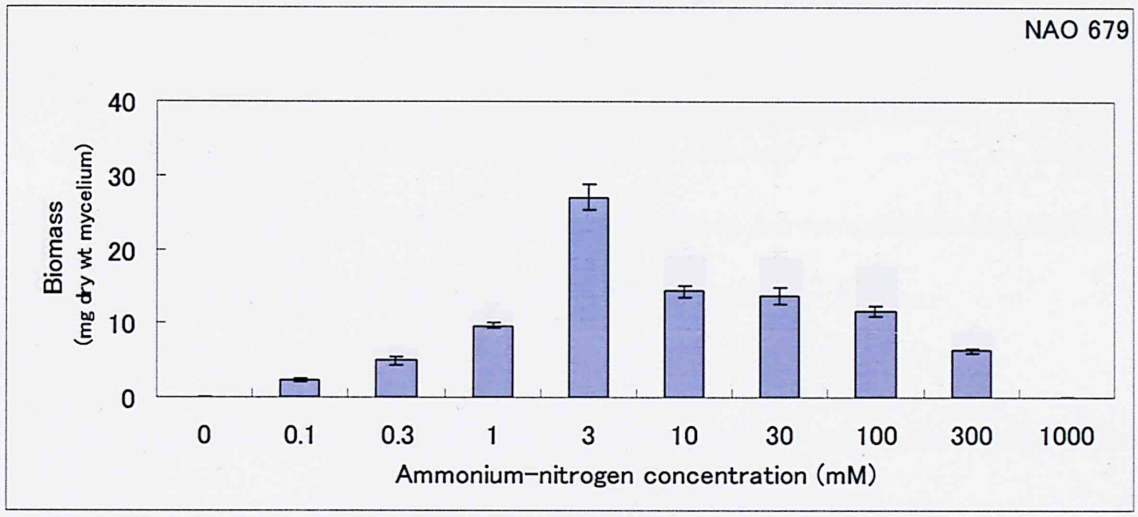


Figure Ⅱ -9 Mycelial yields of *Hebeloma vinosophyllumin* different ammonium-nitrogen concentrations; Bar indicates SE of the means.





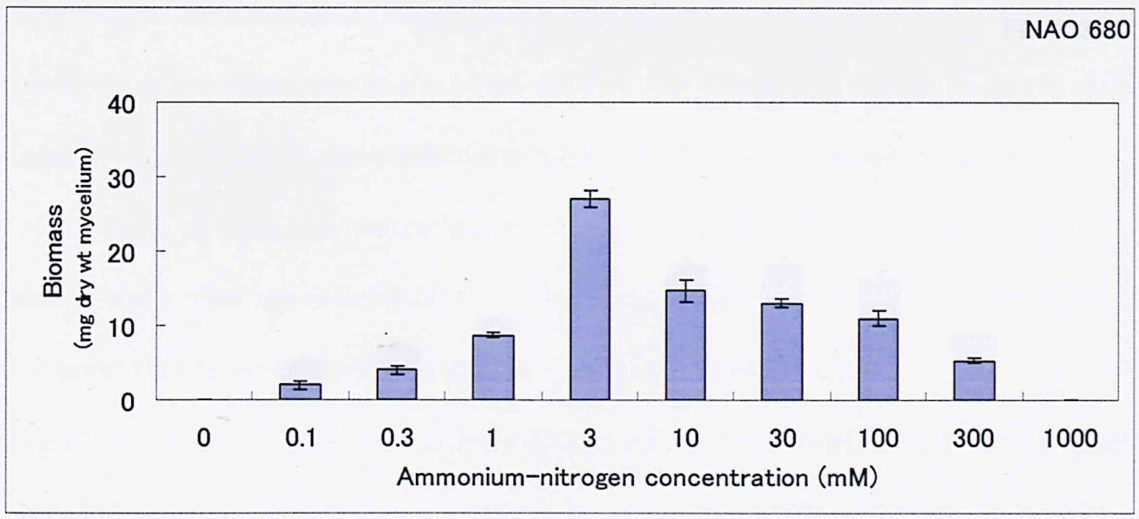
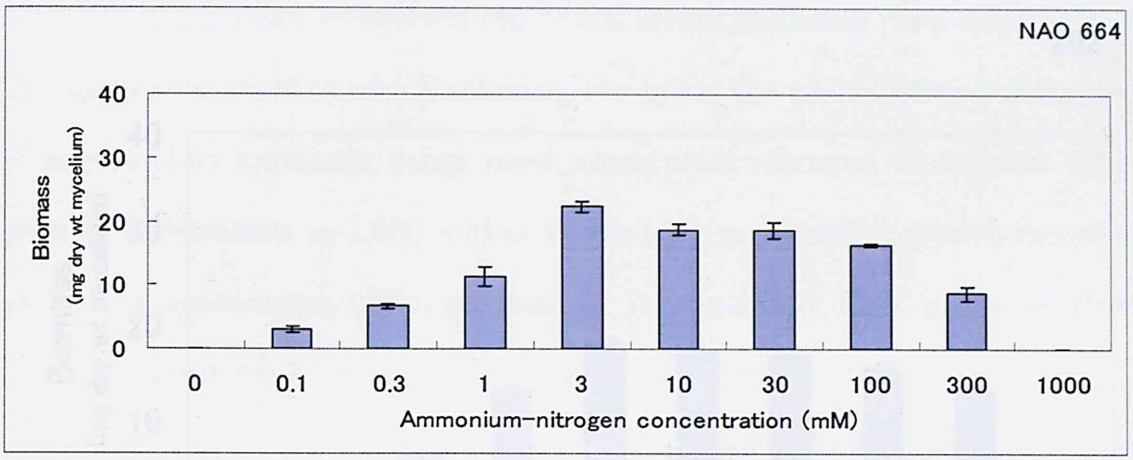
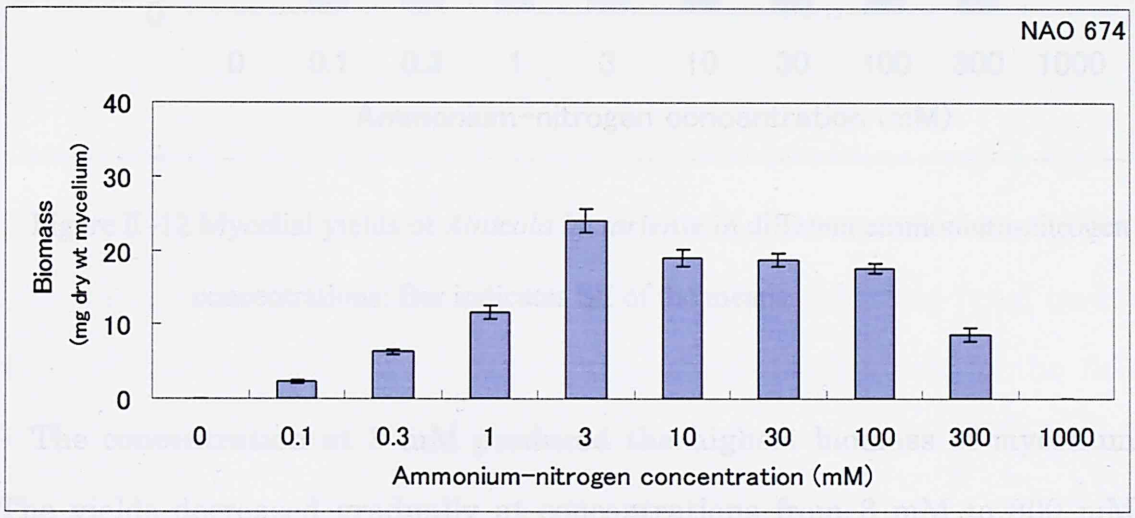


Figure Ⅱ -10 Mycelial yields of *Hebeloma radicosoides* in different ammonium-nitrogen concentrations; Bar indicates SE of the means.





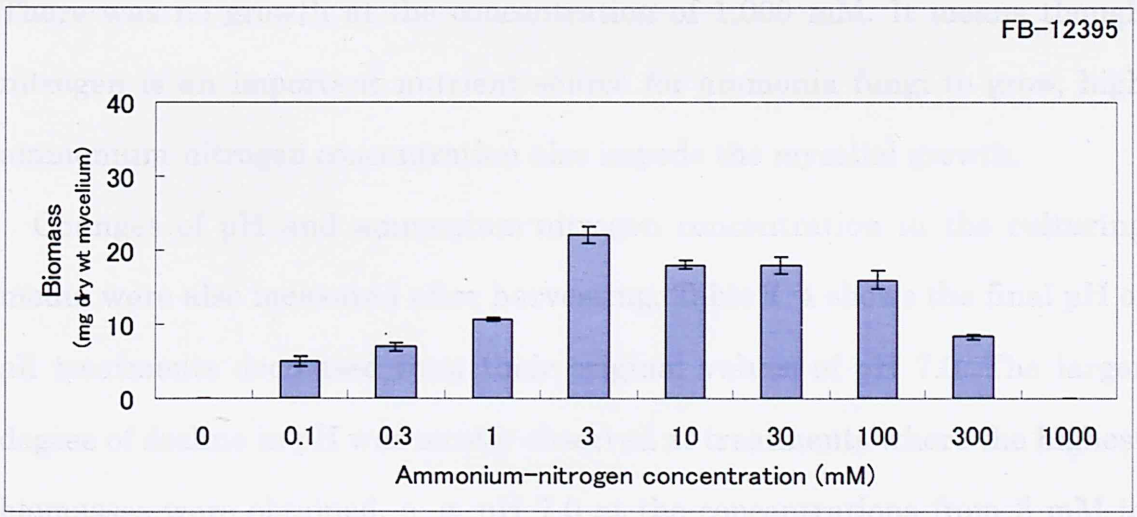


Figure Ⅱ -11 Mycelial yields of *Hebeloma spoliatum* in different ammonium-nitrogen concentrations; Bar indicates SE of the means.

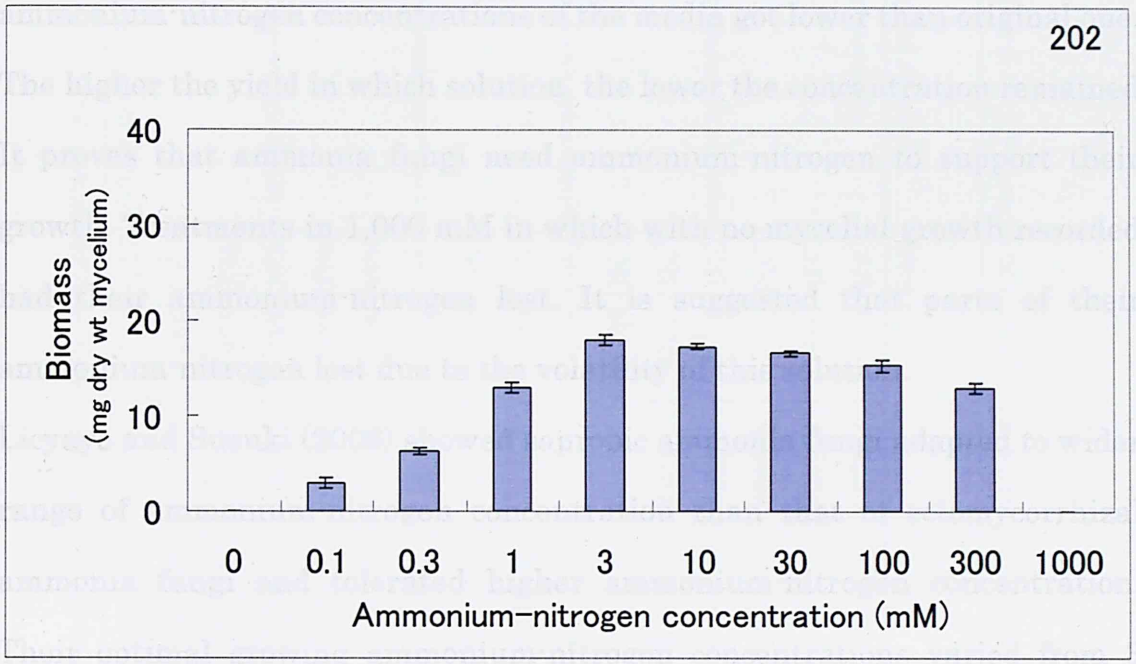


Figure Ⅱ-12 Mycelial yields of *Alnicola lactariense* in different ammonium-nitrogen concentrations; Bar indicates SE of the means.

The concentration at 3 mM produced the highest biomass of mycelium. The yields decreased gradually at concentrations from 3 mM to 300 mM. There was no growth at the concentration of 1,000 mM. It means though nitrogen is an important nutrient source for ammonia fungi to grow, high ammonium-nitrogen concentration also impede the mycelial growth.

Changes of pH and ammonium-nitrogen concentration in the culturing media were also measured after harvesting. Table Ⅱ-4 shows the final pH of all treatments decreased from their original values of pH 7.0. The larger degree of decline in pH was mostly observed at treatments where the highest biomasses were obtained, e. g. pH 7.0 at the concentrations from 3 mM to 100 mM declined to around pH 3.0. On the contrary, pH changed slightly at treatment where low biomasses were obtained. In Table Ⅱ-5, data show that

ammonium-nitrogen concentrations of the media got lower than original one. The higher the yield in which solution, the lower the concentration remained. It proves that ammonia fungi need ammonium-nitrogen to support their growth. Treatments in 1,000 mM in which with no mycelial growth recorded had their ammonium-nitrogen lost. It is suggested that parts of their ammonium-nitrogen lost due to the volatility of this solution.

Licyayo and Suzuki (2006) showed saprobic ammonia fungi adapted to wider range of ammonium-nitrogen concentration than that of ectomycorrhizal ammonia fungi and tolerated higher ammonium-nitrogen concentration. Their optimal growing ammonium-nitrogen concentrations varied from 3 mM to 1000 mM, mostly around 10 mM to 30 mM. Some species were able to grow even under 1300 mM and 1600 mM. Saprobic ammonia fungi invade and colonize earlier than ectomycorrhizal ammonia fungi do in the field when a sudden addition of ammonium materials (Sagara 1975). Following the decomposition and growth of the EP species, decreased concentration of ammonium-nitrogen in the growing environment would be more suitable for the LP species to survive.

Table Ⅱ-4 pH changes of the NH_4Cl synthetic media (initial pH at 7.0) after cultivation at 25°C

Fungal species	Isolates	Ammonium-nitrogen concentrations (mM)								
		0.1	0.3	1	3	10	30	100	300	1000
Hebeloma sp.	CHU 931	6.6±0.1	6.5±0.0	4.9±0.2	3.1±0.0	2.9±0.1	2.8±0.1	3.1±0.1	5.1±0.0	6.5±0.0
	CHU 932	6.7±0.0	6.5±0.0	4.9±0.1	3.1±0.0	2.9±0.0	2.9±0.0	3.2±0.1	5.7±0.2	6.4±0.0
	CHU 2101	6.6±0.0	6.4±0.0	4.7±0.1	3.0±0.1	2.8±0.0	2.8±0.1	3.1±0.0	5.2±0.0	6.4±0.0
Hebeloma aminophilum	CHU 3	6.7±0.0	6.5±0.0	4.7±0.2	3.1±0.0	2.9±0.0	2.9±0.0	3.1±0.0	4.4±0.0	6.4±0.0
	CHU 3-4B	6.6±0.0	6.5±0.0	5.3±0.1	3.1±0.0	3.1±0.1	3.2±0.0	3.4±0.1	4.3±0.0	6.4±0.0
	CHU 6032	6.7±0.1	6.3±0.1	5.4±0.0	3.1±0.0	3.1±0.1	3.2±0.1	3.3±0.1	4.4±0.0	6.4±0.0
Hebeloma vinosophyllu	FB-32636	6.6±0.1	6.3±0.0	4.6±0.1	3.1±0.0	2.8±0.1	2.7±0.0	2.9±0.1	3.4±0.1	6.4±0.0
	CHU Fast	6.7±0.1	6.5±0.0	4.7±0.1	3.1±0.0	3.0±0.0	2.8±0.0	2.9±0.0	3.3±0.0	6.4±0.0
	CHU Hasumi	6.7±0.1	6.5±0.0	4.6±0.0	3.2±0.0	2.9±0.0	2.9±0.1	3.0±0.0	3.4±0.0	6.5±0.0
Hebeloma radicosoides	NAO 659	6.7±0.1	6.4±0.1	4.6±0.0	3.1±0.0	3±0.0	3.0±0.0	3.1±0.0	5.1±0.0	6.3±0.0
	NAO 679	6.6±0.1	6.5±0.1	4.7±0.0	3.1±0.1	3.0±0.1	2.9±0.0	3.1±0.0	5.1±0.1	6.4±0.0
	NAO 680	6.6±0.1	6.5±0.0	4.8±0.1	3.1±0.0	3.0±0.1	2.8±0.0	3.1±0.0	5.2±0.1	6.4±0.0
Hebeloma spoliatum	NAO 664	6.7±0.0	6.6±0.0	4.8±0.0	3.2±0.0	3.1±0.0	3.2±0.3	3.1±0.0	5.6±0.0	6.4±0.0
	NAO 674	6.6±0.1	6.5±0.1	4.8±0.1	3.1±0.0	3.1±0.1	3.2±0.0	3.2±0.0	5.6±0.0	6.3±0.0
	FB-12395	6.7±0.0	6.6±0.0	4.8±0.1	3.1±0.0	3.1±0.0	3.1±0.0	3.2±0.1	5.6±0.0	6.4±0.0
Alnicola lactariense	202	6.6±0.1	6.4±0.0	4.9±0.0	3.2±0.1	3.2±0.1	3.4±0.1	3.3±0.1	3.6±0.0	6.4±0.0

Means and SE calculated from three replicates of each treatment.

