Chemical ecology of the (oxalato)aluminate complex as an antimicrobial substance from the “shiro” of *Tricholoma matsutake*

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Contents

Abbreviations

HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance

Chapter I

General Introduction

1. Introduction to "Matsutake"

1.1. History in Japan

Many mushroom species, which are the fruiting bodies of fungi, are distributed all over the world. Edible mushrooms comprise less than 10% of all known species (Mattila *et al.*, 2000). Mushrooms have been involved in human activities for a long time. Descriptions of mushrooms are found in mythologies and folk stories in Europe and Japan.

The fruiting body of *Tricholoma matsutake*, called "matsutake" in Japan, is one of the most familiar mushrooms to the Japanese, and descriptions about it are found in poems such as "Man'yo-shu," which was written more than 1,300 years ago. According to Ogawa (1978), nobles enjoyed gathering matsutake as a seasonal event in the autumn in the Heian era, and the samurai also enjoyed gathering them in the Azuchi–Momoyama era. People in the upper class such as nobles sent matsutake as gifts, but it was too expensive to eat for most people until the middle Edo era. In the late Edo era, people were able to buy matsutake due to the increase in the crop of matsutake.

The development of civilizations was probably correlated with the increase in matsutake crop. The Kinki region was the most developed region with respect to civilization from the Heian to the Azuchi–Momoyama era, and was the major area yielding matsutake since there were many forests of Japanese red pine, *Pinus densiflora*, which is the main host tree of *T. matsutake* in the Kinki region (Chiba, 1973), probably due to the increase of bare mountains and the occurrence of secondary plant succession. With the advance of civilization, people cut down trees to use as fuel and material for their house and to obtain cultivated lands, resulting in an increase in consumption of broad-leaved trees, which are the dominant species at the climax stage of plant succession (Chiba, 1973). This consumption led to the formation of pine forests that

are known as pioneer trees in plant succession. Red pine forests increased as secondary plant succession proceeded, resulting in an increase in the occurrence of matsutake. This increase may have occurred in an earlier period in the Kinki region.

Bare mountains were found in other regions from the middle Edo era, since the demand for broad-leaved trees rose along with the increase in population. The increase in bare mountains resulted in an increase in the occurrence of matsutake up until the 1940s, after which their production has gradually decreased. The annual production was 12,000 tons in 1941, but only 42 tons in 2014 (Fig. 1) (Ministry of Agriculture, Forestry and Fisheries of Japan, 2016). Two reasons for the decrease have been proposed: the decrease in red pine trees due to the pine wilt disease and eutrophication of the soil (Hosford *et al.*, 1997).

The pine wilt disease, which is caused by pine wood nematodes, *Bursaphelenchus xylophilus*, occurred in 1905 on the southern island of Kyusyu, and has been spread around Japan since the 1970s (Futai, 1979; Futai, 1980). The nematodes are transmitted into the red pine trees by the Japanese pine sawyer, *Monochamus alternatus*. The disease is caused by the obstruction of water and resin movement in the tracheids and resin ducts. Small bubbles from cavitation are responsible for this obstruction (Kuroda *et al.*, 1988; Fukuda *et al.*, 1992). Blockage of the tracheids by bubbles is sometimes observed in healthy trees in dry conditions. In diseased trees, this blockage

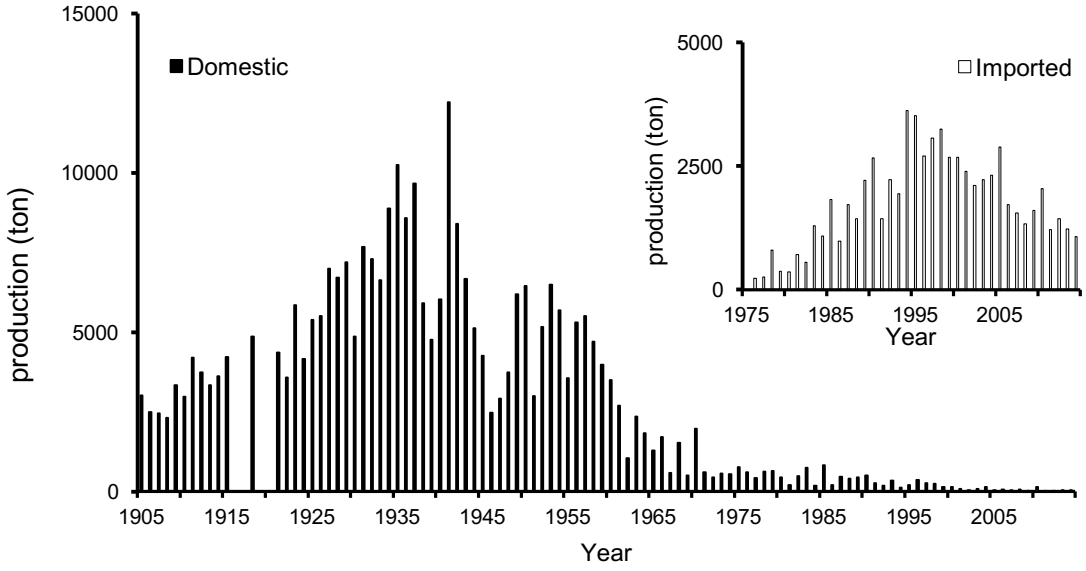

Fig. 1 Change in the annual production of matsutake in Japan. Data were obtained from the Ministry of Agriculture, Forestry and Fisheries of Japan, and edited by K. Nishino.

is a continuous phenomenon, whereas in healthy trees it is transient and reversible. The reason for the difference between diseased and healthy trees is not yet clear. This disease may cause the accumulation of unusual metabolites by necrosis (Melakeberhan and Webster, 1990), but the metabolites have not been identified. Red pine trees resistant to the disease have been found (Toda, 2004), and the damage caused by the nematodes may be decreased in the future by planting these resistant trees.

The eutrophication of soil is caused by insufficient management of red pine forests to keep them under healthy conditions. Litter and woody shrubs that were not used as fuel and compost accompanied the development of the economy in Japan, resulting in eutrophication of the mountain soil. Eutrophication activates wood rotting fungi that suppress the mycorrhizal fungi. If the red pine is not supplied with sufficient water and nutrients through the mycorrhizas, it can barely resist pine wood nematodes from the Japanese pine sawyer (Kikuchi *et al.*, 1991). The eutrophication also promotes the growth of mycorrhizal fungi that compete with *T. matsutake*, *Sarcodon scabrosus,* and *T. flavovirens* (Ogawa, 1977). *Sarcodon scabrosus* often invades the shiro of *T. matsutake*, and forms its own colony on the shiro, which results in disappearance of the shiro (Tsai and Phillips, 1991). The cost of maintaining a forest suitable for matsutake is high, and therefore the occurrence of matsutake is still decreasing and its price is rising.

1.2. Artificial Cultivation

If the artificial cultivation of *T. matsutake* is realized, the price will fall and many people can enjoy it like shimeji, shiitake, and other mushrooms. The artificial cultivation of *T. matsutake* has been attempted for a long time. The primordium that differentiates into a fruiting body was formed on Hamada media containing the mountain soil, and on a medium containing a high concentration of sugar (Ogawa and Hamada, 1975; Takara Bio Inc., 2008). A small fruiting body was formed on vermiculite added to a liquid media containing homogenized mycelium of *T. matsutake* (Kawai and Ogawa, 1976). However, the formation of the primordium and fruiting body was occasional and the results were not reproducible.

As an alternative, a method for the artificial formation of ectomycorrhizas has been developed. The formation of ectomycorrhizas on seedlings of *P. densiflora* has

succeeded under *in vitro* conditions (Yamada *et al.*, 1999). A method of forming the fruiting body using artificial mycorrhizas is still under development. The artificial cultivation of shimeji, *Lyophyllum shimeji*, which acquires nutrients in a similar way to *T. matsutake*, has succeeded (Ohta, 1994; Watanabe, 1994; Yoshida, 1994), and the artificial cultivation of *T. matsutake* might be successful in the future.

2. Biology and Ecology of *Tricholoma matsutake*

2.1 Distribution

T. matsutake is Basidiomycota, and is mainly distributed in East Asia such as Japan, South and North Korea, and the northeastern region of China. The geographical origins of matsutake can be distinguished by using their genome sequence (Joint Genome Institute, 2013), as it is visually difficult to determine their origin due to their highly similar appearances. The retroelement integration sites in the genome are used to distinguish among the matsutake isolated in Asia (Murata *et al.*, 2008). This method is to be applied to tracing the origin of matsutake for making decisions about pricing.

The genome sequences are different even among *T. matsutake* formed in Japan. The sequences of 84 strains of *T. matsutake* obtained in Japan were classified into 8 types, Types A to H, on the basis of their ribosomal DNA (rDNA) in the intergenic spacer 1 region, which is a fungal rRNA operon and varies between intraspecific levels (Bruns *et al.*, 1991; Guerin-Laguette *et al.*, 2002). Type A was the dominant species in Japan, and the genetic diversity was higher in the southern part than in the northern part of Japan.

A method that distinguishes and quantifies the mycelium of *T. matsutake* from other fungi was developed by Yamaguchi *et al.* (2016). The method employs the unique conserved DNA sequence of *T. matsutake*. The mycelium amount of *T. matsutake* in soil can be analyzed using this method, which is also useful for determining the kinetics of fungal growth in soil.

The allied species of matsutake are distributed in Asia including Japan and China, Europe, and north America: *T. bakamatsutake* Hongo, *T. fulvocastaneum*, and *T.*

robustum (Alb. et Schw. : Fr.) Ricken in Japan (Imazeki and Hongo, 1987); *T. zangii* Z.M.Cao, Y.J.Yao & Pegle in China (Cao *et al*., 2003); *T. caligatum* (Viv.) Ricken, *T. anatolicum* H.H. Doğan & Intini, *T. nauseosum* (Blytt) Kyt öv., and *T. dulciolens* Kytöv in Europe (Kyotövuori, 1988; Intini *et al*., 2003); *T. magnivelare* (Peck) Redhead in north America (Hosford *et al*., 1997). The allied species have different rDNA sequences in the internal transcribed spacer (ITS) region that vary significantly between interspecific levels (Kikuchi, *et al*., 2007). *Tricholoma nauseosum* distributed in Finland is the same species as *T. matsutake* based on the comparison of the phenotype and rDNA sequences (Kyotövuori, 1988; Kårén *et al*., 1997; Bergius and Danell, 2000; Matsushita *et al*., 2005). *Tricholoma nauseosum* was the academic name of matsutake before *T. matsutake* was adopted, but "*T. matsutake*" is more commonly used as it is a more familiar name (Bergius and Danell, 2000).

Japanese red pine, the typical host tree of *T. matsutake,* is a widespread tree distributed from Southern Hokkaido to Kyusyu in Japan, and in East Asia such as the Korean peninsula and northeastern China. The tree mainly inhabits dry ridgelines with poor soils. It can grow in a cold climate because the water content and consumption can be lower than that of other trees (Grossnickle, 1988). The red pine tree has an extensive rhizosphere formed by mycelia that extend from ectomycorrhizas with the rootlet, and can obtain water and inorganic nutrients like phosphorus and potassium from a wide area (Horikoshi and Futai, 2003).

Other host trees of *T. matsutake* in Japan are Japanese black pine, *P. thunbergii*, southern Japanese hemlock, *Tsuga sieboldii*, Sakhal in fir, *Abies sachalinensis*, and northern Japanese hemlock, *Tsuga diversifolia* (Imazeki and Hongo, 1987). In China, Yunnan pine, *P. yunnanensis*, and *Castanopsis orthacantha* and *Quercus pannosa*, which belong to the family Fagaceae, are the main host trees (Matsushita *et al.*, 2005).

2.2. Ectomycorrhizal Fungi

Fungi that form fruiting bodies are mainly divided into mycorrhizal fungi and rotting fungi. *Tricholoma matsutake* belongs to the former and forms mycorrhiza, which is a symbiotic association comprising plant rootlets and fungi (Smith, 1974). The host plant is supplied with water, nitrogen, and mineral nutrients such as phosphorus and potassium from the fungus, and the fungus receives photosynthesis

products such as sugars and amino acids from the plant through the mycorrhiza (Donald, 1972; Smith, 1974). Plants with mycorrhizas are more resistant to infections by pathogens, frost and drought damage, and salt stress than those without mycorrhizas (Lehto, 1992; Nikolaou *et al.*, 2003). The relationship between mycorrhizal fungi and host plants is generally considered to be mutualism, but the dependence of the fungi on the plant for its growth is greater than the dependence of the plant on the fungi. This aspect suggests that their relationship may be parasitism or commensalism rather than mutualism (Iwase, 1996).

Mycorrhizas are classified into ectomycorrhizas, ectendomycorrhizas, and arbuscular, ericoid, orchid, arbutoid, and monotropoid mycorrhizas based on their character (Malloch *et al.*, 1980). *Tricholoma matsutake* is an ectomycorrhizal fungus, and most ectomycorrhizal fungi belong to Basidiomycota, Ascomycota, and Zygomycota. The hosts of ectomycorrhizas are woody plants including pine, oak, and eucalyptus, and some rose families (Wang and Qiu, 2006). An ectomycorrhiza comprises a mantle which is a thick covering around the root tip made of the hypha and Hartig net of hypha surrounding the plants cell within the root cortex (Hacquard *et al.*, 2013). The appearance of the rootlet with the mycorrhiza is different from that without the mycorrhiza. The shape of an ectomycorrhiza resembles a letter Y or baculiform (Fig. 2); the mycelium extends into the soil from the mantle to form a wide network.

