Exploring the life cycle and mycelial induction using *Annulohypoxylon* culture filtrate extract to promote the growth of the Japanese white jelly mushroom, *Tremella yokohamensis*

（アンヌロヒポキシロン培養濾液抽出物を用いたシロキクラゲの生育促進と菌糸誘導の検討）

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2024

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A Dissertation for Doctoral Degree

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2024

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Chapter 1

General introduction

The life cycle of mushrooms typically begins with the germination of haploid basidiospores, leading to the formation of haploid yeast or haploid hyphae, commonly referred to as monokaryons. These monokaryons contain one nucleus within their cells. In nature, when two monokaryons with compatible mating types meet, their cells can fuse to create a dikaryon. Each monokaryon contributes nuclei to the other cell, integrating them into its cytoplasm. These nuclei undergo division and are actively transported throughout the entire mycelium until fertilization is complete (Kües & Liu, 2000; Gladfelter & Berman, 2009). The resulting secondary mycelium is characterized by two distinct haploid nuclei per hyphal cell, originating from each parental monokaryon. Fusion of secondary hyphae occurs through broken septa between haploid hyphae (Casselton & Olesnicky, 1998). Under appropriate environmental conditions, the dikaryon proceeds to develop fruiting bodies containing basidiospores (Fig. 1.1).

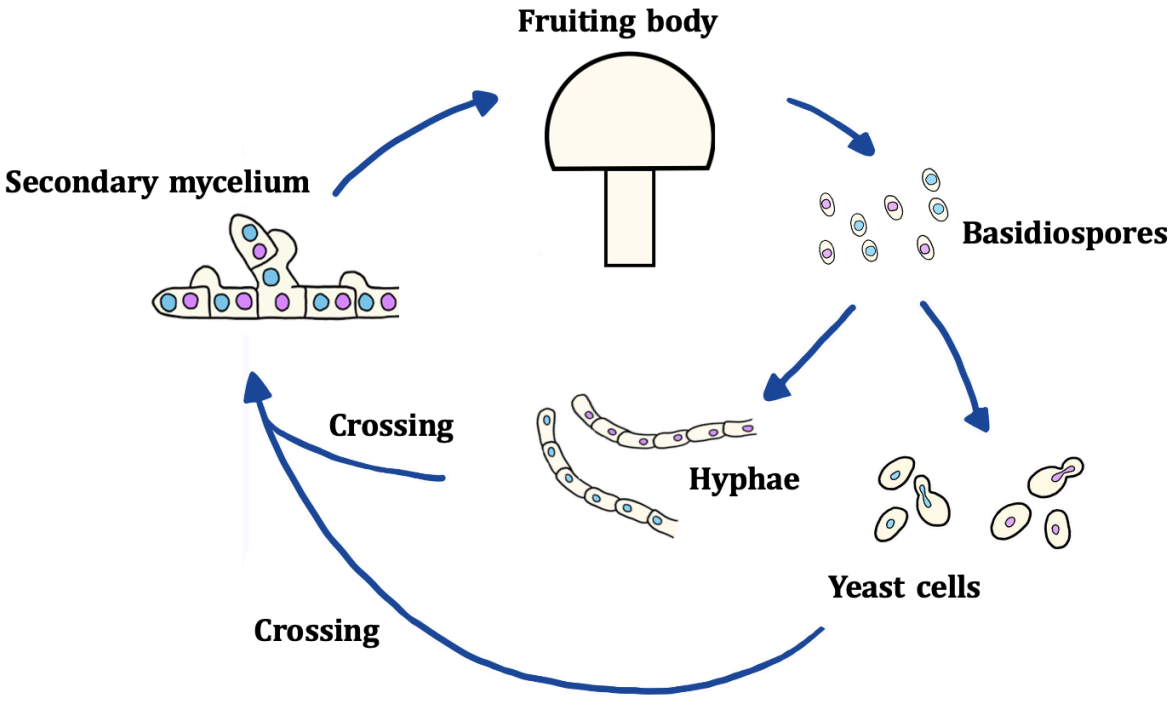


Fig. 1.1 Life cycle of basidiomycetes mushroom.

Basidiomycetes mushrooms exhibit two distinct sexual reproduction systems: homothallism and heterothallism. In homothallism, fungi can develop fruit bodies and undergo sexual reproduction without requiring mating. Homothallic sexuality can manifest in two forms: primary and secondary. Primary homothallism occurs when a single spore containing a meiotic nucleus germinates to produce a homokaryotic individual capable of dikaryotization (Raper, 1966). Secondary homothallism begins with a heterokaryotic individual arising from two basidiospores with compatible nuclei, ultimately self-fertile (Foulongne-Oriol et al. 2021). In heterothallism, there are two variations: bipolar and tetrapolar. In bipolar, mating is controlled by one pair of *A* factors. Basidiospores from a single fruiting body can have two different mating types, and only monokaryons of different mating types can mate to complete the sexual life cycle. In tetrapolar, the mating-type system is governed by both *A* and *B* factors. From one strain, four basidiospores with different mating types are isolated, and the sexual life cycle can only be completed through mating between monokaryons possessing different *A* and *B* factors (Rapper, 1966)

The order Tremellales represents the largest group of fungi within the class Tremellomycetes, which belongs to the phylum Basidiomycota. Tremellales contain a diverse range of organisms, including teleomorphic yeast species such as *Tremella*, anamorphic yeast species like *Bullera*, *Cryptococcus*, and *Fellomyces*, taxa that completely lack basidiocarps, and species associated with lichens (Bandoni & Boekhout, 2011; Millanes et al., 2011; Weiss et al., 2014). The life cycle of *Tremella* mushrooms is dimorphic, encompassing two distinct morphological stages. In the teleomorphic state (diploid), basidiocarps develop following the conjugation of yeast cells on suitable substrates under appropriate conditions. Conversely, in the anamorphic state (haploid), basidiospores germinate through budding, giving rise to a haploid yeast stage (Bandoni & Boekhout, 2011; Weiss et al., 2014). It's noteworthy that all known heterothallic *Tremella* species employ a tetrapolar mating system (Hanson & Wells, 1991; Wells, 1994). However, certain teleomorphic species within the Tremellales, such as *Auriculibuller fuscus* (Sampaio et al., 2004), *C. neoformans*, and *C. gattii* (Fraser et al., 2004; Keller et al., 2003; Kwon, 1975; Lengeler et al., 2002; Loftus et al., 2005; Nielsen et al., 2003), have been ported to employ bipolar mating systems.

Tremella mushrooms have garnered significant interest among researchers due to their diverse biological activities (Huang et al., 2010; Cheung, 1996; Cho et al., 2006; Du et al., 2010; Reshetnikov et al., 2000; Chen, 2010; Yu et al., 2019; Iturbe et al., 2016). Among

the *Tremella* species frequently utilized for their nutritional and medicinal properties are *T. fuciformis* and *T. aurantialba*, which are cultivated commercially. The fruiting bodies of these species are rich in the composition of polysaccharides, triterpenoids, protein, dietary fiber, vitamins, minerals, and chitin (Zhang et al., 2011), with *T. fuciformis*, also known as white jelly mushroom, being particularly noteworthy. The polysaccharides derived from *T. fuciformis* have attracted attention for their diverse bioactive properties, including antioxidant, anti-inflammatory, anti-tumor, anti-quorum sensing, and anti-aging effects. Moreover, they are recognized for their immunomodulatory capabilities and their role in blood sugar regulation (Wu et al., 2019; Xu et al., 2021; Yang et al., 2019; Zhang et al., 2014; Zhu & Sun, 2008). Additionally, in China and Japan, *T. fuciformis* is utilized in the production of beauty products, owing to its ability to enhance skin moisture retention and counteract age-related microvascular changes, thereby reducing wrinkles. Park et al. (2007) even suggested its potential use as a preventative agent in neurodegenerative diseases.

The production of white jelly mushrooms has traditionally involved a substrate inoculation technique using a mixed culture (Huang & Chen., 2002). Due to some unusual characteristics of white jelly mushrooms, which cannot degrade and use either cellulose or lignin efficiently, it is necessary to use a mixed culture of white jelly mushrooms, a basidiomycete, and its host, an ascomycete, to obtain a rich yield. Whether in natural habitats or under artificial cultivation, the successful cultivation of white jelly mushrooms is greatly dependent on the presence of a companion, *Annulohypoxylon* (Huang & Chen., 2002; Deng et al., 2016; Hsieh et al., 2005). These *Annulohypoxylon* species possess lignin and carbohydrate degradation capabilities, exhibiting activities such as β-glucosidase, pectinase, xylanase, and β-glucanase (Robl et al., 2015), thereby providing residual nutrition for the growth and development of the white jelly mushroom.

However, the industrial production of white jelly mushrooms faces significant challenges due to the lack of high-quality species, leading to potential risks in production and economic losses. The dimorphism of the mushroom presents great difficulties in the breeding process, particularly in the difficulty of mycelial formation when basidiospores transition into yeast-like conidia (Li et al., 2022). Recent studies focusing on the dimorphism of white jelly mushrooms primarily explore the impact of various factors, including nitrogen and carbon sources, carbon-to-nitrogen ratio, pH levels, temperature, and culture duration, on the transition from yeast-like conidia to hyphae (Liu et al., 2008). Investigating the hosts associated with white jelly mushrooms provides crucial insights for cultivation practices. As a significant biological contributor, we aim to investigate whether *Annulohypoxylon*

produces relevant compounds or engages in interactions with the Japanese white jelly mushroom, known as “Shiro kikurage," to analyze its role as a facilitator in the transition from yeast to hyphal forms. Through isolating and elucidating the structure of mycelialinducing compounds, we can select suitable conditions to maintain mycelial growth and promote the cultivation of the white jelly mushrooms. Additionally, the phylogeny and life cycle of the Japanese white jelly mushroom will be illustrated here.

Chapter 2

Isolation of Japanese white jelly mushrooms and analysis of the mating system

2.1 Introduction

The order Tremellales is the largest known group of fungi in the class Tremellomycetes (Basidiomycota). The order contains teleomorphic yeast species (*Tremella*), anamorphic yeast species (*Bullera*, *Cryptococcus*, and *Fellomyces*), taxa that completely lack a basidiocarp and grow in the hymenium of other fungi, and taxa associated with lichens (Bandoni & Boekhout, 2011; Millanes et al., 2011; Weiss et al., 2014). *Tremella* is a highly polyphyletic genus that contains a diverse array of teleomorphic yeast species and lichenicolous species. This categorization is supported by phylogenetic analyses of various *Tremella* species that are based on the D1/D2 region of the nuclear large subunit ribosomal rRNA (LSU) and the internal transcribed spacer (ITS) region (Alshahni et al., 2011; Scorzetti et al., 2002; Weiss et al., 2014). *Tremella yokohamensis* was discovered in 2011 at the Kanazawa Zoological Park in Yokohama, Japan, was initially described as the anamorphic yeast species *Cryptococcus yokohamensis*, a member of the Fuciformis clade (Alshahni et al., 2011; Scorzetti et al., 2002). In 2015, the sexual stage of *C. yokohamensis* (=*T. yokohamensis*) was discovered through corroboration with DNA sequence data. Interestingly, a specimen collected in the Russian Far East (VLA M-11700) formed a cluster in the same clade as *C. yokohamensis* (voucher JCM 16991). This evidence led to the fungus being reclassified as *T. yokohamensi*s (Malysheva et al., 2015).

The mating systems employed by the Tremellales are varied, and all known heterothallic *Tremella* species employ a tetrapolar mating system (Hanson & Wells, 1991; Wells, 1994). However, other teleomorphic species in the Tremellales, such as *A. fuscus* (Sampaio et al., 2004), *C. neoformans*, and *C. gattii* (Fraser et al., 2004; Keller et al., 2003; Kwon, 1975; Lengeler et al., 2002; Loftus et al., 2005; Nielsen et al., 2003), have been reported to employ bipolar mating systems. In this study, fruiting bodies of a white jelly mushroom that were similar in morphological appearance to *T. fuciformis* were collected on the log of a broadleaved tree in central Tottori Prefecture, Japan. The isolates were identified by the nucleotide sequences of the rDNA-ITS region and were used to analyze the mating compatibility of the collected fungi.