Table Ⅱ-5 Changes of ammonium-N concentration of the media after harvesting

Fungal species	Isolates	Initial ammonium-N concentration (mM)		
		3	10	1000
		Final ammonium-nitrogen concentration (mM)		
Hebeloma sp.	CHU 931	0.7	2.9	723
	CHU 932	1	3.4	745
	CHU 2101	0.9	2.8	734
Hebeloma aminophilum	CHU 3	0.8	4.3	698
	CHU 3-4B	1.3	4.6	677
	CHU 6032	1.2	4	711
Hebeloma vinosophyllum	FB-32636	0.6	2.3	659
	CHU Fast	0.8	3.1	703
	CHU Hasumi	0.9	3.7	676
Hebeloma radicosoides	NAO 659	0.7	3.9	751
	NAO 679	1	4.1	732
	NAO 680	0.9	4.6	755
Hebeloma spoliatum	NAO 664	1.2	4.2	691
	NAO 674	1	4.4	603
	FB-12395	1.2	4	686
Alnicola lactariense	202	1.3	4	724

※ Only the two concentrations which had maximum mycelial growth (3 mM, 10 mM) and the one had no mycelial growth (1000 mM) being measured as comparison; samples were chosen randomly in no replicate.

Conclusion

Fungi generally grow well in acidic condition (Dix & Webster, 1995), but some species favor neutral to slightly alkaline conditions. In the case of ammonia fungi, optimal pHs for saprobic / EP species and ectomycorrhizal / LP species were a little different (Yamanaka 2003). For ectomycorrhizal fungi, they grew on acidic soil. Mycorrhizal fungi usually tolerated a broader range of pH in symbiosis (Erland et al. 1990). This study shows the ectomycorrhizal ammonia fungi were able to grow well at the range of pH 5.0 to 7.0, and survived from a wide range of pH 4.0 to 8.0.

In addition to the tolerable pH range, nitrogen source was also an important factor for their survival. Table Ⅱ-3 shows no growth did in pure water but grew from a very low concentration of nitrogen at 0.1 mM. Ectomycorrhizal species had different responses to a nitrogen source in pure culture from saprobic species. They grew well not only on ammonium-nitrogen but also on nitrate-nitrogen (Yamanaka 1999). Ammonium-nitrogen in soil increased at an early phase after the treatment with urea, and nitrate-nitrogen increased at the late phase (Yamanaka 1995c).

Unfortunately, there were no more evidences from the changes of pH or ammonium-nitrogen concentration can differentiate the species from East Asia to Southern Hemisphere since the tendency of changes of pH and ammonium-nitrogen concentration in the culturing media was all the same to 16 isolates, irrespective of their biogeographic distributions.

With the application of urea in the field, *A. lactariolens* has been collected

from the central part of Honshu Island, Japan to Taiwan, and *H. radicosoides* is known from the central part of Honshu Island to Iriomote Island in the subtropical region of Japan. *H. vinosophyllum* has been recorded from Japan (Sagara 1975, 1976, 1992, 1995; Suzuki 1992; Yamanaka 1995a, b, c; Fukiharu & Horigome 1996) and China (Hongo 1996). *H. spoliatum* has been obtained in European Continent, East Asia and Siberia even Central Africa. Laccaria spp. were recorded from many areas around the world. In New Zealand and Australia, only *H. aminopilum* and *Laccaria* spp. have been recorded (Suzuki et al. 2003). It seems to mean that the biogeograpic distribution of these *Hebeloma* spp. are not mainly determined by the factors such as acidic or alkaline conditions and the richness of nitrogen sources in soil. It is suggested that ecological environment especially the formation of vegetation will be an important factor affecting the biogeographic distribution of ectomycorrhizal fungi because different vegetation has different host plants.

CHAPTER Ⅲ

Effects of pH, ammonium-nitrogen concentration, temperature, and storage period on basidiospore germination in an ectomycorrhizal ammonia fungus *Hebeloma vinosophyllum*

Introduction

"Ammonia fungi" are defined as a chemoecological group of fungi that sequentially develop reproductive structures exclusively or relatively luxuriantly on soil after a sudden addition of ammonia, or of other nitrogenous materials that react as bases by themselves or on decomposition, or alkalis (Sagara 1975). According to the sequential occurrence of ammonia fungi in the field, they are divided into two types, early phase fungi (EP fungi) and late phase fungi (LP fungi). The former type species are saprobic fungi and most of the latter type species are ectomycorrhizal fungi (Sagara 1995).

Invasion sequence of the saprobic ammonia fungi (EP fungi) was examined by the cultivation of packed soils, which had been collected from the forest floor at different days after urea application, in sterilized test tubes with cotton plugs (Suzuki et al. 2002b). However, invasion sequence of ectomycorrhizal ammonia fungi has not been examined by this method, since the ectomycorrhizal ammonia fungi never form basidiomata by the

urea-treated soil cultivation without their host plant(s) (Suzuki et al. 2002b; Suzuki 2006).

Suzuki et al. (1982) found that basidiospore germinations in EP fungi *Coprinopsis cinerea* and *C. phlyctidospora* were stimulated by the presence of ammonium-nitrogen under alkaline to slightly acidic conditions. Suzuki (1978) found that basidiospore germination in LP fungus *Hebeloma vinosophyllum* was also stimulated by 0.1 –100 mM aqua ammonia. Later, it was confirmed that the spore germination of the three EP fungi, namely conidium germination of *Amblyosporium botrytis*, ascospore germination of *Ascobolus denudatus*, and basidiospore germination of *C. phlyctidospora*, were markedly stimulated by the water extract of soil obtained 6 days after urea application. Moreover, it was revealed that basidiospore germination in the LP fungi *Hebeloma spoliatum* and *H. vinosophyllum* was also markedly stimulated by the water extract of the urea-treated soil (Suzuki 2006). These results suggest that the spore germination of ammonia fungi would be stimulated by the presence of ammonium-nitrogen under a weak alkaline to a weak acidic conditions.

The appearance of reproductive structures of ammonia fungi results either from mycelial growth that prefers or tolerates high concentrations of ammonium-nitrogen under a weak alkaline to a weak acidic conditions, and/or from spore germination that is stimulated by ammonium-nitrogen under the same conditions (Suzuki 1989, 2006; Yamanaka 1999, 2003; Suzuki et al. 2002b; Licyayo and Suzuki 2006).

The latent form(s) of ammonia fungi in the field has not yet been fully

examined, but Sagara (1976) speculated that the spores and/or the fragment of the hyphae would be principal propagules for their rapid colonization. There are some preliminary data about the spatial distribution of living propagules of several EP fungi in the fields (Suzuki et al. 2002b; Suzuki 2006) and the spore longevity of a LP fungus *H. spoliatum* (Suzuki 2003). There are enough data to elucidate the propagation mechanism of each ammonia fungus. Therefore, it may be valuable to examine the longevity of spores in various environmental conditions and the effects of different environmental factors such as pH, ammonium-nitrogen concentration and temperature, which would be principal factors for their colonization in the field, on the spore germination in order to elucidate the colonizing mechanism of ammonia fungi.

On this background, as the first step to elucidate the colonization mechanism of ammonia fungi, the longevity of basidiospores of *H. vinosophyllum* was examined as well as its basidiospore germination under different environmental conditions such as pH, ammonium-nitrogen concentration and temperature, which would be principal factors to elucidate the colonization mechanism of ammonia fungi in the field.

Materials and Methods

Organism

Stock culture of a dikayotic isolate of *Hebeloma vinosophyllum* Hongo (isolate CHU Kiyosumi) was used in this study. The stock culture had been isolated from a *Quercus* and *Castanopsis* dominating forest in Chiba, Japan (Licyayo and Suzuki 2006).

Preparation of spore suspension

The stock culture was inoculated on MY agar slant [10 g malt extract (Difco, Becton, Dickinson and Co., USA), 2 g yeast extract (Difco, Becton, Dickinson and Co., USA), 15 g agar (Nacalai tesque), 1,000 ml pure water] and the slants placed upright were incubated at 25.0±0.5°C in the dark until basidiomata were formed. Then, a piece of sterilized filter paper (No.1, Advantec) was placed into the slant facing the pilei. Basidospores were obtained aseptically from the filter paper having spore prints. Only the basidospores discharged in the first three days were used for this study. Twenty milliliter of the spore suspension was centrifuged at 500 rpm for 5 minutes at 5°C and the supernatant solution was decanted. After addition of another 50 ml of sterilized pure water, the spore suspension was again centrifuged and the supernatant decanted. This procedure was repeated three times. Finally, the water was removed and basidospores precipitated in the bottom of a centrifugation tube were collected for the following experiments. The basidiospores used for storage experiment were not rinsed

with pure water.

Cultivation and sampling methods for spore suspension

Aqueous solution of ammonium chloride (NH_4Cl) for culturing basidiospores was sterilized by filtration (acetate cellulose, 0.2 μm pore size, Advantec). The basidiospores were suspended in these solutions. As a negative control, the basidiospores were suspended into pure water sterilized by the membrane filter filtration. Basidiospore densities were adjusted as described below. Twenty milliliter of each suspension was poured into a sterile glass bottle (4 cm in diameter, 6.5 cm in height) sealed with screwed caps with silicon rubber and incubated in different conditions as described below. Just after the stirring of basidiospore germination, 0.5 ml each of suspension was taken out for microscopic examination at different incubation periods.

Incubation of spore suspension under various environmental conditions

1. Spore density

Aqueous solution containing 100 mM NH_4Cl was adjusted to pH 8.0 with KOH and the rinsed basidiospores were suspended at different spore densities (5.0×10^5 - 2.0×10^7 spores/ ml). Spore densities were determined with a hemocytometer by the dilution method. Twenty milliliter of each suspension was poured into the glass bottle as described above and incubated for 14 days in the dark at different temperatures from 5.0°C to 30.0°C at 5.0°C intervals. The precision of the each designated temperature

was ±0.5°C. The incubation and sampling of the spores were done by the same procedure as described above.

2. Ammonium-nitrogen concentration

For the effects of ammonium-nitrogen concentration on spore germination, NH_4Cl aqueous solution was adjusted at different concentrations (10 mM - 1000 mM) to pH 8.0 with KOH using a glass electrode. The rinsed basidiospores were suspended at spore density of 1.0×10^6 - 2.0×10^6 spores/ ml. Meanwhile, as a positive control, the rinsed basidiospores were also suspended in 50 mM dibasic ammonium phosphate aqueous solution [$(NH_4)_2HPO_4$, (pH 8.0)] at the density of 1×10^6 - 2×10^6 spores/ ml. The basidiospore suspensions were incubated for 14 days in the dark at different temperatures from 5.0°C to 30.0°C at 5.0°C intervals. The precision of the each designated temperature was ±0.5°C. After the end of the incubation, the final ammonium-nitrogen concentration of the spore suspensions was measured by HPLC (Licyayo and Suzuki 2006) and the final pH was measured with a glass electrode.

3. pH

The aqueous solution containing 100 mM NH_4Cl was adjusted to pH 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, or 9.5 with KOH, NaOH, or NH_4OH (aqua ammonia) for pH above 6.0, and with HCl or H_2SO_4 for pH below 5.0. The precision of each designated pH was ±0.05. The rinsed basidiospores were suspended at a spore density of 1.0×10^6 - 2.0×10^6 spores/ ml, and incubated

at 25.0±0.5°C in the dark for 12 days. After the end of incubation, the final pH of the suspensions was measured with a glass electrode.

4. Temperature

The rinsed basidiosores suspended at the spore density of $1.0 \times 10^6 \cdot 2.0 \times 10^6$ spores/ ml in 100 mM NH_4Cl aqueous solution at pH 8.0, were incubated at various temperatures from 5.0°C to 40.0°C at 5.0°C intervals in the dark for 14 days. Other incubation methods and sampling methods were done by the same way described above. After the end of incubation, the final pH of suspensions was measured with a glass electrode.

5. Spore longevity

The spore prints made on the sterilized filter paper (No. 1, Advantec) were placed in a glass bottle of the same size as described above. A smaller glass bottle (1.5 cm in diameter, 4.0 cm in height) was put into the bottle (4.0 cm in diameter, 6.5 cm in height) and fixed by filling the gap between the two bottles with paper. For the dry condition, the small bottle (inner bottle) was filled with silica gel as a desiccant. Containers were sterilized by dry heating at 140°C for 40 minutes. For the wet condition, after sterilization by dry heating, 8 ml of sterilized pure water was poured into the small bottle. After placing the spore prints into the gap between the small and large bottles (outer bottle), the large bottle was sealed with a silicon rubber cap sterilized by autoclaving and stored at various temperatures from 5.0°C to 30.0°C at 5.0°C intervals in the dark. The precision of each designated temperature

was ±0.5°C. During the stored period, a part of the non-rinsed spores (intact spores) were taken out at different sampling dates and then suspended at a spore density of 1.0×10^6 - 2.0×10^6 spores/ ml in 100 mM NH_4Cl aqueous solutions adjusted to pH 8.0 with KOH. Thereafter, the spore suspensions were incubated for 12 days at 25.0±0.5°C in the dark.

Microscopic observation of spore germination

Germination of a basidiospore in the present study was defined as the protrusion of hypha(e) [germ tube(s) discernible (Fig.Ⅲ-1) under a microscope. In each treatment, 100 spores were examined in random microscope fields to count the germination percentage. All results are shown as the average of three replications.

Spore germination percentage computation:

$$\text{Germination (\%)} = \frac{\text{Number of spore germinated}}{\text{Total number of spore observed}} \times 100$$

Observation of mycelial growth under different temperatures

Stock dikayotic isolate and monokaryotic isolates of four mating types of this fungus was inoculated on the center of the MY agar plates then incubated at various temperatures from 5.0°C to 40.0°C at 5.0°C intervals in the dark. The precision of each designated temperature was ±0.5°C. For the measurement of vegetative growth speed of the isolates, the radii of four cross directions at 90° interval of each mycelial colony were measured at a 15-day incubation

and then the average of the four radii in three replicates was calculated to be the index of vegetative growth ability of each culture.