Many ectomycorrhizal fungi secrete auxin, which is a plant hormone, in the early stages of ectomycorrhiza formation (Ek *et al.*, 1983; Gay and Debaud, 1987). Auxin regulates plant development, morphogenesis, and expression of transcription factors

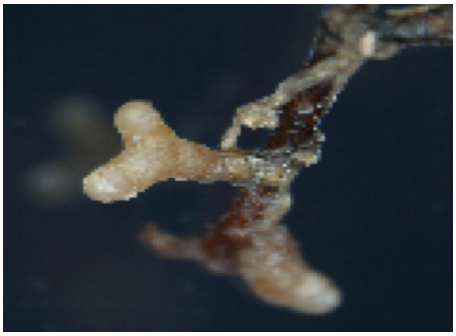

Fig. 2 A micrograph of *T. matsutake* ectomycorrhiza formed on a red pine tree (×20, photo by K. Oizumi).

(Abel *et al.*, 1994; Kim *et al.*, 1997), suggesting that auxin produced by fungi prepares suitable conditions for mycorrhizal formation, since a morphological change is necessary for its initiation (Podila, 2002).

The ectomycorrhizal fungi such as *Paxillus* spp. were proposed to suppress bacteria by decreasing the uptake of thymidine and leucine (Olsson *et al.*, 1996). The mycorrhizas might produce antimicrobial substances. The aqueous ethanol extract from the rhizosphere soil of seedlings of Norway pine, *Pinus resinosa*, inoculated with *Paxillus involutus* contained oxalic acid, which inhibited the growth of the plant pathogen *Fusarium oxyporum* (Duchesne *et al.*, 1989). The oxalic acid may be produced by the mycorrhizal fungi, the pine, or the both.

2.3. Life Cycle and Growth Conditions

The life cycle of *T. matsutake* has three stages, i.e., spore germination, mycelium growth, and fruiting-body formation, which is the same as other fungi.

The spores produced in the pileus of a fruiting body are released into the surroundings. The optimum temperature for germination is from 16 °C to 26 °C, and the spores do not germinate at 30 °C (Ogawa, 1978). The optimum pH for the germination is from 4.0 to 5.0, which is close to the soil pH in red pine forests (Ohta, 1990). The spores germinate on standard media such as malt extract agar and soil decoction agar, but the germination rate is very low (Nishikado and Yamauchi, 1935). The rate significantly increased on an agar medium with a hot-water extract of Japanese red pine needles (Hiromoto, 1960). Ohta identified *n*-butyric acid as a germination-promoting substance in the extract (Ohta, 1986), but *n*-butyric acid was not detected in soils from red pine forests.

The spores of fungi are monokaryotic or dikaryotic. The monokaryon is important for breeding, since a new dikaryon is made by hybridization of monokaryons that have different genomes. Tamada and Lian attempted to obtain monokaryotic spore from the fruiting body of *T. matsutake*, but the obtained spores were dikaryotic (Tamada and Lian, 2004). The DNA sequence of mycelia from a novel colony of *T. matsutake* was different to that from the original colony, suggesting that *T. matsutake* has monokaryotic spores (Murata, 2005; Narimatsu *et al.*, 2016); however, monokaryotic spores have not been found so far.

Information about the optimal conditions and essential nutrients for the mycelium growth of *T. matsutake* is accumulating. *Tricholoma matsutake* can grow between 5 °C and 28 °C, but growth slows under 15 °C (Ogawa, 1978). Glucose, mannose, and fructose were used as carbon resources (Kawai and Abe, 1976). The growth of hypha was promoted on the agar media with glucose and fructose at ratios from 1:4 to 2:3. Much glucose and fructose were contained in the leaves and roots of red pine trees (Ogawa, 1978). *Tricholoma matsutake* could grow by obtaining the sugars from the host trees through ectomycorrhizas. Maltose and trehalose were also utilized as carbon resources (Kawai and Abe, 1976). Mycorrhizal fungi used amino acids and ammonia as nitrogen resources instead of nitrate and nitrite, which had either no effect or a negative effect on growth (Kawai and Abe, 1976). Corn steep liquor, dry yeast, casamino acid, and polypeptone, whose main nitrogen source is the amino group of amino acids, are often added to media for *T. matsutake* (Kawai and Abe, 1976). Some vitamins and metal ions promoted the growth of *T. matsutake* (Kawai and Terada, 1976). Vitamin B_1 could be essential for the growth, since hyphae hardly grew on agar without this vitamin. Thiamin and nicotinic acids promoted the growth of hyphae (Kawai and Terada, 1976). Possible sources of these vitamins are host trees or biosynthesis by *T. matsutake* itself. Iron and magnesium ions were effective metal ions for the growth. Tween 80, detergent, and olive oil had positive effects on elongation of hyphae (Guerin-Laguette *et al.*, 2003). Many substances promoting mycelium growth have been reported. The mycelium amount required for the formation of one fruiting body has been suggested to be approximately 100 grams (Ogawa, 1978; Suzuki, 2005). More information about the optimal growth conditions for *T. matsutake* should be accumulated for artificial cultivation.

Mycelia aggregate to form primordia, and their formation begins at ground temperatures under 19 °C. The aggregate was able to grow between 13 °C and 19 °C (Kinugawa, 1965). The growth stopped if the temperature rose above 20 °C after the formation started. A sudden drop in temperature suppressed the growth of the primordium. This information strongly suggests that the primordium is sensitive to changes in temperature. Timing of rainfall is important as well as annual precipitation. Production of matsutake decreased when there was little precipitation in the season for

fruiting-body formation (Kareki, 1972). Other climatic factors also seem to be important for the formation of *T. matsutake*.

2.4. Formation and Properties of Shiro

The fruiting bodies of *T. matsutake* are formed in a circle called a fairy ring, which is the same as other mycorrhizal fungi (Fig. 3a). The ring, whitish mycelium, and soil aggregated zone corresponds to an active mycorrhizal zone (Fig. 3b). The fairy ring and its interior are called “shiro” in Japanese (Ogawa, 1975). The active mycorrhizal zone corresponds to the shiro front (Fig. 4). The horizontal width of the shiro front is approximately 15 cm, its vertical depth ranges from 10 to 20 cm, and its front advances outwards at a rate of 10–15 cm per year.

The shiro front of *T. matsutake* has been known to have antimicrobial activity (Ohara, 1966). Bacteria and actinomycetes were not detected, and only *Mortierella* sp. (=*Umbelopsis* sp.) was detected in the shiro front, although they were abundant in the soil outside the shiro front and detected inside the shiro front of *T. matsutake* (Fig. 4) (Ohara and Hamada, 1967). Elimination of micro-organisms from the shiro front was also observed in the shiro front at another site (Yoshimura, 2003). This strongly suggests that the shiro front produced an antimicrobial substance, and the antimicrobial substance was involved in protecting *T. matsutake* from soil micro-organisms. Investigation of this antimicrobial substance is described later.

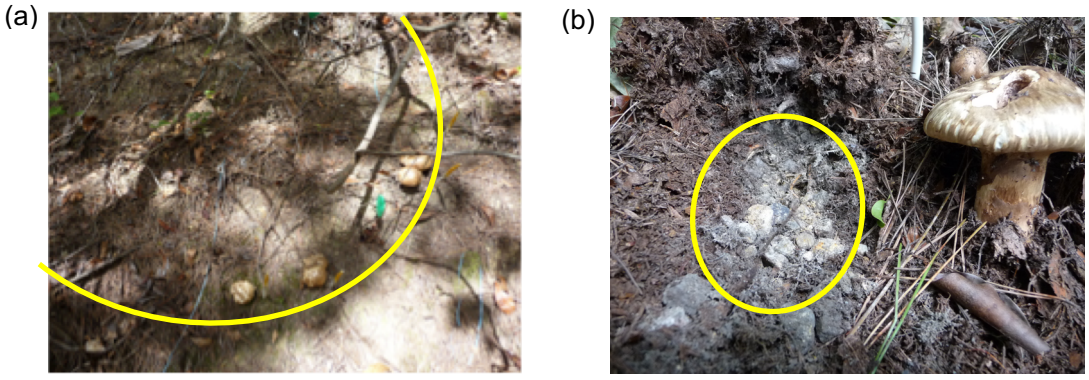

Fig. 3 Photographs of the fairy ring and active mycorrhizal zone of *T. matsutake* (Photo by N. Hirai). a: Fairy ring. The line indicates the ring. b: Active mycorrhizal zone. Inside the circle is a shiro front, an active mycorrhizal zone, which is an aggregate of whitish mycelium and soil.

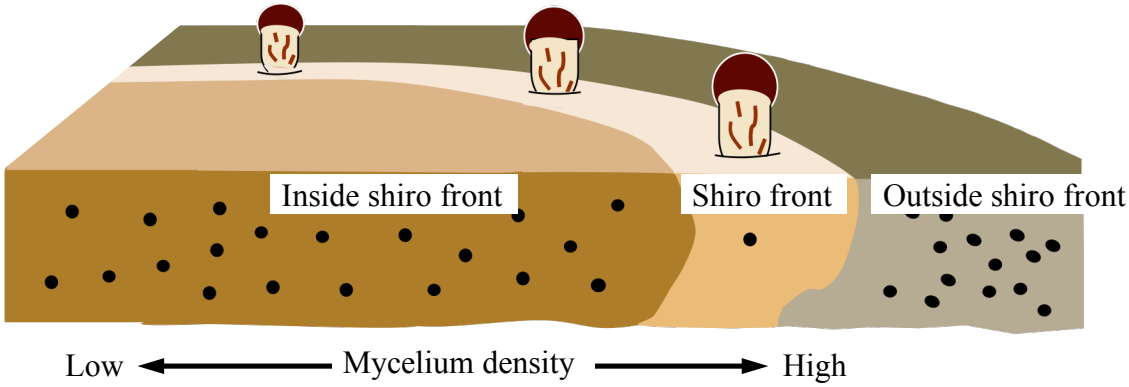

Fig. 4 Illustration of the structure of a shiro for *T. matsutake*.
●: Micro-organisms.

2.5. Odor Substances

The fruiting body of *T. matsutake* emits an odor that is a good smell for Japanese people, but not for western people. The major odor substances are methyl cinnamate, 1-octene-3-ol called matutake-ol, and (*E*)-2-octen-1-ol (Murahashi, 1936; Murahashi, 1937; Iwade, 1937). Methyl cinnamate is biosynthesized in lamella and spores in the pileus of a fruiting body, and its content increases as the body grows (Ohta, 1983; Cho *et al.*, 2006). These substances keep collembolans away from the fruiting body (Sawahata *et al.*, 2008), while methyl cinnamate attracts Euglossine bees, a pollinator of some plants (Eltz and Lunau, 2005; Hentrich *et al.*, 2010). The physiological roles of these substances in *T. matsutake* have not been confirmed.

Cinnamic acid is biosynthesized from L-phenylalanine by phenylalanine ammonia lyase (PAL). PAL is found in vascular plants and fungi including *T. matsutake* (Dixon and Reddy, 2003; Hyun *et al.*, 2011). This enzyme is involved in the growth, development, and defense system in plants (Mauch-Mani and Slusarenko, 1996). The transcription level of PAL gene was high in the primordium, stipe, and gills of *T. matsutake* (Yoon *et al.*, 2013; Tasaki and Miyakawa, 2015), suggesting that formation of cinnamic acid was active in those organs.

Two biosynthetic pathways from cinnamic acid to methyl cinnamate in *T. matsutake* have been proposed (Hattori *et al.*, 2016). One is the direct conversion of cinnamic acid to methyl cinnamate by cinnamate carboxyl methyltransferase, and the other is an indirect conversion via cinnamoyl CoA. The indirect pathway is supposed

to be successively catalyzed by a cinnamate:CoA ligase and an acyltransferase. The activity of cinnamate carboxyl methyltransferase has not been detected in *T. matsutake* although it was detected in the brown rotting fungus, *Lentinus lepideus*, Basidiomycota (Ohta *et al.*, 1991), and the activities of the enzymes in the indirect pathway and the gene coding enzymes have also not been detected in Basidiomycota. Methyl cinnamate in *T. matsutake* might be biosynthesized through an alternative pathway.

1-Octene-3-ol is formed through cleavage of linoleic acid with hydroperoxide lyase in some mushrooms (Wurzenberger and Grosch, 1984; Assaf *et al.*, 1997). The biosynthetic pathway to 1-octene-3-ol for *T. matsutake* may be similar to that in these mushrooms. (*E*)-2-Octen-1-ol is biosynthesized from arachidonic acid in a moss (Wichard *et al.*, 2005). *Tricholoma matsutake* seems to form (*E*)-2-octen-1-ol through a similar pathway, although this pathway has not been found in mushrooms.

Matsutake imported from foreign countries hardly emit the characteristic odor in the Japanese market. The loss of odor is caused by washing before import and the long transportation time. If a technique for keeping the odor after harvesting is developed, matsutake will become more popular in Japan.

2.6. Saprotrophic Ability

Mycorrhizal fungi are supplied with photosynthetic products from host plants. However, a supply of only vegetative mass from host plants is probably insufficient for their growth, and the fungi may obtain nutrients saprophytically in specific conditions.

Tricholoma matsutake showed an ability to decompose tree bark (Vaario *et al.*, 2002) and a high activity of β-glucosidase, which catalyzes the hydrolysis of the β-1,4-glycosidic linkage in D-cellobiose and cellotriose (Kusuda *et al.*, 2008). The transcriptional level of the gene encoding glucoamylase of the glycoside hydrolase family 15, and the extracellular glucoamylase activity in mycelia of *T. matsutake* increased in a culture medium with amylose (Wan *et al.*, 2012). The mycelial biomass increased on the media containing hemicellulose, and xylosidase, which is a hemicellulolytic enzyme, exhibited a significantly high activity on an agar plate with bark (Vaario *et al.*, 2012). *Tricholoma matsutake* might be able to acquire nutrients saprotrophically in addition to symbiotically.