2.2 Materials and methods

2.2.1 Fungal strains and culture conditions

Fruiting bodies of a white jelly mushroom were discovered on a log of a broadleaved tree in a kindergarten in Tottori College, Kurayoshi, Tottori Prefecture, Japan, in 2020. Fresh basidiocarp tissue was attached to the cover of a petri dish with Vaseline, and the spores were allowed to fall on the CJM-1 agar medium in the Petri dish below (2 g/L glucose, 2 g/L Difco Soytone, 15 g/L agar; Bandoni et al., 1975). The position of the fallen spores on the plate were checked using a microscope and spore locations were recorded by marking the bottom of the petri dish with a marker pen. Subsequently, small agar pieces containing a single spore, the locations of which had been marked using a marker pen, were excised from the gel and transferred to the new agar medium. The spores were successfully germinated, grown in yeast form, and 15 basidiospore isolates (A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O) were obtained and maintained on 2% malt agar medium at room temperature. Among the basidiospore isolates, isolates G and C, were analysis and deposited in the Tottori University Fungal Culture Collection (TUFC). The collection number of the voucher specimen is TUMH65418 and those of the monospore isolates G and C are TUFC101924 and TUFC101925, respectively.

2.2.2 Mating compatibility test

The yeast cells of the 15 basidiospore isolates were crossed with each other by spotting a small amount of each inoculum on Kagome vegetable juice (KVJ) agar medium (50 mL/L Kagome Low Salt Vegetable Juice, 0.5 g/L KH_2PO_4, 40 g/L agar, pH 7.0) with a sterile loop and then mixing the spots, as described by Wickes et al. (1996). For this medium, "Kagome Low Salt Vegetable Juice" (Kagome Co. Ltd., Nagoya, Aichi, Japan) was used instead of "V8 juice" to observe mycelium development and clamp cell formation between compatible strains, such as TUFC 101924 and TUFC 101925.

2.2.3 DNA extraction and amplification

A single colony of yeast cells was inoculated into 20 mL minimal liquid medium [1.5 g/L $(NH_4)_2HPO_4$, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 1 mL/L thiamine hydrochloride (50 mg/mL), pH 5.6]. The yeast cells were grown at 25 °C with shaking at 120 rpm for 2 d, then harvested as yeast pellets by centrifugation. Genomic DNA was extracted from the yeast cells cultured in minimal media using the cetyltrimethylammonium

bromide method (Dellaporta et al., 1983; Riffiani et al., 2021). The DNA was purified using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The purified DNA was used for polymerase chain reaction (PCR) amplification of ribosomal DNA. The partial small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and partial large subunit ribosomal RNA gene (i.e., the rDNA ITS region), which are considered to be effective for species identification and molecular phylogenetic analysis, were amplified with the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al., 1990). Each 20 μL PCR reaction mixture contained 20 ng of genomic DNA, 20 pmol of each primer, 2.5 mM dNTP mixture, 10 × Ex *Taq* buffer with 2 mM $MgCl_2$, and 0.5 U Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan). DNA amplification was performed using the following PCR protocol: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel in 1 × TAE buffer (40 mM Tris-acetate pH 8, 1 mM EDTA). The gel was stained with ethidium bromide and visualized under UV light. The PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen, CA, USA), and analysis of the nucleotide sequences of the PCR products was outsourced (Eurofins Genomics Japan, Tokyo, Japan). The nucleotide sequence data of the rDNA ITS region of isolates G (TUFC 101924) and C (TUFC 101925), which are reported in this paper, were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC784256 and LC784255, respectively.

2.2.3 Phylogenetic analysis

Nucleotide sequence data were analyzed and edited with the GENETYX software package (version 13, GENETYX, Tokyo, Japan). The nucleotide sequences of the rDNA-ITS region of isolates G and C determined in this study, and nucleotide sequences of those of other *Cryptococcus* and related species extracted from the DNA Data Bank of Japan (DDBJ) DNA database were aligned with MEGA 11 software (Tamura et al., 2021). The accession numbers of these sequences are shown in Table 2.1. A phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou & Nei, 1987). Data consistency was tested by bootstrapping the alignments using 1000 replicates. For the molecular phylogenetic analysis, DNA sequences downloaded from the National Center for Biotechnology Information (NCBI, USA; https://www.ncbi.nlm.nih.gov/) were added to the

sequences obtained in this study, and *Ramaria pallidissima ZT Myc 55616* (NR_171869.1) was used as an outgroup.

2.3 Results

2.3.1 Phylogenetic analysis

Nucleotide sequences of the rDNA-ITS region, which had an average length of 510 base pairs (bp), were aligned. A phylogenetic tree was constructed by the NJ method and based on the nucleotide sequences of the rDNA ITS region (Fig 2.1) including those of basidiospore isolates G (TUFC 101924) and C (TUFC 101925). Strain G (TUFC 101924), C (TUFC 101925), *T.* yokohamensis KHU20230328 01, *T. yokohamensis* CBS:11776, *T. yokohamensis* VLA:M-11700, *C. yokohamensis* JCM 16989, *C. yokohamensis* JCM 16991 were all grouped in the same clade as *C. yokohamensis* JCM 16990. Moreover, a similarity search of the ITS regions using BLAST revealed that *C. yokohamensis* JCM 16990 was most closely related to strains G and C, with only one nucleotide difference. Based on these findings, we classified the basidiospore isolate strains G (TUFC 101924) and C (TUFC 101925) as *T. yokohamensis*.

2.3.2 Polarity of mating type

To investigate the mating system of this fungus, we crossed 15 basidiospore isolates with each other and examined clamp cell formation under a light microscope. In the case of compatible yeast cell combinations, hyphal filaments were observed at the periphery of the yeast colony. Conversely, incompatible yeast cell combinations remained as yeast cells. The results of the mating compatibility tests involving the 15 basidiospore isolates (A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O) are shown in Table 2.2 The 15 basidiospore isolates could clearly be divided into two incompatibility groups depending on the production of clamp cells after crossing with each other. Group 1 comprised isolates A, C, E, F, H, K, L, M, and O, while Group 2 comprised isolates B, D, G, I, J, and N. Based on this grouping, we concluded that *T. yokohamensis* is a heterothallic bipolar mushroom, and the mating types of Group 1 and Group 2 were defined as "*a*" and "*α*", respectively.

The polarity of mating type in each species is also shown in Fig. 2.1. Previous studies have been conducted on the mating behavior of several Tremellales yeast species, and their *MAT* loci structures have been investigated (Guerreiro et al., 2013; Hsueh et al., 2011). These studies showed that tremellaceous yeasts, including *C. amylolentus*, *C. heveanensis*,

Kwoniella mangroviensis, and *T. mesenterica*, employ tetrapolar mating systems (Hsueh et al., 2011). This is the first report of a bipolar mating system in the genus *Tremella* and the second in the genus *Cryptococcus*. The latter genus includes *C. neoformans* and *C. gattii*, both of which are reported to employ bipolar mating systems comprising two opposite mating types, defined as a and α (Fraser et al., 2003; Keller et al., 2003; Kwon, 1975, 1976a, 1976b; Nielsen et al., 2003).

2.4 Discussion

Considering the results of the phylogenetic analysis and the polarity of the mating type, although *T. yokohamensis* has a very similar life cycle to *Tremella* and is a member of the Fuciformis clade (Alshahni et al., 2011; Scorzetti et al., 2002), which contains tetrapolar mushrooms (Chang & Miles, 2004), *T. yokohamensis* employs a bipolar mating system. Maia et al. (2015) investigated the mating system of the yeast *Leucosporidium scottii*, and concluded that the tetrapolar mating type is the ancestral state of all basidiomycetes. Hsueh et al. (2011) reported that *C. amylolentus* and *C. heveanensis* employ tetrapolar mating systems, which provides additional support for the tetrapolar-to-bipolar evolutionary model. Fraser et al. (2004) conducted a study on the *MAT* structure of *C. neoformans* and proposed an evolutionary model of the *MAT*-specific regions and linkage of the two loci from a hypothetical tetrapolar ancestor to form the large, derived locus of the bipolar *Cryptococcus* species. Other studies have examined the composition and function of the mating-type loci in bipolar basidiomycetes. Aimi et al. (2005) characterized the bipolar mating system of the mushroom *Pholiota microspora*, synonym *P. nameko*) ("nameko" in Japanese) by linkage mapping and DNA sequencing. Although both *A* and *B* mating-type homologs are found in bipolar mushrooms, they are found on different chromosomes, and only the *A* mating-type homologs are related to mating compatibility. Yi et al. (2010) investigated the role of homeodomain protein genes in regulating clamp formation in the bipolar basidiomycete *P. microspora*. Their results suggested that the high expression levels of these genes are necessary for clamp formation, and that altered expression of the *A* mating-type genes alone can drive true clamp formation. According to James et al. (2006), these occurrences were caused by a loss of a mating-type-specific pheromone receptor function.

Table 2.1 Nucleotide sequence with GenBank accession numbers used in the molecular phylogenetic analysis, ITS data were obtained from the National Center for Biotechnology Information website. Bold text indicates the species identified in this study.

Species	Strain/Voucher No.	GenBank accession No.
Tremella yokohamensis	KHU20230328_01	OR791291.1
Tremella yokohamensis	CBS:11776	KY105698.1
Tremella yokohamensis	**TUFC 101924**	**LC784256**
Tremella yokohamensis	**TUFC 101925**	**LC784255**
Cryptococcus yokohamensis	JCM 16990	HM222930.1
Cryptococcus yokohamensis	JCM 16989	HM222926.1
Tremella yokohamensis	VLA:M-11700	KP986529.1
Cryptococcus yokohamensis	JCM 16991	HM222928.1
Tremella fuciformis	CBS 6970	NR_155936.1
Tremella globispora	CBS 6972	NR_155889.1
Tremella mesenterica	CBS 6973	NR_155937.1
Tremella brasiliensis	CBS 6966	NR_119454.1
Cryptococcus amylolentus	CBS 6039	NR_111372.1
Cryptococcus wingfieldii	CBS:7118	KY105769.1
Cryptococcus neoformans	CBS:7827	KY102836.1
Cryptococcus gattii	AFLP4_CBS919	JN939459.1
Ramaria pallidissima	ZT Myc 55616	NR_171869.1

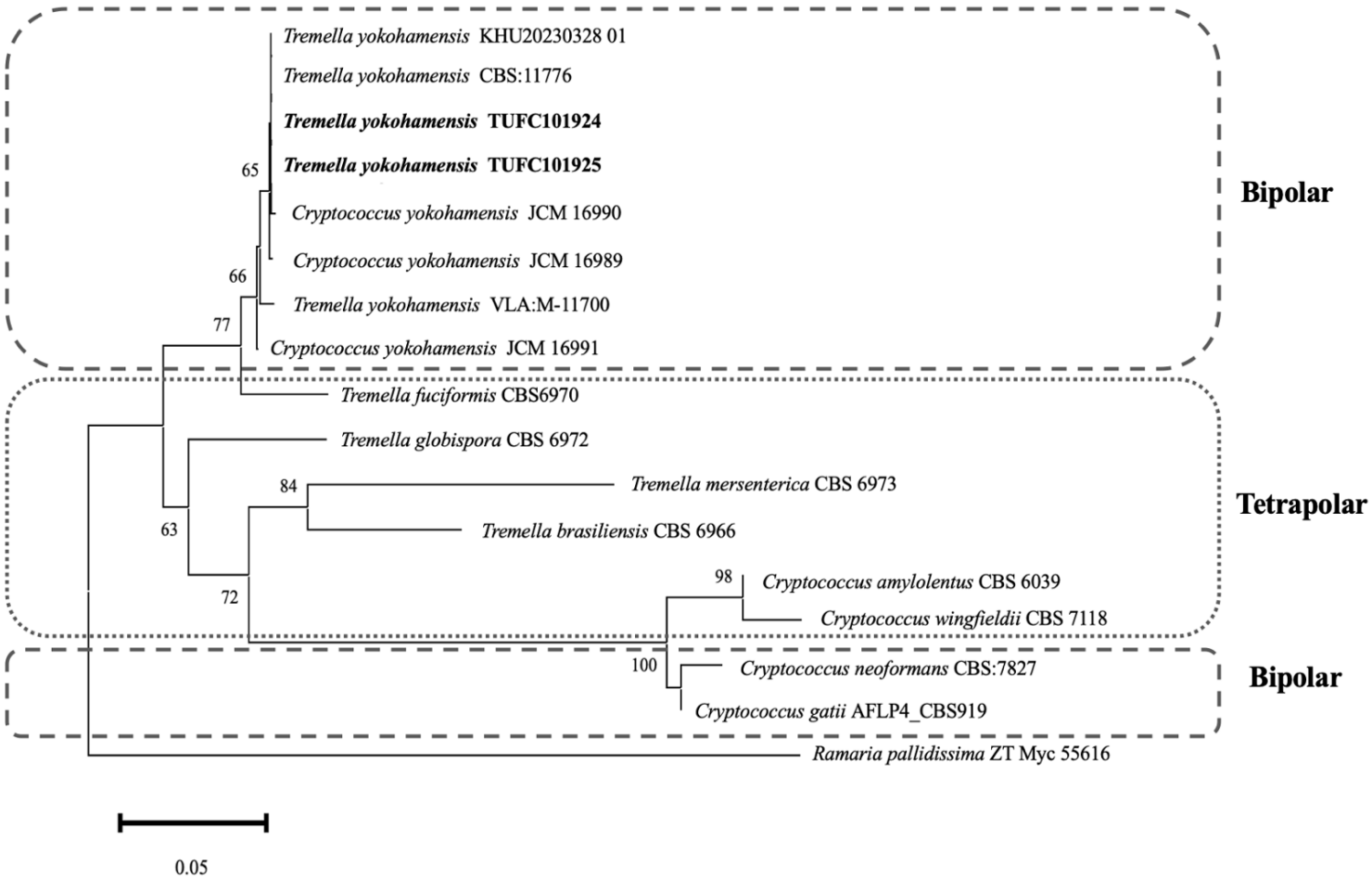


Fig. 2.1 Phylogenetic tree constructed from the internal transcribed spacer sequences of *Tremella yokohamensis* and closely related species using the Neighbor-joining method implemented in the MEGA software package (version 11). Bootstrap values were calculated from 1,000 replicates (≥ 50%). The scientific names and the accession numbers of the sequences of the species used in this study are indicated in bold. *Ramaria pallidissima ZT Myc 55616* was used as an outgroup. The dashed box shows the bipolar mating type and the dotted box shows the tetrapolar mating type. The bar indicates a patristic distance of 0.05 substitutions per site.