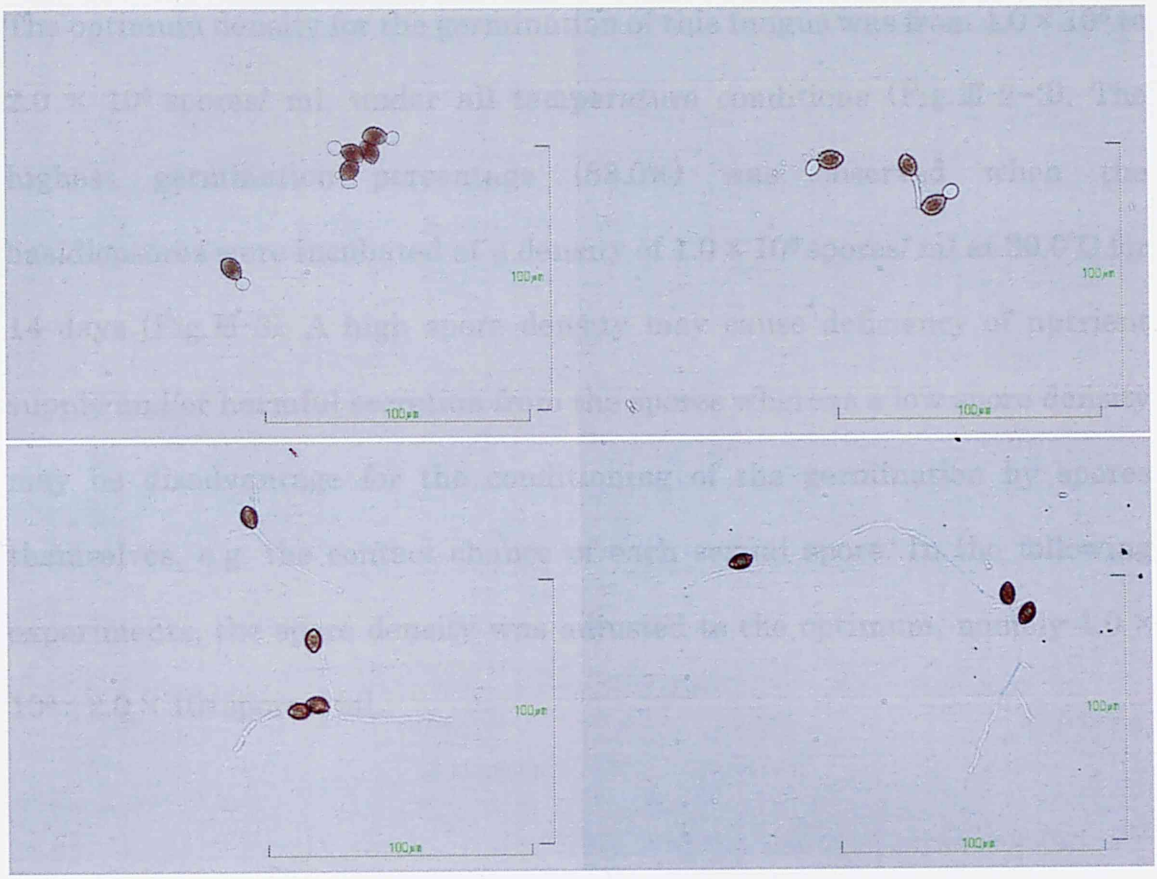


Figure Ⅲ-1 The germinating morphologies in basidiospores of *Hebeloma vinosophyllum* observed under a microscope

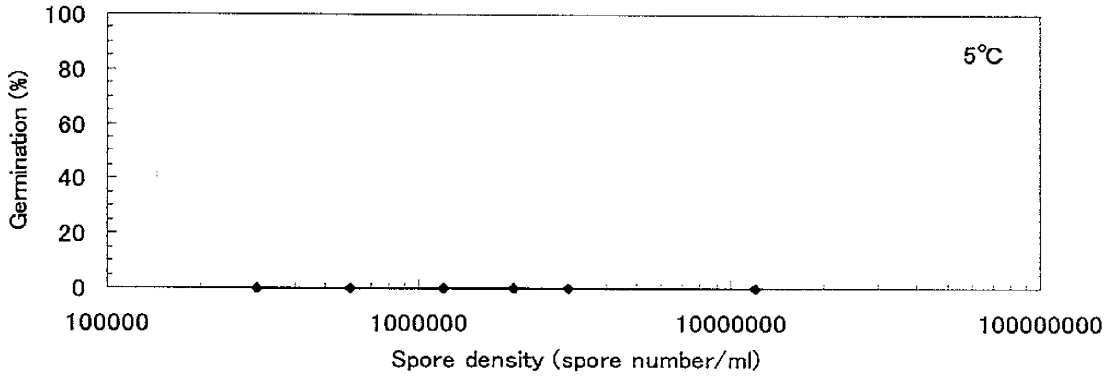
Statistical analysis

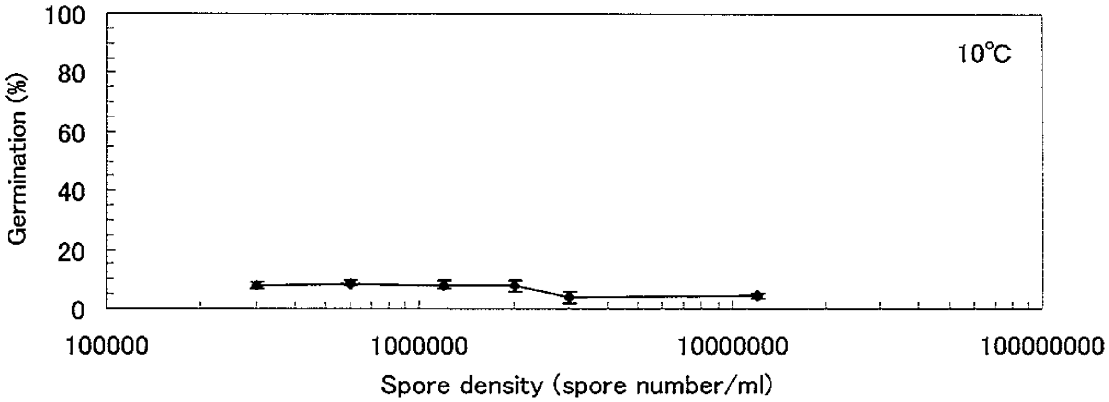
All statistical analyses were performed by the Tukey-Kramer method.

Results and Discussion

Effect of spore density on germination

The optimum density for the germination of this fungus was from 1.0×10^6 to 2.0×10^6 spores/ ml, under all temperature conditions (Fig.Ⅲ-2~3). The highest germination percentage (83.0%) was observed when the basidiopsores were incubated at a density of 1.0×10^6 spores/ ml at 30.0°C for 14 days (Fig.Ⅲ-3). A high spore density may cause deficiency of nutrient supply and/or harmful secretion from the spores whereas a low spore density may be disadvantage for the conditioning of the germination by spores themselves, e.g. the contact chance of each sexual spore. In the following experiments, the spore density was adjusted to the optimum, namely 1.0×10^6 - 2.0×10^6 spores/ ml.





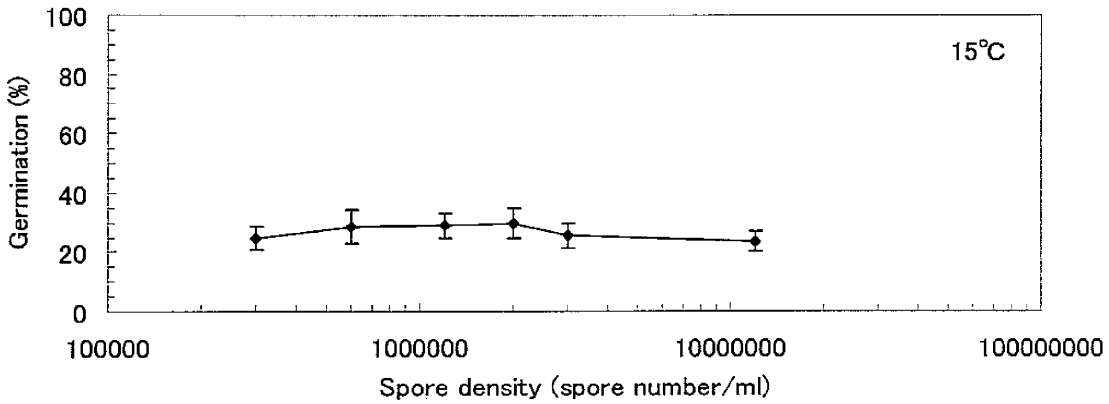
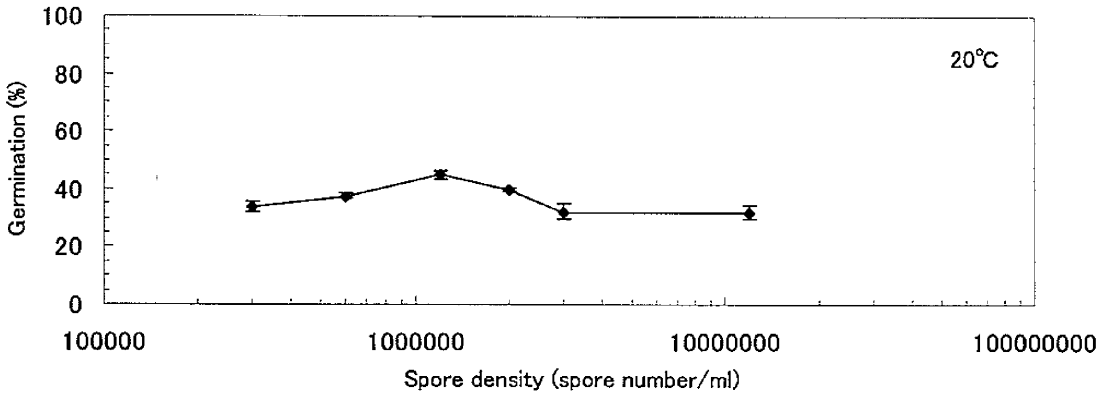
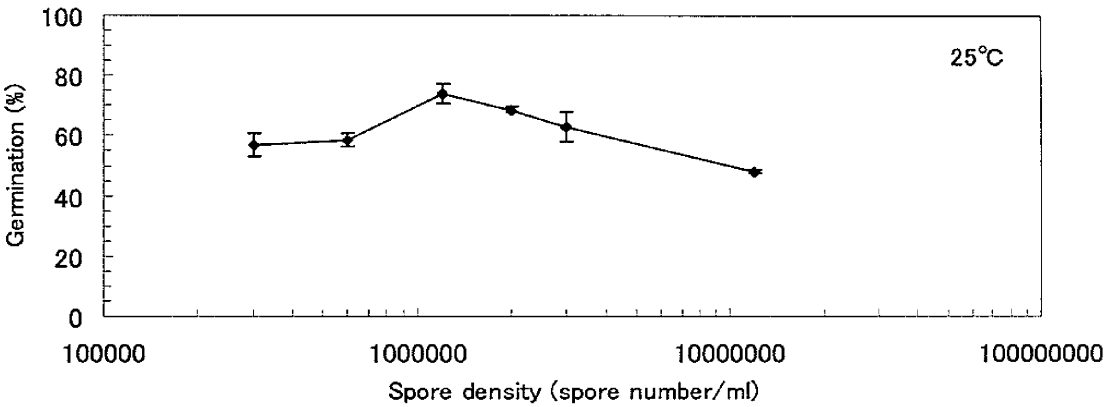


Figure Ⅲ-2 Germination rate of *Hebeloma vinosophyllum* at different densities under different temperatures; values indicate means ±SE.





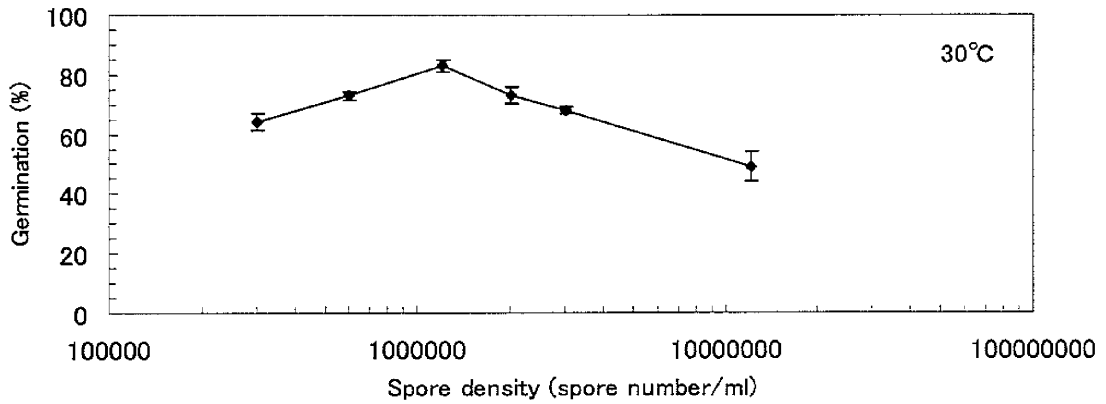
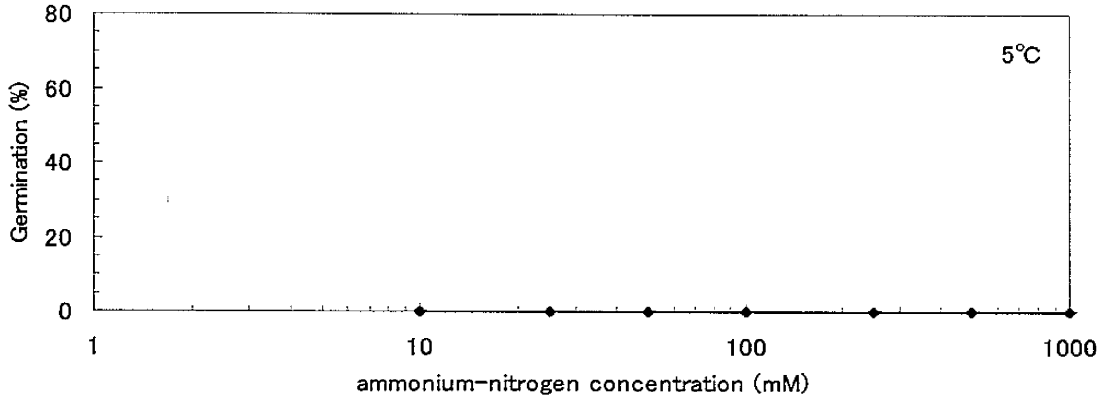
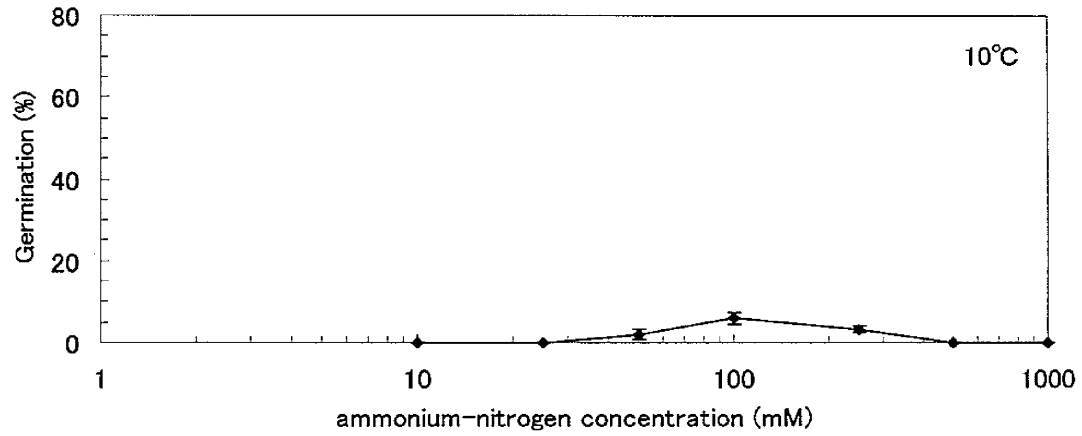


Figure Ⅲ-3 Germination rate of *Hebeloma vinosophyllum* at different densities under different temperatures; values indicate means ±SE.

Effect of ammonium-nitrogen concentration on germination

Basidiospore germination of *H. vinosophyllum* was stimulated by 10 mM to 500 mM NH_4Cl aqueous solution, but not by 1000 mM NH_4Cl aqueous solution adjusted to pH 8.0, irrespective of temperature conditions (10.0°C - 30.0°C), (Fig.Ⅲ-4~5). The highest germination percentage, 74.3%, was obtained in 100 mM NH_4Cl solution. The concentration of ammonium-nitrogen decreased with increasing germination percentage (Table Ⅲ-1). The 50 mM $(NH_4)_2HPO_4$ aqueous solution (pH 8.0) also stimulated the spore germination (80.7% by a 14-day incubation at 25.0°C in the dark). Suzuki (1978) reported that basidiospore germination of *H. vinosophyllum* was markedly stimulated by 0.5 - 5 mM aqua ammonia, but not in pure water. These results indicate that basidiospore germination of *H. vinosophyllum* is stimulated by ammonium-nitrogen, but not by chloride ions. The ammonium-nitrogen concentration markedly decreased at the concentration which gave a higher germination percentage, although the ammonium-nitrogen concentration decreased in all experiments (Table Ⅲ-1). This suggests that the weak decrease in ammonium-nitrogen concentrations may be caused by the volatilization of ammonia. Decline of ammonium-nitrogen concentrations became more pronounced when germination percentage became higher. This may mainly derived from the absorption of ammonium-nitrogen by the basidiospore as a nitrogen source for germ tube growth and not simply as a germination triggering substance.





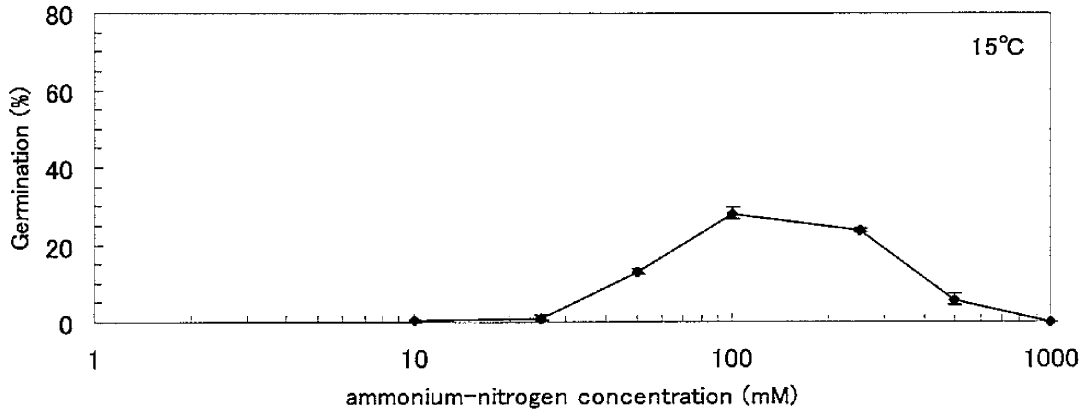


Figure Ⅲ -4 Germination rate of *Hebeloma vinosophyllum* at different ammonium-nitrogen concentrations under different temperatures; values indicate means ±SE.

