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Ergosterol and Water Changes in *Tricholoma matsutake* Soil Colony during the Mushroom Fruiting Season

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The purpose of this study is to understand spatio-temporal changes of active fungal biomass and water in *Tricholoma matsutake* soil colonies during the mushroom fruiting season. The active fungal biomass was estimated by analyzing ergosterol content at four different points within four replicated locations in a single circular *T. matsutake* colony at Ssanggok valley in the Sogri Mt. National Park in Korea during 2003 to 2005. The four points were the ahead of the colony, the front edge of the colony and 20 cm and 40 cm back from the front edge of the colony. Ergosterol content was 0.0 to 0.7 μg per gram dried soil at the ahead, 2.5 to 4.8 μg at the front edge, 0.5 to 1.8 μg at the 20 cm back and 0.3 to 0.8 μg at the 40 cm back. The ergosterol content was very high at the front edge where the *T. matsutake* hyphae were most active. However, ergosterol content did not significantly change during the fruiting season, September to October. Soil water contents were lower at the front edge and 20 cm back from the front edge of the colony than at the ahead and 40 cm back during the fruiting season. Soil water content ranged from 12 to 19% at the ahead, 10 to 11% at the edge, 9 to 11% at the 20 cm back and 11 to 15% at the 40 cm back. Our results suggest that the active front edge of the *T. matsutake* soil colony could be managed in terms of water relation and *T. matsutake* ectomycorrhizal root development.

KEYWORDS : Ergosterol, Fungal biomass, Soil water, Spatio-temporal change, *Tricholoma matsutake* soil colony

Tricholoma matsutake forms ectomycorrhizas with *Pinus densiflora* roots (Ka *et al*., 2006; Guerin-Laguette, *et al*., 2005; Koo *et al*., 2005; Bak *et al*., 2006) and rarely with *P. rigida* in Korea (Park *et al*., 2004). It requires a site specific niche for fruiting (Suzuki, 2005; Hosford *et al*., 1997; Yamada *et al*., 1999). *T. matsutake* is economically very significant in Korea, Japan and China. The price is 200 to 600 US$/kg. However, the production of *T. matsutake* is gradually decreasing in Korea. In mid 1980s the export to Japan was 1,000 to 1300 tons (30 to 50 million US$) per year, but in mid 2000s the amount was only 36 to 130 tons (7 to 14 million US$) (http://www.forest.go.kr). *T. matsutake* production decreased between 1985 and 1995 by an average of 7% per year in Korea (Koo and Bilek, 1998) due to changes in the pine forest vegetation and in the *T. matsutake* colony life cycle itself (Ogawa, 1991).

It has been suggested that a cause of the long-term decrease in *T. matsutake* production is due to the decline in pine forests due to insect damage and the succeeding deciduous shade tolerant species (Kim *et al*., 1999). *T. matsutake* colony disappeared due to its biological characteristics, that is, the outwardly growing colonies degenerated when they met together (Kyoto Forest Research Institute, 1982). *T. matsutake* mycorrhizal colonies also disappeared due to the invasion of other mycorrhizal species and due to highly accumulated soil organic matter from deciduous trees (Ogawa, 1991).

One of the causes of short-term variation in *T. matsutake* production was due to the variation of annual weather condition, such as precipitation and daily minimum temperature, during its fruiting season (Park *et al*., 1995). Irrigation has improved *T. matsutake* production but it has not been entirely efficient. Inefficiency of irrigation may be because the live and active zone within *T. matsutake* soil colony is only in limited zone (Ogawa, 1975). This suggests that the active zone and its water regime should be identified. Then, irrigation efficiency for *T. matsutake* production can be improved by the direct irrigation of the active zone. This active zone can be identified with ergosterol content.