Table 2.2 Results of the mating compatibility test among 15 basidiospore isolates (A–O) obtained from the same fruiting body of *Tremella yokohamensis.*

	Mating type	*a*									*α*					
Mating type		A	C	E	F	H	K	L	M	O	B	D	G	I	J	N
a	A		-	-	-	-	-	-	-	-	+	+	+	+	+	+
	C	-		-	-	-	-	-	-	-	+	+	+	+	+	+
	E	-	-		-	-	-	-	-	-	+	+	+	+	+	+
	F	-	-	-		-	-	-	-	-	+	+	+	+	+	+
	H	-	-	-	-		-	-	-	-	+	+	+	+	+	+
	K	-	-	-	-	-		-	-	-	+	+	+	+	+	+
	L	-	-	-	-	-	-		-	-	+	+	+	+	+	+
	M	-	-	-	-	-	-	-		-	+	+	+	+	+	+
	O	-	-	-	-	-	-	-	-		+	+	+	+	+	+
α	B	+	+	+	+	+	+	+	+	+		-	-	-	-	-
	D	+	+	+	+	+	+	+	+	+	-		-	-	-	-
	G	+	+	+	+	+	+	+	+	+	-	-		-	-	-
	I	+	+	+	+	+	+	+	+	+	-	-	-		-	-
	J	+	+	+	+	+	+	+	+	+	-	-	-	-		-
	N	+	+	+	+	+	+	+	+	+	-	-	-	-	-	

+: clamp cells were observed

−: clamp cells were not observed

Chapter 3

Nuclear phase and life cycle of *Tremella yokohamensis*

3.1 Introduction

Tremella is a dimorphic genus that comprises two morphologically distinct life cycles: a teleomorphic state (diploid), in which basidiocarps resulting from the conjugation of yeast cells develop on appropriate substrates under suitable conditions, and an anamorphic state (haploid), in which basidiospores germinate by budding to establish a haploid yeast stage (Bandoni & Boekhout, 2011; Weiss et al., 2014). *Tremella* mushrooms are widespread and can be easily found on the decayed trunks of hardwood trees and recently fallen branches of broadleaf trees (Wu et al., 2019). The mushrooms are characterized by their tremella-like basidiomata. Many morphological characteristics have been used in taxonomic studies of *Tremella*, including the shape, color, and size of basidiomata, basidia, and basidiospores, as well as other features such as length of the stalks and sterigmata, spore formation of the basidia, conidia, swollen cells, and hyphidia (Chen, 1998). The life cycle of *Tremella* species, notably *T. fuciformis*, is complicated by the fact that basidiospores may germinate in more than one way and that there are several means of asexual reproduction involving both monokaryotic and dikaryotic phases of the life cycle. Basidiospores can germinate in two ways: one involves the formation of germ tubes followed by the development of mycelia, while the other entails budding to produce yeastlike conidia. These conidia can, under suitable conditions, germinate to form monokaryotic hyphae and mycelia. The mycelium may originate from conidia, yeastlike cells, or germlings that initially formed from basidiospores. (Chang & Miles, 2004). Understanding the life cycle of *Tremella* mushroom is critical for their successful cultivation and breeding. In this study, the morphology and life cycle of *T. yokohamensis*, a Japanese white jelly mushroom, were examined to optimize growth conditions and reproduction for further cultivation.

3.2 Materials and methods

3.2.1 Fungal strains and culture conditions

Tremella yokohamensis TUFC101924 and TUFC101925, obtained from the Fungus/Mushroom Resource and Research Center at Tottori University, Japan (TUFC). These strains were observed on mycelial progression by transferring one loop amount of

each inoculate to minimal liquid medium, followed by mixing with a vortex mixer for a couple minutes. Subsequently, 10 μL of the mixed strains were applied onto KVJ agar medium by dropping with a micropipette and incubated at 25 °C.

3.2.2 Microscopic analysis

The mycelial progression resulting from the crossing of compatible strains (TUFC 101924 and TUFC 101925) was observed at the edge of the colony on the KVJ agar plate using a light microscope (Nikon Eclipse 80i; Nikon, Tokyo) equipped with a digital camera (Nikon DS-L2; Nikon) following incubation from days 1 to 21 after inoculation. The nuclear phase of the basidiospores, yeast cells, and hyphae was examined under a microscope (Nikon Eclipse 80i; Nikon) fitted with a UV excitation apparatus (Nikon C-SHG1, Nikon) and a digital camera (Nikon DS-L2; Nikon). 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Osaka, Japan) was used for nuclear staining according to the method described by Chen et al. (2021). Briefly, yeast cells were cultivated in liquid minimal medium at 25 °C for 2 d and harvested in microtubes. The yeast pellets were then fixed by suspending in Carnoy's solution (acetic acid: ethanol = 1: 3, v/v) for 20 min, followed by centrifugation at 10000 × *g* for 60 s and removal of Carnoy's solution. Then, one drop of DAPI (1 ppm) solution was to the pellet and the samples were incubated at room temperature for 30 min. A piece of mycelium that had been growing on KVJ agar medium for 14 d after crossing the compatible strains was then picked using a toothpick and transferred to a slide glass. Carnoy's solution was then dropped onto the mycelium to fix the sample, followed by one drop of DAPI solution (1 ppm) and one drop of Calcofluor White (Fluka, Buchs, Switzerland) solution (2 ppm) to stain the septa. The slide glass was then incubated overnight in the dark at room temperature before the nuclei were observed under a fluorescence microscope.

3.3 Results

3.3.1 Mycelium induction

The morphology of the 15 basidiospore colonies were observed on KVJ agar medium; those of isolate C (TUFC101925) and isolate G (TUFC101924) are shown in Fig. 3.1A and Fig. 3.1B, respectively. Although this fungus produced a foliose, irregularly branched, and visible fruiting body, all of the basidiospore isolates germinated into the yeast form. Therefore, the condition of mycelial development from yeast was examined. Initially,

basidiospore isolates C and G were maintained separately on CJM-1 agar medium, and no mycelium developed around each isolate's yeast colony. Then, the G and C isolates were mixed and inoculated on mycelium-inducing media, such as malt extract agar (20 g/L malt extract, 15 g/L agar, pH 5.6), minimal medium agar [1.5 g/L $(NH4)_2HPO_4$, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 1 mL/L thiamine hydrochloride (50 mg/mL), 15 g/L agar, pH 5.6], potato dextrose agar (200 g/L potato, 20 g/L glucose, 15 g/L agar, pH 5.6), yeast extract-peptone-dextrose agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar, pH 5.6), and KVJ agar medium. Unfortunately, mycelium development was observed only on the KVJ agar medium (Fig. 3.1C, 3.3E). Figure 3.2 shows mycelium extending beyond the edge of a yeast colony. When a mixture of two compatible strains (G and C) was inoculated on the KVJ agar medium, the mycelium began to extend beyond the edge of the yeast colony after 7 d. However, when these mycelia were transferred to fresh KVJ agar medium, the mycelia did not grow well any more, and the mycelia colony occasionally reverted to yeast form. Thus, other types of media need to be screened to determine whether the mycelial form of this fungus can be maintained. Moreover, developing mycelium from the homokaryotic yeasts without mating was difficult, and mating and heterokaryon formation were necessary in order to develop the mycelial form.

3.3.2 Nuclear phase

Basidiospores and yeast cells germinated from each basidiospore were observed under a light microscope (Fig. 3.3A and 3.3C, respectively). In order to clarify the life cycle of this fungus, the number of nuclei in the basidiospores, the yeast form of basidiospore isolates, and the developed mycelium were observed by fluorescent microscopy after staining with DAPI. The basidiospore (Fig. 3.3B) and yeast form of basidiospore isolates (Fig. 3.3D) were mononucleate (monokaryon). Germinated mycelium (Fig. 3.3G) from a mixed culture of strains C and G was binucleate (dikaryon), and clamp cells were observed (Fig. 3.3E, 3.3F). Based on these observations, it is strongly suggested that the isolates produced from basidiospores were homokaryotic monokaryons. Conversely, the mycelium that germinated from the mixed culture was a heterokaryotic dikaryon. Furthermore, strains C and G were identified as being sexually compatible and the proposed life cycle is shown in Fig. 3.4. The transition from the nuclear phase in basidiospores, through the germinated yeast form of basidiospore isolates, to the development of dikaryotic mycelium with clamp cells in *T. yokohamensis*, collectively indicates a heterothallic life cycle for this species.

2.4 Discussion

The basidiospore of *T. yokohamensis* germinates into the yeast form, but cannot develop into mycelia by themselves; however, mixed cultures of compatible basidiospore isolates can develop into mycelia on a limited medium. Wickes et al. (1996) reported that the concentration of agar was crucial for mycelium formation and that a concentration of 4% was optimal. According to Kent et al. (2008), no single factor is responsible for the effects of the V8 juice medium. Rather, the unique composition of V8 juice medium provides the proper nutrient composition for promoting and preserving complete sexual development, with copper playing a vital role in the activation of the mating pheromone genes *MFa* and *MFα* in *C. neoformans*. In this study, maintaining the mycelium form during subculture failed on agar plates, as KVJ medium is deficient in nutrients and cannot support and maintain mycelium growth. Nevertheless, some rich-nutrient media, such as potato dextrose agar and yeast extract-peptone-dextrose agar, can induce an increase in the yeast form, but inhibit yeast-mycelium conversion. In order to maintain and enhance the mycelium form of this fungus, other media and supplements must be investigated. For example, supplementing media with inositol has been demonstrated to increase the likelihood of inducing mating structure development, successful sexual reproduction, and mycelium growth. The presence of quorum sensing peptides, as well as using glucosamine as the sole carbon source in nutrient-rich conditions, has been demonstrated to induce filamentation in *Cryptococcus* (Kent et al., 2008; Tian et al., 2018; Xu et al., 2017; Xue et al., 2007). Enjalbert and Whiteway (2005) reported that quorum sensing molecules, such as farnesol in the growth medium of *Candida albicans* cells, play a significant role in triggering the induction of hyphal formation during the resumption of growth. In addition, two hormones, tremerogens A-9291-1 and A-9291-11, were isolated from the culture filtrate of *A*-type cells of *T. brasiliensis*. These pheromones have been shown to induce conjugation tube formation in *a*-type cells of the same species (Ishibashi et al., 1983).