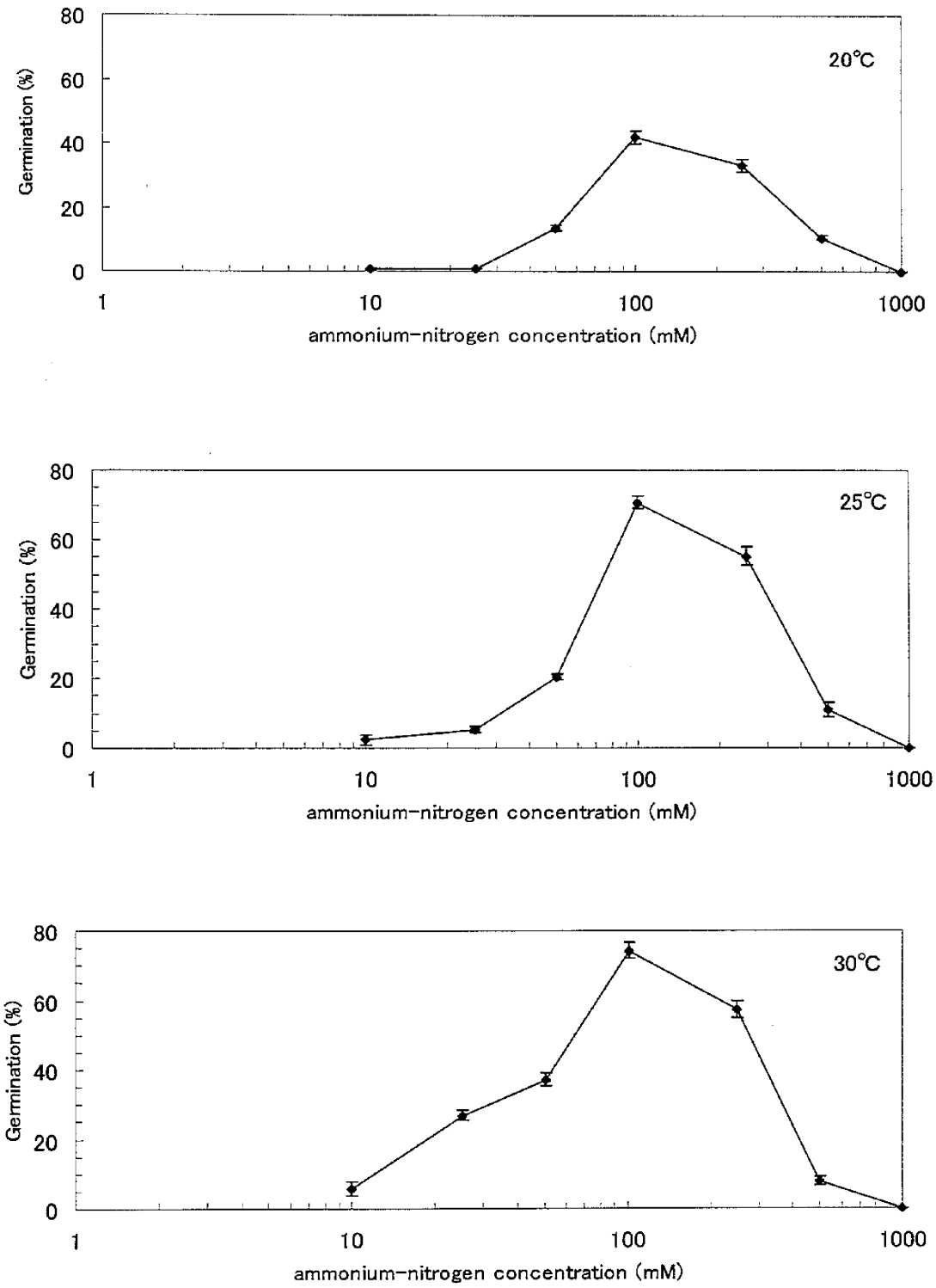


Figure Ⅲ -5 Germination rate of *Hebeloma vinosophyllum* at different ammonium-nitrogen concentrations under different temperatures; values indicate means ±SE.

Table Ⅲ-1. Changes in pH and ammonium-nitrogen concentration of the basidiospore suspension of *Hebeloma vinisiphyllum* after a 14-day incubation

The basidiospores were suspended (1.0×10^6 - 2.0×10^6 spores/ ml) in NH_4Cl aqueous solution at different ammonium-nitrogen concentrations (initial pH 8.0). The spore suspensions were incubated at different temperatures in the dark.

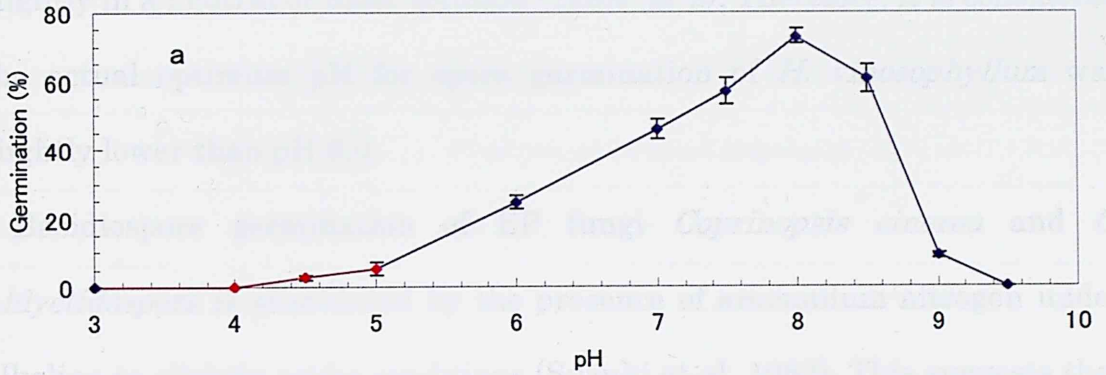
Initial ammonium-nitrogen concentration	Final pH[a]/Final ammonium-nitrogen[b] concentration (mM) of the spore suspensions incubated at different temperatures (°C)					
(mM)	5.0	10.0	15.0	20.0	25.0	30.0
10	6.5[a]/7[b]	6.9/7	6.8/6	6.8/5	7.1/5	6.9/4
25	6.9/14	7.1/14	7.0/10	7.0/10	7.2/9	7.2/8
50	7.1/25	7.3/24	7.2/23	7.2/21	7.4/20	7.3/14
100	7.4/65	7.3/55	7.4/53	7.3/48	7.4/43	7.4/25
250	7.5/149	7.5/120	7.5/118	7.4/112	7.5/106	7.4/100
500	7.5/496	7.6/424	7.5/376	7.5/323	7.5/241	7.4/351
1000	7.7/886	7.7/946	7.6/818	7.7/837	7.9/758	7.5/608

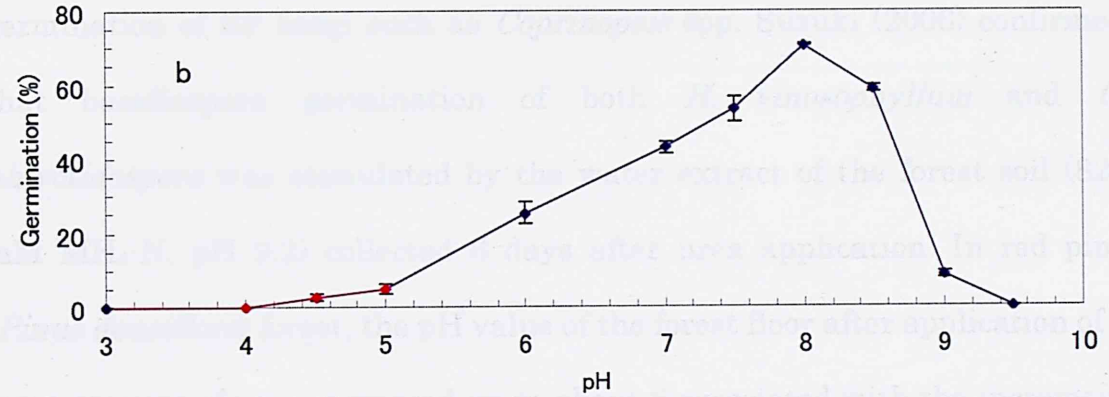
[a]Final pH

[b]Final ammonium-N

Effects of pH on basidiospore germination

Spore germination was stimulated at pH 4.5 - 9.0 with the optimum at pH 8.0, in the presence of 100 mM (optimum concentration) of NH_4Cl (Fig.Ⅲ-6). The highest germination percentage, 73.0%, was observed at pH 8.0 after a 12-day incubation at 25.0°C. There was no significant difference in percentage germination of the basidospores among reagents used for pH





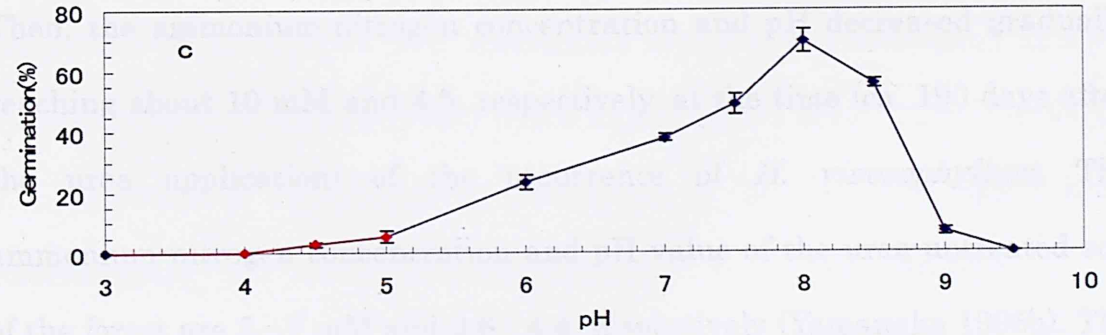


Figure Ⅲ-6 Germination rate of *Hebeloma vinosophyllum* at different pHs under 25.0°C; values indicate meas±SE; a: pH adjusted by HCl for 3.0-5.0 and KOH for 6.0-9.5; b: pH adjusted by H_2SO_4 for 3.0-5.0 and NaOH for 6.0-9.5; c: pH adjusted by HCl for 3.0-5.0 and NH_4OH for 6.0-9.5.

adjustments. The final pH value rose slightly in acid solution, and decreased slightly in a neutral or basic solution (Table Ⅲ-2). Therefore, it is considered the actual optimum pH for spore germination of *H. vinosophyllum* was slightly lower than pH 8.0.

Basidiospore germination of EP fungi *Coprinopsis cinerea* and *C. phlyctidospora* is stimulated by the presence of ammonium-nitrogen under alkaline to slightly acidic conditions (Suzuki et al. 1982). This suggests that the effect of pH on spore germination of LP fungi is similar to that on spore germination of EP fungi such as *Coprinopsis* spp. Suzuki (2006) confirmed that basidiospore germination of both *H. vinosophyllum* and *C. phlyctidospora* was stimulated by the water extract of the forest soil (820 mM MH_4-N, pH 9.2) collected 6 days after urea application. In red pine (*Pinus densiflora*) forest, the pH value of the forest floor after application of a large amount of urea increased up to about 8 associated with the increment of ammonium-nitrogen concentration up to about 1000 mM at the 7th day. Then, the ammonium-nitrogen concentration and pH decreased gradually reaching about 10 mM and 4.5, respectively, at the time (ca. 190 days after the urea application) of the occurrence of *H. vinosophyllum*. The ammonium-nitrogen concentration and pH value of the urea-untreated soil of the forest are 3 - 7 mM and 3.6 - 4.4, respectively (Yamanaka 1995b). The ammonium-nitrogen concentrations and pH values of the urea-treated soils of the mixed forests (*Abies firma* and *Quercus* sp dominating forests) sometimes reached above 1000 mM and 8.5, respectively, and then gradually

Table Ⅲ-2. Changes in pH of basidiospore suspension of *H. vinosophyllum* after a 12-day Incubation. The basidiospores (1.0×10^6 - 2.0×10^6 spores/ml) suspended in 100 mM NH_4Cl aqueous solutionat different initial pHs were incubated at 25.0°C in the dark.

		Final pHs of the spore suspension						
Initial pH		3.0	4.0	4.5	5.0			
Reagent[a]	HCl	3.2	4.1	4.6	5.4			
	H_2SO_4	3.1	4.1	4.6	5.4			
		Final pHs of the spore suspension						
Initial pH		6.0	7.0	7.5	8.0	8.5	9.0	9.5
Reagent[a]	KOH	5.7	6.9	7.3	7.6	8.1	8.9	9.2
	NaOH	5.9	6.3	6.9	7.8	8.2	8.9	9.4
	NH_4OH	5.6	6.4	7.3	7.7	8.1	8.8	9.3

[a]Chemical reagents used for pH adjustment

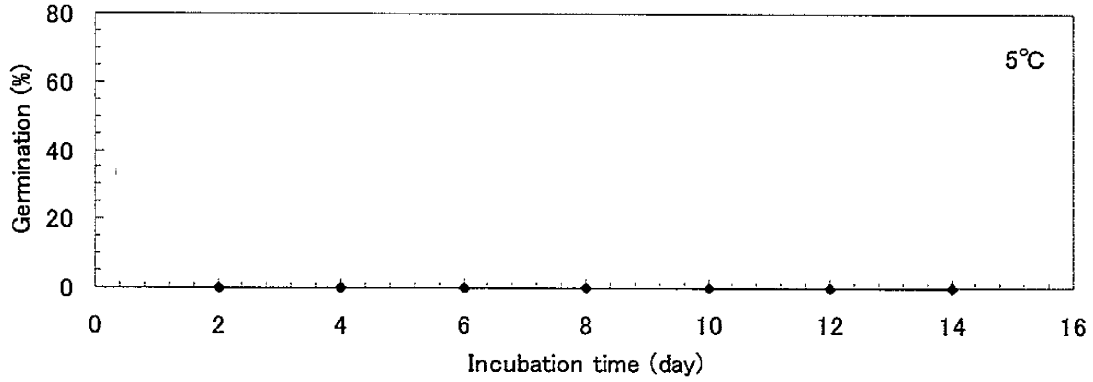
declined to less than 100 mM and about 5.5, respectively by the time of the occurrence of LP fungus. The ammonium-nitrogen concentrations and pH values of the urea-untreated soils of the mixed forests are 2 - 20 mM and 4.5 - 5.5, respectively (Suzuki 2000; Suzuki et al. 2002b). The upper limit concentration of ammonium-nitrogen for vegetative growth of *H. vinosophyllum* is 600 mM and the optimum is 3 mM. It grows faintly even at 0.1 mM ammonium-nitrogen (Licyayo and Suzuki 2006). Vegetative mycelia of *H. vinosophyllum* grow at pH 4 - 8 (Yamanaka 2003; Suzuki 2006). Moreover, *H. vinosophyllum* grows vigorously on the γ-ray-sterilized soils collected 3 months after the urea application, but hardly grows on the

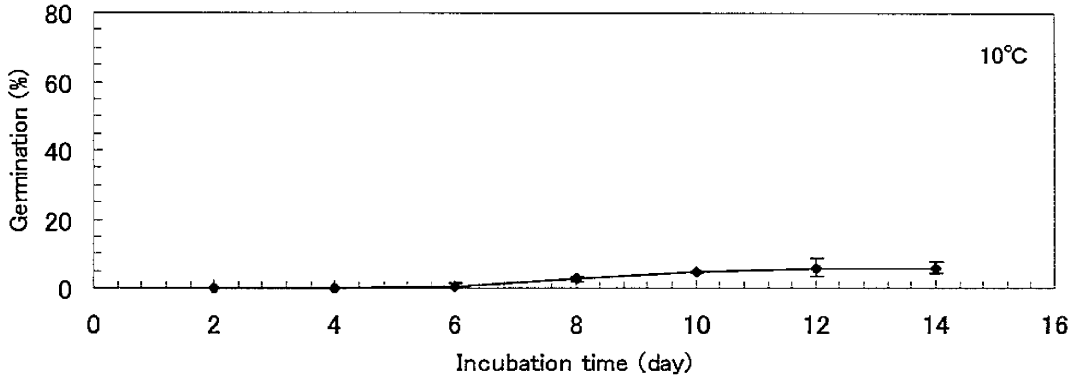
γ-ray-sterilized soils collected half month after the urea application (Suzuki 2006). The colonization of EP fungi is speculated to initiate within 1.5 months after the urea application in the field (Suzuki 2006). These findings suggest that the EP fungi *Coprinopsis* spp. and LP fungus *H. vinosophyllum* germinate one after another when both ammonium-nitrogen concentration and pH of the urea-treated soil decline below 500 - 800 mM and 8 - 9, respectively, in the field and establish their territory by their vegetative growth.

Effect of temperature on basidiospore germination and mycelial growth

The basidiospores germinated at 10.0°C to 35.0°C with optima at 25.0°C to 30.0°C (Fig. Ⅲ-7~9). The basidiospores did not germinate at 5.0°C (Fig. Ⅲ-7) and 40.0°C (Fig. Ⅲ-9). At the optimum temperature, the spore germination initiated on the second day after the treatment and increment of the germination percentage reached a plateau about 12 days after the start of the treatment.

Meanwhile, growth of vegetative mycelia under different temperature was observed. The mycelia grew at 10.0°C to 30.0°C with optima at 25.0°C (Fig. Ⅲ-10), irrespective of dikaryon or monokaryon/ and of mating types. Mycelia did not grow at 5.0°C and 35.0°C. Table Ⅲ-3 shows the growth speed of the vegetative mycelia. At the optimum temperature of 25.0°C, there were non significant differences among all kinds of vegetative mycelium for their growth speed.