Ergosterol is a biological precursor (a provitamin) to Vitamin D2 and a fungus specific biomaker (Nylund and Wallander, 1992). Ergosterol ($C_{28}H_{44}O$, ergosta-5,7,22-trien-3β-ol, 396.66 g/mol), a sterol, was first discovered in the ergot fugus *Claviceps pupurea* (Hart and Brokkes, 1996). It is the primary sterol in the phospholipid bilayer of the fungal cell membranes, but it is either absent or a minor component in the majority of higher plants (Weete, 1989; Parsi and Gorecki, 2006). This sterol serves the same

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function in fungal cells that cholesterol serves in animal cells (Antibus and Sinsabaugh, 1993). It correlates with fungal surface area (West *et al*., 1987) and hyphal length, and is more sensitive to live fungal biomass than chitin (Matcham *et al*., 1985). Because of these characteristics, ergosterol has been used to estimate fungal biomass in decomposing plant material (Bentz and Six, 2006), soil (Hart and Brookes, 1996) and mycorrhizae (Antibus and Sinsabaugh, 1993).

Ergosterol varies in its concentration in mycelia by species, age of cultures, developmental stage and growth conditions (Pasanen *et al*., 1999). It degraded by up to 95% within two weeks after fumigation and death of the fungal cells (Davis and Lamar, 1992). Therefore, this compound was analyzed to estimate live fungal biomass changes in *T. matsutake* ectomycorrhizal colonies.

The objectives of this study were to investigate water changes and the live active hyphal zone within *T. matsutake* soil colony, and to understand the spatio-temporal changes of ergosterol and water contents during *T. matsutake* fruiting season.

Materials and Methods

Experiment site and colony samples. The experiment site is located at a *T. matsutake* production pine stand at Ssanggok valley in the Sogri Mt. National Park in central Korea. The site has two partly combined, partly circular colonies, each about 7 m diameter (Fig. 1, Fig. 2). Because

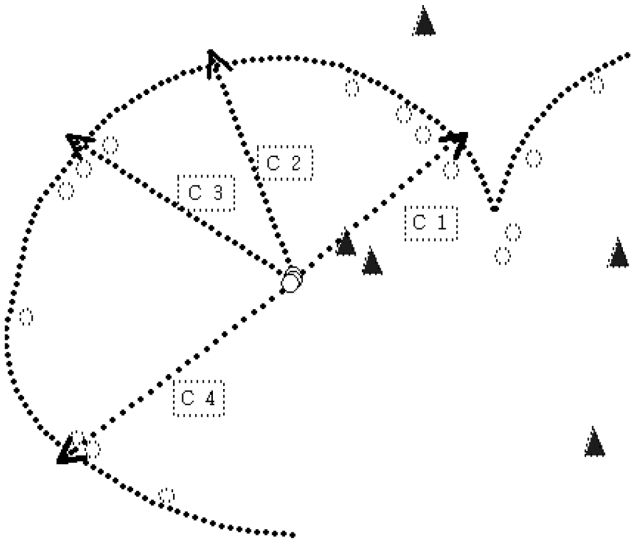

Fig. 1. *Tricholoma matsutake* colony investigated for ergosterol and water change in Ssanggok Valley in the Sogni National Park in 2005. The colony is composed of 7 m diameter of 3/4 circular colony and a 3 m long arc colony. Small circles are *T. matsutake* fruiting points and arrows are sampling directions.

Fig. 2. Experimental plot in the *Tricholoma matsutake* production pine forest at Ssanggok Valley in the Sogni Mt National Park. *T. matsutake* fruiting has been recorded since 2002.

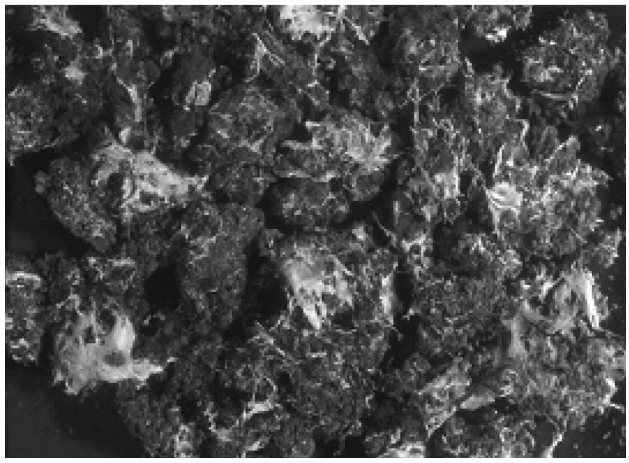

Fig. 3. Soil, white hyphae and ectomycorrhizae at front edge of the *Tricholoma matsutake* colony at Ssanggok valley in the Sogni Mt National Park.