Strains of *L. shimeji*, a mycorrhizal fungus, have high saprotrophic ability, resulting in the successful artificial cultivation for shimeji on mushroom beds (Ohta, 1994; Watanabe, 1994; Yoshida, 1994). Finding a high saprotrophic strain of *T. matsutake* might be the key to its artificial cultivation.

2.7. Bioactive Substances

Bioactive substances from the fruiting body of *T. matsutake* are known. The peptide having the sequence WALKGYK had antihypertensive effect (Geng *et al.*, 2016). Polysaccharides extracted from the fruiting body with ethanol showed functional activation of macrophages, and antioxidative activity in scavenging tests employing the 1,1-diphenyl-2-picrylhydrazyl radical (Young *et al.*, 2008; Byeon *et al.*, 2009; Tong *et al.*, 2013). α-D-Glucan-protein, whose ratio of sugar to protein is 94.3:5.7, had an immunomodulatory effect (Hoshi *et al.*, 2005; Hoshi *et al.*, 2007).

Pyranose oxidase that catalyzed the oxidation at C-2 of sugar and generated hydrogen peroxide was found in the fruiting body of *T. matsutake*, and the hydrogen peroxide showed antifungal activity against phytopathological fungus, *Rhizoctonia solani* (Takakura and Kuwata, 2003; Takakura, 2015). Low molecular-weight compounds exhibiting biological activities have not been found in the fruiting body of *T. matsutake*.

3. Objective

The shiro front of *T. matsutake* has antimicrobial activity as mentioned in section 2.4. Ohara (1966) investigated the shiro in Iwakura, Kyoto, and demonstrated that the active mycorrhizas and soil crusts from the shiro front inhibited the growth of Gram-negative bacteria from outside of the shiro, *Arthrobacter* spp., *Agrobacterium* spp., *Psudomonas* spp., *Bacillus subtilis*, and *Serratia marcescens* (Fig. 5a). This result clearly showed that an antimicrobial substance was present in the active mycorrhizas. The antimicrobial activity was not found in the decayed zone of the shiro, newly developed roots with no mycorrhizas, the roots of red pine seedlings that were two years old, a culture filtrate of *Mortierella* sp., and mycelia of *T. matsutake*

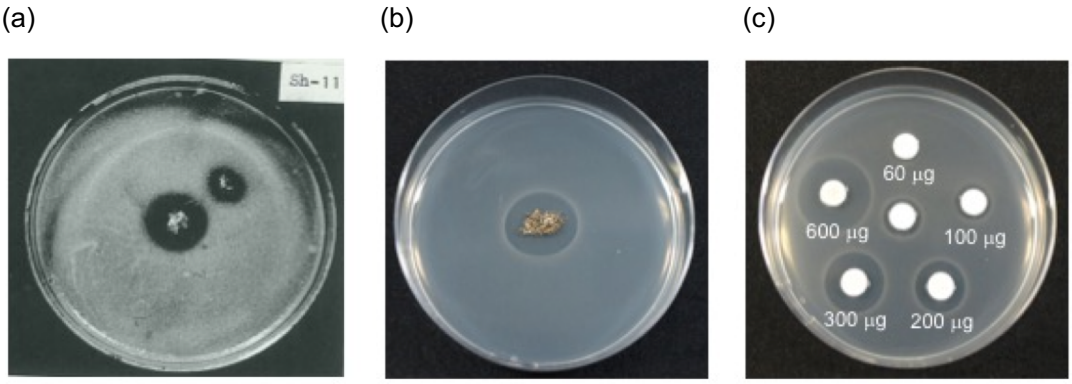

Fig. 5 Antimicrobial activity of the soil containing mycorrhiza from shiro front (a, b) and paper discs containing the extract from the shiro front soil (c). Test bacteria in (a) were bacteria from outside of the shiro, and in (b) and (c) was *B. subtilis*. Photo (a) by H. Ohara (1968), and (b) and (c) by M. Shiro (2013). The center disc contained totrol as a standard antimicrobial compound in (c).

(Ohara, 1966). This suggested that the antimicrobial activity was closely associated with the active mycorrhizas. The antimicrobial substance may protect the mycorrhizas from soil micro-organisms and contribute to the preservation and development of the shiro. The antimicrobial substance seemed to be unstable or volatile, since the antimicrobial activity was believed to disappear from the active mycorrhiza and the shiro soil after incubation for 48 h at 30 °C (Ohara, 1968).

Identification of the antimicrobial substance from the shiro front was attempted using *B. subtilis* as a test bacterium (Tsuruta and Kawai, 1979). Volatile monoterpenes, α- and β-pinene, were proposed to be the antimicrobial compounds by analysis of the extract from the shiro front and the antibacterial activity of pure monoterpenes without isolation of the antimicrobial substance. Oizumi (2007) traced the antimicrobial activity of monoterpenes, α- and β-pinene, α-pinene oxide, (+)- and (-)-limonene, (+)-limonene oxide, and α-terpineol against *B. subtilis* using a paper-disc method at a dose of 1 mg. However, antimicrobial activity of the monoterpenes except for (-)-limonene was not observed. (-)-Limonene was not detected in the extract from the shiro (Tsuruta and Kawai, 1979), and its low antimicrobial activity could not account for the antimicrobial activity of the shiro front. These results strongly suggest that the active substance was not a monoterpene.

The antimicrobial substance was reexamined using the shiros of *T. matsutake* at Sakai Research Forest, Kyoto (Oizumi, 2010; Shiro, 2013). The soils containing the mycorrhiza of *T. matsutake* showed clear antimicrobial activity against *B. subtilis*, as

did those from the shiro of Iwakura (Fig. 5b). The substance was hydrophilic, heat-stable, and did not show UV absorption. However, chemical identification of the substance was not accomplished due to difficulty isolating it.

The major objective of this study is to identify the antimicrobial substance from the shiro front that has remained unidentified for 50 years. The chemical properties of the antimicrobial substance, and its distribution in the shiro along with the mycelia density, micro-organisms are also investigated. Finally, the mechanism of preservation and development of the shiro by the antimicrobial substance is proposed.

Chapter II

Identification of the Antimicrobial Substance from the Shiro of *Tricholoma matsutake*

1. Introduction

The antimicrobial substance from the shiro front soil of *T. matsutake* has been shown to be hydrophilic, acidic, and heat-stable (Shiro, 2013). The antimicrobial activity of an extract from soil in a 20% MeOH aqueous solution was dose-dependent between 60 and 600 μg (Fig. 5c), and was not degraded by heating at 100˚C for 60 min. Analysis of the extract by HPLC using an ODS column did not yield a significant peak at a detection wavelength at 254 nm, although a small peak that eluted in the void volume was detected at 220 nm. The antimicrobial substance was not retained by an ODS column, but was retained by an anion-exchange resin and then eluted with 100 mM HCl. This information on the properties of the antimicrobial substance is very helpful for its isolation and identification. The shiro front of *T. matsutake* at Sakai Research Forest was used to isolate the antimicrobial substance.

2. Materials and Methods

Materials

The shiros at the Sakai Research Forest that have been conserved by Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center were used. The soils from the front of shiro No. 11, where fruiting bodies were 178 formed from 2009 to 2014, were collected in October 2013 for the isolation of the antimicrobial substance. The soil from shiro No. 32, where fruiting bodies were formed 15 from 2009 to 2014, was collected in October 2014, and used for analysis of the content of the (oxalato)aluminate complex. *Bacillus subtilis* FKU101 was provided from Emeritus Professor K. Yamamoto, Kyoto University, and the other micro-organisms were

purchased from RIKEN BioResource Center (Tsukuba, Japan). Rice (*Oryza sativa* L. cv. Nipponbare) seeds were provided from the Experimental Farm, Kyoto University, and lettuce (*Lactuca sativa* L. cv. Melbourne MT) seeds were purchased from a local supplier.

Chemicals

All chemicals were reagent grade. (Oxalato)aluminate hydrate as standard oxalato(aluminate) complex was purchased from Alfa Aesar (UK). Aluminum phosphate was purchased from Kanto Chemical (Japan). Yeast extract, tryptone, and potato dextrose agar (PDA) were purchased from Becton, Dickinson and Company (USA). Others were purchased from Wako Pure Chemical (Japan).

Antimicrobial assay

The antimicrobial activity against *B. subtilis* FKU101 was assayed at 30˚C for 24 h by the paper-disc method (Beer and Sherwood, 1945). All media were autoclaved for 15 min at 120°C before use. *Bacillus subtilis* was pre-incubated in a liquid medium consisted of 0.1% bonito extract, 0.1% yeast extract, and 0.05% NaCl overnight at 30°C before the tests.

The pre-incubated media and an autoclaved new liquid media with 1.5% agar were mixed at 1:19 to prepare agar media. Tested material was added into paper disc (diameter of 8 mm). The disc was placed on the agar media. Diameter of inhibitory zone was measured for evaluation of antimicrobial activity after incubation.

The agar media containing 5.5 mM aluminum phosphate were used for the antimicrobial assay of organic acids, which was added into a paper disc at 1 μmol. The agar media without aluminum phosphate were used as control. This assay was conducted in triplicate.

HPLC Analysis of the Material Eluted with 100 mM HCl from the DEAE Column

The material dissolved in water was analyzed by HPLC: YMC Pack ODS-AQ (5 μm, 100×6.0 mm, YMC, Japan); acetonitrile: 20 mM ammonium acetate=5:95; 1.0 mL/min; 220 nm. The concentration of oxalic acid was calculated with a calibration curve between the weight and peak area of standard oxalic acid.

^{1}H, ^{13}C, and ^{27}Al-NMR measurement

AVANCE III 500 (500 MHz for ^{1}H, Bruker BioSpin, Kanagawa, Japan) was used for the ^{1}H and ^{13}C NMR measurement, and AVANCE III 400 (104 MHz for ^{27}Al) was used for the ^{27}Al NMR measurement. $AlCl_3(H_2O)_6$ was used as an external standard in the ^{27}Al-NMR spectra. The ratio of individual speciation of the (oxalato)aluminate complex was calculated with the signal area for ^{13}C, and ^{27}Al-NMR measurement. Deuterium water was used for ^{1}H and ^{13}C NMR measurements, and distilled water for ^{27}Al NMR as solvents.

Isolation of the antimicrobial substance

The soil (35 g) from the front of shiro No. 11 was extracted with 100 mL of 20% MeOH aqueous solution. The extract was filtered through a filter paper. The filtrate was concentrated to give a viscous liquid (353 mg), and the liquid was dissolved in a small amount of water. The solution was applied to an activated charcoal column (20×145 mm), and eluted with 100 mL of water and 10, 30, 60, and 100% MeOH. The material eluted with water was concentrated, and purified with a 32×380-mm column of TOYOPEARL DEAE-650M (Tosoh, Japan) eluting with 600 mL of 0, 10, 30, 100, and 300 mM HCl aqueous solutions. The material (34 mg) eluted with 100 mM HCl was dried in vacuo at 50°C for 3 h. The material was applied to an Oasis WAX column (150 mg/6 mL) that was preliminary washed with 10% NH_4OH in MeOH (10 mL) and water (10 mL). The material dissolved in 10 mL water was applied on the column, and eluted with aqueous solution of 2% formic acid (10 mL), MeOH (15 mL), and 5 and 10% NH_4OH in MeOH (15 mL). The material (21 mg) eluted with the 5% NH_4OH in MeOH was concentrated, and dissolved in water. Slow evaporation of water yielded colorless needles, m.p. >300°C. This was subjected to X-ray structural analysis.

X-ray structural analysis

Intensity data from the crystalline substance were obtained using a Rigaku Saturn 70 CCD diffractometer with a VariMax Mo Optic using Mo Kα radiation (λ = 0.71075Å) (RIGAKU Corporation, Tokyo, Japan). Structures were solved by a direct method (SIR2004) (Burla, *et al.*, 2005) and refined by the full-matrix least squares

method on F^2 for all reflections (SHELXL-97) (Sheldrick, 2008). All hydrogen atoms of H_2O and H_3O^+ were placed at appropriate positions using DFIX instructions, while all other atoms were refined anisotropically. The crystalline $[Al(C_2O_4)_3^{3-} \bullet (H_3O^+)_3(H_2O)_3]$: M = 402.16, T = -170 ºC, triclinic, P-1 (no.2), a = 7.8364(2) Å, b = 10.5104(5) Å, c = 10.5729(5) Å, α = 84.087(2)º, β = 70.4649(15)º, γ = 70.058(3)º, V = 771.43(6) Å^3, Z = 2, D_{calc} = 1.731 g cm^{-3}, μ= 0.231 mm^{-1}, $2\theta_{max}$ = 51.0º, measured/independent reflections = 11752/2837 (R_{int} = 0.0512), 271 refined parameters, GOF = 1.084, R_1 = 0.0795 [$I>2\sigma(I)$], wR_2 = 0.2147 [for all data], largest diff. Peak and hole 1.003 and -0.909 e.Å^{-3} is contained in the supplementary crystallographic information for this paper. The data (CCDC-1432762) can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Analysis of the content of the (oxalato)aluminate complex

The soil (4 g) from the front of shiro No. 32 was extracted with 20 mL of water. After shaking for 30 min and filtration with filter paper, the filtrate was lyophilized and dissolved in 0.6 mL of water. This solution was used for ^{27}Al-NMR measurement to analyze the content of the (oxalato)aluminate complex, and for antimicrobial assay with paper-disc method without concentration. The concentrations of the (oxalato)-aluminate complex were calculated with a calibration curve between concentrations of 0.5 and 10 μM and the ^{27}Al signal area of tris(oxalato)aluminate.