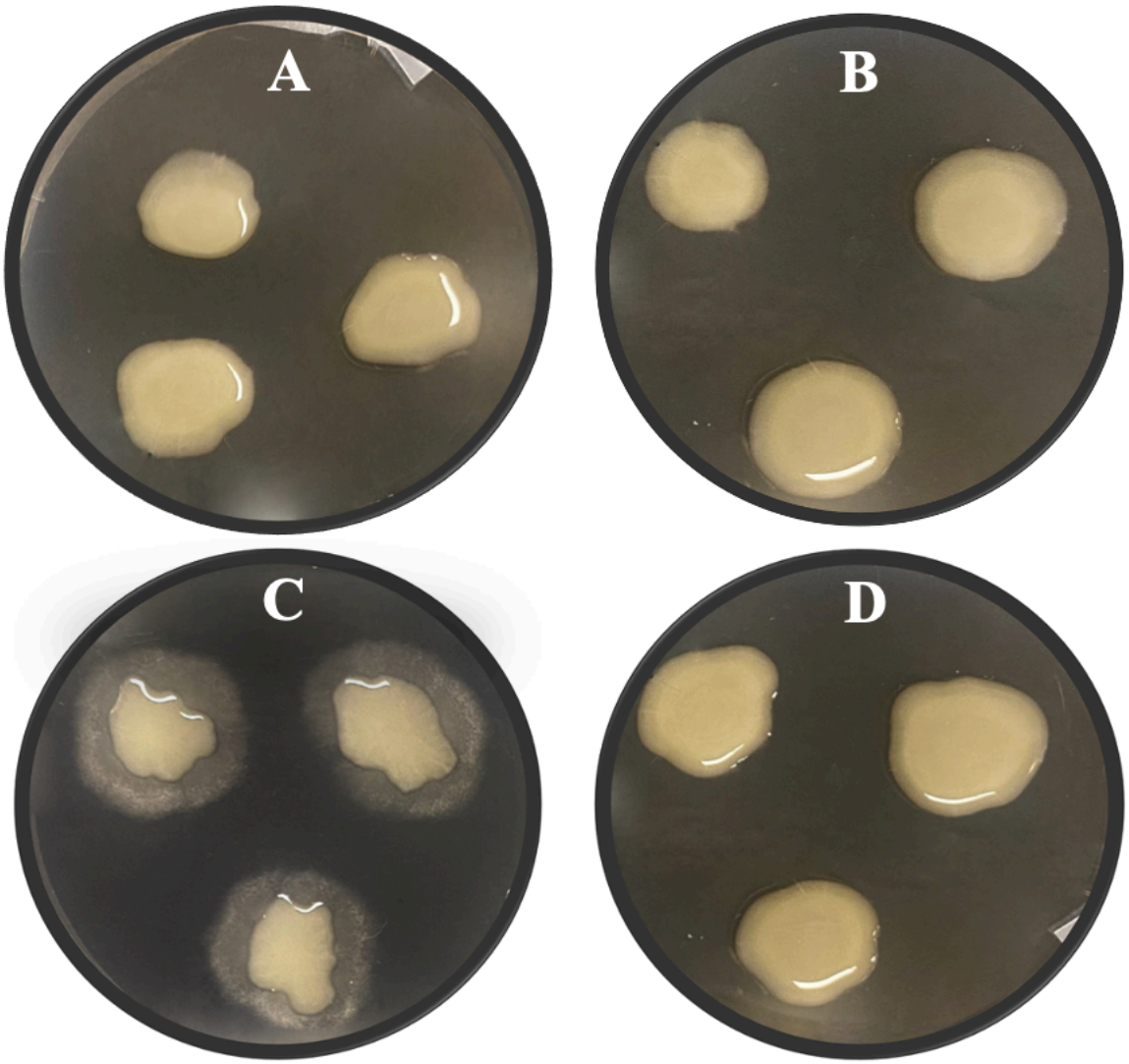


Fig. 3.1 Colony morphology of *Tremella yokohamensis* cultured on KVJ agar medium. A and B: Basidiospore isolates TUFC101925 and TUFC101924, respectively. C: Cross between compatible yeast cells (TUFC101924 × TUFC101925), with whitish hyphae appearing at the periphery of the yeast colony. D: Cross between incompatible yeast cells; no hyphae appear at periphery of the yeast colony.

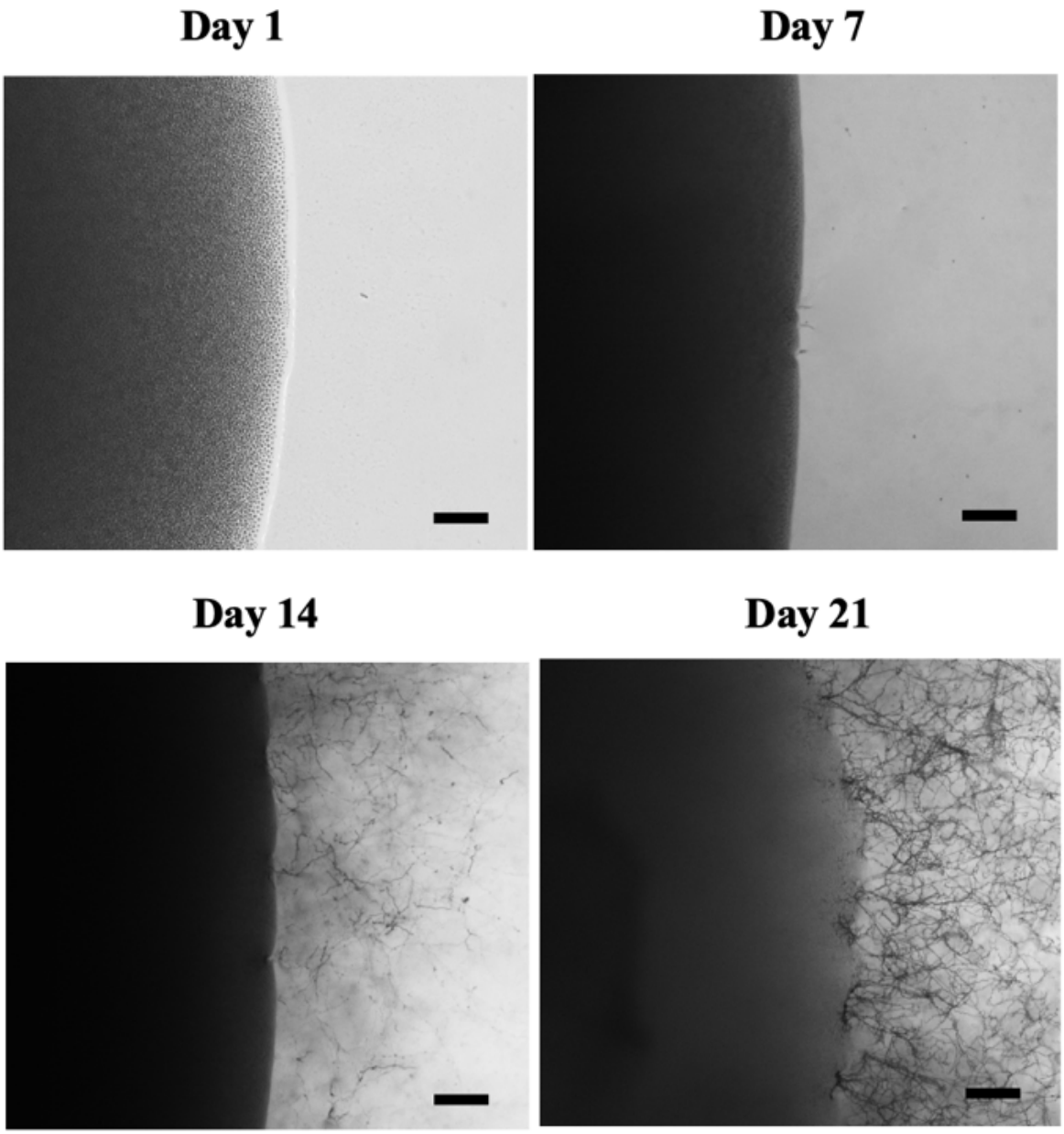


Fig. 3.2 Mycelial elongation in a cross of *Tremella yokohamensis* TUFC101924 × TUFC101925 strains on KVJ agar medium observed under a light microscope. Scale bars represent 100 μm.

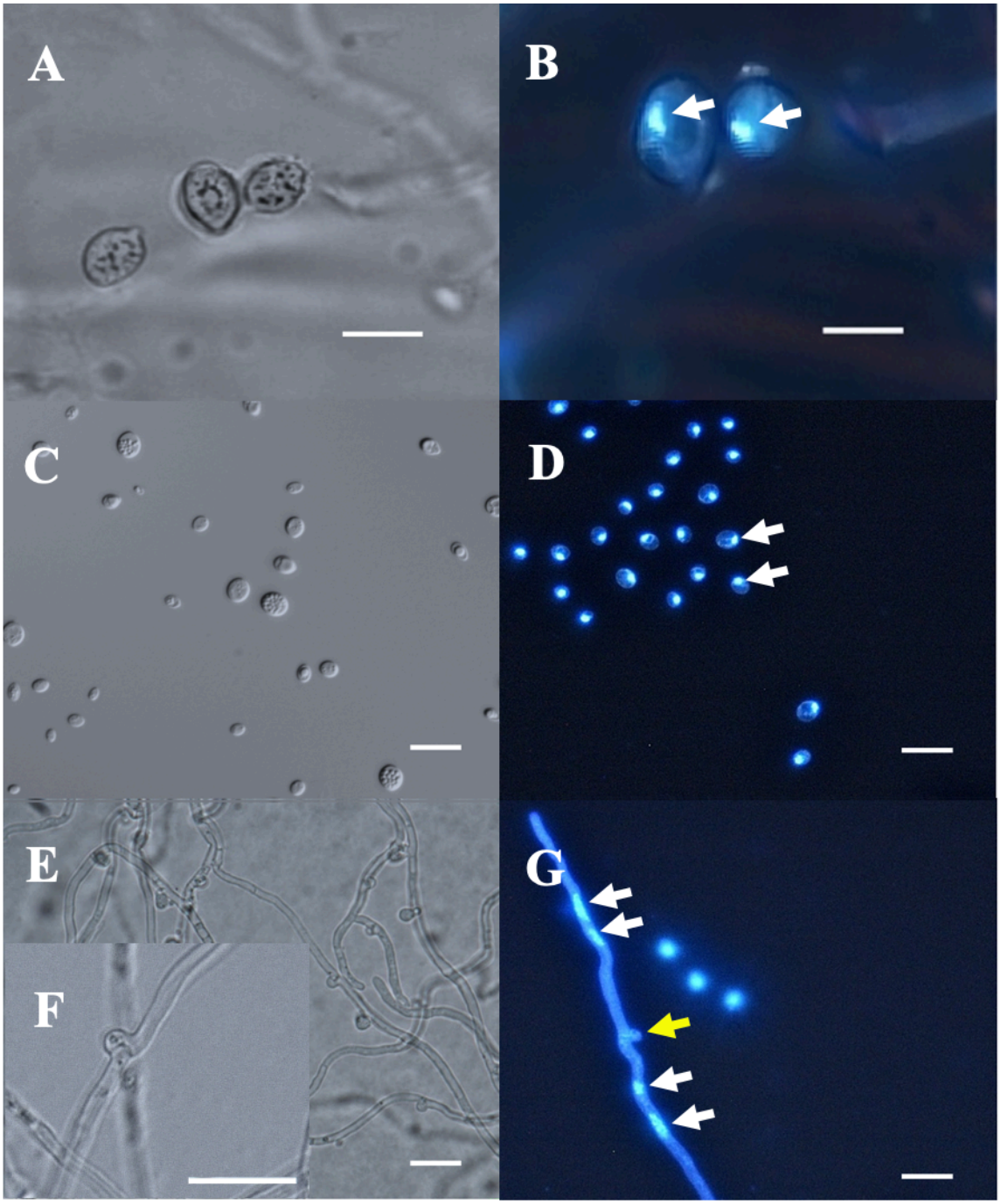


Fig. 3.3 Morphological characteristics of *Tremella yokohamensis*. A: Light micrograph of basidiospores. B: Fluorescence micrograph of basidiospores after staining with DAPI, the white arrows indicate the position of nuclei. C: Light micrograph of yeast cells. D: Fluorescence micrograph of yeast cells, the white arrows indicate the position of nuclei. E, F: Light micrograph of secondary mycelia with clamp connection after crossing between *T. yokohamensis* TUFC101924 × TUFC101925. G: Fluorescence micrograph of staining with DAPI and Calcofluor White, the yellow arrow indicates the position of a septum and the white arrows indicate the position of nuclei. Bars: 10 μm.