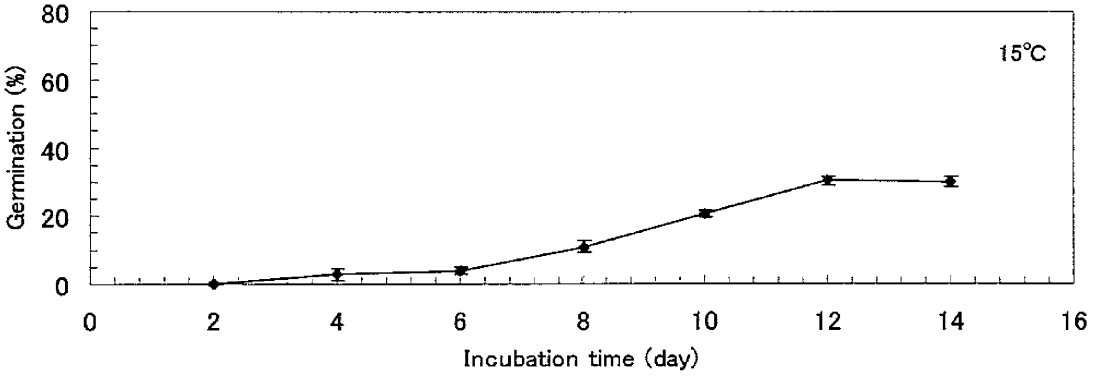


Figure Ⅲ-7 Germination rate of *Hebeloma vinosophyllum* at different temperatures; values indicate meas±SE.

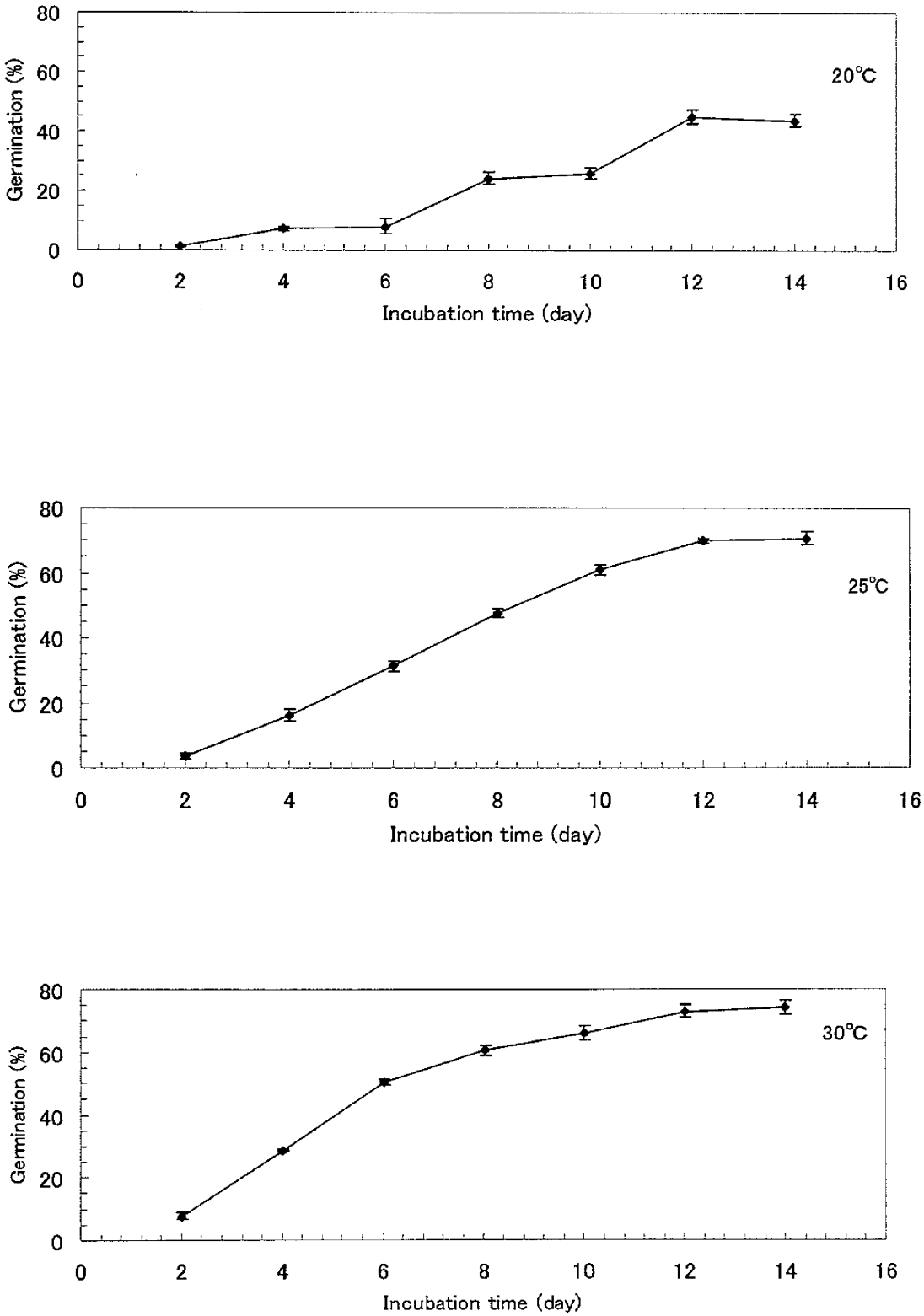


Figure Ⅲ-8 Germination rate of *Hebeloma vinosophyllum* at different temperatures; values indicate meas±SE.

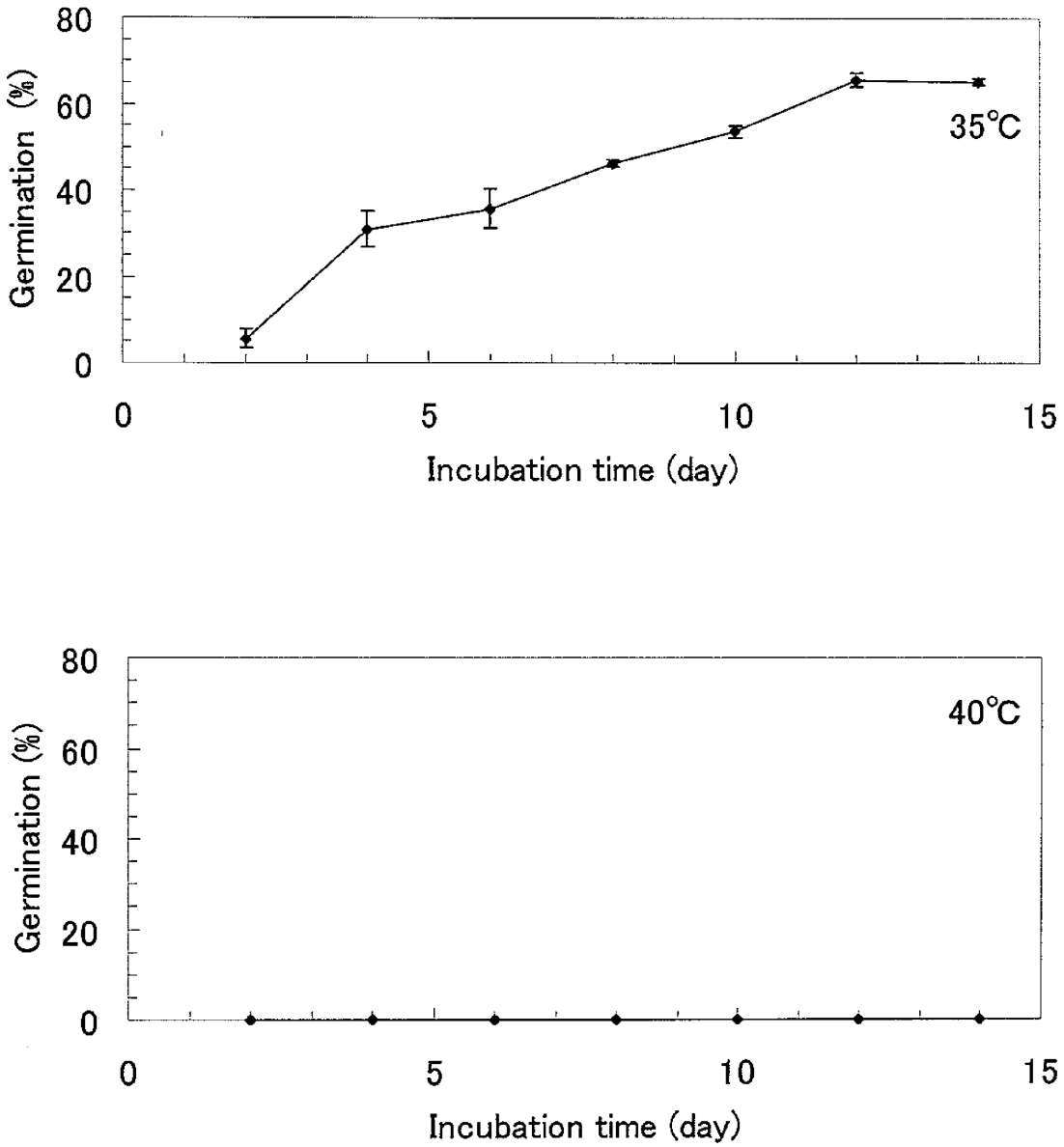


Figure Ⅲ-9 Germination rate of *Hebeloma vinosophyllum* at different temperatures; values indicate meas±SE.

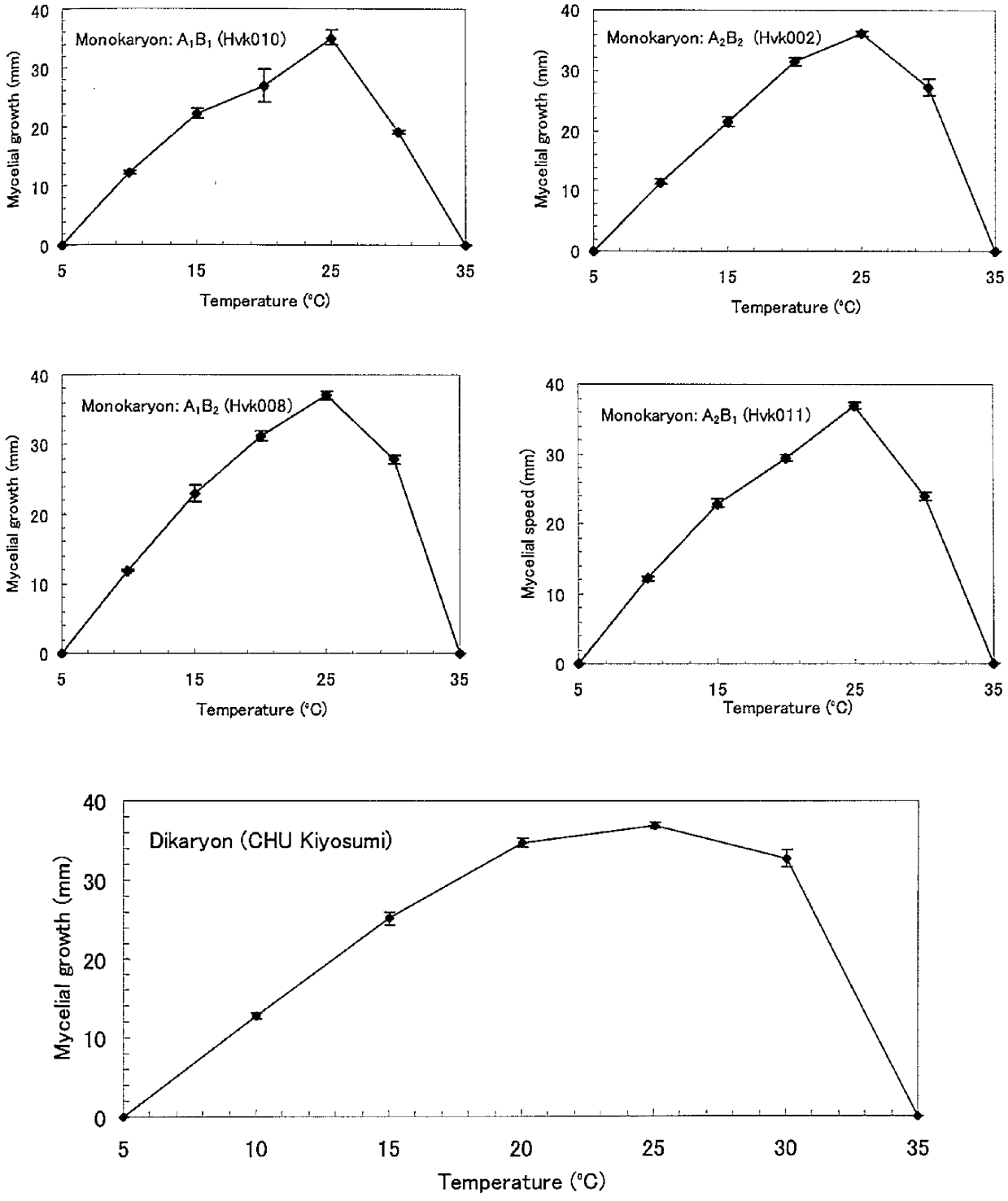


Figure Ⅲ-10 Mycelial growth radius of *Hebeloma vinosophyllum* on MY agar plate under different temperatures in 15 days; values indicate meas±SE.

Table Ⅲ-3. Mycelial growth radius (mm) of *Hebeloma vinosophyllum* on MY agar medium under different temperatures for a 15-day incubation.

Isolate	Incubation temperature (°C)						
	5	10	15	20	25	30	35
Dikaryon (CHU Kiyosumi)	0	12.8±0.3 a	25.1±0.8 a	34.7±0.5 b	36.9±0.3 a	32.8±1.1 c	0
A_1B_1 (Hvk010)	0	12.3±0.3 a	22.2±0.9 a	27.0±2.8 a	35.1±1.3 a	19.2±0.2 a	0
A_2B_2 (Hvk002)	0	11.6±0.3 a	21.4±0.8 a	31.4±0.7 ab	36.1±0.4 a	27.2±1.3 bc	0
A_1B_2 (Hvk008)	0	11.9±0.1 a	23.0±1.2 a	31.2±0.7 ab	37.1±0.6 a	27.9±0.6 bc	0
A_2B_1 (Hvk011)	0	12.2±0.4 a	22.9±0.6 a	29.4±0.4 ab	36.9±0.4 a	23.9±0.5 ab	0

Different alphabetical letters in each column indicate significant difference at $P<0.05$ according to the Tukey - Kramer test;

Means and SE are calculated from three replicates of each treatment.

H. vinosophyllum occurs after the urea application both in summer and winter, although a few of them occur at high frequency only after the urea application in summer or in winter (Sagara 1975). The occurrence of *H. vinosophyllum* in the field, irrespective of the urea application season, may be partially explained by the wide temperature range for spore germination of *H. vinosophyllum*. Though the basidiospore of *H. vinosophyllum* was able to germinate at 35°C, its mycelium was not able to survive any more, irrespective of the monokayon sprouting from a single spore or later being a dikaryon. Moreover, the above results suggest that *H. vinosophyllum* has potential ability to propagate even in the sub-tropical and cool temperate

region. However, the occurrence of *H. vinosophyllum* has been recorded in the area from Kanto district to the middle Kyushu Island in Japan and San Ming in China, but not in the cool temperate and sub-tropical regions in Japan (Sagara 1975; Suzuki 1992, 2000; Yamanaka 1995b; Fukiharu and Horigome 1996; Hongo 1996; Fukiharu et al. 2000a, b). The biogeographic distribution of *H. vinosophyllum* would be affected by the temperature through each stage of its morphogenesis including spore germination, vegetative growth and interaction with other microbes including other ectomycorrhizal ammonia fungi in the field. The biogeographic distribution of host trees would be mainly determined by several environmental factors such as temperature, rainfall and pH.