the annual outward growth of *T. matsutake* colony was 11 to 14 cm, the age of the colony was approximately 30 years old. From the 3/4 circular colony, samples at four points (ahead of the colony, front edge, 20 cm back, 40 cm back) each of 4 replicated colony locations (ridge, down slope, upslope and contour) were collected with a 3.2 cm diameter × 10 cm long core sampler. The samples were collected every two weeks from 31 August to 28 October 2005, during *T. matsutake* fruiting season and stored in a ziplock bag at −79°C until analysis. Samples from the ahead of colony did not have *T. matsutake* mycorrhizae, front edge ones include the most active *T. matsutake* mycorrhizae with white mycelia (Fig. 3), 20 cm back ones have some decayed dark *T. matsutake* mycorrhizae and 40 cm back ones had powdered *T. matsutake* hyphae with black roots (Table 1).

Table 1. Characteristics of the *Tricholoma matsutake* soil colony where soil samples were collected

T. matsutake colony	Characteristics of *T. matsutake* soil colony, mycorrhizas and soils
Sampling points (cm)	
Ahead −10~−5	Noncolony soil, outside of *T. matsutake* colony front that *T. matsutake* hyphae grow into later; has higher water content but lower root density than *T. matsutake* colony.
Front edge 0~5	White fungal colony mass with the most actively growing hyphae, feeder roots and soil; water content is lower than the ahead but higher than the other positions.
Back 20~25	White powdery colony; *T. matsutake* hyphae decays; generally has lower water content than any other position; shrunk black feeder roots peeled to show light brown surface: just outside the previous year *T. matsutake* fruiting position.
Back 40~45	Gray powdery colony; *T. matsutake* ectomycorrhizas decayed mostly; has lower root density than the front edge colonies; root tips are very thin and weak.

Ergosterol analysis. Ergosterol analysis was done using the method by Nylund and Wallander (1992), which we slightly modified. Five g of soil and 10 *ml* of 10% KOH in 95% methanol were placed into 20 × 150 mm glass tubes and homogenized using a vortex mixer for ten seconds. The tubes were placed in a 90°C water bath for 1 hr for saponification. Each tube was cooled, the 3 *ml* of deionized water plus 2 *ml* of n-hexane was added. The mixture was then vortexed for a few seconds. The sample was allowed to settle for 2 minutes, then approximately 1.5 *ml* of the supernatant (n-hexane + ergosterol) was removed with a 2 *ml*-syringe. It was placed in a new glass tube and evaporated under N_2 gas. The dried sample was dissolved in 2 *ml* of methanol and filtered through a 0.2 μm syringe filter tip (Millipore) into a micro-centrifuge tube. The tubes with the filtered sample solution were stored in a dark, 4°C refrigerator. The extraction sample solution (20 μl) was injected into HPLC (Waters 510) with C-18 Nova-Pak column at the Pharmaceutical analysis lab in Chungbuk National University. The column was eluted with HPLC grade 100% methanol at 1.5 *ml*/min flow rate under 40°C oven temperature and the ergosterol peak was detected at 280 nm at 6.5 min.

Soil water and temperature measurements. Soil water content (% by v/v) was measured using a 15 cm long probe of a portable Hydrosensor every 20 cm from 10 cm ahead of the colony to 250 cm back of the colony front edge at the four replicated colony locations (ridge, down slope, upslope and contour) in the 3/4 circular colony every month from May 2003 to June 2004 (Fig. 1). During the *T. matautake* fruiting season from 31 August to 28 October 2005, soil water using the Hydrosensor and soil temperature using a 15 cm long probe of a portable Testo 915-1 thermometer were measured at four sampling points (ahead of colony, front edge, 20 cm back, 40 cm back) in the same four replicated colony locations in the 3/4 circular colony every two weeks.