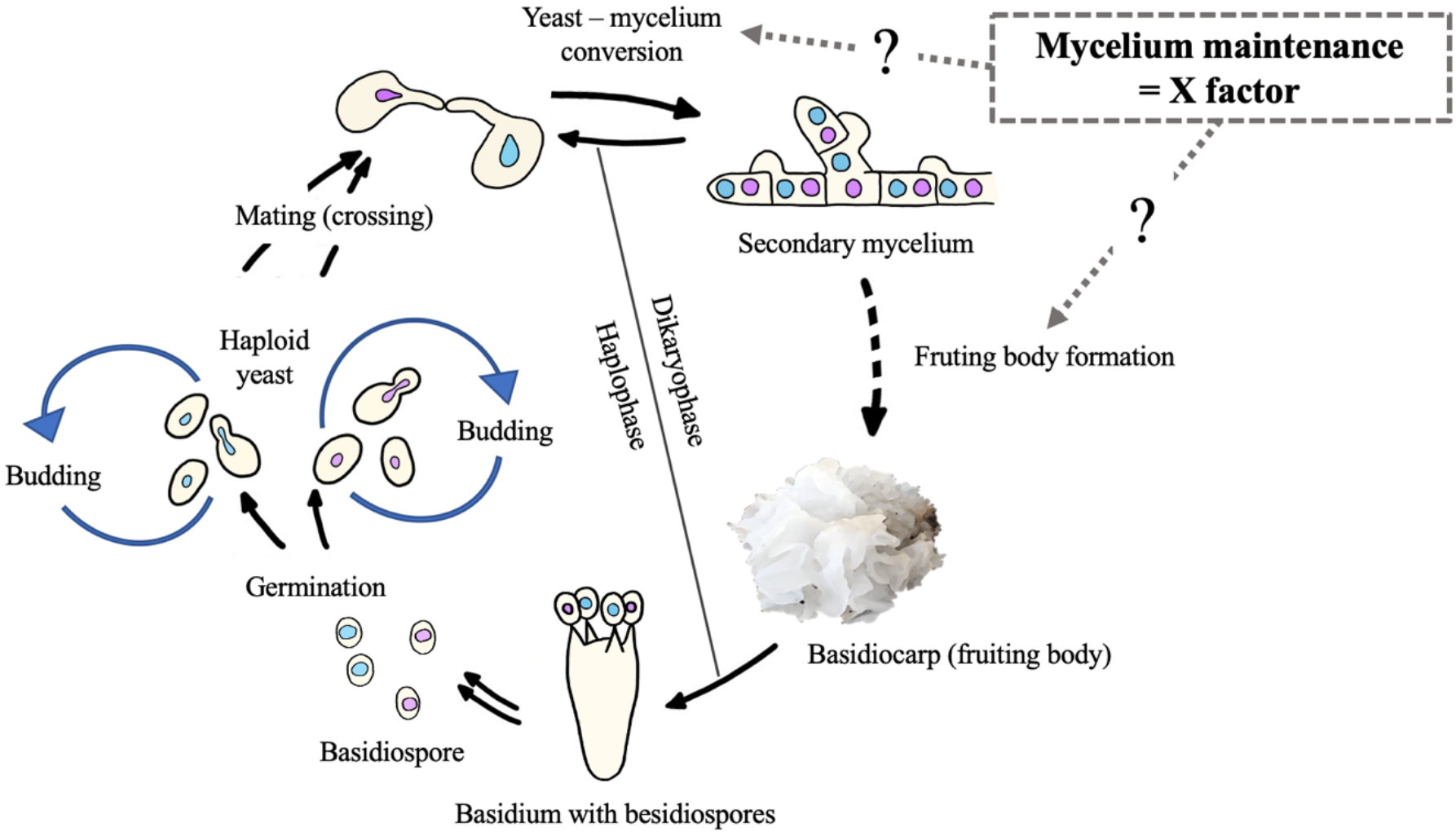


Fig. 3.4 Proposed life cycle of *Tremella yokohamensis*.

Chapter 4

Identification of tyrosol in the culture filtrate of *Annulohypoxylon truncatum* as a mycelial inducer of *Tremella yokohamensis*

4.1 Introduction

Mushrooms of the genus *Tremella*, which is part of the jelly fungus Tremellaceae family, are white jelly mushrooms that have been receiving much interest due to their various biological activities (Huang et al., 2010; Cheung, 1996; Cho et al., 2006; Du et al., 2010; Reshetnikov et al., 2000). They are renowned for their culinary appeal and numerous significant medicinal properties (Chen, 2010; Yu et al., 2019; Iturbe et al., 2016). The genus *Hypoxylon* belonging to the Hypoxylaceae family of fungi underwent taxonomic revision in 2005 (Hsieh et al.), with some species being reclassified into the new genus *Annulohypoxylon*. The fungi of this new genus are known to produce remarkably diverse secondary metabolites (Kuhnert et al., 2016; Helaly et al., 2018).

In recent years, *Annulohypoxylon* species have gained increasing attention for their beneficial effects in the production of white jelly mushrooms. Whether in natural habitats or under artificial cultivation, the successful growth of white jelly mushrooms is greatly dependent on the presence of *Annulohypoxylon* as a companion (Huang & Chen., 2002; Deng et al., 2016; Hsieh et al., 2005). White jelly mushrooms exhibit a dimorphic fungal life cycle, initially appearing as a slow-growing yeast with a gelatinous texture (yeast form), then suddenly transitioning to a filamentous form (hyphal form) in response to environmental cues, particularly its preferred substrate (Chen & Huang, 2001; Liu et al., 2007). The production of white jelly mushrooms has traditionally involved a substrate inoculation technique using a mixed culture (Huang & Chen., 2002). This method incorporates helper mycelia, such as mycelia from *Annulohypoxylon* species, which have been reported to be useful as companion fungi. These helper species can degrade lignin and carbohydrates, and they exhibit β-glucosidase, pectinase, xylanase, and β-glucanase activities, among others; these capabilities enable them to provide required nutrients for the growth and development of white jelly mushrooms (Robl et al., 2015).

The industrial production of white jelly mushrooms faces significant challenges due to the lack of high-quality species, which pose certain risks to production and cause

economic losses. The dimorphic life cycle of the mushrooms is problematic in the breeding process, particularly the difficulty in inducing mycelial formation when basidiospores transition into yeast-like conidia (Li et al., 2022). Current research on the dimorphism of white jelly mushrooms is primarily focused on the influences of different factors, such as the nitrogen source, carbon source, carbon/nitrogen ratio, pH value, temperature and culture duration, on the transition from the yeast form to the hyphal form (Liu et al., 2008).

To gain a deeper understanding of how *Annulohypoxylon* species, as companion fungi and a biological factor, help white jelly mushrooms to complete their life cycle under specific environmental conditions, we investigated whether *A. truncatum* produces mycelia-inducing compounds or interacts with the Japanese white jelly mushroom *T. yokohamensis*. We also analyzed its role in inducing the transition from the yeast form to the hyphal form as well as the suitable conditions for maintaining mycelial growth for the cultivation of white jelly mushrooms.

4.2 Materials and methods

4.2.1 General Procedures

Silica gel (Silica gel 60N, spherical neutral, particle size 63 – 210 μm; Kanto Kagaku Co., Ltd., Japan) was used for column chromatography. For thin-layer chromatography (TLC), a TLC plate (SiO_2 glass plate, 60 F254 silica gel; Merck, Darmstadt, Germany) was used. Detection under ultraviolet (UV) light was performed using a wavelength of 254 nm, and spots were detected by using Hanessian staining solution as indicators of separated compounds. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra and 2D spectra [Correlation Spectroscopy (COSY), Heteronuclear multiple quantum coherence (HMQC), and Heteronuclear multiple-bond coherence (HMBC)] were recorded using a Bruker Avance II 600 spectrometer (Bruker, Billerica, MA, USA).

4.2.2 Fungal strains and culture condition

The strains used in the present study were *T. yokohamensis* TUFC101924, *T. yokohamensis* TUFC101925, and *A. truncatum* TUFC65001; all were obtained from the Fungus/Mushroom Resource and Research Center at Tottori University, Japan (TUFC). The strains were cultivated and maintained at 25°C on minimal medium agar (1.5 g/L $(NH_4)_2HPO_4$, 1.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 20.0 g/L glucose, 1.0 mL/L thiamine hydrochloride (50 mg/mL), and 15.0 g/L agar; pH of 5.6). After 14 d of incubation, the mycelia of *A. truncatum* TUFC65001 were inoculated into 100-mL conical flasks containing

20 mL of minimal liquid medium, and incubated without shaking at 25°C for 14 d. The culture broth was filtered through a filter paper (Whatman #1, Toyo Roshi Kaisha, Ltd., Japan), and used for the mycelia-inducing activity test and the isolation of the active compound.

4.2.3 Active compound extraction and purification

The culture filtrate (5 L) of *A. truncatum* TUFC65001 was extracted with ethyl acetate three times, and the pooled organic layer was dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, the resulting dark brown crude product of *A. truncatum* TUFC65001 (872 mg) was subjected to column chromatography using silica gel eluted with mixtures of ethyl acetate and hexane (Fig. 4.1). The concentration of ethyl acetate was increased from 25% to 100% in increments of 25%; the volume of each fraction was 300 mL, and the remaining silica gel was eluted with methanol in 300-mL volumes. The mycelia-inducing activity was detected in fraction **1** (211.0 mg), and fraction **1** was thus subjected to a silica-gel column (φ 20 × 150 mm) with ethyl acetate-hexane as an eluent (v/v = 1/1, 8 mL/test tube). All fractions were examined by TLC analysis with precoated silica gel plates and a UV lamp at 254 nm. The UV-positive areas (No. 8 to 16) were combined and concentrated to obtain Fraction **1D**. Fraction **1D** was purified by preparative TLC (toluene/acetone = 1/2). The UV-positive area (retention factor, 0.3) was collected, extracted with ethyl acetate, and concentrated to obtain compound **1D-3 (**3.0 mg) as a colorless powder.

4.2.4 Bioassay

For the mycelia-inducing activity test, the yeast suspension was prepared by the cross of a single colony yeast between compatible strains, *T. yokohamensis* (TUFC101924 × TUFC101925), in minimal medium broth, and the cells were cultured at 25°C with shaking at 120 rpm for 2 d. The culture filtrate, crude extract, and isolated fractions of *A. truncatum* TUFC65001 were tested. The culture filtrate was used directly for the inoculation of the yeast suspension. The crude extract and isolated fractions were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/mL. The samples were tested in a 24-well plate, with each well containing 10 µL of a sample (10 µL DMSO was used as a negative control), 890 µL of minimal medium broth (1.5 g/L (NH4)2HPO4, 1.0 g/L KH2PO4, 0.3 g/L MgSO4.7H2O, 5.0 g/L glucose, and 1 mL/L thiamine hydrochloride (50 mg/mL); pH 5.6), and 100 µL of the yeast suspension. The 24-well plate was incubated at 25°C with

shaking at 120 rpm. Mycelia-inducing activity was determined by transferring 10 μL from each well onto a slide for observation under a light microscope (Nikon Eclipse 80i; Nikon, Tokyo) equipped with a digital camera (Nikon DS-L2; Nikon).

4.3. Results

4.3.1 Determination of the chemical structure

The culture filtrate of *A. truncatum* TUFC65001 was extracted with ethyl acetate, and this crude extract was fractionated by silica-gel column chromatography. Fraction **1**, which was eluted by 25% ethyl acetate in hexane, contained the mycelia-inducing compound (Fraction **1**). Thus, Fraction **1** was re-chromatographed through silica gel and eluted with ethyl acetate-hexane, then the resulting fraction **1D** was purified by preparative TLC. Compound **1D-3** was isolated, and was identified to be 2-(4-hydroxyphenyl)ethanol (Fig. 4.2) by the chemical shift value and coupling splitting pattern of the characteristic 1H-NMR signals in comparison to the measurement results (δ H 2.80, δ H 3.82, δ H 6.79, δ H 7.09) of Gautschi et al. (2007) shown in Table 4.1

4.3.2 Mycelial induction

The culture filtrate and isolated fractions of *A. truncatum* TUFC65001 were used for the mycelia-inducing activity test in the single colony yeast suspension from the cross between the *T. yokohamensis* strains (TUFC101924 × TUFC101925). DMSO in minimal medium broth was used as a negative control (Fig. 3A). By microscopic observation, we detected mycelia-inducing activity in the culture filtrate of *A. truncatum* TUFC65001, *i.e.,* germ tubes germinated in which growth occurs by apical extension and division occurs without leaving constrictions at the septa and elongated into hyphae (Fig. 3B). In contrast, in minimal medium broth containing 0.5 mg/mL of 2-(4-hydroxyphenyl)ethanol, germ tubes germinated but were unable to sustain hyphal growth (Fig. 3E, 3F). Furthermore, the presence of 2-(4-hydroxyphenyl)ethanol led to the formation of pseudohyphae, which the buds did not separate, producing the chains of cells (Fig. 3E, 3G). Germ tube formation in *T. yokohamensis* was quantified as the percentage of cells that formed germ tubes, and we found that the culture filtrate was the most effective in inducing mycelial growth, with 15% of cells forming germ tubes; in contrast, the presence of 2-(4-hydroxyphenyl)ethanol resulted in germ tube formation in only 0.5% of cells.