Effect of the coordination number on antimicrobial activity of the (oxalato)aluminate complex

$AlCl_3(H_2O)_6$ and disodium oxalate were mixed at molar ratios of 1:1 for (oxalato)aluminate, 1:2 for bis(oxalato)aluminate, and 1:3 for tris(oxalato)aluminate in water (Ma, *et al.*, 1998). Each solution was diluted with the liquid medium controlled at pH 5 to make test solutions whose concentration of Al was between 0 and 320 μM. The broth microdilution method was used to measure antimicrobial activity of the mixtures against *B. subtilis* (Cockerill, *et al.*, 2012). The pre-incubated liquid medium was diluted with saline to 0.5 McFarland standard. The diluted medium was diluted to 1:10 with saline to prepare an inoculum medium. The inoculum medium and test

solutions were put into 96-well microplate in the ratio of 1:19. A multi-length microplate reader (Multiskan JX, Thermo Fisher Scientific, USA) was used to measure the optical density of samples at 630 nm after incubation at 30°C for 24 h. This test was conducted in triplicate.

Effect of pH on the activity of antimicrobial (oxalato)aluminate complex

The (oxalato)aluminate complex was added to the liquid media at pH 5, 6, and 7 to yield a final concentration of 300 ppm. The pH of media changed from 5 to 4.3, 6 to 4.9, and 7 to 5.5, respectively. These media were used for ^{27}Al-NMR measurement, and added 1.5% agar to prepare agar media for antimicrobial assay against *B. subtilis* after the measurement. The agar plates containing 0, 30, 100, or 300 ppm of the (oxalato)aluminate complex were also prepared. The 2 μL inoculum medium of *B. subtilis* were inoculated directly on the agar plates (Cockerill, *et al.*, 2012). The inoculum medium was prepared with the same manner in "*Effect of the coordination number on antimicrobial activity of the (oxalato)aluminate complex*".

Bacteriocidal activity of the (oxalato)aluminate complex

Bacillus subtilis was pre-incubated in a liquid medium for 3 h at 30°C. The incubated medium was divided equally. The (oxalato)aluminate complex was added to one of them to yield a concentration of 300 ppm. The pH of the other medium was adjusted to pH 6, which was the same as that of the 300 ppm broth. The two media were incubated for 2.5 h at 30°C. The bacteria were washed with a new medium three times, and suspended in a new medium so that the optical density at 630 nm of the medium was 0.040. The suspensions were diluted 250 times by a new medium. The number of colonies was counted after incubation of 5 μL of the final suspensions on the agar plate for 18 h at 30°C.

Effect of the (oxalato)aluminate complex on the growth of T. matsutake

Blocks (5 mm×5 mm), which cut from another colony of *T. matsutake* incubated for six months, were placed on 1/2 Hamada media, which consisted of 1% glucose, 0.1% yeast extract, 0.05% KH_2PO_4, and 1.5% agar, at pH 5 containing 30, 100, or 300 ppm of the (oxalato)aluminate complex (Ogawa, 1978). After incubation at 23°C for

one month, the areas of colonies were measured by Image J (NIH, USA). This test was conducted in triplicate.

Seedling elongation test for rice

Seeds of rice were soaked in EtOH for 5 min, and washed three times with tap water (Todoroki, *et al.*, 1994). The seeds were sterilized with 1% sodium hypochlorite for 1 h, and washed with running tap water for 3 h. The sterilized seeds were allowed to germinate in water for 2 days at 30°C. Seven of the seedlings were placed in a glass tube containing 2 mL of a test solution, and grown in the tube capped with vinyl film for 1 week at 30°C under continuous fluorescent light of 6,000 lx. The length of the second leaf sheath was measured.

Lettuce germination assay

Fifty seeds of lettuce were placed on two filter papers (diameter of 55 mm) soaked in 3 mL of a test solution and allowed to germinate at 25°C under consecutive 2,500 lx for 48 h (Todoroki, *et al.* 1994). The germination ratio was defined as (number of germination seeds/50)×100. This assay was conducted in triplicate.

Antimicrobial activity of the (oxalato)aluminate complex

The (oxalato)aluminate complex was added to the liquid media at pH 5 to yield a final concentration of 0, 30, 100, or 300 ppm, and added 1.5% agar to prepare agar media for antimicrobial assay. The 2 μL inoculum media of bacteria were inoculated directly on the agar plates. The inoculum medium was prepared with the same manner in "*Effect of the coordination number on antimicrobial activity of the (oxalato)-aluminate complex*".

Spores obtained from fungi incubated for 6 days on plates of PDA were suspended in water. The suspensions were filtered through two layers of KimWipes S-200 (Nippon paper crecia, Japan), and centrifuged at 1,100×g for 1 min to collect spores. The spores were washed by saline and centrifuged. This washing was done three times. Spore concentration was adjusted to 0.5 McFarland standard with saline. The suspensions were diluted to 1:1000 with saline for inoculum suspensions.

The suspension of *S. cerevisiae* obtained from one-day-old PDA plate, whose

concentration is 0.5 McFarland, was diluted to 1:1000 with saline for inoculum suspension. The inoculum suspensions of fungi and yeast were added 10 μL into paper discs. The discs were placed on the agar media, which were the same as the test for bacteria, for the assay (Zhang, *et al.*, 2010, Dota, *et al*., 2011, Cockerill, *et al.*, 2012).

3. Results

3.1. Isolation and identification of the antimicrobial substance

The extract with the 20% MeOH aqueous solution was applied to activated charcoal column. The antibacterial material eluted with water was purified with anion exchange resin of a DEAE type, and the antibacterial activity was detected in the material eluted with a 100 mM HCl aqueous solution. The material contained oxalic acid at 46% by weight. Oxalic acid has antimicrobial activity (Yamamoto, *et al.*, 1984), but the antimicrobial activity of the material was higher than that of oxalic acid contained in the material, suggesting that the material contained a substance promoting the activity of oxalic acid, or another antibacterial substance. The material was further purified with another type of anion exchange resin, Oasis WAX, to yield an antibacterial fraction by elution with 5% NH_4OH in MeOH, although the minimum inhibitory dose of the active material increased to 90 μg from 38 μg of the active material from the DEAE column. The ^{1}H NMR spectrum of the antibacterial material from the Oasis WAX column showed no signals, but its ^{13}C NMR spectrum showed signals at 165.71 and 165.80 ppm assignable to a carboxyl group of oxalic acid at an intensity ratio of 34:66, suggesting that the material had two forms of oxalic acid. Crystallization of the material from the aqueous solution produced colorless needles that were identified as ammonium tris(oxalato)aluminate(III) trihydrate(III) by X-ray analysis (Fig. 6) (Bulc, *et al.*,1984). Ammonia was derived from the eluent, so the major substance from the shiro front would be tris(oxalato)aluminate(III) trihydrate(III). The ^{27}Al NMR spectrum of the antibacterial material from the Oasis WAX column showed a minor signal at 12.4 ppm and a major signal at 16.1 ppm at an intensity ratio of 29:71 assignable to ^{27}Al of bis(oxalato)aluminate and tris(oxalato)aluminate,

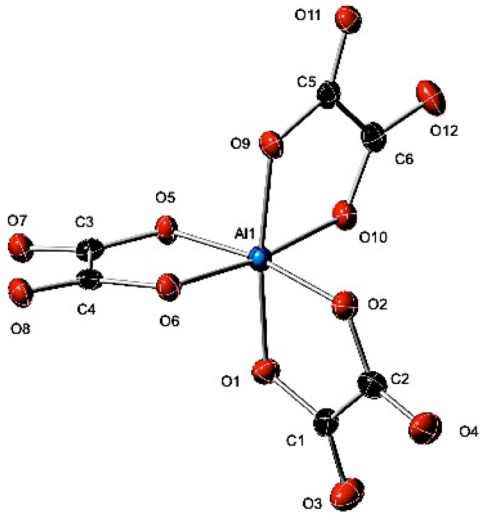

Fig. 6 Molecular form of the crystalline structure with atomic displacement parameters set at 50% probability. Only the $Al(C_2O_4)_3^{3-}$ moiety is shown here.

respectively (Kerven, *et al.*, 1995), assigning the ^{13}C signal at 165.71 and 165.80 ppm as ^{13}C of bis(oxalato)aluminate and tris(oxalato)aluminate, respectively. This indicated that only tris(oxalato)aluminate from the mixture of bis(oxalato)aluminate and tris(oxalato)aluminate crystallized. A standard (oxalato)aluminate complex that is a mixture of (oxalato)aluminate, bis(oxalato)aluminate, and tris(oxalato)aluminate at a ratio of 23:63:14 showed antimicrobial activity with the inhibitory zone diameter 20 mm at 320 μg. The extract from the No. 32 shiro front contained 388 μg of the (oxalato)aluminate complex with the three speciations ratio 22:71:7, as shown in Fig. 7,

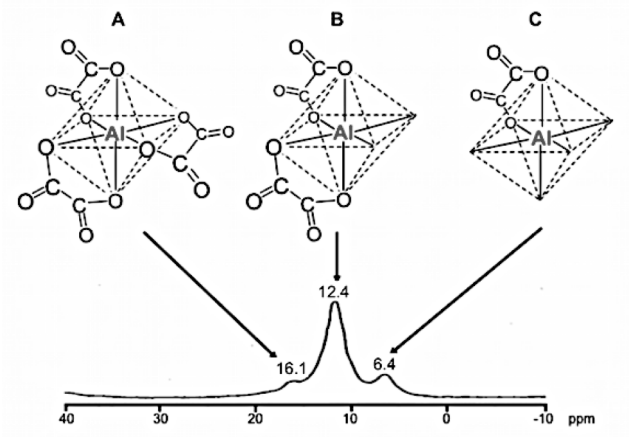

Fig. 7 The steric structure and ^{27}Al NMR spectrum of the extract from the shiro front of shiro No. 32. A: Tris(oxalato)aluminate. B: Bis(oxalato)aluminate. C: (Oxalato)aluminate.

similar to that of the standard (oxalato)aluminate complex. This extract showed the inhibitory zone diameter 21 mm corresponding to that of the standard (oxalato)-aluminate complex. Thus, the antimicrobial substance of the shiro front was identified as the (oxalato)aluminate complex.

3.2. The chemical and antimicrobial properties of the (oxalato)aluminate complex

The antimicrobial activity of the (oxalato)aluminate complex was higher than that of oxalic acid, as described above. The minimum inhibitory dose of the (oxalato)-aluminate complex was 0.1 μmol containing 0.2 μmol of oxalic acid, while that of oxalic acid which had antimicrobial activity was 1 μmol (Table 1). Metal aluminum did not show antimicrobial activity.

Bacillus subtilis did not grow after washing the bacteria incubated in a medium containing the (oxalato)aluminate complex (data not shown). This result meant that the antimicrobial activity of the (oxalato)aluminate complex was bacteriocidal, and not bacteriostatic.

Aluminum adopts a regular octahedral complex where one to three molecules of oxalic acid can coordinate (Kerven, *et al.*, 1995). The number of oxalic acid molecules coordinating to aluminum depends on the molar ratio of aluminum to oxalic acid (Ma, *et al.*, 1998). The antimicrobial activity could be different among one, two, and three molecule coordination. This possibility was examined using culture media containing mixtures of aluminum chloride and disodium oxalate with molar ratios of 1:1, 1:2, and 1:3, respectively. The ratio of (oxalato)aluminate, bis(oxalato)aluminate, and

Table 1 The antimicrobial activity of oxalic acid and the (oxalato)aluminate complex.

Compound	μmol	DIZ (mm)
Oxalic acid	0.1	ND
	0.3	ND
	1.0	14
(Oxalato)aluminate complex	0.1	10
	0.3	16
	1.0	24

DIZ: Diameter of inhibitory zone. ND: Not detected.

tris(oxalato)aluminate in the mixture with molar ratio of 1:1 was 80:20:0, that of 1:2 was 14:57:29, and that of 1:3 was 0:0:100. The activity of the molar ratio of 1:1 was the highest, and that of 1:2 was medium, while that of 1:3 was almost inactive (Fig. 8). This result showed that the order of the antimicrobial activity was tris(oxalato)aluminate, bis(oxalato)aluminate, and (oxalato)aluminate from lowest to highest.

The dissociated type of oxalic acid coordinates to aluminum, and so the number of oxalic acid molecules coordinating to aluminum increases as the pH increases (Lee, *et al.*, 2005). The antimicrobial test at three pHs showed that the order of the antimicrobial activity of the (oxalato)aluminate complex was pH 4.3, 4.9, and 5.5. The ^{27}Al NMR analysis of the culture media containing the standard (oxalato)aluminate complex indicated that the signal ratios of (oxalato)aluminate: bis(oxalato)aluminate: tris(oxalato)aluminate were 0:90:10 at pH 4.3, 0:85:15 at pH 4.9, and 0:68:32 at pH 5.5. These results confirmed that the antimicrobial activity of the (oxalato)aluminate complex decreased as the pH increased since inactive tris(oxalato)aluminate increased. This explains the reason why the antimicrobial activity of the active fraction decreased after purification with the Oasis WAX column. The pH of the active material from the DEAE column was *ca.* 1.0, and the ^{27}Al NMR analysis showed that the content of the active bis(oxalato)aluminate complex was 69%, while the pH of the active fraction from the Oasis WAX column was *ca.* 6.0, and the content of the inactive tris(oxalato)-aluminate complex was 71%.