Longevity of basidiospores of *H. vinosophyllum* at different temperatures in both wet and dry conditions

Germination ability of the spores gradually decreased with increasing storage period in both dry and wet conditions. Storage at 25.0°C and 30.0°C markedly lowered their germination ability. The germination ability of the basidiospores was maintained for a longer period in a dry condition than in a wet condition (Fig. Ⅲ-11~12). In a wet condition, a high temperature caused conspicuous loss of the spore viability. The basidiospores completely lost their germination ability within a 150-day storage in a wet condition. However, the basidiospores stored in a dry condition for 150 days at 10.0°C and 15.0°C still germinated at the rate of 13.0% and, of 19.3%, respectively. In a wet condition, a lower temperature seems to be more favorable for the

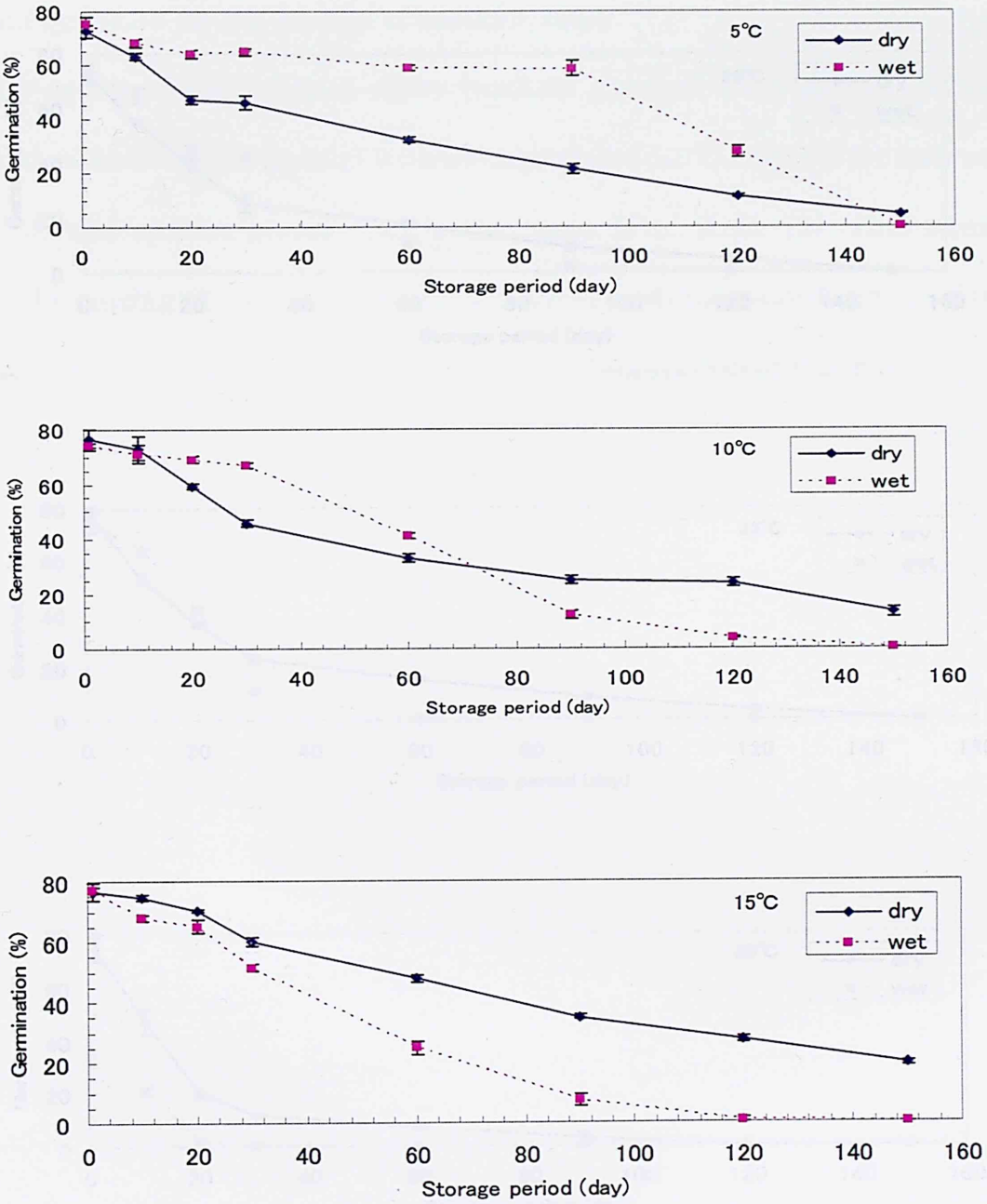


Figure Ⅲ-11 Longevity of basidiospores of *Hebeloma vinosophyllum* under different temperatures in wet and dry conditions. The stored spores were suspended in 100 mM NH4Cl solution at pH 8.0 by KOH; the density of the spore suspension was adjusted to 1.0×10^6 - 2.0×10^6/ ml; the spore suspensions were incubated at 25.0±0.5°C in the dark for 12 days; bar: Standard error.

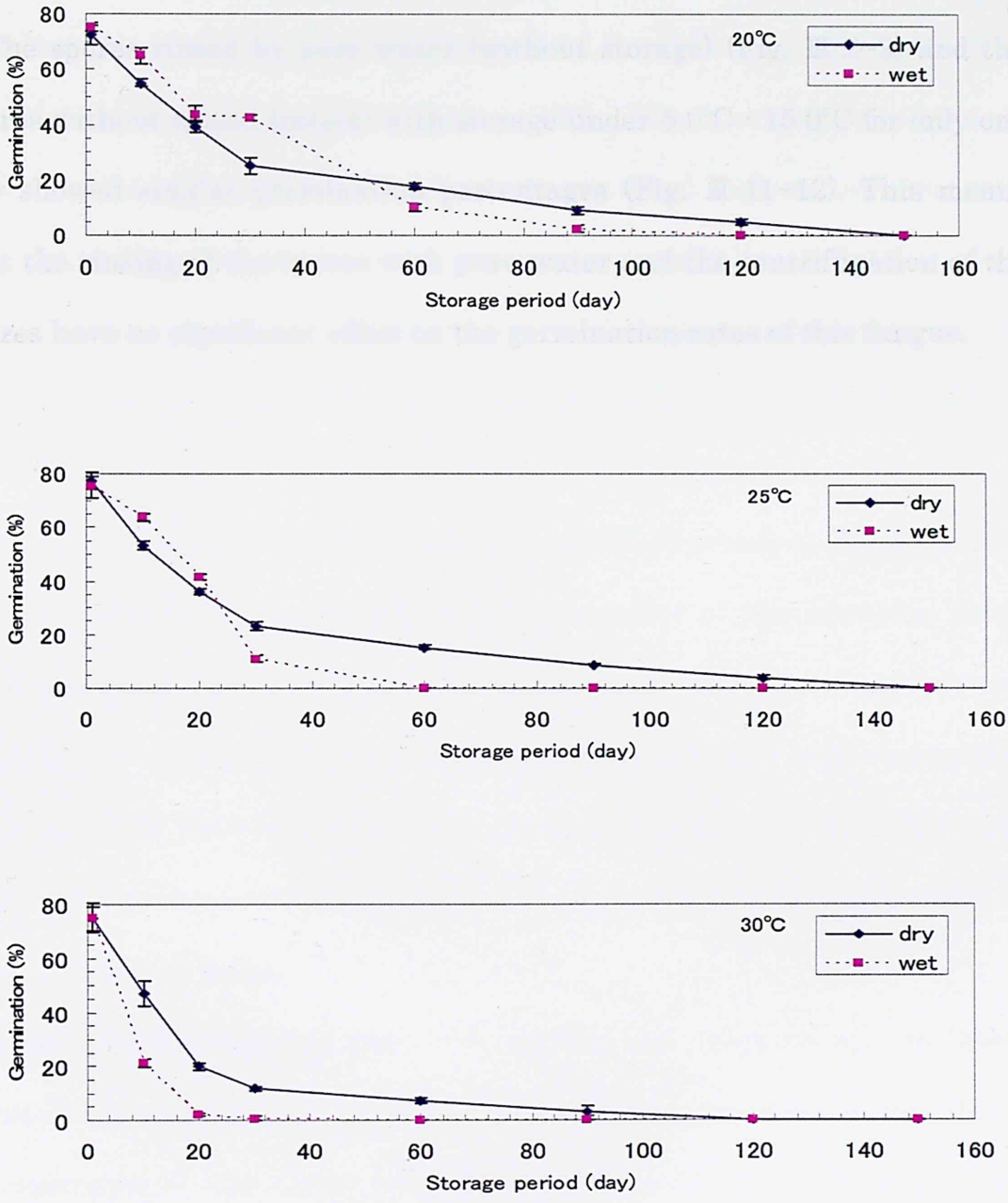


Figure Ⅲ-12 Longevity of basidiospores of *Hebeloma vinosophyllum* under different temperatures in wet and dry conditions. The stored spores were suspended in 100 mM NH4Cl solution at pH 8.0 by KOH; the density of the spore suspension was adjusted to 1.0×10^6 - 2.0×10^6/ ml; the spore suspensions were incubated at 25.0±0.5°C in the dark for 12 days; bar: Standard error.

storage of the basidiospores. In a dry condition, a temperature around 15.0°C would be better for the storage of basidiospores.

The spores rinsed by pure water (without storage) (Fig. Ⅲ·2~9) and the spores without rinsed (intact) with storage under 5.0°C - 15.0°C for only one day showed similar germination percentages (Fig. Ⅲ·11~12). This means that the rinsing of the spores with pure water and the centrifugation of the spores have no significant effect on the germination rates of this fungus.

Conclusion

Basidiospore germination of ectomycorrhizal fungi is not easily induced or stimulated (Horikoshi and Suzuki 1990). The germination rates of basidiospores of ectomycorrhizal fungi do not reach high percentages even when stimulated by specific environmental factor(s) (Ohta 1986; Horikoshi and Suzuki 1990). In contrast, the germination percentage of an ectomycorhizal ammonia fungus *H. vinosophyllum* reached above 70.0% by the incubation under optimum conditions. This suggests that the stage of basidiospore germination would be at least one of principal characteristics for categorizing *H. vinosophyllum* into a member of the ammonia fungi. Ammonia fungi may not always have access to an ammonium-nitrogen-rich condition in the field. In other words, *H. vinosophyllum* may successfully colonize in the field even when the fungus is exposed by chance to a large amount of ammonium-nitrogen derived from urea or the decomposition of faces, corpses, or urine.

Sagara (1976) supposed that both hyphae and spores would be latent forms of ammonia fungi in the field, but no research has been done to clarify his assumption. The short longevity of the basidiospores suggests that principal form of colonization of *H. vinosophyllum* would be hyphae, but not the basidiospores. Probably, the principal role of basidiospore of *H. vinosophyllum* would be the establishment of genetic diversity of this species, and not the resistance to severe environmental conditions.

Sood and Sackston (1971, 1972) found that day length and light intensity

had little effect on the germination of some basidiospores, but higher light intensity had an adverse effect on the germination of the spores. Maddison and Manners (1973) proposed that the nucleic acids and proteins of spores were affected by high intensity light. The light intensity on the forest floor and in the litter may have no significant inhibitive effects on the spore germination of *H. vinosophullum*, although we cannot completely deny the possibility that sunlight filtering down through the trees affects the spore germination of this fungus, e.g., shortening of the longevity of the spores.

CHAPTER Ⅳ

Homokaryotic fruiting of an ectomycorrhizal ammonia fungus, *Hebeloma vinosophyllum*

Introduction

Hebeloma vinosophyllum was firstly identified by Hongo (1965) and later reported as ectomycorrhizal fungus by Fukiharu (1991) and Sagara (1995). *H. vinosophyllum* only occurs following ammonium-nitrogen application in the *Castanopsis*, *Pinus*, and *Quercus* forests (Sagara 1995; Yamanaka 1999; Fukiharu 1991; Imamura & Yumoto 2004). *H. vinosophyllum* occurs from about half to two years following a large amount of ammonium-nitrogen application in the field (Yamanaka 1995). Sagara (1976) speculated that there would be sites in the field where all spores and hyphae were completely destroyed by a large amount of ammonium-nitrogen application. These suggest that late phase fungi such as *H. vinosophyllum* would have the colonization strategy adapting to a large amount of ammonium-nitrogen disturbance which happens occasionally in the field.

Homokaryotic fruiting has been reported in various saprobic mushroom species in Basidiomycota (Stahl and Eesser 1976), but not in ectomycorrhizal mushroom species. However, no report has been done on the homokaryotic fruiting of ammonia fungi even in saprobic species, although it is

indispensable to examine the whole life cycle of each ammonia fungus in order to reveal its colonization strategies in the field.

Based on this background, monokaryotic fruiting ability of *H. vinosophyllum* was examined as the step to reveal the colonization strategy of ectomycorrhizal ammonia fungi in the field.

Materials and Methods

Twelve monokaryotic isolates having different mating types (genotypes) were selected at random, i.e., A_1B_1 (Hvk001, Hvk010, Hvk018); A_2B_2 (Hvk002, Hvk003, Hvk029); A_1B_2 (Hvk004, Hvk08, Hvk012); A_2B_1 (Hvk011, Hvk022, Hvk028), as the isolates for the examination of homokaryotic fruiting.

Vegetative growth and fruiting

Monokaryotic isolates having different kinds of incompatibility factor alleles were selected for the following fruiting experiments.

Isolates of dikaryon and monokaryon having four mating types were separately inoculated on the center of the MY agar [malt extract(Difco, Becton, Dickinson and Co., USA)10 g; yeast extract(Difco, Becton, Dickinson and Co., USA) 2 g;, 50mL] plates and the MY agar slants and then incubated at 25.0±0.5°C in the dark. For the measurement of vegetative growth speed of the isolates, the radii of four cross directions at 90° interval of each mycelial colony were measured at the 15-day incubation and then the average of the four radii in three replicates was calculated to be the index of vegetative growth ability of each culture. For the examination of fruiting, each isolate was inoculated for 50 slants and then morphogenetic processes of fruiting and the time required for basidioma formation were investigated in 25 days. The fruiting time of each isolate was expressed as the average of

the time required for the basidioma primorium formation of all cultures.

Mycelial growth under different ammonium-nitrogen concentrations

Different concentrations of NH_4Cl aqueous solution was adjusted to pH 7.0 by KOH and sterilized by filtration (acetate cellulose, 0.2 μm pore size, Advantec). MY medium was autoclaved to sterilize for 15 minutes under 121°C. Then MY medium was mixed with NH_4Cl aqueous solution for each concentration by 1: 19. Twenty milliliter mixed medium of each concentration was poured into a 50 ml-conical flask. Four mating type isolates growing on MY agar plates were cut with a cork borer and inoculated into the liquid medium. Meanwhile, a dikaryotic isolate was also inoculated as an isolate control. Pure water was used as a medium control. Three replicates were prepared for each treatment. Incubation was at 25.0 ± 5°C in the dark for 15 days.

Basidiospore productivity and germination ability

For the dikayotic isolate, the basidiospores collected from the spore prints were suspended in the sterilized 100 mM ammonium chloride (NH_4Cl) aqueous solution to examine the basidiospore productivity of the basidioma, i. e. number of basidiospores per pileus. Meanwhile, the suspension was adjusted at a density of $1.0 \times 10^6 \cdot 2.0 \times 10^6$ spores/ ml to examine the spore germination ability. For the monokaryotic isolates, since the basidiospores were not able to collected from spore prints, they were directly collected from

the excised pileus embedding in the 100 mM NH_4Cl aqueous solution. The density of the spore suspensions was about 1.6×10^5 spores/ ml due to smaller number of basidiospores. All spore suspensions were incubated for 12 days at 25.0±0.5°C in the dark. The numbers of basidiospore were determined with a hemocytometer by the dilution method.

Results and Discussion

Basidioma formation

The patterns in the basidioma formation of the dikaryotic and the monokayotic isolates were categorized into three types (Deng & Suzuki 2008b).

1) Basidioma formation in dikaryotic isolates

Type I (Fig. Ⅳ-1)

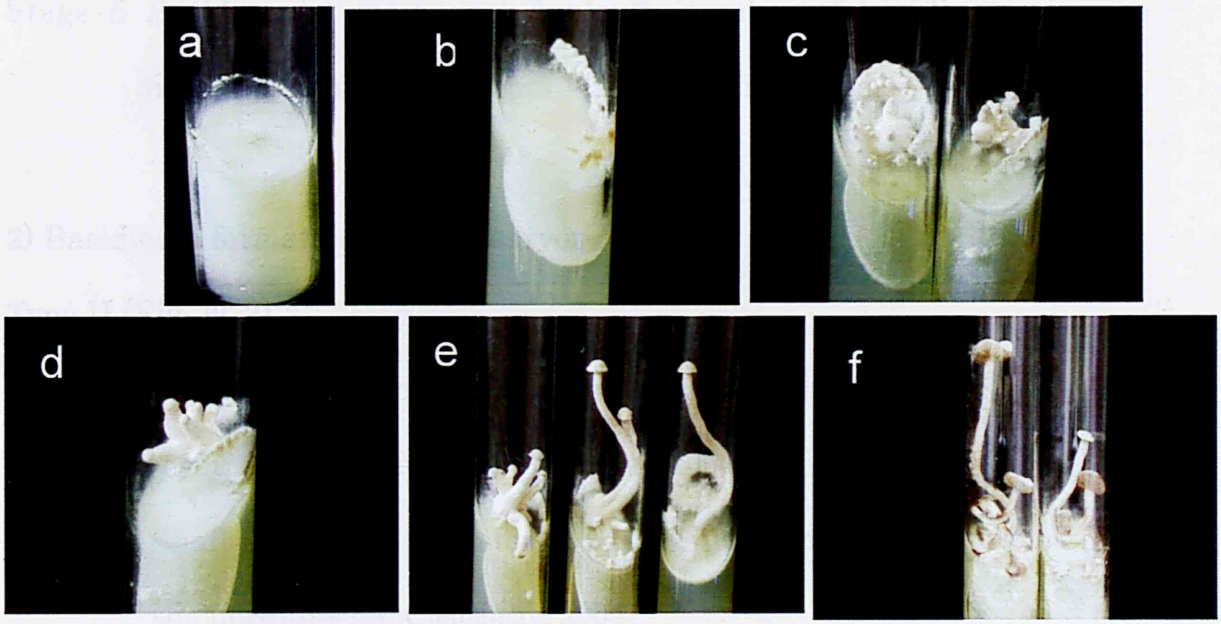


Figure Ⅳ-1 Dikaryotic fruiting of *Hebeloma vinosophyllum* (Type Ⅰ)

a: Stage 1, b: Stage 2, c: Stage 3, d: Stage 4-1, e: Stage 4-2, f: Stage 5

Stage 1 Nodulus formation: a tuft of entangled hyphae produced by vegetative mycelium (cf. Clémençon 2004)

Stage 2 Shaft formation: a cluster of hyphae in a conical shape issued from the nordulus (cf. Clémençon 2004)

Stage 3 Primordium formation: Pileus formation issued by hymenium formation

Stage 4 Basidioma development: Pileus and stipe steadily developed accompanying basidiospore formation. Pileus and stipe became in pale pink. Stipe was usually 20 to 60 mm in length. Pileus was round and became ca. 10 mm in diameter. Gills became reddish brown.