4.4 Discussion

The growth and development of mycelia in white jelly mushrooms are regulated by various signals. Recent investigations into white jelly mushroom dimorphism have focused primarily on how factors, such as the nitrogen source, carbon source, pH value, temperature, and culture duration, influence the transition from the yeast form to the hyphal form (Liu et al., 2008). The cultivation of white jelly mushrooms relies on the digestive capabilities of companion fungi, such as *Annulohypoxylon* species, which provide essential nutrients for fruiting body growth and development (Chen & Huang, 2001; Deng et al., 2018). In the present study, we explored the relationship between the Japanese white jelly mushroom *T. yokohamensis* and the companion fungus *A. truncatum*, which is a biological factor for mycelial induction in *T. yokohamensis*. *Annulohypoxylon* species are known for their remarkably diverse secondary metabolites (Kuhnert et al., 2016; Helaly et al., 2018), including azaphilones (Surup et al., 2013), naphthalene (Bitzer et al., 2007), diterpenes (Borgschulte et al., 1991), cytochalasins (Edwards et al., 1989), benzo[j]fluoranthene derivatives, such as truncatone and hypoxylonols (Koyama et al., 2002), and various aromatic compounds (Edwards et al., 1991). In addition, Wu et al. (2008) have reported that *A. stygium* is a melanin producer, and although melanin is not essential for fungal growth and development, it has been shown to be a very efficient scavenger for peroxide free radicals. Most of these compounds exert cytotoxic and antiangiogenic activities. In the present study, we discovered that the culture filtrate of *A. truncatum* TUFC65001 could effectively induce mycelial growth in *T. yokohamensis*. This marks the first time that the culture filtrate from an *Annulohypoxylon* species has been shown to be useful as an inducer of mycelial growth.

The active compound responsible for inducing mycelial growth was isolated from the culture filtrate of *A. truncatum,* and its chemical structure was determined. The active compound was identified to be 2-(4-hydroxyphenyl)ethanol, which is also known as tyrosol, and its chemical structure was previously reported by Gautschi et al. (2007). This is the first report of tyrosol production by *A. truncatum*. Tyrosol plays a crucial role in ascomycetous yeasts, notably in *C. albicans*, as a quorum-sensing molecule that influences biofilm development and yeast cell morphological transitions. It accelerates germ tube and hypha formation (Alem et al., 2006; Chen et al., 2004). In *C. albicans*, tyrosol, along with farnesol, regulates the germ tube formation mechanism; tyrosol promotes filamentation while farnesol inhibits germ tube formation. These processes involve complex gene regulation, including the activation of genes, such as *CaEDT1* (Chen et al., 2004). In the same basidiomycetous

yeast, Albuquerque et al. (2014) examined whether farnesol and tyrosol, which recognize signaling molecules in *C. albicans*, could function as signals for *C. neoformans*; however, they found that neither farnesol nor tyrosol had any effect on *C. neoformans*. The present study revealed tyrosol's ability to induce germ tube formation in a basidiomycetous yeast, suggesting a possible quorum-sensing relationship between the ascomycetous fungus and basidiomycetous yeast.

While fungal relationships are frequently established through antagonistic mycelial interactions that affect the morphology of mycelia, metabolic processes, secondary metabolite release, and extracellular enzyme patterns (Menezes et al., 2014), our findings indicated that *A. truncatum* may play a role in inducing hypha formation in white jelly mushrooms. According to Li et al. (2019), the interaction between white jelly mushrooms and *A. stygium* resulted in whiter mycelia rather than antagonistic mycelia. The production of secondary metabolites in *Annulohypoxylon* species may therefore enhance the potential for survival of white jelly mushrooms.

White jelly mushrooms typically remain in the yeast form, and it is challenging to induce transformation into the hyphal form. Nonetheless, cultivation of these mushrooms requires the induction and maintenance of the hyphal form. In the present study, the purified compound 2-(4-hydroxyphenyl)ethanol, which exhibited mycelia-inducing activity in *T. yokohamensis*, proved to be less effective than the culture filtrate of *A. truncatum* in inducing mycelial growth. Although 2-(4-hydroxyphenyl)ethanol could induce the initiation of germ tube formation, it did not lead to the sustained maintenance and improvement of mycelial growth, which are required for cultivation purposes. Our results suggest that not only a single compound, but rather a specific combination of components that are present in the *A. truncatum* culture filtrate are needed for the induction of mycelial growth. This also indicates that the initiation, maintenance, and growth of *T. yokohamensis* mycelia may be regulated by different compounds, and that the mycelia-inducing activity of *Annulohypoxylon* species in white jelly mushrooms may be dependent on its ability to produce all of the necessary compounds.

Further research is needed with a focus on the discovery of compounds produced and released prior to and during the interaction between *Annulohypoxylon* species and white jelly mushrooms that can help maintain and accelerate mycelial development. Additionally, as previous reports have indicated the usefulness of other *Annulohypoxylon* species, such as *A. archeri* (Wingfield et al., 2018), *A. stygium* (Deng et al., 2016; Deng et al., 2018), and *A.*

annulatum (Chun, 2010), various *Annulohypoxylon* species should also be considered in future studies.

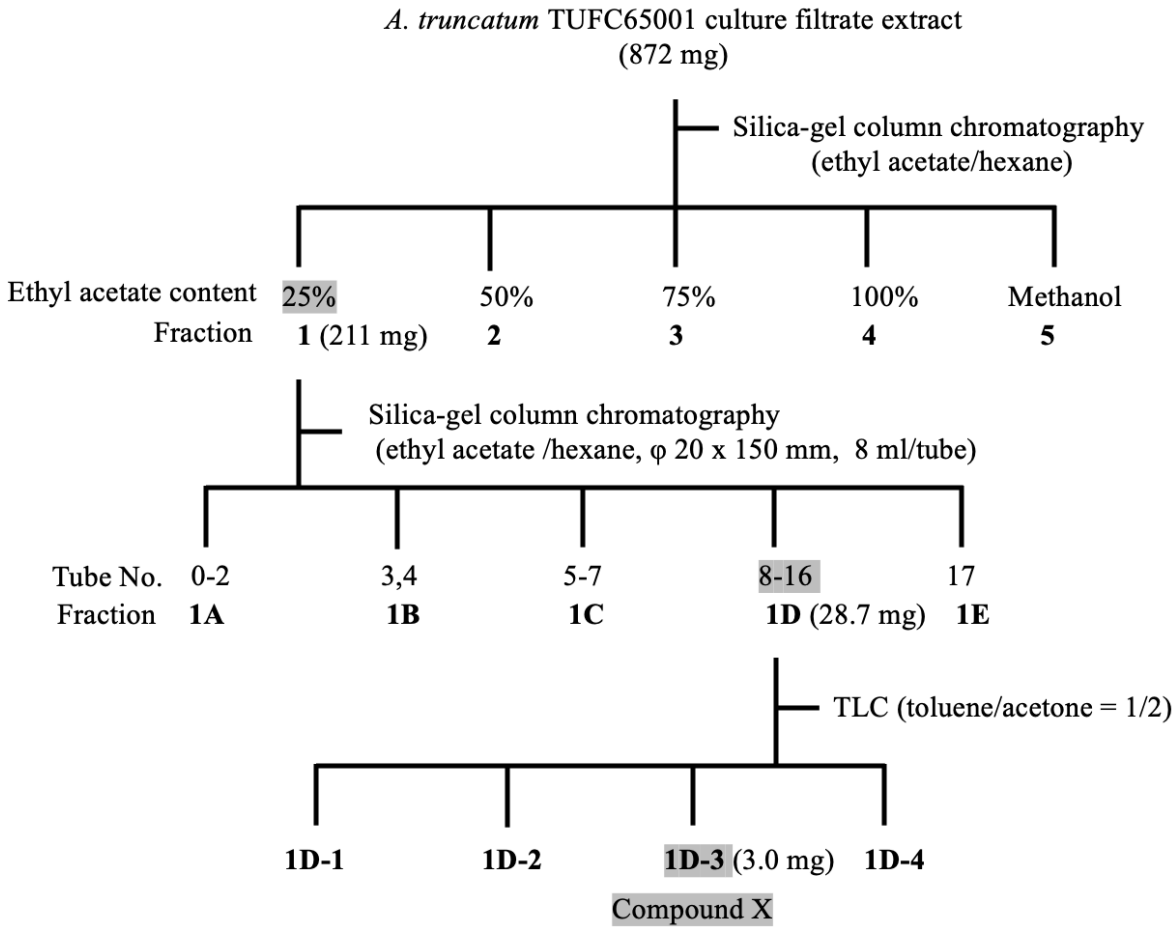
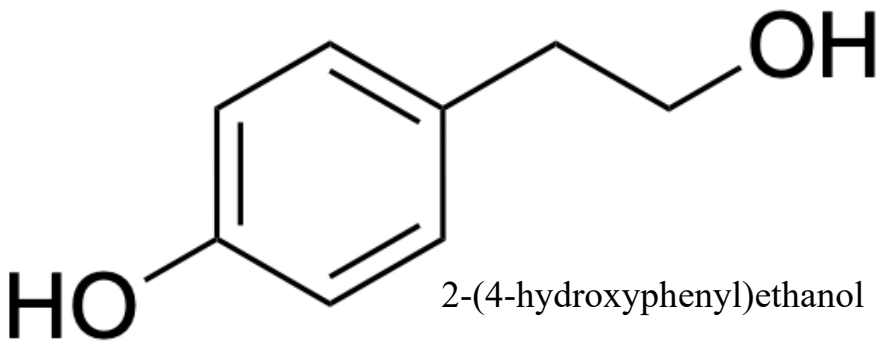


Fig. 4.1 Isolation process of compounds that induce mycelial growth in *Tremella yokohamensis* from the ethyl acetate extract of the *Annulohypoxylon truncatum* TUFC65001 culture filtrate.

Table 4.1 comparison of the characteristic H-NMR signals

Position	δ_H (multi, *J* in Hz)	
	Compound 1D-3	2-(4-hydroxyphenyl)ethanol (Gautschi et al., 2007)
1	3.67 (*t*,7.2)	3.68 (*t*,7.0)
2	2.70 (*t*,7.3)	2.72 (*t*,7.0)
2',6'	7.02 (*d*,8.4)	7.00 (*d*,8.5)
3',5'	6.69 (*d*,8.5)	6.69 (*d*,8.5)



2-(4-hydroxyphenyl)ethanol

Fig. 4.2 Structure of the isolated mycelia-inducing compound.

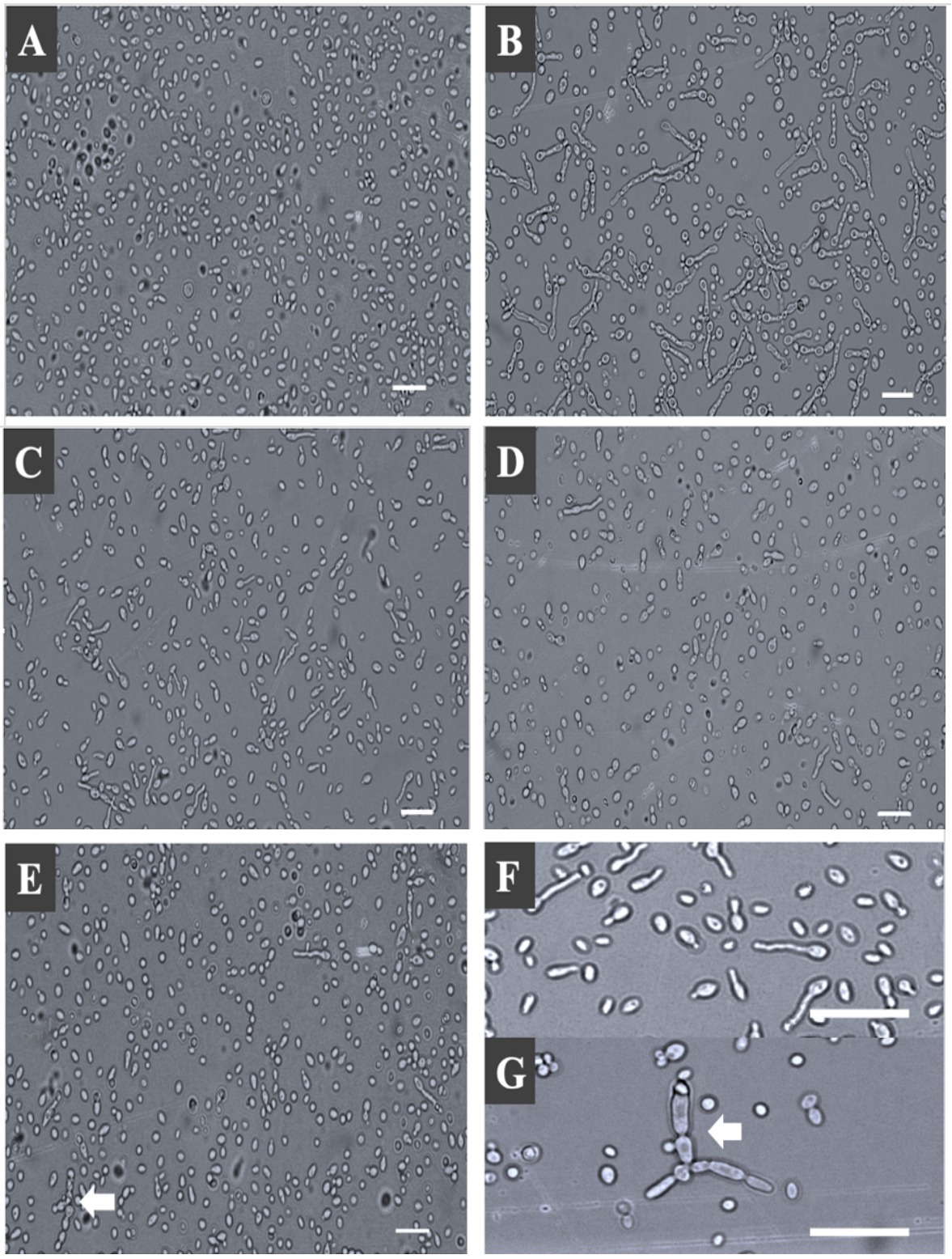


Fig. 4.3 Mycelia-inducing activity in the cross between *T. yokohamensis* strains (TUFC101924 × TUFC101925) incubated with minimal medium broth (A), the culture filtrate of *A. truncatum* TUFC65001 (B), Fraction **1** (C), Fraction **1D** (D) and Fraction **1D-3** [2-(4-hydroxyphenyl)ethanol] (E, F, and G) as observed under a light microscope. The white arrows indicate pseudohypha formation. Scale bars indicate 20 μm.