Aluminum of the (oxalato)aluminate complex would be derived from insoluble aluminum phosphate or aluminum hydroxide in soil. Oxalic acid secreted from the

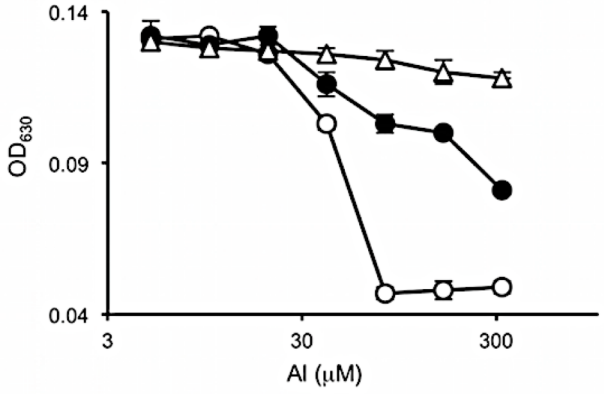

Fig. 8 The antimicrobial activities of the mixtures of aluminum chloride and disodium oxalate with molar ratios of 1:1 (○), 1:2 (●), and 1:3 (△).

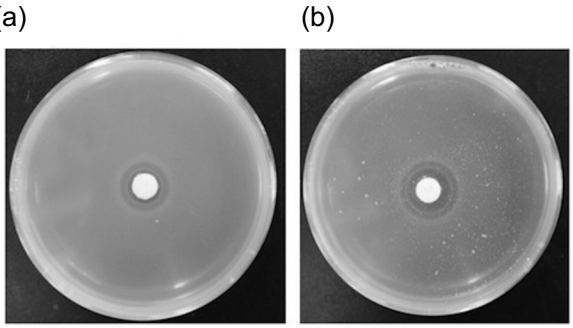

Fig. 9 The antimicrobial activity of oxalic acid against *B. subtilis* in the absence (a) and presence (b) of aluminum phosphate (5.5 mM). The discs contained 1 μmol of oxalic acid.

mycorrhizas probably reacts with aluminum phosphate or aluminum hydroxide to form a water-soluble (oxalato)aluminate complex. As a model of this process, antimicrobial activity of oxalic acid was tested using bacterial agar plates containing dispersed aluminum phosphate. Oxalic acid had a higher activity on agar plates containing aluminum phosphate than that on agar plates containing no aluminum phosphate (Fig. 9), while aluminum phosphate itself did not have any activity.

Aluminum may enhance the antimicrobial activity of other short chain organic acids. This possibility was examined with the same method as that for oxalic acid. Antimicrobial activity of lactic, malic, and succinic acid on bacterial agar plates was

Table 2 The antimicrobial activity of organic acids (1 μmol) against *B. subtilis* in the presence or absence of aluminum phosphate (5.5 mM).

Organic acid	$AlPO_4$ in media	DIZ (mm)
Lactic acid	−	20
	+	29
Malic acid	−	9
	+	11
Succinic acid	−	9
	+	11
Malonic acid	−	ND
	+	ND
Citric acid	−	11
	+	11

DIZ: Diameter of inhibitory zone. ND: Not detected

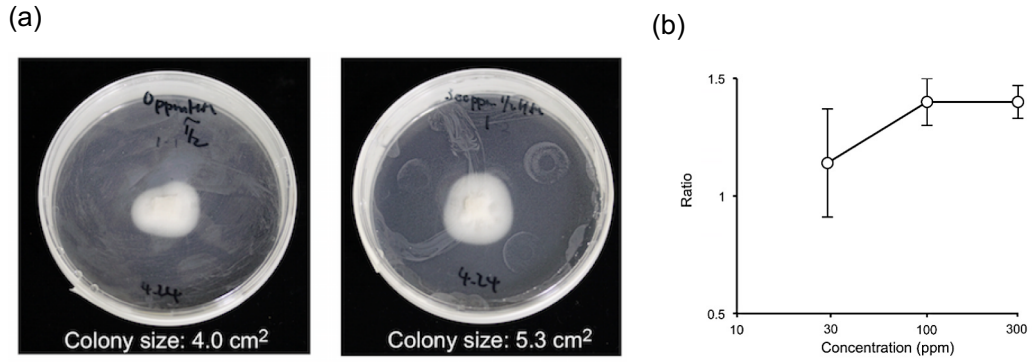

Fig. 10 The effect of the (oxalato)aluminate complex on the mycelia growth of *T. matsutake*. a: Photo of the colonies of *T. matsutake*. Left: Control medium. Right: Medium containing the complex at 300 ppm. b: Ratio means magnification of the colony size on the medium containing the oxalato(aluminate) complex on comparison with the control.

enhanced by impregnation of the media with aluminum phosphate, while that of malonic, and citric acid was not enhanced by it (Table 2).

The occurrence of the (oxalato)aluminate complex in the shiro front suggested that *T. matsutake* was resistant to the (oxalato)aluminate complex. In fact, the effect of the (oxalato)aluminate complex at 30–300 ppm on the mycelium growth of *T. matsutake* was promotion rather than inhibition (Fig. 10). The colony size of *T. matsutake*

Table 3 The antimicrobial activity of the (oxalato)aluminate complex.

Microorganism	MIC (ppm) or inhibitory rate (%) at 300 ppm
Bacillus subtilis FKU101	30 ppm
B. subtilis JCM1465	300 ppm
Arthrobacter chlorophenolicus	300 ppm
Pseudomonas aeruginosa	300 ppm
Rhizobium radiobacter	300 ppm
Escherichia coli JCM18426	300 ppm
E. coli JCM20135	300 ppm
Saccharomyces cerevisiae	30 ppm
Penicillium citrinum	81%
Aspergillus niger	59%
Trichoderma reesei	>300 ppm
Rhizopus oryzae	>300 ppm

MIC: Minimum inhibitory concentration

increased to 1.4 times on 1/2 Hamada medium containing 100 ppm of the (oxalato)-aluminate complex on comparison with the control. The antimicrobial activity of the (oxalato)aluminate complex against other micro-organisms is summarized in Table 3. The antibacterial activity was not so high, but growth not only of Gram-positive but also of Gram-negative bacteria was inhibited at 300 ppm and pH 5. *Bacillus subtilis* FKU101 used for the antimicrobial test in this experiment, and yeast were more sensitive to the (oxalato)aluminate complex than those bacteria. The growth of *Penicillium citrinum* and *Aspergillus niger* was partially inhibited at 300 ppm, and *Trichoderma reesei* and *Rhizopus oryzae* were resistant to the (oxalato)aluminate complex. The (oxalato)aluminate complex did not inhibit the growth of rice seedlings, or the germination of lettuce seeds up to 1,000 ppm, indicating that it is not toxic to plants (data not shown).

4. Discussion

The antimicrobial substance of the shiro front that has remained unidentified 50 years was identified as the (oxalato)aluminate complex. The (oxalato)aluminate complex is known as a byproduct of the reaction of oxalic acid and aluminum phosphate to release soluble phosphorus as an important mineral nutrient (Graustein, *et al.*, 1977), or as a byproduct formed through the detoxification of aluminum derived from aluminum phosphate and/or aluminum hydroxide in the acidic soil (Zheng, *et al.*, 1998). However, the antimicrobial activity of the (oxalato)aluminate complex has never been reported. This is the first report on its antimicrobial activity.

Aluminum appeared to enhance the antimicrobial activity of oxalic acid, since metal aluminum did not show antimicrobial activity (Table 1). However, the inactivity of tris(oxalato)aluminate suggested that vacant coordination position of the (oxalato)-aluminate complex was important for the antimicrobial activity, although the coordination of oxalic acid to aluminum is essential. The bacteriocidal activity of the (oxalato)aluminate complex suggests that the vacant coordination position of (oxalato)-aluminate might bind with cell wall components like peptidoglycan of bacteria to lose its function.

Oxalic acid secreted from the mycorrhizas may react with aluminum phosphate in soils to form the antimicrobial (oxalato)aluminate complex. The result of the model experiment (Fig. 9) supported the above process.

Antimicrobial activity of lactic, malic, and succinic acid on bacterial agar plates was enhanced by impregnation of the media with aluminum phosphate, while that of malonic, and citric acid was not enhanced by it (Table 2). This result showed that aluminum may enhance the antimicrobial activity of other short chain organic acids, and that not only a carboxyl group but also the chemical structure would be important for the antimicrobial activity. This may be coincident with the antimicrobial activity of the aluminum complex itself.

The effect of the (oxalato)aluminate complex on the mycelium growth of *T. matsutake* (Fig. 10) suggested that the (oxalato)aluminate complex promoted the growth of *T. matsutake* under natural conditions. Antimicrobial activity of the complex may have a wider range against bacteria and yeast than against fungi (Table 3), and the complex was not toxic to plants.

Chapter III

Distribution of the (Oxalato)aluminate Complex, Mycelium Density of *Tricholoma matsutake*, Oxalic Acid, pH, Antimicrobial Activity, Micro-organisms, and Soluble Phosphorus in the Shiro

1. Introduction

The antimicrobial substance isolated from the shiro front soil of *T. matsutake* was identified as an (oxalato)aluminate complex as described in Chapter II. If the (oxalato)aluminate complex is involved in protecting the shiro from soil micro-organisms, its distribution should correlate with the mycelium density of *T. matsutake* and the antimicrobial activity of the shiro, whereas the distribution of bacteria and fungi should show a negative correlation with that of the (oxalato)aluminate complex. The pH value of the shiro front should be lower than those outside and inside the shiro front, since the shiro front would contain oxalic acid secreted from the active mycorrhizas. The contents of oxalic acid and soluble phosphorus in the shiro front should be higher than inside and outside the shiro front. To examine these expectations, two shiros, No. 7 and No. 32, at the Sakai Research Forest were chosen, and analyzed.

2. Materials and Methods

Materials

The shiros at the Sakai Research Forest were used. The soils from No. 32 and 7, where fruiting bodies were formed 15 and 48 from 2009 to 2014, respectively. Soil samples from 30 cm inside the shiro front, the shiro front, and 30 cm outside the shiro front at a depth of 15 cm were collected, and named as SI, SF, and SO, respectively. The soils from shiro No. 32 were taken in October, the season of fruiting body formation. Soil samples, SI, SF and SO, were also taken from shiro No. 7 in November as well as shiro No. 32. The soil samples were taken from one spot in each

zone, and analyzed once, since soil is highly heterogeneous and it is difficult to obtain statically significant average values from plural spots even in the same zone. *Bacillus subtilis* FKU101 was provided from Emeritus Professor K. Yamamoto, Kyoto University.

Chemicals

Nutrient agar was purchased from Becton, Dickinson and Company (USA). For others, see "*chemicals*" in Chapter II.

Antimicrobial assay

See "*Antimicrobial assay*" in Chapter II.

^{27}Al-NMR measurement

See "*^{1}H, ^{13}C, and ^{27}Al-NMR measurement*" in Chapter II.

Analysis of the mycelium density

The soils of SI, SF, and SO from the both shiros were lyophilized, pulverized with a Multi-beads Shocker (Yasui Kikai Corporation, Osaka, Japan), and their DNA content was extracted using CTAB lysis buffer containing 0.6% skim milk (Yamaguchi, *et al.*, 2009, Ugawa, *et al.*, 2012). The DNA extracts were stored in TE buffer at -20°C until use. qPCR analysis was performed using a LightCycler system (Roche Life Science, Mannheim, Germany), and the primer sets MY201f: 5'-GAGACACAACGGCG-AGATT-3'/MY101r: 5'-ACCCTTACCCGCTCAGT-3', which amplify, respectively, the 202-bp DNA fragment used to quantify *T. matsutake* mycelia. Plasmids for standard curve were constructed by inserting 202 bp of target DNA into the pT7Blue cloning vector (Novagen, Darmstadt, Germany) (Yamaguchi, *et al.*, 2016). DNA amplification and detection were performed in glass capillaries in a total volume of 20 μL containing 2 μL of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Risch-Rotkreutz, Switzerland). The reaction mixture was composed of 3.2 μL (5 mM) of $MgCl_2$, 1 μL (0.25 μM) of each primer, 10.8 μL of H_2O (sterile PCR grade), and 2 μL of template DNA. To detect the signal derived from *T. matsutake* mycelia, the qPCR was performed using the following program as recommended by the

manufacturer (Roche Life Science): 1 cycle at 95°C for 10 min, followed by 50 cycles at 95°C for 10 s, Ta for 2 s, 72°C for 1 s, and 83°C for 1 s, followed by signal detection. Unless stated otherwise, the standard curve was constructed using a 1.0-ng/μL sample of the respective plasmid containing the target DNA, which was serially diluted and added to the 20-μL reaction mixture. PCR products with a single melting curve that fitted the respective standard curve were considered to be authentic, quantified PCR products.

Analyses of the (oxalato)aluminate complex, antimicrobial activity, oxalic acid, and pH

Water (20 mL) was added to each soil (4 g) from shiro No. 32 and 7. After shaking for 30 min and filtration with filter paper, pHs of the filtrates were measured with a pH meter. The filtrates were lyophilized and dissolved in 0.6 mL of water. This solution was used for ^{27}Al-NMR measurement to analyze the content of the (oxalato)aluminate complex and for antimicrobial assay with paper-disc method. The concentrations of the (oxalato)aluminate complex were calculated with a calibration curve between concentrations of 0.5 and 10 μM and the ^{27}Al signal area of tris-(oxalato)aluminate. The same filtrates were used for quantitation of oxalic acid with HPLC: Shodex RSpak KC-811 (300×8.0 mm, Showa Denko, Japan); 50 mM perchloric acid; 1.0 mL/min; 220 nm.