Stage 5 Basidiospore discharge: A plenty number of basidiopsores were discharged and a dense spore print was formed.

2) Basidioma formation in homokaryotic isolates

Type II (Fig. Ⅳ-2)

Stage 1 Nodulus formation: a tuft of entangled hyphae produced by vegetative mycelium (cf. Clémençon 2004)

Stage 2 Shaft formation: a cluster of hyphae in a conical shape issued from the nordulus (cf. Clémençon 2004)

Stage 3 Primordium formation: Pileus formation issued by hymenium formation

Stage 4 Basidioma development: Irregular developments of pileus and stipe. The pileus and stipe became pale pink. Basidiospores were formed during pileus developmet. The surface of the developed

stipe was not smooth and lopsided or malformed compared with that of dikaryotic one. The length of stipe was apparently shorter (ca. 20 to 30 mm) than that of dikaryon but the diameter of stipe became largrer than dikaryotic one. Sometimes basidioma became fluffy by dedifferentiation of the surface of the basidioma. Pileus development often ceased although stipe development proceeded. The pileus was usually not very round appearing malformed and small. The margin of the gill rolled up a little and was observed by taking a side view of the pileus. Gills became pale brownish pink.

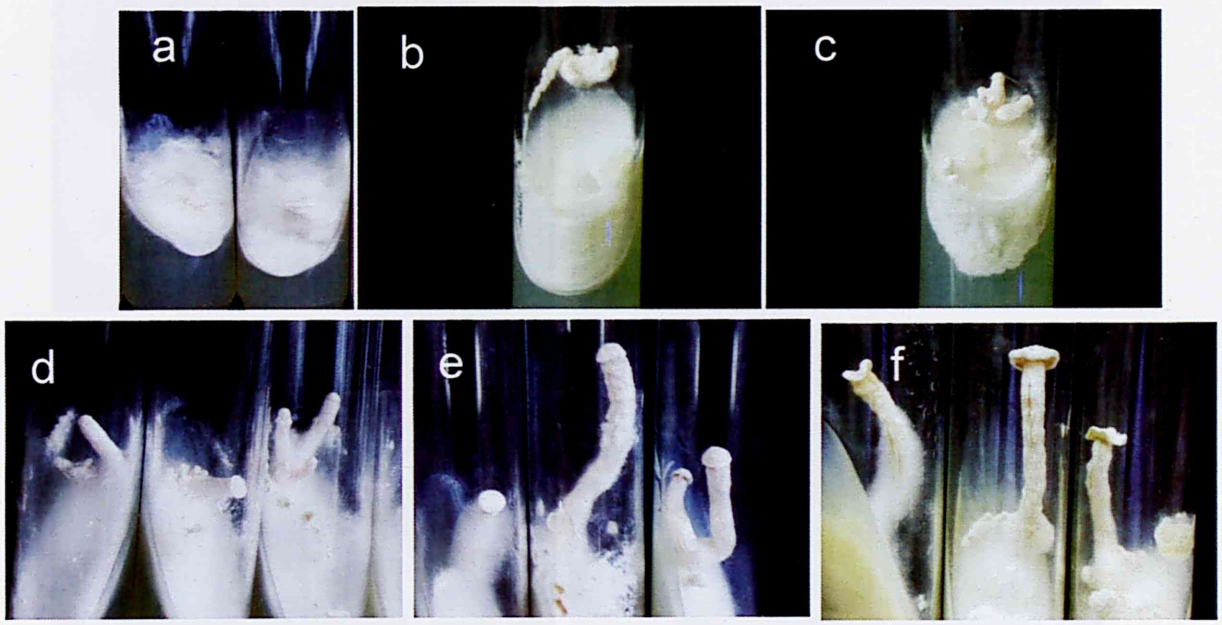


Figure Ⅳ-2 Homokaryotic fruiting of *Hebeloma vinosophyllum* (Type Ⅱ)

a: Stage 1, b: Stage 2, c: Stage 3, d: Stage 4-1, e: Stage 4-2, f: Stage 5

Stage 5 Basidiospore discharge: There was no basidiospore discharge to form a spore print though basidiospores were formed (Table Ⅳ-2).

Type III (Fig. Ⅳ-3):

Stage 1 Nodulus formation: a tuft of entangled hyphae produced by vegetative mycelium (cf. Wessels 1994)

Stage 2 Primordium formation: Promordium was formed by the nodulus without distinct shaft formation. Namely, nearly round-shaped primordium looks like sessile was formed.

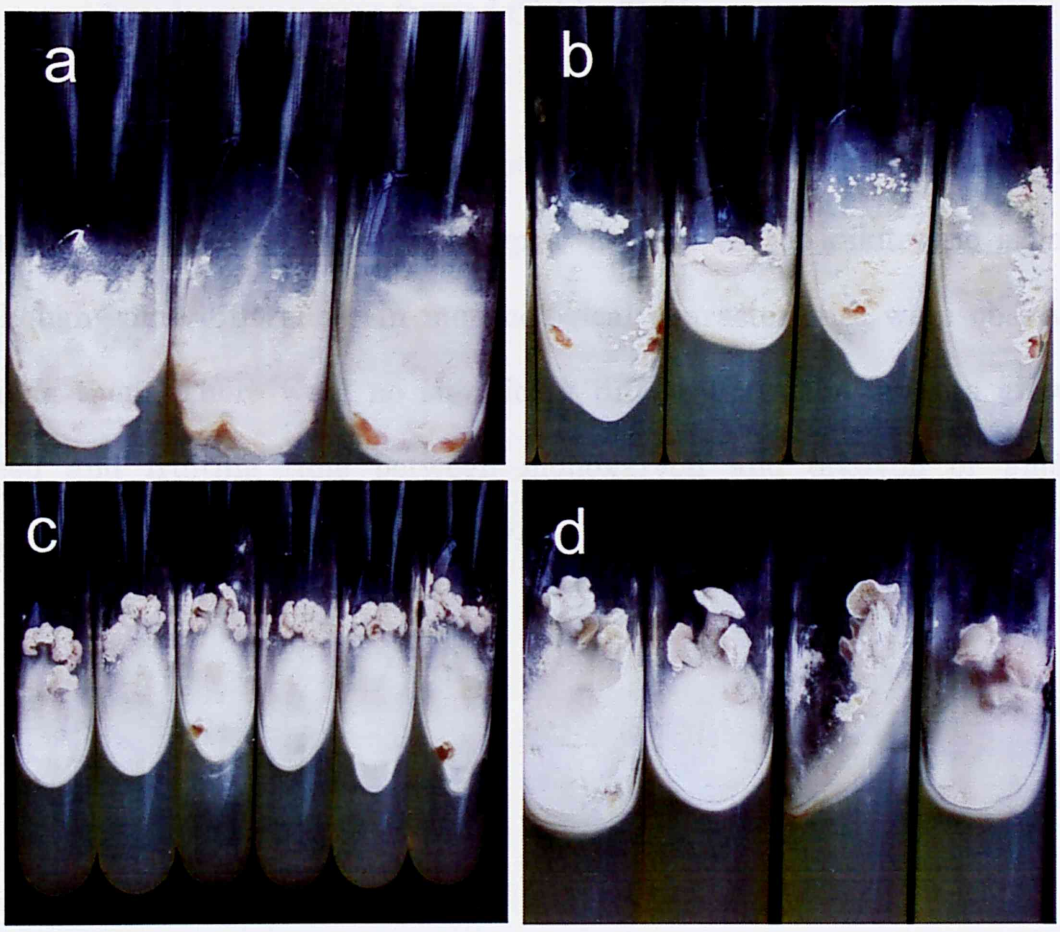


Figure Ⅳ-3 Homokaryotic fruiting of *Hebeloma vinosophyllum* (Type Ⅲ)

a: Stage 1, b: Stage 2, c: Stage 3, d: Stage 4

Stage 3 Basidioma development: Pileus and stipe developed and became pale pink. Basidiospores were formed during pileus development. Stipe of the basidioma was very short, and most of them were ca. 10 mm in length. Pileus was also small (ca. 5 mm in diameter). Gills became pale brownish pink.

Stage 4 Basidiospore discharge; There was no basidiospore discharge to form a spore print though a relatively a large number of basidiospores were formed (Table Ⅳ-2).

Basidioma formation was observed in all monokaryotic isolates having four different mating types (genotypes) as well as the dikaryotic isolate, although some differences in morphological characteristics were observed among them. There were no significant difference in the mycelia growth speed and fruiting time between monokaryon and dikayon or among monokaryotic mating types (Table Ⅳ-1).

Table Ⅳ-1. Fruiting types of *Hebeloma vinosophyllum*.

		Basidioma formed (%)			Fruiting	Mycelium
		Type Ⅰ	Type Ⅱ	Type Ⅲ	time (day)[*1]	radius (mm)
Dikaryon (CHU Kiyosumi)		92[*2]			12.0 ± 1.0[*3] a	39.0 ± 0.9[*3] a
Monokaryotic isolates						
Genotype	Strain No.					
A_1B_1	Hvk001		90			
	Hvk010		92		11.8 ± 0.4 a	37.0 ± 1.9 a
	Hvk018		68			
A_2B_2	Hvk002			96		
	Hvk003			100	12.9 ± 0.7 a	33.0 ± 1.7 a
	Hvk029			62		
A_1B_2	Hvk004		2			
	Hvk008		96		15.2 ± 3.3 a	36.0 ± 1.2 a
	Hvk012		90			
A_2B_1	Hvk011			96		
	Hvk022			88	12.3 ± 0.6 a	33.0 ± 0.9 a
	Hvk028			80		

Same alphabetical letters in each column indicate no significant difference at $P < 0.05$ according to the Tukey- Kramer test.

*1: The time required for the basidia primordium (cf., Figs. 3-5) formation.

*2: Numerals in this column are percentage of basidioma formed in 50 inoculated slants.

*3: Means and SE are calculated from three replicates of each treatment.

Table Ⅳ-2. Basidiospore productivity and germination ability in *Hebeloma vinosophyllun*

Basidioma type		Spore numbers per basidioma	Spore germination (%)
Dikaryon	Type Ⅰ	$1.0\times10^7 \pm 2.7\times10^6$* b	72.1 ± 2.0* c
Monokaryon	Type Ⅱ	$1.2\times10^5 \pm 6.4\times10^4$ a	13.7 ± 2.4 a
	Type Ⅲ	$2.1\times10^5 \pm 1.2\times10^5$ a	22.6 ± 1.3 b

Different alphabetical letters in each column indicate significant difference at $P<0.05$ according to the Tukey- Kramer test

*: Means and SE are calculated from 6 basidiomata for each type

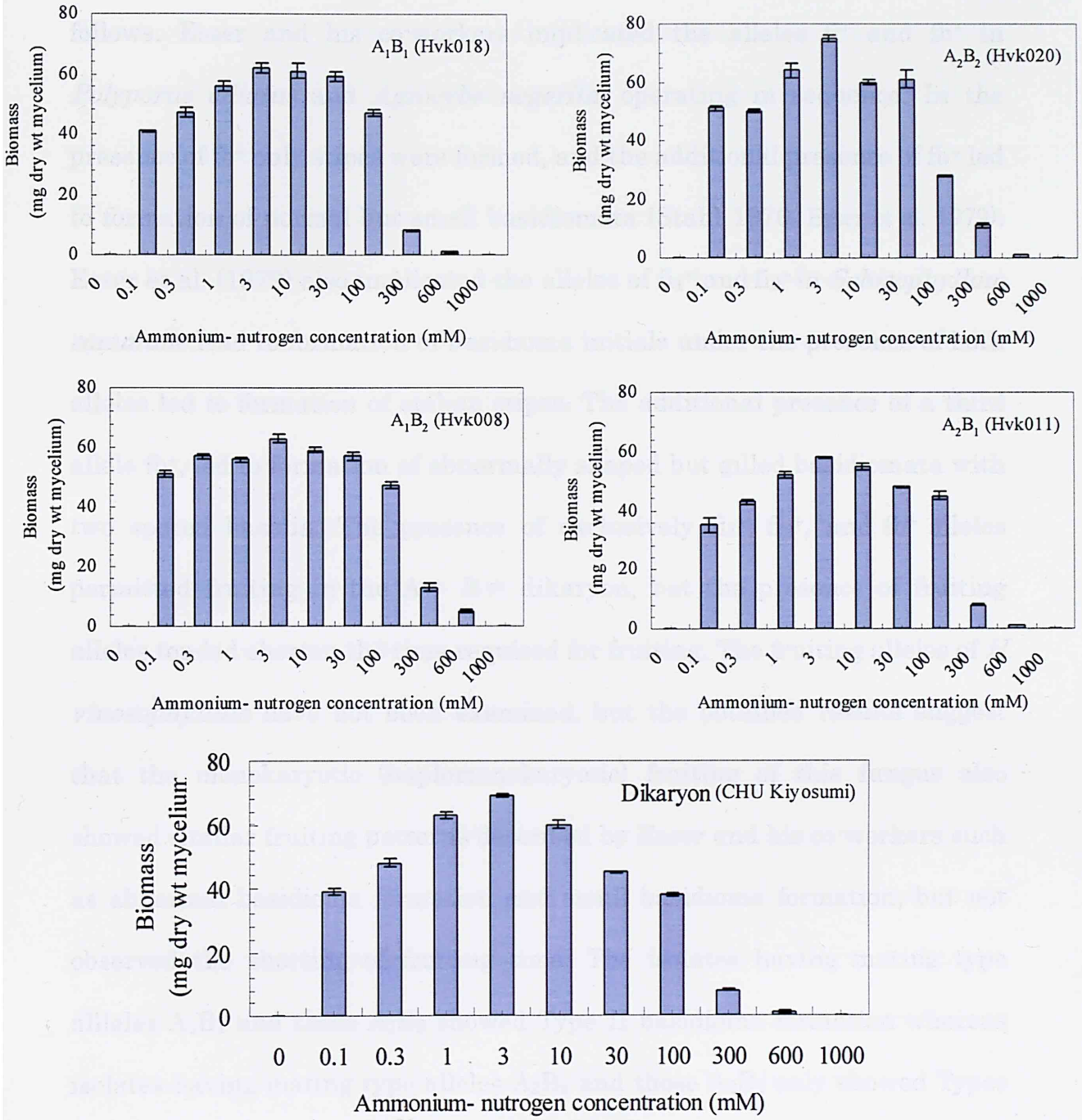
The responses of monokaryotic isolates to ammonium-nitrogen concentration based on their biomasses

Isolates of monokaryon having four mating types grew in NH_4Cl aqueous solution from 0.1 mM to 600mM with the optimum concentration in 3 mM as well as the isolate of dikaryon did. Table Ⅳ-3 and Figure Ⅳ-4 show the biomass for these isolates growing under various ammonium-nitrogen concentrations. All mycelia grew well from 0.1 mM to 100 mM. In the concentration of 600 mM, mycelia were able to survive but did not grow well. Mycelia did not grow in the concentration of 1,000 mM and distilled water. Though the biomasses show significant differences among these isolates in each concentration, the responses for these isolates to all concentrations show nearly the same tendency. It is suggested that mycelial growth of four different mating types of *H. vinosophyllum* shows the same response to different ammonium-nitrogen concentrations as well as the dikaryotic one did. Therefore, it is considered the fruiting response of these isolates under various ammonium-nitrogen concentrations would be the same.