Chapter 5

Conclusion and Discussion

Tremella yokohamensis was initially described as the anamorphic yeast species *C. yokohamensis*, a member of the Fuciformis clade (Alshahni et al., 2011; Scorzetti et al., 2002). In 2015, the sexual stage of C. *yokohamensis* was discovered through corroboration with DNA sequence data. This evidence led to the fungus being reclassified as *T. yokohamensi*s (Malysheva et al., 2015). In Chapter 2, fruiting bodies of a white jelly mushroom, which are referred to as "*Shiro kikurage*" in Japanese,that were similar in morphological appearance to *T. fuciformis* were collected on the log of a broadleaved tree in central Tottori Prefecture, Japan. The basidiospores were isolated and used for mating compatibility test. Among the basidiospore isolates, isolates G and C were identified by the rDNA-ITS region and deposited in the Tottori University Fungal Culture Collection (TUFC). The collection numbers of the monospore isolates G and C are TUFC101924 and TUFC101925, respectively. Considering the results of the phylogenetic analysis and the polarity of the mating type, although *T. yokohamensis* has a very similar life cycle to *Tremella*, which contains tetrapolar mushrooms (Chang & Miles, 2004), *T. yokohamensis* employs a bipolar mating system. Maia et al. (2015) investigated the mating system of the yeast *L. scottii* and concluded that the tetrapolar mating type is the ancestral state of all basidiomycetes. Hsueh et al. (2011) reported that *C. amylolentus* and *C. heveanensis* employ tetrapolar mating systems, which provides additional support for the tetrapolar-to-bipolar evolutionary model. Fraser et al. (2004) conducted a study on the *MAT* structure of *C. neoformans* and proposed an evolutionary model of the *MAT*-specific regions and linkage of the two loci from a hypothetical tetrapolar ancestor to form the large, derived locus of the bipolar *Cryptococcus* species. Other studies have examined the composition and function of the mating-type loci in bipolar basidiomycetes. Aimi et al. (2005) characterized the bipolar mating system of the mushroom *P. microspora*, synonym *P. nameko* ("nameko" in Japanese) by linkage mapping and DNA sequencing. Although both *A* and *B* mating-type homologs are found in bipolar mushrooms, they are found on different chromosomes, and only the *A* mating-type homologs are related to mating compatibility. Yi et al. (2010) investigated the role of homeodomain protein genes in regulating clamp formation in the bipolar basidiomycete *P. microspora*. Their results suggested that the high expression levels of these genes are necessary for clamp formation and that altered expression of the *A* mating-type

genes alone can drive true clamp formation. According to James et al. (2006), these occurrences were caused by a loss of a mating-type-specific pheromone receptor function.

Tremella is a dimorphic genus that comprises two morphologically distinct life cycles: a teleomorphic state (diploid) and an anamorphic state (haploid) (Bandoni & Boekhout, 2011; Weiss et al., 2014). Understanding the life cycle of *Tremella* species is critical for their successful cultivation and breeding. In Chapter 3, *T. yokohamensi*s TUFC101924 and TUFC101925, which were mating-compatible strains, were used to analyze the life cycle. The number of nuclei in the basidiospores, the yeast form of basidiospore isolates, and the developed mycelium were observed by fluorescent microscopy after staining with DAPI. The basidiospore and yeast form of basidiospore isolates were mononucleate (monokaryon). Germinated mycelium from a mixed culture of strains C and G was binucleate (dikaryon), and clamp cells were observed. Based on these observations, it is strongly suggested that the isolates produced from basidiospores were homokaryotic monokaryons. Conversely, the mycelium that germinated from the mixed culture was a heterokaryotic dikaryon. The transition from the nuclear phase in basidiospores, through the germinated yeast form of basidiospore isolates, to the development of dikaryotic mycelium with clamp cells in *T. yokohamensis* collectively indicates avheterothallic life cycle for this species.

The morphology of *T. yokohamensis* colonies was observed on KVJ agar medium. Unfortunately, the mycelia did not grow well, and the mycelia colony occasionally reverted to yeast form. Moreover, developing mycelium from the homokaryotic yeasts without mating was difficult, and mating and heterokaryon formation were necessary in order to develop the mycelial form. Thus, other types of media need to be screened to determine whether the mycelial form of this fungus can be maintained. Wickes et al. (1996) reported that the concentration of agar was crucial for mycelium formation and that a concentration of 4% was optimal. According to Kent et al. (2008), no single factor is responsible for the effects of the V8 juice medium. Rather, the unique composition of V8 juice medium provides the proper nutrient composition for promoting and preserving complete sexual development, with copper playing a vital role in the activation of the mating pheromone genes *MFa* and *MFα* in *C. neoformans*. In this study, maintaining the mycelium form during subculture failed on agar plates, as KVJ medium is deficient in nutrients and cannot support and maintain mycelium growth. Nevertheless, some rich-nutrient media, such as potato dextrose agar and yeast extract-peptone-dextrose agar, can induce an increase in the yeast form but inhibit yeast-mycelium conversion. In order to maintain and enhance the mycelium form of

this fungus, other media and supplements must be investigated. For example, supplementing media with inositol has been demonstrated to increase the likelihood of inducing mating structure development, successful sexual reproduction, and mycelium growth. The presence of quorum sensing peptides, as well as using glucosamine as the sole carbon source in nutrient-rich conditions, has been demonstrated to induce filamentation in *Cryptococcus* (Kent et al., 2008; Tian et al., 2018; Xu et al., 2017; Xue et al., 2007). Enjalbert and Whiteway (2005) reported that quorum sensing molecules, such as farnesol in the growth medium of *C. albicans* cells, play a significant role in triggering the induction of hyphal formation during the resumption of growth. In addition, two hormones, tremerogens A-9291-1 and A-9291-11, were isolated from the culture filtrate of *A*-type cells of *T. brasiliensis*. These pheromones have been shown to induce conjugation tube formation in *a*-type cells of the same species (Ishibashi et al., 1983).

To gain a deeper understanding of this mushroom's growth and life cycle, we proposed conducting experiments where we cultivated dikaryotic hyphae formed by mating compatible strains (TUFC101924 and TUFC101925). These hyphae were then maintained on a specific nutrient medium, which was described in Chapter 4. As we know, the cultivation of white jelly mushrooms relies on the digestive capabilities of companion fungi, *Annulohypoxylon* species, which provide essential nutrition for fruiting body growth and development (Chen & Huang, 2001; Deng et al., 2018). This Chapter explores the relationship between white jelly mushroom and the companion fungus, *A. truncatum*, as a biological factor for mycelial induction in *T. yokohamensis*. The culture filtrate of *A. truncatum* (TUFC65001) was extracted to determine the chemical structure of the active compound exhibiting mycelial induction activity. The active compound, produced and released into the culture broth of *A. truncatum* TUFC 65001, was identified as 2-(4-hydroxyphenyl) ethanol, also known as tyrosol, whose chemical structure was previously identified by Gautschi et al. (2007). This is the first report of tyrosol production by *A. truncatum*. Tyrosol plays a crucial role in Ascomycetous yeast, notably *C. albicans*, acting as a quorum-sensing molecule that influences biofilm development and yeast cell morphological transitions. It accelerates germ tube and hypha formation (Alem et al., 2006; Chen et al., 2004). In *C. albicans*, tyrosol, along with farnesol, regulates the germ tube formation mechanism, where tyrosol promotes filamentation while farnesol inhibits germ tube formation. These processes involve complex gene regulation, including the activation of genes such as CaEDT1 (Chen et al., 2004). In the same basidiomycetous yeast, Albuquerque et al. (2014) reported whether farnesol and tyrosol, recognized signaling

molecules in *C. albicans*, could function as signals for *C. neoformans*. However, they found no effects in *C. neoformans*. The present study revealed tyrosol's ability to induce germ tube formation in a basidiomycetous yeast, suggesting a possible quorum-sensing relationship between the ascomycetous fungus and basidiomycetous yeast.

The white jelly mushroom typically remains in its yeast form and is challenging to transform into its hyphal form. Cultivating this mushroom requires the induction and maintenance of the hyphal form initially. However, using the purified compound 2-(4-hydroxyphenyl)ethanol, which exhibits mycelial-inducing activity in *T. yokohamensis*, proved to be less effective than using culture filtrate. Although 2-(4-hydroxyphenyl)ethanol could initiate germ tube formation, it did not facilitate the sustained maintenance and improvement of mycelial growth for cultivation purposes. Our research suggests that mycelial induction requires not only a single compound but rather a specific combination of components present in *Annulohypoxylon* culture filtrate. These findings imply that the initiation, maintenance, and growth of *T. yokohamensis* mycelia are also regulated by distinct compounds. Therefore, the mycelial induction activity of *Annulohypoxylon* in the white jelly mushroom depends on its ability to produce all necessary compounds.

Further research ought to focus on discovering compounds that help maintain and accelerate mycelial development that was produced and released prior to and during the interaction. Additionally, utilizing various species of *Annulohypoxylon*, as suggested by previous reports such as *A. archeri* (Wingfield et al., 2018), *A. stygium* (Deng et al., 2016; Deng et al., 2018), and *A. annulatum* (Chun, 2010), could be considered to maintain mycelial growth and promote the cultivation of the white jelly mushroom.

Abstract

Exploring the life cycle and mycelial induction using *Annulohypoxylon* culture filtrate extract to promote the growth of the Japanese white jelly mushroom, *Tremella yokohamensis*

In this study, fruiting bodies of a white jelly mushroom, referred to as “Shiro kikurage” in Japanese, that were similar in morphological appearance to *Tremella fuciformis* were collected on the log of a broadleaved tree in central Tottori Prefecture, Japan. The basidiospores were isolated from fruiting bodies. To investigate the mating system of this mushroom, we crossed 15 basidiospore isolates (A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O) with each other and examined clamp cell formation under a lightmicroscope. The results of the mating compatibility tests could clearly be divided into two incompatibility groups depending on the production of clamp cells after crossing with each other. Group 1 comprised isolates A, C, E, F, H, K, L, M, and O, while Group 2 comprised isolates B, D, G, I, J, and N. Based on this grouping, we concluded that this mushroom is a heterothallic and bipolar mushroom, and the mating types of Group 1 and Group 2 were defined as "a" and "α", respectively.

Among the basidiospore isolates, isolates G and C were identified and deposited in the Tottori University Fungal Culture Collection (TUFC). The collection numbers of the monospore isolates G and C are TUFC101924 and TUFC101925, respectively. Nucleotide sequences of the rDNA-ITS region revealed that *Cryptococcus yokohamensis* JCM 16990 was most closely related to strains G and C. Based on these findings, we classified the basidiospore isolate strains G (TUFC 101924) and C (TUFC 101925) as *T. yokohamensis*, which was initially described as the anamorphic yeast species *C. yokohamensis*, a member of the Fuciformis clade, and was reclassified as *T. yokohamensis* in 2015. Considering the results of the phylogenetic analysis and the polarity of the mating type, although *T. yokohamensis* has a very similar life cycle to *Tremella*, which contains tetrapolar mushrooms such as *T. fuciformis, T. mesenterica, T. globispora, T. brasiliensis*, and other tremellaceous yeasts, including *C. amylolentus, C. heveanensis*, and *C. wingfieldii, T. yokohamensis* employs a bipolar mating system. This is the first report of a bipolar mating system in the genus *Tremella* and the second in the genus *Cryptococcus*. The latter genus includes *C. neoformans* and *C. gattii*, both of which are reported to employ bipolar mating systems.