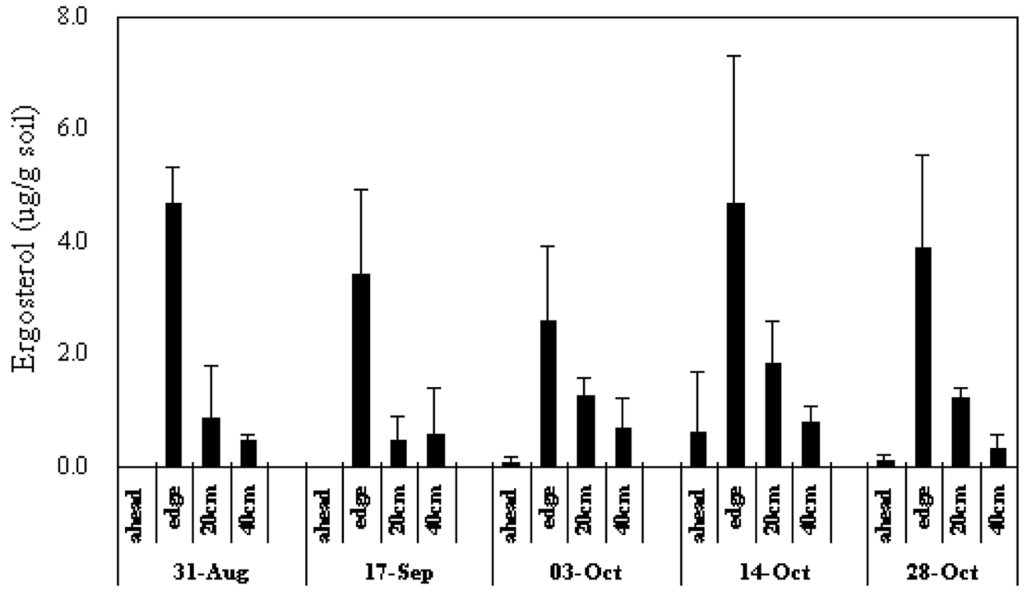

Fig. 4. Changes of ergosterol content in the *Tricholoma matsutake* soil colony by points (ahead, front edge, 20 cm back and 40 cm back) during September and October in 2005. Error bars represent one standard deviation.

Results and Discussion

Ergosterol content change. The most active hyphal zone of the *T. matsutake* soil colony was only about 15 cm wide from the front. The ergosterol content ranged from 2.5 to 4.8 μg/g dried soil in the ahead of colony, active zone, versus 0 to 1.8 μg/g dried soil in the other points (Fig. 4). The spatio-temporal changes of ergosterol contents in *T. matsutake* soil colony were related to the points within colony, but was not related to season or to water content.

While the ergosterol concentrations were very low at the ahead where colony had not formed or at the 40 cm back where the colony was more than two years old, the content was very high at the front edge of the colony where the *T. matsutake* hyphae were most active. The ergosterol content was 0.0 to 0.7 μg/g dried soil at the ahead, 2.5 to 4.8 μg/g dried soil at the front edge, 0.5 to 1.8 μg/g dried soil at the 20 cm back and 0.3 to 0.8 μg/g dried soil at the 40 cm back. This observation supports the result that ergosterol content is high within 20 cm range from the colony front edge (Koo *et al*., 2003) where *T. matsutake* colony is active. This ergosterol change can explains Ogawa's finding (1975) that *T. matsutake* colony grows outwardly about 10 to 15 cm per year, and that the previous year colony content is used for the mushroom fruiting. Ogawa (1975) estimated that *T. matsutake* mushroom requires 100 to 200 cm^2 surface area and 1500 to 2000 cm^3 colony volume including soil, ectomycorrhizae and hyphae.