Table Ⅳ-3. Biomass of isolates of *Hebeloma vinosophyllum* cultivated in MY medium under different ammonium-nitrogen concentrations

Mating tyoe of isolates	Biomass (mg dry wt mycelium) under different ammonium-nitrogen concentrations (mM)										
	0	0.1	0.3	1	3	10	30	100	300	600	1000
A_1B_1 (Hvk018)	0	41±0.3 b	47±1.6 ab	56±1.8 a	62±1.5 ab	61±2.3 b	59±1.7 b	47±1.1 c	8±0.3 a	1±0.2 a	0
A_2B_2 (Hvk020)	0	51±0.6 c	50±0.6 b	64±2.7 b	75±1.3 d	60±0.8 b	61±3.1 b	28±0.4 a	11±0.6 ab	1±0.0 a	0
A_1B_2 (Hvk008)	0	51±1.2 c	57±0.9 c	56±0.8 a	63±1.2 b	59±0.9 ab	57±1.6 b	47±1.1 c	13±1.6 b	5±0.5 b	0
A_2B_1 (Hvk011)	0	35±2.3 a	43±0.8 a	52±0.9 a	58±0.3 a	55±1.2 a	48±0.3 a	45±1.3 c	8±0.3 a	1±0.0 a	0
Dikaryon (as control)	0	39±1.2 ab	48±1.5 b	63±1.1 b	69±0.4 c	60±1.5 b	45±0.3 a	38±0.7 b	8±0.3 a	1±0.3 a	0

Means and SE calculated from three replicates of each treatment;
Different letters following yields in the same row indicate significant difference at $P<0.05$ according to the Tukey-Kramer test.



FigureIV-4 Mycelial yields of monokaryotic and dikaryotic isolates of *Hebeloma vinosophyllum* CHU Kiyosumi cultivated under different ammonium-nitrogen concentrations; Bar indicates SE of the means.

Wessels (1994) summarized monokaryotic fruiting of Basidioamata as follows. Esser and his co-workers implicated the alleles fi^+ and fb^+ in *Polyporus ciliatus* and *Agrocybe aegerita*, operating in sequence. In the presence of fb^+ only stipes were formed, and the additional presence of fb^+ led to formation of normal but small basidiomata (Stahl 1976; Esser et al. 1979). Esser et al. (1979) also implicated the alleles of fi_1^+ and fi_2^+ in *Schizophyllum commune* lead to formation of basidioma initials under the presence of both alleles led to formation of epilate stipes. The additional presence of a third allele fb^+, led to formation of abnormally shaped but gilled basidiomata with two spored basidia. The presence of exclusively fi_1^+, fi_2^+, and fb^+ alleles permitted fruiting in the A≠ B≠ dikaryon, but the presence of fruiting alleles tended shorten the time required for fruiting. The fruiting alleles of *H. vinosophyllum* have not been examined, but the obtained results suggest that the monokaryotic (haplomonokaryotic) fruiting of this fungus also showed similar fruiting patterns described by Esser and his co-workers such as abnormal basidioma formation and small basidioma formation, but not observed the shorting of fruiting time. The isolates having mating type allleles A_1B_1 and those A_1B_2 showed Type II basidioma formation whereas isolates having mating type alleles A_2B_2 and those A_2B_1 only showed Types III basidioma formation (Table Ⅳ-1). These suggest that probably more than two genes is participating the basidioma formation of *H. vinosophyllum*.

Basidiospore productivity and germination ability of the basidiospores produced by the monokaryotic and dikaryotic isolates were also investigated

(Table Ⅳ-2). The germination ability was examined under the optimum conditions for the germination of the basidiospores produced by the dikaryotic isolates (Deng & Suzuki 2008a). The basidioma formed on the dikaryotic isolate produced more than 1×10^7 spores/ pileus. Whereas Types II and III basidiomata formed on the monokaryotic isolates having different kinds of mating types produced significantly smaller number of basidiospores comparing to those produced on the dikaryotic isolate. No significant difference in basidiospore productivity was observed between the basidioma of stage 5 in Type II and those of stage 4 in Type III (Table Ⅳ-2). Although the basidiospores produced from both dikaryotic and monokaryotic isolates, the germination percentage of the former was above 70% and the latter was less than 25%, irrespective of fruiting types (Table Ⅳ-2). The density of spore suspension affects the germination ability of basidiospores obtained from dikaryotic basidioma of *H. visnophyllum,* but is not so drastic (Fig. Ⅲ -2). The optimum density for the spore germination of *H. vinosophyllum* is 1.0×10^6 spores/ ml. In this paper, spore suspension made from monokaryotic basidiomata was 1.6×10^5 spores/ml (Deng & Suzuki, 2008a). Therefore, it is expected that the actual germination percentage of the basidiospores obtained from the monokaryotic basidiomata would show somewhat higher value at its optimum spore density.

Conclusion

An ectomyorrhizal ammonia fungus, *H. vinosophyllum*, has the ability to colonize and disperse by the monokaryotic mycelium derived from basidiospores formed on monokaryotic basidiomata even when it has no chance to come across another monokaryotic mycelia having different incompatible factor alleles or dikaryotic mycelia of the same species. The propagation strategy in this assumption would be an advantage for the sustaining of higher population of the latent propagules in *H. vinosophyllum* which occurs only after disturbances such as a large amount of ammonium-nitrogen application, since the distribution of such kind of disturbance would be sparse and not always happen in the same site. *H. vinosophyllum* is a facultative ectomycorrhizal fungus because its dikayotic isolate forms basidiomata easily on nutrient rich natural media such as MY agar and litter in pure culture (Suzuki 2006), but it can not develop mature basidiomata in the litter without the host plant in pure culture condition. Therefore, further experiments about fruiting ability of the monokaryotic isolates under ectomycorrhiza synthesis in pure culture as well as those about genes for the homokaryotic fruiting are needed.

GENERAL DISCUSSION

The community of ammonia fungi is considered to be a basic component of the normal fungus community in forest and grassland ecosystems. It is important to survey the species composition of ammonia fungi in different vegetation types in different regions, since this will aid understanding of the role of ammonia fungi in the global ecosystem.

Examination of ammonia fungi following application of urea in the field and/or laboratory began with many studies in Japan. Later studies have been undertaken in New Zealand, Europe, North America, Taiwan, and Australia.

In the biogeographic distribution of ectomycorrhizal ammonia fungi, *Hebeloma vinosophyllum* and *H. aminophilim* were recorded separately from Japan and Australia by applying urea on the plots. A *Hebeloma* sp. was also recorded from New Zealand by applying urea on the plot. They belong to *Hebeloma* subgenus *Porphyrospora.*

In this paper, studies began from the identification of *Hebeloma* sp. from New Zealand because the identification of fungal species belonging to *Hebeloma* subgenus *Porphyrospora* is controversial due to only subtle morphological differences between taxa. To elucidate phylogenetic relationships among *Hebeloma* spp. belonging to *Porphyrospora*, i.e., *Hebeloma* sp. from New Zealand, *H. aminophilum* from Australia and *H. vinosophyllum* from Japan, this study evaluated sequences of ß-tubulin genes (long and short sequences). The phylogenetic trees indicated that specimens examined were segregated into two groups, one comprising

Hebeloma sp. from New Zealand and *H. aminophilum,* and another containing *H. vinosophyllum.* To clarify the taxonomic rank of the two groups, di-mon and mon-mon mating tests were undertaken among *Hebeloma* sp. from New Zealand, *H. aminophilum* and *H. vinosophyllum.* Results of mating experiment were consistent with the groupings based on their sequences of β-tubulin genes. Therefore, the results of the phylogenetic and hybridization studies as well as morphological studies presented in this paper revealed that the *Hebeloma* species from New Zealand was the same species to *H. aminophilum,* but different species from *H. vinosophyllum.*

The species composition of ectomycorrhizal ammonia fungi in each area might be more strongly related to the distribution of host mycorrhizal trees (Suzuki et al. 2003). New Zealand and Australia have very different native flora and fauna from that of Northern Hemisphere (Alexander 1996; Taylor & Smith 1997). This is why species of ectomycorrhizal ammonia fungi from Japan are quite different from both New Zealand and Australia by applying urea on the plots. These features make the study of species composition of ammonia fungi in New Zealand and Australia as well as in Japan, especially relevant to knowledge of biogeographic distribution of these fungi.

Based on the result of identification for the isolates from the three areas, further study on vegetative growth was examined among these isolates under the media at different pHs and different ammonium-nitrogen concentrations. Isolates showed different growth responses at different pH values from pH 4.0 to pH 8.0. Relatively, these ectomycorrhizal ammonia fungi grew well at pH ranges from weak acid to weak alkaline condition with pH 6.0 to 7.0 as their optimum. Fries (1956) reported that *Coprinopsis*

species grew well at above pH 8.0. *Coprinopsis* species are belonging to saprobic/ EP ammonia fungi. Yamanaka (2003) reported these ammonia fungi had their optimal pH range from pH 7.0 to 8.0. Data in this study and Chapter Ⅲ show that pH of the growing environment in neutral or alkaline always declined a little following the spore germinating or mycelial growing. Therefore, the optimum pH for these fungi is correlated with the succession of saprobic/ EP → ectomycorrhizal/ LP fungi in the field where they sporulate. Sagara (1992), Yamanaka (1995) and Suzuki (2002b) also proved that EP species sporulated in neutral to slightly alkaline conditions and LP grew on acidic soil. It suggests that the succession of ammonia fungi causes the change of soil pH.

Dikaryotic isolates of *H. vinosophyllum* showed different growth responses on different ammonium-nitrogen concentrations from 0.1 mM to 300 mM. Distilled water as a control showed no growth for all isolates. The higher the yield in which solution, the lower the concentration remained. It proves that ammonia fungi need ammonium-nitrogen to support their growth. Licyayo and Suzuki (2006) showed saprobic ammonia fungi adapted to wider range of ammonium-nitrogen concentration than that of ectomycorrhizal ammonia fungi and tolerated higher ammonium-nitrogen concentration. Their optimal growing ammonium-nitrogen concentrations varied from 3 mM to 1000 mM, some species were able to grow even under 1300 mM and 1600 mM. In the field, saprobic ammonia fungi invade and colonize earlier than ectomycorrhizal ammonia fungi do after a sudden addition of ammonium materials (Sagara 1975). Results support that following the earlier decomposition and growth of the EP species, decrease concentration of

ammonium-nitrogen in the growing environment would be more suitable for the LP species to survive later.

H. vinosophyllum from the warm, northern, temperate zone of East Asia, and *H. aminophilum* from the warm, southern, temperate zone of New Zealand and Australia showed the similar responses to different pHs and ammonium-nitrogen concentrations for their vegetative growth.

With the application of urea in the field, *H. vinosophyllum* has been recorded from Japan (Sagara 1975, 1976, 1992, 1995; Suzuki 1992; Yamanaka 1995a - c; Fukiharu & Horigome 1996), and *H. radicosoides* is known from the central part of Honshu Island to Iriomote Island in the subtropical region of Japan. *H. spoliatum* has been obtained in European Continent, East Asia and Siberia even Central Africa. In New Zealand and Australia, only *H. aminopilum* and *Laccaria* spp. have been recorded (Suzuki et al. 2003). It seems to mean that the biogeographic distribution of these *Hebeloma* species are not mainly decided by the factors such as acidic or alkaline conditions and the richness of nitrogen sources in soil basing on this physiological study. It is suggested that ecological environment especially the formation of vegetation will be an important factor affecting the biogeographic distribution of ectomycorrhizal fungi because different vegetation has different host plants.

Basidiospore germination of ectomycorrhizal ammonia fungi was able to be stimulated by ammonium-nitrogen. *H. vinosophyllum* was stimulated by the presence of ammonium-nitrogen at 10 - 500 mM. The hyphae of this fungus were able to grow even under 0.1 mM of ammonium-nitrogen. It seems to mean ammonium-nitrogen is used not only as a nitrogen source for

germinating and growing, but also as a germination triggering substance. The optimum pH for the germination and the vegetative growth were 8.0 and 7.0, separately. The optimum ammonium-nitrogen concentration was 100 mM for the germination and 3 mM for the vegetative growth. This is consistent with the changes of pH and ammonium-nitrogen concentration in a culturing medium following the spore germination to hyphal growth. Although the spore germination was observed from 10 - 35°C, the hyphal growth was recorded from 10 - 30°C. *H. vinosophyllum* occurs after the urea application both in summer and winter (Sagara 1975). The occurrence of *H. vinosophyllum* in the field, irrespective of the urea application season, may be partially explained by the wide temperature range for spore germination of *H. vinosophyllum*. Though the basidiospores of *H. vinosophyllum* were able to germinate at 35°C, its hyphae were not able to survive any more, irrespective of a monokayon or a dikaryon. Moreover, the above results suggest that *H. vinosophyllum* has potential ability to propagate even in the sub-tropical and cool temperate region. However, the occurrence of *H. vinosophyllum* has not been recorded in the cool temperate and sub-tropical regions in Japan (Sagara 1975; Suzuki 1992, 2000; Yamanaka 1995b; Fukiharu and Horigome 1996; Hongo 1996; Fukiharu et al. 2000a, b). The biogeographic distribution of *H. vinosophyllum* would be affected by the temperature through each stage of its morphogenesis including spore germination, vegetative growth, and interaction with other microbes including other ectomycorrhizal ammonia fungi in the field.

Spore longevity is one of the important factors to speculate the colonization strategy of fungi in the field. The germination ability of the

spores remained only 4.7 - 19.3% after 150 days storage at 5 - 10°C under the dry condition and lost in all temperatures (5 - 30°C) under the wet condition. The short longevity of the basidiospores suggests that principal form of colonization of *H. vinosophyllum* would be hyphae, but not the basidiospores. Probably, the principal role of basidiospore of *H. vinosophyllum* would be the establishment of genetic diversity of this species, and not the resistance to severe environmental conditions. The result assumes that spores, as well as hyphae, are the main form to invade and colonize in the field.

Most mycorrhizal fungi do not fruit in pure culture. In this study, the dikaryotic isolate of *H. vinosophyllum* cultured on malt extract-yeast extract agar media formed basidiomata. Unfortunately, *H. aminophilum* either from Australia or New Zealand failed to do so under the same culturing conditions. It may suggest that *H. aminophilum* did not fruit under the conditions where *H. vinosophyllum* did because *H. aminophilum* needs the nutritious sources concerned in its host specificity in which it is different from *H vinosophyllum,* for both exist in two different ecosystems. It may suggest that biogeographic distribution of ectomycorrhizal ammonia fungi may be mainly decided by the formation of vegetation that causing different species of fungi have their more rigid host specificity.

H. vinosophyllum formed basidiomata on MY agar medium in *vitro* not only from its dikaryon but also from its monokaryon. This fungus was tetrapolar. Monokaryons of four mating types were able to fruit. Monokaryotic basidiomata of this fungus also produced basidiospores though their quantity was smaller (less than 1×10^6 spores per basidioma) and the germination percentage was lower (less than 25%) compared with dikaryotic

one (more than 1×10^7 spores per basidioma with the germination percentage of more than 70%). Results from mycelia growth experiment under the different ammonium-nitrogen concentrations also showed isolates of monokaryon having four mating types grew as well as the isolate of dikaryon did. Therefore, it was consistent with the fruiting response of these isolates on MY agar medium. This indicates ammonia fungi as pioneer fungi in forests or grass lands would have propagation strategies for a sudden colonization following a large amount of ammonium-nitrogen disturbance.

The study of ammonia fungi in Japan has been undertaken in various habitats at various geographic locations for about half a century, but studies of ammonia fungi in other countries began only about 20 years ago in one to several habitats of very restricted parts of Taiwan, Europe, North America, New Zealand, and Australia. Urea treatments have not yet been studied in many parts of the world, especially in regions of the Southern Hemisphere, such as eastern Australia, Africa, Madagascar, Indonesia, New Guinea, and South America, as well as in the tropical zone of both Hemispheres. Therefore, further surveys are needed for ammonia fungi in presently unexplored areas.

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ABSTRACT IN JAPANESE (和文要旨)

本実験は、*Hebeloma* 属 *Porphyrospora* 亜属の菌根性アンモニア菌の生物地理的分布と同亜属菌の増殖戦略の解明を目的としたものである。アンモニア菌に属する同亜属菌は、日本、ニュージーランド、オーストラリアから報告されている。これらのアンモニア菌は、βチューブリン遺伝子領域の塩基配列による系統解析の結果、ニュージーランド産の *Hebeloma* sp.とオーストラリア産の *H. aminophilum* からなるグループと日本産の *H. vinosophyllum* のみからなるグループに二分された。これら 3 菌の交配試験の結果が分子系統解析の結果と一致したことから、ニュージーランド産の *Hebeloma* sp.は、*H. aminophilum* と同定した。これら3菌の栄養菌糸は、いずれも pH 4.0 - 8.0 に調製した 0.1 - 300 mM 塩化アンモニウム水溶液を用いた培養で生長し、pH 6.0 - 7.0、3 mM 塩化アンモニウム水溶液を用いた 25℃での培養で良好な生長を示した。*H. vinosophyllum* の担子胞子は、pH 4.5 - 9.0 に調製した 10 - 500 mM 塩化アンモニウム水溶液処理によって、10 - 35℃で発芽が誘起され、pH 8.0 に調製した 100 mM、塩化アンモニウム水溶液処理、25－30℃で高い発芽率が得られた。同担子胞子は、15℃、暗黒、乾燥条件下で最も長期間発芽能が維持され、150 日間の保存でも 19.3%のものが発芽能を有していた。また、*H. vinosophyllum* は単核発芽能を有していた。単核子実体は、通常の複核子実体に較べて担子胞子生産能が低かったが、同胞子は発芽能を有しており、同菌は複核化を伴わない分散・増殖戦略も有していることが判明した。以上のように、同位種と推察された菌根性アンモニア菌２種の高窒素条件下での増殖戦略の一端を明らかにした。