T. yokohamensis TUFC101924 and TUFC101925, which were mating compatible strains, were used to analyze the life cycle. The number of nuclei in the basidiospores, the yeast form of basidiospore isolates, and the developed mycelium were observed by fluorescent microscopy after staining with DAPI. The visualization of nuclei in each cell revealed that the basidiospores isolated from the fruiting body and the yeast form of basidiospore isolates were mononucleate (monokaryon). Germinated mycelium from a mixed culture of strains C and G was binucleate (dikaryon), and clamp cells were observed. Based on these observations, it is strongly suggested that the isolates produced from basidiospores were homokaryotic monokaryons. Conversely, the mycelium that germinated from the mixed culture was a heterokaryotic dikaryon. The transition from the nuclear phase in basidiospores, through the germinated yeast form of basidiospore isolates, to the development of dikaryotic mycelium with clamp cells in *T. yokohamensis* and the mating system collectively indicate a heterothallic life cycle for this species. Crossing between compatible yeast strains, such as TUFC 101924 and TUFC 101925, successfully induced the development of the filamentous stage bearing clamp connections after 7 d of incubation on Kagome vegetable juice agar medium (50 mL/L Kagome Low Salt Vegetable Juice, 0.5 g/L KH2PO4, 40 g/L agar, pH 5.6). Unfortunately, the mycelia did not grow well, and the mycelia colony occasionally reverted to yeast form. Moreover, developing mycelium from the homokaryotic yeasts without mating was difficult. KVJ medium is deficient in nutrients and cannot support and maintain mycelium growth. Nevertheless, some rich-nutrient media, such as potato dextrose agar and yeast extract-peptone-dextrose agar, can induce an increase in the yeast form but inhibit yeast-mycelium conversion.

It is well known that the cultivation of white jelly mushrooms relies on the digestive capabilities of companion fungi, *Annulohypoxylon* species, which provide essential nutrition for fruiting body growth and development. We explored the relationship between the white jelly mushroom and *A. truncatum*. As a biological factor, we investigated how *Annulohypoxylon* supports the life cycle of the Japanese white jelly mushroom, focusing on its ability to induce the transition from yeast form to hyphal form in *T. yokohamensis*. By using the culture filtrate of *A. truncatum* TUFC65001, we found compounds that maintain and promote the mycelial growth of *T. yokohamensis*. The culture filtrate was extracted with ethyl acetate, and after decompression and concentration, the dark brown crude product was fractionated through silica-gel column chromatography, giving a fraction of the mycelial-inducing compound, which was eluted with a solvent of 75% hexane and 25% ethyl acetate (Fraction 1). Fraction **1** was re-chromatographed through silica gel, and the activity was

found in fraction **1D**, which was eluted with ethyl acetate. This fraction was purified by preparative TLC, giving a colorless powder compound. The chemical structure of this compound was determined using the chemical shift values of the characteristic 1H-NMR signal and the coupling partitioning pattern. As a result, the active compound found in the culture filtrate of *A. truncatum* (TUFC 65001) that exhibited mycelial induction activity was identified as 2-(4-hydroxyphenyl)ethanol, also known as tyrosol. This is the first report of tyrosol production by *A. truncatum*; no such effect was found in *C. neoformans*, a basidiomycetous yeast identical to *T. yokohamensis*, and the first report that tyrosol promotes germ tube formation in the basidiomycetous yeast *T. yokohamensis*, suggesting quorum sensing between ascomycetous fungi and basidiomycetous yeast.

However, using the purified compound 2-(4-hydroxyphenyl)ethanol, which exhibits mycelial-inducing activity in *T. yokohamensis*, proved to be less effective than using culture filtrate. Although 2-(4-hydroxyphenyl)ethanol could initiate germ tube formation, it did not facilitate the sustained maintenance and improvement of mycelial growth for cultivation purposes. Our research suggests that mycelial induction requires not only a single compound but rather a specific combination of components present in *Annulohypoxylon* culture filtrate. These findings imply that the initiation, maintenance, and growth of *T. yokohamensis* mycelia are also regulated by distinct compounds. Therefore, the mycelial induction activity of *Annulohypoxylon* in the white jelly mushroom depends on its ability to produce all necessary compounds.

Further research ought to focus on discovering compounds that help maintain and accelerate mycelial development that was produced and released prior to and during the interaction. Additionally, utilizing various species of *Annulohypoxylon* to select suitable conditions, maintain mycelial growth, and promote the cultivation of the white jelly mushrooms.

和文摘要

アンヌロヒポキシロン培養濾液抽出物を用いたシロキクラゲの生育促進と菌糸誘導の検討

本研究では、日本産シロキクラゲの子実体を鳥取県中部の広葉樹の原木で採取し，そこから 15 種の担子胞子分離株（A、B、C、D、E、F、G、H、I、J、K、L、M、N、O）を分離した。このきのこの交配システムを調べるため、総当たりで交配し，クランプ細胞の形成を光学顕微鏡で観察したところ，交配和合性は、2 つのグループに分けることができ，第 1 グループは A，C，E，F，H，K，L，M，O の各菌株，第 2 グループは，B，D，G，I，J，N の各菌株であった。この結果から，このきのこはヘテロサリックな生活環をもつ二極性きのこであると結論付け，第 1 グループの交配型を「*a*」，第 2 グループの交配型を「*α*」と定義した。担子胞子分離株のうち、G 株と C 株の rDNA-ITS 領域の塩基配列から、*Cryptococcus yokohamensis* JCM 16990 と最も近縁であることが明らかになったことから、担子胞子分離株 G 株（TUFC 101924）および C 株（TUFC 101925）を *Tremella yokohamensis* と同定し，鳥取大学カルチャーコレクション（TUFC）に寄託した。*T. yokohamensis* は当初、Fuciformis クレードに属する無形麹菌種 *C. yokohamensis* として記載されていたが、2015 年に *T. yokohamensis* に再分類された。系統解析の結果と交配型の極性を考慮すると、*T. yokohamensis* は、*T. fuciformis*、*T. mesenterica*、*T. globispora*、*T. brasiliensis* などの四極性きのこや、*C. amylolentus*、*C. heveanensis*、*C. wingfieldii* などの *Tremella* 属酵母と生活環がよく似ているが、*T. yokohamensis* は二極性の交配システムを有しており，これは *Tremella* 属では初めての報告であり、クリプトコッカス属では 2 例目である。後者にはクリプトコッカス・ネオフォルマンス（*C. neoformans*）とクリプトコッカス・ガッティ（*C. gattii*）が含まれ、いずれも二極性交配システムを有していることが報告されている。

生活環の解析には交配和合株である *T. yokohamensis* C株とG株を用いた。DAPI で染色後、蛍光顕微鏡で担子胞子の核数、担子胞子分離株の酵母型細胞、発達した菌糸を観察した。その結果、子実体から分離された担子胞子および担子胞子から分離された酵母型細胞はモノカリオンであった。C 株と G 株の混合培養から発達した菌糸は二核（二核）であり，クランプ細胞が観察された。これらのことから、担子胞子から生成した酵母型細胞はホモカリオン性のモノカリオンであることが強く示唆された。一方，混合培養から発芽した菌糸体はヘテロカリオン・ダイカリオン体であった。これらのことから，ホモカリオン性のモノカリオンである担子胞子から、酵母型細胞が発芽し，和合性の株が交配し，クランプ細胞を伴うヘテロカリオン性のダイカリオンへと移行することが推察され，本菌は，ヘテロサリックなライフサイクルを示している。C 株と G 株のような和合性の酵母型細胞間の交配により、カゴメ野菜ジュース寒天培地（KVJ 培地，50 mL/L カゴメ低塩野菜ジュース、0.5 g/L KH2PO4、40 g/L 寒天、pH 5.6）上で 7 日間培養後、クランプ結合を有する糸状菌糸体の発生を誘導することに成功した。残念ながら、菌糸体はうまく成長せず、菌糸体コロニーは酵母の形に戻った。また，交配せずにホモカリオティック酵母から菌糸体を発育させることは困難であった。KVJ 培地は栄養素が不足しており、菌糸体の成長を維持することができない。それにもかかわらず、ポテトデキストロース寒天培地や酵母エキス-ペプトン-デキストロース寒天培地のような栄養豊富な培地は、酵母型の増加を誘導することはできるが、酵母から菌糸体への変換を阻害した。従って，この菌の菌糸体を維持・増殖させるためには、他の培地やサプリメントを検討しなければならない。

シロキクラゲの栽培は、子実体の成長と発育に不可欠な物質を供給する *Annulohypoxylon* 属菌の存在に依存していることはよく知られている。我々は、シロキクラゲと *Annulohypoxylon truncatum* との関係を探った。生物学的要因として、*Annulohypoxylon* 属菌の *T. yokohamensis* の酵母型から菌糸型への移行を誘導する能力に着目し、*A. truncatum* TUFC65001 の培養濾液を利用することで、*T. yokohamensis* の菌糸成長を維持・促進する化合物を見出し、その生活環をどのよ

うに支えているのかを検討した。*Annulohypoxylon* 属菌の培養濾液を抽出し、酢酸エチルで抽出し減圧濃縮後の濃褐色の粗生成物をで分画し、ヘキサン 75%-酢酸エチル 25%の溶媒で溶出した画分 **1** に菌糸誘導活性を見出した．この画分を再度分取薄層クロマトグラフィーで分画したところ，画分 **1D** に活性が認められ，この物質は無色粉末であった。この化合物の化学構造を特徴的な 1H-NMR シグナルの化学シフト値とカップリング分割パターンを用いて決定した。その結果，*A. truncatum* TUFC 65001 の培養濾液中に見出された菌糸誘導活性を示す活性化合物は 2-(4-ヒドロキシフェニル)エタノール、別名チロソールであることが同定できた．これは *A. truncatum* によるチロソールの生産に関する最初の報告である。*T. yokohamensis* と同じ担子菌酵母 *C. neoformans* では、このような影響は見られなかった。このことは、チロソールが担子菌酵母 *T. yokohamensis* の生殖管形成を促進するという初めての報告であり、子嚢菌と担子菌酵母の間のクオラムセンシングを明らかにしたことになる。

しかし、*T. yokohamensis* の菌糸誘導活性を示す精製化合物 2-(4-ヒドロキシフェニル)エタノールを用いた場合、培養濾液を用いた場合よりも効果が低いことが判明した。2-(4-ヒドロキシフェニル)エタノールは生殖管の形成を開始させることができたが、培養目的での菌糸の持続的な維持・成長を促進することはできなかった。我々の研究は、菌糸の誘導には単一の化合物だけでなく、むしろ *Annulohypoxylon* 培養濾液中に存在する特定の成分の組み合わせが必要であることを示唆している。

Acknowledgments

I wish to express my appreciation to Professor Tadanori Aimi for his generous support and guidance throughout my studies at Tottori University. His patience, insightful feedback, and unwavering commitment to my development have been invaluable. I am also grateful to Professors Norihiro Shimumura and Hiroyuki Azakami for their assistance. Special thanks to Professor Kozue Sotome for providing the white jelly mushroom strains, to Professor Tsuyoshi Ichiyanagi for his expert assistance with compound extraction, and to Mr. Taiyo Kobayashi for his collaboration. Furthermore, I express my thanks to the staff at UGSAS and the Japanese Government Scholarship (MEXT) for their financial support.

My sincere thanks go to my friends, Ms. Rini Riffiani and Mr. Frank Chen, who served as my initial mentors. I also appreciate Ms. Weitong Zhang, Mr. Tomoki Hirata, and all lab members for their unwavering support.

Special gratitude is extended to my partner in Japan, Mr. Tatsuki Nakanishi, and his wonderful family, whose constant encouragement, especially emotional support, has been a source of my strength. I am eternally grateful to my family and friends in Thailand for their constant belief in me and endless support. Finally, I want to acknowledge my own endurance in the face of numerous challenges. Despite the difficulties and moments of doubt, I am proud to have reached this significant milestone.

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List of publications

1. Nanthawan Kaeoniwong, Kozue Sotome, Tsuyoshi Ichiyanagi, Norihiro Shimomura, and Tadanori Aimi: Life cycle and mating compatibility in the Japanese white jelly mushroom, *Tremella yokohamensis*. *Mycoscience*, Vol. 65 (2024) 208-215 (The corresponding content is in Chapter 2 and 3)
2. Nanthawan Kaeoniwong, Taiyo Kobayashi, Kozue Sotome, Tsuyoshi Ichiyanagi, Norihiro Shimomura, and Tadanori Aimi: Identification of tyrosol in the culture filtrate of *Annulohypoxylon truncatum* as a mycelial inducer of the Japanese white jelly mushroom *Tremella yokohamensis*. *Mushroom science and biotechnology*, Vol. 32(3) (Accepted, June 7, 2024) (The corresponding content is in Chapter 4)