Densities of bacteria and fungi

Each soil (500 mg) from shiro No. 32 and 7 was passed through a sieve with a mesh size of 1 mm. The sieved soils were put into mortars and grinded. The grinded soils (100 mg) were transferred to glass tubes. Water (10 mL) containing 0.01% Tween 80 was added to the tubes. The tubes were shaken for 30 min. The supernatant (100 μL) was diluted to 1:10 by water, and spread on the nutrient agar media with 50 ppm iprodione for bacteria, and complete medium with 50 ppm kanamycin and 50 ppm cromycin for fungi. The complete medium was consisted of 1% glucose, 0.1% yeast extract, 0.1% tryptone, 0.15% $Ca(NO_3)_2(H_2O)_4$, 0.05% $MgSO_4(H_2O)_7$, 0.05% KCl, 0.4% KH_2PO_4, 0.003% K_2HPO_4, and 1.5 % agar. The number of colonies (CFU/g soil) were counted after incubation for 3 days at 23°C. CFU meant “Colony Forming Unit”.

Quantification of phosphorus

Each soil (1 g) from shiro No. 32 and 7 was added to the 20 mL Bray 2 extractant consisted of 0.03 M NH_4F and 0.1 M HCl (Bray and Kurtz, 1945). The suspensions were shaken for 1 min and filtered through filter papers. The filtrates were analyzed by the ascorbic-molybdenum blue method with UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) (Murphy and Riley, 1962). Boric acid was used to eliminate possible interface from fluorides (Kurtz, 1942).

3. Results

The results of the two shiros were compared to know whether there is the same relationship among the items between the two shiros. The mycelium contents of SI and SF were 4.8 and 11.7 μg/g soil in No. 32, and 0 and 65 μg/g soil in No. 7, respectively (Fig. 11a). The mycelium was not detected in both SO. The results confirmed that SF was at the shiro front, and SI and SO were inside and outside the shiro front, respectively. The results of analyses are summarized in Fig. 11b-h.

The contents and speciation of the (oxalato)aluminate complex in SI, SF, and SO were analyzed with the ^{27}Al NMR spectra. The ^{27}Al signals from the (oxalato)-aluminate complex were detected only in SF, and the contents of SF were 388 μg/g soil in No. 32, and 92 μg/g soil in No. 7, respectively. The ratio of (oxalato)aluminate, bis-(oxalato)aluminate, and tris(oxalato)aluminate were 22:71:7 for No. 32 and 31:65:4 for No. 7. The contents of oxalic acid in SI, SF, and SO from No. 32 were 26 μg/g soil, 433 μg/g soil, and less than the detection limit of 1.0 μg/g soil, respectively. Oxalic acid was detected at 150 μg/g soil in SF whereas not detected in SI, and SO from No. 7. The pHs of SI, SF, and SO in No. 32 were 5.0, 4.5, and 5.2, respectively, and in No. 7 were similar to those of No. 32.

For the antimicrobial activity of both shiros, SF showed high activity, SI low or no activity, and SO did not any activity. The densities of bacteria and fungi in SF were clearly lower than those of SI and SO. The distributions of antimicrobial activity and micro-organisms were closely related with those of the (oxalato)aluminate complex.

Oxalic acid secreted from the mycorrhizas can react with insoluble aluminum

phosphate and/or insoluble calcium phosphate to release soluble phosphorus (Graustein, *et al.*, 1977). The quantitation of soluble phosphorus showed that the content of

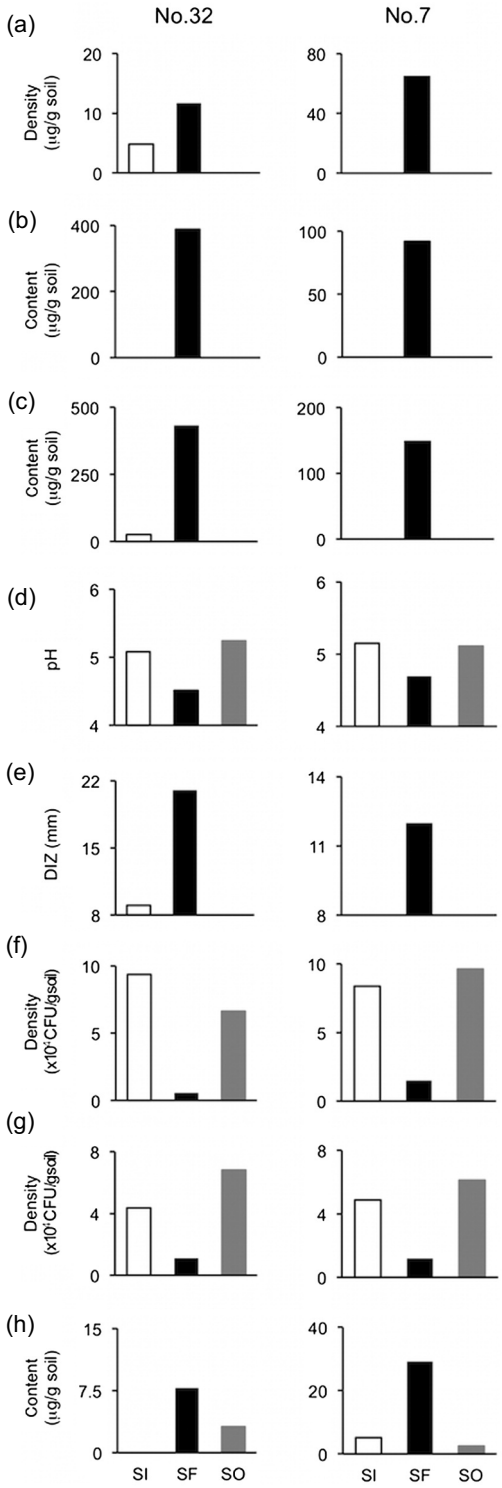

Fig. 11. The distribution of *T. matsutake* mycelium (a), the (oxalato)aluminate complex (b), oxalic acid (c), pH (d), antimicrobial activity (e), bacteria (f), fungi (g), and phosphorus (h) inside the shiro front (SI), at the shiro front (SF), and outside the shiro front (SO) of No.32 and 7. DIZ: Diameter of inhibitory zone

phosphorus in SF was higher than SI and SO.

4. Discussion

Distribution of the mycelium content of *T. matsutake*, the (oxalato)aluminate complex, and others are important to discuss the ecological function of the (oxalato)-aluminate complex. As shown in Fig. 11b, the ^{27}Al signals from the (oxalato)-aluminate complex were detected only in SF, and the antimicrobial complex, (oxalato)-aluminate, and bis(oxalato)aluminate, were dominant. Oxalic acid was detected in SF of both shiros, and SI of No. 32 (Fig. 11c). The presence of oxalic acid in SI of No. 32 suggested that SI contained the (oxalato)aluminate complex, but its content would be too low to detect the ^{27}Al signals. The pHs of SF in both shiros were lower than those of SI and SO (Fig. 11d), being similar to those reported previously (Isram and Ohga, 2012). The pH of the shiro is probably affected by the content of oxalic acid in the soil, since oxalic acid was detected only in SF in No. 7 and 32. Oxalic acid would be secreted from the mycorrhizas, and the producer is likely to be *T. matsutake* as well as wood-rotting basidomycetes (Shimada, *et al.*, 1997), but we cannot deny that *P. densiflora* produces oxalic acid. These results revealed that the shiro front contained a high level of the (oxalato)aluminate complex while the soils inside and outside of the shiro front contained a trace amount and none of the (oxalato)aluminate complex, respectively. When SI was the shiro front 1–2 years ago, it would have contained more of the (oxalato)aluminate complex than that of SI at present. The (oxalato)-aluminate complex inside the shiro front may have been leached out by rain.

The distributions of antimicrobial activity and micro-organisms were closely related with those of the (oxalato)aluminate complex (Fig. 11b, 11e-g). The low anti-microbial activity of SI from No. 32 was coincident with the suggested occurrence of a trace amount of the (oxalato)aluminate complex.

Oxalic acid secreted from the mycorrhizas can react with insoluble aluminum phosphate and/or insoluble calcium phosphate to release soluble phosphorus (Graustein *et al.*, 1977). The content of phosphorus in SF was more than three times as high as that of SO (Fig. 11h). The low content of soluble phosphorus in SO suggested that

most phosphorus in SO remained as the insoluble form of aluminum phosphate and/or calcium phosphate. The soluble phosphorus in SF may be utilized by *T. matsutake* as an important nutrient for formation of the fruiting body.

These results clearly showed a close correlation between the distribution of the (oxalato)aluminate complex and that of mycelium of *T. matsutake*, antimicrobial activity, and phosphorus. This correlation strongly suggests that the (oxalato)-aluminate complex contributes to preservation and development of the shiro by protecting it from other micro-organisms, and through the supply of phosphorus. I propose a third role, acting as an antimicrobial agent, to the (oxalato)aluminate complex in addition to its roles as a byproduct formed by the release reaction of phosphorus, and as a byproduct of the detoxification of aluminum. For mycorrhiza, the secretion of oxalic acid is like "killing three birds with one stone" (Fig. 12). Chapter IV describes the seasonal change in the content of the (oxalato)aluminate complex in the shiro, and identification and sensitivity to the (oxalato)aluminate complex of the micro-organisms isolated from SF, SI, and SO.

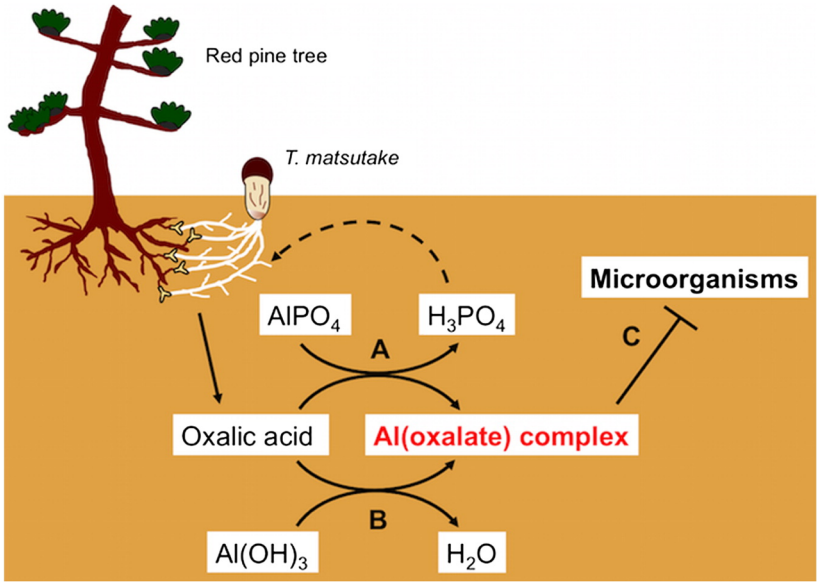

Fig. 12 Three functions of oxalic acid secreted from the mycorrhiza. A: Release of soluble phosphorus from aluminum phosphate. B: Detoxification of aluminum in acidic soil. C: Formation of the antimicrobial (oxalato)aluminate complex. Al(oxalate) complex: the (oxalate)aluminate complex.

Chapter IV
Seasonal Changes in the Content of the (Oxalato)aluminate Complex and the *Tricholoma matsutake* mycelium, and in the Bacterial Community Structure in the Shiro Area

1. Introduction

The (oxalato)aluminate complex was detected only in the shiro front, not inside or outside the shiro front in the season for fruiting bodies, as described in Chapter III. The distribution of the complex in the shiro area showed a positive correlation with those of the antimicrobial activity and the mycelium density of *T. matsutake*, and a negative correlation with that of bacteria. The shiro front advances outwards at 10–15 cm annually (Ohara, 1966), so that bacteria sensitive to the (oxalato)aluminate complex outside the shiro front may be expelled along with the extension of the shiro, with resistant bacteria remaining at the shiro front. Bacteria sensitive to the complex inside the shiro may recover.

The content of the (oxalato)aluminate complex should be high in the fruiting body season since oxalic acid secretion may be promoted to obtain soluble phosphorus for growth. This chapter describes the seasonal changes over a year in the content of the (oxalato)aluminate complex, mycelium density of *T. matsutake*, antimicrobial activity, and pH inside, outside, and the shiro front. The bacteria in the shiro area in October were identified, and their sensitivity to the complex was examined to confirm the above hypothesis.

In this study, we did not calculate the average values of the results for the following two reasons: First, shiro is very valuable, so we refrained from taking many soil samples from one shiro. Second, soil is highly heterogeneous, and it is difficult to obtain statistically significant average values even from the same zone of one shiro. Marked variation could occur even if the interval between sampling spots is less than 1 cm. To obtain average values with small standard errors, many soil samples must be taken from one shiro, and this may seriously damage it. Because of the difficulty in taking several samples from one shiro, I collected samples from three shiros, and

assessed whether the same relationships between the parameters existed among the shiros.

2. Materials and Methods

Materials

Soils were taken from shiro No. 7, 11, and 39 at the Sakai Research Forest, which has been conserved by Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center. The number of fruiting bodies formed from 2010 to 2014 were 46, 130, and 36 at shiro No. 7, 11, and 39, respectively, and in 2015 were 1, 12, and 3, respectively. The whitish soil (5 g) from the shiros at a depth of 10 cm were collected as the shiro front with a soil sampler (ϕ 1.4 × H 20 cm, International Scientific Instrument Supply, Japan), and termed SF. Soil samples (5 g) obtained 30 cm inside and 30 cm outside SF were also collected and termed SI and SO, respectively. Soil samples were collected every two months, April 23rd, June 9th, August 5th, October 2nd, and December 9th, 2015, and February 16th and April 11th, 2016. The soil samples were taken from one spot in each zone, and analyzed once. The sampling spots were located at more than 10-cm intervals. *Bacillus subtilis* FKU 101 was provided by Emeritus Professor K. Yamamoto of Kyoto University.

Chemicals

All chemicals were of reagent grade. (Oxalato)aluminate hydrate was purchased from Alfa Aesar (UK). Yeast extract was purchased from Becton, Dickinson and Company (USA). Primers were purchased from Fasmac (Japan), and the reagents for PCR were from Takara Bio (Japan). Others were purchased from Wako Pure Chemical (Japan).

^{27}Al-NMR measurement

See "*^{1}H, ^{13}C, and ^{27}Al-NMR measurement*" in Chapter II.

pH measurement, and quantitative analysis of the (oxalato)aluminate complex by ^{27}Al NMR spectrometry

Water (20 mL) was added to each soil (4 g). After shaking and filtration, the pH of the filtrates was measured with a pH meter. Six milliliters of the filtrates were concentrated and dissolved in 0.6 mL of distilled water for ^{27}Al-NMR measurement. AVANCE III 400 (104 MHz for ^{27}Al, Bruker Biospin, Japan) was used for the ^{27}Al NMR measurement. $AlCl_3(H_2O)_6$ was used as an external standard in the ^{27}Al-NMR spectra. The content of the (oxalato)aluminate complex was calculated with a calibration curve between 0.5 and 10 μM using the ^{27}Al signal area of tris-(oxalato)aluminate, which was made by mixing $AlCl_3(H_2O)_6$ and disodium oxalate at a molar ratio of 1:3. The ratio of three speciations of the (oxalato)aluminate complex, (oxalato)aluminate, bis(oxalato)aluminate, and tris(oxalato)aluminate, were calculated using the signal areas corresponding to those speciations (Kerven et al. 1995): 6.4 ppm for (oxalato)aluminate, 11.4 ppm bis(oxalato)aluminate, and 16.0 ppm tris(oxalato)-aluminate.

Antimicrobial assay

See "*Antimicrobial assay*" in Chapter II.

Analysis of the mycelium density in T. matsutake

See "*Analysis of the mycelium density*" in Chapter III.

Densities of bacteria in the presence and absence of the (oxalato)aluminate complex

Soils at the shiro front, and inside and outside the shiro front of No. 39 shiro were collected on Oct 2nd, 2015, which is the high season for the fruiting bodies of *T. matsutake* in Kyoto, Japan, and were passed through a sieve with a mesh size of 1 mm. The sieved soils (200 mg) were transferred to glass tubes, and 10 mL of water was added to the tubes. After shaking, 10 μL of the supernatant was spread on the agar media containing the (oxalato)aluminate complex at 300 ppm, and on the agar media without the complex (control media). These agar media contained 50 ppm fludioxonil. The number of colonies (CFU/g soil, CFU: colony forming units) was counted after incubation for 3 d at 23°C.

Isolation and identification of the bacteria

One hundred ninety-two colonies of bacteria from each soil were picked from the control media used for measurement of the bacterial density, and streaked onto new control media. Twenty bacteria each in SI, SF, and SO were identified by 16s rRNA analysis (Jannasch and Nelson, 1984). The 16s rDNA sequences were amplified by direct PCR using 16S universal primers 27F and 926R. The total volume of the solution for the PCR was 50 μL (distilled water 40.55 μL, 0.1 μL each of 100 pmol primers, Takara Ex Taq HS® 0.25 μL, 10 × buffer 5 μL, dNTP Mixture 4 μL). The thermal cycler was programmed as follows: initial denaturation, 1.5 min at 95°C; then 35 cycles of 0.5 min at 95°C, 0.5 min at 50°C, 1 min at 72°C. Amplification of 16s rDNA was confirmed by electrophoresis in 1% agarose gel. The amplified DNA was purified with Mag Extractor™ -PCR & Gel Clean up- (Toyobo, Japan) according to the manufacturer's direction. The purified DNA was used for sequence reaction with 16S rRNA primers 357F and 534R, and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's recommendation. All sequences were aligned using Codon Code aligner (Hulinks, Japan). Isolated bacteria were identified using the NCBI database with the alignments. At least 97% similarity was used as the limit for classifying an operational taxonomic unit (Wayne, *et. al.*, 1987; Stackebrandt and Goebal, 1994).

Evaluation of sensitivity to the (oxalato)aluminate complex

For the sensitivity test, agar media containing 30, 100, or 300 ppm of the complex were prepared. Two microliters of the inoculum medium of the identified bacteria with a turbidity 0.05 on the McFarland scale was added directly onto the agar media (Cockerill, *et al.*, 2012). Colony formation of the bacteria was used for judgment of sensitivity after incubation for a week at 30°C.

Ground temperature and rainfall

The ground temperature of No.11 shiro was recorded with a data logger (T&D Corporation, Japan). The data were obtained from Apr 15th, 2015 to Apr 30th, 2016. Data on rainfall in Sonobe, Kyoto, about 20 km from the Sakai Research Forest were used (Japan Meteorological Agency).

3. Results

3.1. Seasonal changes in the mycelium density of *T. matsutake*, the content of the (oxalato)aluminate complex, antimicrobial activity, and pH

The results are summarized in Fig. 13. Mycelium densities of *T. matsutake* from SF collected in April, 2015 were 6.9, 21.7, and 21.4 μg/g soil in No. 7, 11, and 39 shiros, respectively, and increased from April to August in No. 11 and to October in No. 7 and 39 shiro (Fig. 13a). The mycelium densities of SF were low in winter, from December to February, in all shiros except for the density of No. 39 shiro in December. The mycelium was not detected in any SI or SO.

The (oxalato)aluminate complex was detected only in SF, and not in SI or SO, in all seasons (Fig. 13b). The contents of the complex in SF were higher than 200 μg/g soil in June and October in all shiros, and in August in No. 11 shiro. The content was also high in December in No. 39 but low from December to April, 2016 in all shiros. The (oxalato)aluminate complex has three speciations, which are (oxalato)aluminate,

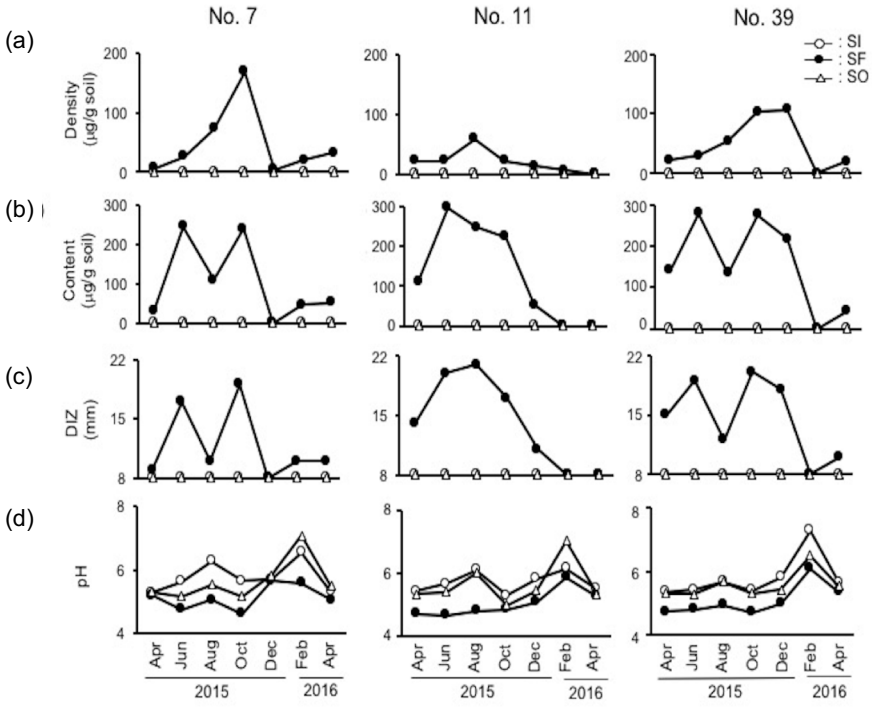

Fig. 13 Seasonal changes in the mycelial density of *T. matsutake* (a), the content of the (oxalato)aluminate complex (b), antimicrobial activity (c), and pH (d) inside the shiro front (SI), at the shiro front (SF), and outside the shiro front (SO). DIZ: Diameter of growth-inhibitory zone of *B. subtilis*. Apr: April. Jun: June. Aug: August. Oct: October. Dec: December.

bis(oxalato)aluminate, and tris(oxalato)aluminate (Kerven *et al.*, 1995). Table 4 summarizes the ratios of the speciation of the (oxalato)aluminate complex in SF, showing that the major speciation was bis(oxalato)aluminate.

Antimicrobial activity was observed only in SF, with no activity in any season (Fig. 13c). The magnitude of the activity of SF was closely correlated with amount of the (oxalato)aluminate complex.

The change in the pH of SF was similar to those of SI and SO, but the pH of SF was lower than those of SI and SO in all seasons (Fig. 13d). The pH values of SF, SI, and SO in February from all shiros were the highest among all seasons.

Table 4. The speciation contents (μg/g soil) of the (oxalato)aluminate complex in shiro fronts.

Shiro no.	Month, year	(Oxalato) aluminate	Bis(oxalato) aluminate	Tris(oxalato) aluminate	Total
No. 7	April 2015	0	30	0	30
	June 2015	18	188	38	244
	August 2015	0	95	15	110
	October 2015	51	187	0	238
	December 2015	0	0	0	0
	February 2016	9	38	0	47
	April 2016	0	52	0	52
No. 11	April 2015	15	94	1	110
	June 2015	61	201	36	298
	August 2015	42	192	14	248
	October 2015	41	183	0	224
	December 2015	0	52	0	52
	February 2016	0	0	0	0
	April 2016	0	0	0	0
No. 39	April 2015	12	120	10	142
	June 2015	52	205	22	279
	August 2015	31	104	0	135
	October 2015	61	214	0	275
	December 2015	50	168	0	218
	February 2016	0	0	0	0
	April 2016	0	42	0	42

3.2. Bacterial density in the shiro

The bacterial density of SF from No. 39 shiro was clearly lower than those of SI and SO on the control media (Fig. 14). The density was 39,500 CFU/g soil for SF, 99,300 CFU/g soil for SI, and 105,000 CFU/g soil for SO. The bacterial density of SO on the media containing the (oxalato)aluminate complex was lower than that on the control media. For SF and SI, the bacterial density on the media containing the (oxalato)aluminate complex were also lower than on the control media, but the difference was small.

3.3. Identification of bacteria and their sensitivity to the (oxalato)aluminate complex

Bacteria showing good growth on the control media were isolated from SI, SF, and SO and identified. Figure 15 shows the composition of bacteria in SI, SF, and SO and their sensitivity to the complex. Most of the bacteria were identified as *Burkholderia*. The species could not be identified, but their 16s rDNA sequences were different. They were tentatively distinguished as "*Burkholderia* sp. 1" to "*Burkholderia* sp. 5". Six bacteria were isolated from SI (identified as *Burkholderia ambifaria*, *Burkholderia arboris*, *Burkholderia cepacia*, *Burkholderia xenovorans*, *Burkholderia* sp. 1, and *Dyella terrae*), four from SF (*B. xenovorans*, and *Burkholderia* sp. 2, 3, and 4), and

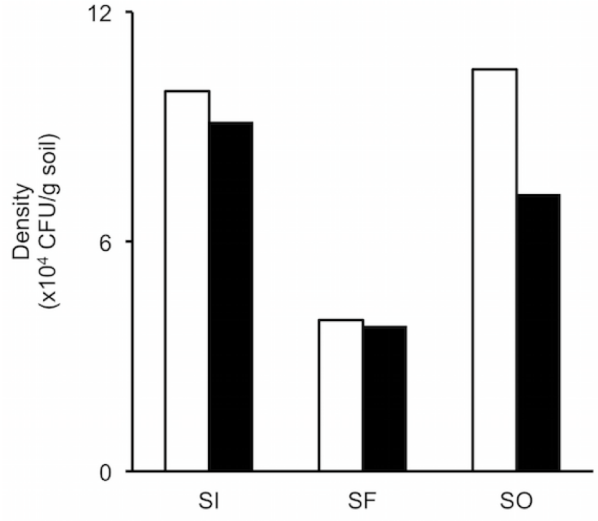

Fig. 14 The density of bacteria that were isolated from soils inside the shiro front (SI), at the shiro front (SF), and outside the shiro front (SO) in shiro No. 39 and then cultured on agar media with (black bars) or without the (oxalato)-aluminate complex (white bars) was measured.

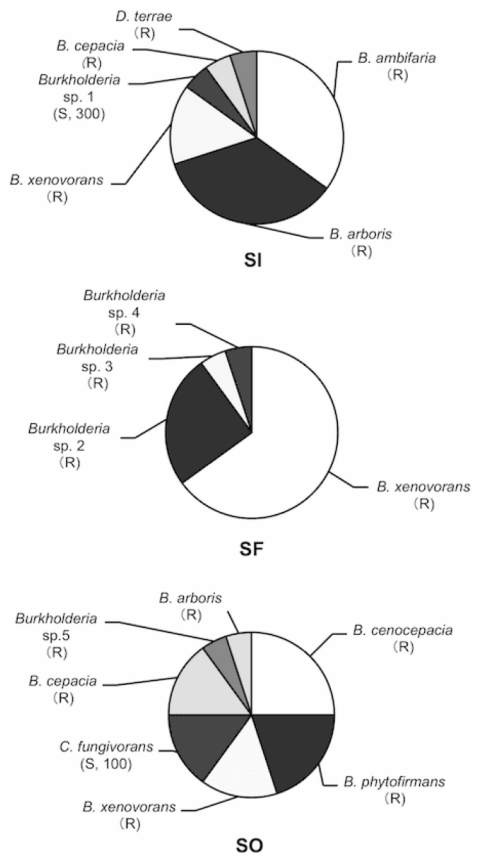

Fig. 15 The abundance ratios of bacteria isolated from inside the shiro front (SI), at the shiro front (SF), and outside the shiro front (SO) in shiro No. 39.
R: resistant to the (oxalato)aluminate complex. S: sensitive to the (oxalato)-aluminate complex. The values show the minimum inhibitory concentration of the (oxalato)aluminate complex (ppm).

seven from SO (*B. arboris*, *Burkholderia cenocepacia*, *B. cepacia*, *B. phytofirmans*, *B. xenovorans*, *Burkholderia* sp. 5, and *Collimonas fungivorans*). *Burkholderia xenovorans* was dominant in SF, although it was present in SI and SO. *Burkholderia ambifaria* and *B. arboris* were major in SI, but no dominant bacterium existed in SO.

Burkholderia sp. 1 from SI and *C. fungivorans* from SO were sensitive to the (oxalato)aluminate complex, with their growth was respectively inhibited at 300 and 100 ppm of the complex, respectively (Table 5). Other bacteria were resistant to the complex at 300 ppm.

3.4. Ground temperature and rainfall

The ground temperature of No. 11 shiro rose from April to August 2015. After

Table 5 The antimicrobial activity of the (oxalato)aluminate complex against the bacteria isolated from shiro No. 39.

Bacteria	Accession no.	MIC (ppm)
Burkholderia ambifaria	CP009798.1	>300
B. arboris	KM487703.1	>300
B. cenocepacia	CP013397.1	>300
B. cepacia	CP013732.1	>300
B. phytofirmans	KC537741.1	>300
B. xenovorans	CP008762.1	>300
Burkholderia sp.1	CP014578.1	300
Burkholderia sp.2	AJ884797.1	>300
Burkholderia sp.3	KM253200.1	>300
Burkholderia sp.4	KU83944.1	>300
Burkholderia sp.5	JQ518344.1	>300
Collimonas fungivorans	AJ496444.1	100
Dyella terrae	LN890126.1	>300

MIC: Minimum inhibitory concentration.

that, the temperature fell to February (Fig. 16), and then rose again. There was much rain from June to July in Sonobe, because of the rainy season (Fig. 16). The rainfall at the Sakai Research Forest would be similar to that in Sonobe, since they are close.

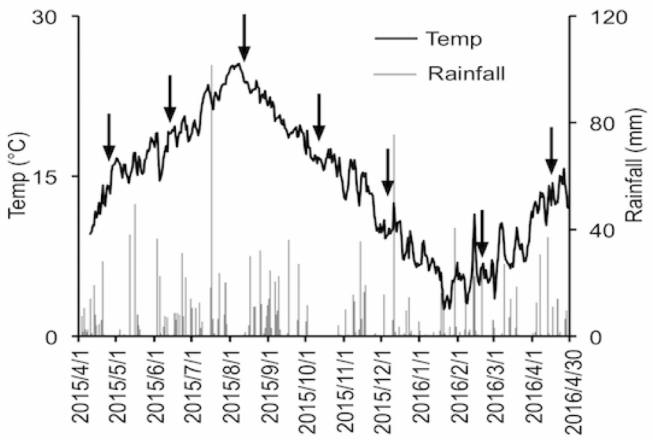

Fig. 16 The ground temperature in shiro No. 11 at the Sakai Research Forest, and rainfall in Sonobe, Kyoto. Arrows show sampling dates.

4. Discussion

Mycelium of *T. matsutake* was found only in SF, and not in SI or SO (Fig. 13a), indicating that the sampling of the soil of SF, SI, and SO had been correctly carried out. It was previously reported that the mycelium density of *T. matsutake* was high in October, in the high season in which the fruiting bodies are formed (Yamaguchi, *et al.*, 2016). The high mycelium density of SF in October from No. 7 and 39 shiros was consistent with the former observation. However, the mycelium density of SF from No. 11 shiro was not high in October, while the content of the (oxalato)aluminate complex was high. This might have occurred because the mycelium density was probably not uniform due to heterogeneity of the soil. The density of the mycelium in all shiros was also low in June regardless of the high content of the (oxalato)aluminate complex (Fig. 13a). This result may have been due to another reason. Both the complex and soluble phosphate, which may be utilized by *T. matsutake* as an important nutrient for growth, are released by the reaction of oxalic acid secreted from the mycorrhizas and insoluble aluminum phosphate and/or insoluble calcium phosphate (Graustein, *et al.*, 1998). The secretion of oxalic acid could be active in June, since the ground temperature is over 15°C, which is suitable for the growth of *T. matsutake* (Fig. 16). The content of the complex may increase due to secretion, while *T. matsutake* is considered to grow gradually when using the soluble phosphate. The difference might be reflected in the results. The mycelium densities of SF from No. 7 and No. 11 were low in winter, from December to February. *Tricholoma matsutake* can grow between 5°C and 28°C, but the rate of the growth slows under 15°C (Ogawa, 1978). The ground temperature at a 10-cm depth was under 15°C after November (Fig. 16), so the mycelium growth for this period would be slow due to the low temperature. The high mycelium density of SF in December in No. 39 shiro might be due to the heterogeneity of soil. The mycelium density of SF of No. 7 and 39 shiros in April 2016 increased compared with that in February. The activity of mycorrhiza seemed to increase as the ground temperature rose, while it did not increase in SF of No. 11 shiro, suggesting that the shiro does not always concentrically extend past obstacles such as stones and roots in soil.

The (oxalato)aluminate complex was detected in only SF, and not in SI or SO, as

well as the mycelium of *T. matsutake* (Fig. 13b). This distribution was the same as in our previous study (Nishino, *et al.*, 2017). The content of the complex was high in June and October in all shiros. This result is consistent with the observation that June is another fruiting season in Kyoto (Ogawa, 1978). The content of the complex decreased in August in No. 7 and 39 shiros, although the mycelium density increased. This result could be due to the water solubility of the complex. There was much rain from June to July, the rainy season, in Kyoto (Fig. 16). SF soil cannot absorb much water due to its water repellency, which might be caused by mycelium aggregation (Ogawa, 1978). However, a litter layer and humus, which generally cover mountain soil, can retain water, and gradually supply water to the undersoil (Putuhena and Cordery, 1996; Ohta, 2012). This water could cause the leaching of the (oxalato)-aluminate complex from SF soil, which could have led to a decrease in the content of the (oxalato)aluminate complex in No. 7 and 39 shiros in August. There was little difference in the content of the complex between June and August in No. 11 shiro. However, a litter layer and humus hardly covered the soil of SF in No. 11 shiro, resulting in preventing the complex from exposure to retained rainwater in August. The change in the content of the complex after December correlated positively with that of the mycelium density. The secretion of oxalic acid could have been activated as the temperature rose, resulting in an increase in both the content of the (oxalato)aluminate complex in April 2016 and the mycelium density of *T. matsutake*.

Seasonal changes in the antimicrobial activity in SF were closely correlated with those in the (oxalato)aluminate complex (Fig. 13c), supporting the antimicrobial activity in SF being derived from the (oxalato)aluminate complex. The major speciation of the complex in all SF samples was bis(oxalato)aluminate (Table 4), suggesting that bis(oxalato)aluminate was responsible for the antimicrobial activity.

The pH of SF may be negatively correlated with the content of the (oxalato)-aluminate complex (Fig. 13d). Previous results showed that the pH of SF was closely correlated with the content of oxalic acid and the (oxalato)aluminate complex (Nishino, *et al.*, 2017), suggesting that the pH of SF is mainly dependent on the content of oxalic acid secreted from the *T. matsutake* mycorrhiza. Organic acids of the TCA cycle may partially affect the pH of SF, but the organic acids (citric acid, lactic acid, malic acid, malonic acid, and succinic acid) were not detected in SF by HPLC analysis (data not

shown). The high pH values in August and February suggest a decrease in the metabolic activity of the mycorrhiza in summer and winter.

The bacterial density of SF on the control media was lower than those of SI and SO (Fig. 14). This result agrees with the previous data (Nishino *et al.*, 2017), supporting that the antimicrobial complex suppresses the bacterial density in SF. The bacterial densities of SF, SI, and SO on the media containing the complex were lower than those on the control media, but the difference between them was smaller in SF and SI than in SO, showing that the majority of the bacteria in SF and SI were resistant to the complex. This is consistent with the outward development of the shiro front expelling the micro-organisms.

The bacteria identified from SF, SI, and SO were all Gram-negative, and the diversity of bacterial species was high in SO and SI, and low in SF (Fig. 15). Seven species of bacteria were detected in SO and six species in SI, but four species in SF. The bacteria sensitive to the complex were detected more frequently in SO and SI than in SF (Table 5). This suggested that the complex expels sensitive bacteria near the shiro front in the process of shiro extension, and that sensitive bacteria gradually recovered after SF shifted to SI.

The bacterial community structures of SI, SF, and SO were different from each other (Fig. 15). It is difficult to understand the reason for difference, but one possible explanation is that it might be caused by the difference in the nutritional, organic, and antimicrobial properties of each soil (Fig. 17). It has been reported that rootlets of Japanese red pine secrete glucose, which is probably absorbed by *T. matsutake* for its growth (Ogawa, 1978). Our preliminary analysis showed that glucose occurred at 0.3 mg/g soil in SF, but not in SI or SO. The content of soluble phosphorus was also

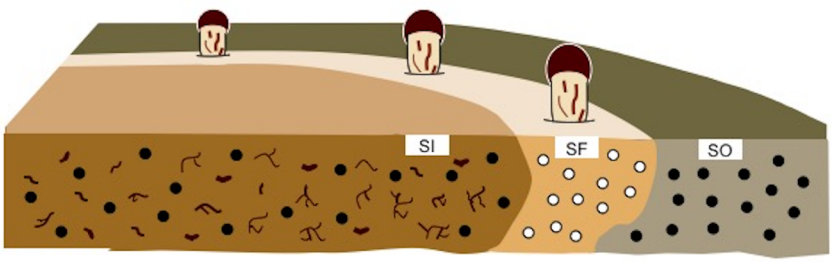

Fig. 17 A vertical section illustration of the shiro structure of *T. matsutake*.
～: mycorrhiza or decomposition products of roots.
○: the (oxalato)aluminate complex. ●: micro-organism.
SI: shiro, inside. SF: shiro front. SO: shiro, outside.

higher in SF than in SI and SO (Nishino, *et al.*, 2017). These observations mean that the nutrient condition in SF was better than that in SI or SO. The mass of organic matter in SI would be higher than that in SF or SO, since there are decomposed mycorrhizas and rootlets of Japanese red pine in SI after the shiro front has passed (Ogawa, 1978). Considering the outward development of the shiro, in SO there is no antimicrobial complex produced and nutrition is poor, for SF there is the antimicrobial substance produced and nutrition is rich, and for SI, the antimicrobial complex had been produced, nutrition is poor, but organic matter is rich. These differences seem to correspond to the difference in the bacterial community structures in SI, SF, and SO. The bacteria in SO may be common ones in mountain soil, the bacteria resistant to the antimicrobial complex may grow under good nutrient conditions in SF, and the bacteria resistant to the antimicrobial complex and adapted to a poor nutrient condition and much organic matter may grow in SI. We need to conduct further analysis to examine this hypothesis.

Twelve species of bacteria from the shiro in Shitumi, Iwakura, Kyoto, and others, Japan (Ohara, 1966), 28 species of bacteria from Iwaizumi and Iwate in Japan (Yoshimura, 2003), and 16 species of bacteria from Nuuksio, Kourajärvi, and Alkkia, Finland have been identified (Vaario, *et al.*, 2011). All bacteria from the shiro of Shitumi, Iwakura, and the locations other than Kyoto (Ohara, 1966), and 25 bacteria from the shiro of Iwaizumi were Gram-negative (Yoshimura, 2003). The main bacteria of the shiros Iwaizumi were *Achromobacter* and *Pseudomonas* (Yoshimura, 2003). All bacteria from the shiro of Nuuksio, Kourajärvi, and Alkkia were Gram-positive, the main bacteria of which were *Actinobacterium* and *Thermomonosporeceae* (Vaario, *et al.*, 2011). Thus, the bacteria from the shiro of Sakai Research Forest and other shiros in Japan, though there were no common species among them, were all Gram-negative, while those from the shiros in Finland were all Gram-positive. Gram-negative bacteria seem to be more common than Gram-positive bacteria in the shiros of Japanese forests. The (oxalato)aluminate complex may also affect the distribution of the bacteria in other shiros in Japan and Finland. The distribution of the complex in those shiros and the sensitivity of the bacteria from the shiros to the complex should be analyzed to better understand their relationship.

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List of Publications

Original Papers

1) Katsutoshi Nishino, Misao Shiro, Ryuki Okura, Kazuya Oizumi, Toru Fujita, Takahiro Sasamori, Norihiro Tokitoh, Akiyoshi Yamada, Chihiro Tanaka, Muneyoshi Yamaguchi, Syuntaro Hiradate and Nobuhiro Hirai: The (oxalato)aluminate complex as an antimicrobial substance protecting the “shiro” of *Tricholoma matsutake* from soil micro-organisms. *Biosci. Biothecnol. Biochem.* 2017, **81**, 102-111.

2) Katsutoshi Nishino, Kaya Matsubara, Chihiro Tanaka, Muneyoshi Yamaguchi, Toru Fujita, Akiyoshi Yamada and Nobuhiro Hirai: Seasonal change in the content of the (oxalato)aluminate complex, the antimicrobial substance of the shiro of *Tricholoma matsutake*, and the bacterial community structure in the shiro area. *Mushroom Sci. Biotechnol.* 2017, in press.