The ergosterol concentration by colony locations did not change significantly during the fruiting season. The average ergosterol concentration in the colony front edge was 3.8 μg/g dried soil, and ranged from 1.4 to 6.7 μg/g dried soil. These values are quite low compared with other studies. When grown in a sterilized and nutrient enriched soil, the ergosterol content of *T. matsutake* hyphae was ca 50 μg/g dried soil, but in natural *T. matsutake* soil colonies, it was 15.5 μg/g dried soil (Bak *et al*., 2006). Ergosterol content in pure *T. matsutake* hyphae from liquid culture was about 0.85% and it was high at lag and stationary phases during the culture period (Lee *et al*., 2003). In various ectomycorrhizal fungal hyphae, ergosterol contents ranged from 0.86 mg to 17.55 mg per gram fungal dry weight, with an average of 5.45 mg (Satomura *et al*., 2006). This highly variable ergosterol content in the *T. matsutake* colony suggests that the hyphal distribution in the colony front edge is highly heterogenous. On the other hand, the high ergosterol content can mean the hyphae in the front continuously grow and may not be related to current fruiting, while energy and nutrients from the hyphal tissues of the previous year are used for fruiting.

These lower ergosterol contents in older colony strongly support the conclusion of Ekblad *et al.* (1998) that ergosterol as a component of membranes is considered to give a good measure of metabolic activity. They found that aging had a marked effect on ergosterol concentration and that ergosterol content of 7 month-old brown, shrunken Pine-*Paxillus* ectomycorrhizas was only 10% of white turgid 1 to 4 month old specimens.

Soil water change. During the fruiting season, the cumulative precipitation for the previous 10 days was 28.5 mm for the 31 August, 103.5 mm for the 17 September, 105.0 mm for the 1 October, 8.5 mm for the 14 October and 7.0 mm for the 28 October (Fig. 5). The changes soil water content followed the previous rainfall amount closely. Thus, soil water content was as high as 19% on 3 October with 105 mm rainfall within three days. However, rainfall five days before did not seem to affect the soil water on steep slopes or in a dense tree stand. The soil water content varied 12 to 19% at the ahead, 10 to 11% at the edge, 9 to 11% at the 20 cm back and 11 to 15% at the 40 cm back (Fig. 6 and Fig. 8). That is, the soil water change due to rainfall was lower in the colony front edge and 20 cm back points than in the ahead and the 40 cm back points.

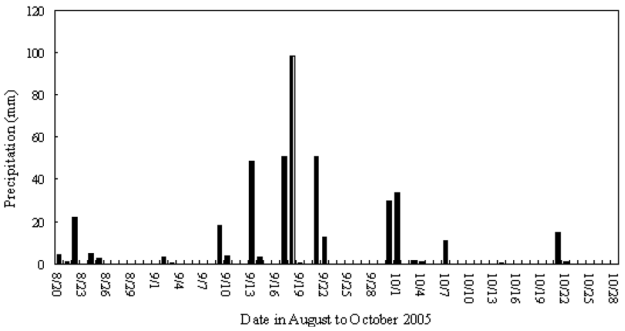

Fig. 5. Precipitation record at Chungju weather station near the Ssanggok Valley *Tricholoma matsutake* research site from 20 August to 28 October in 2005.

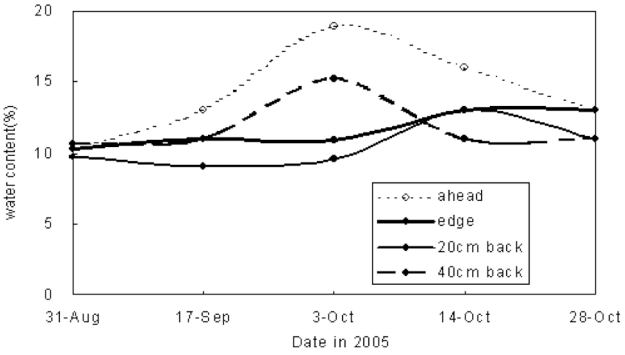

Fig. 6. Changes in water content within the *Tricholoma matsutake* colony at Ssanggok Valley in the Sogri Mt. National Park in 2005 (data from Koo *et al*., 2007).

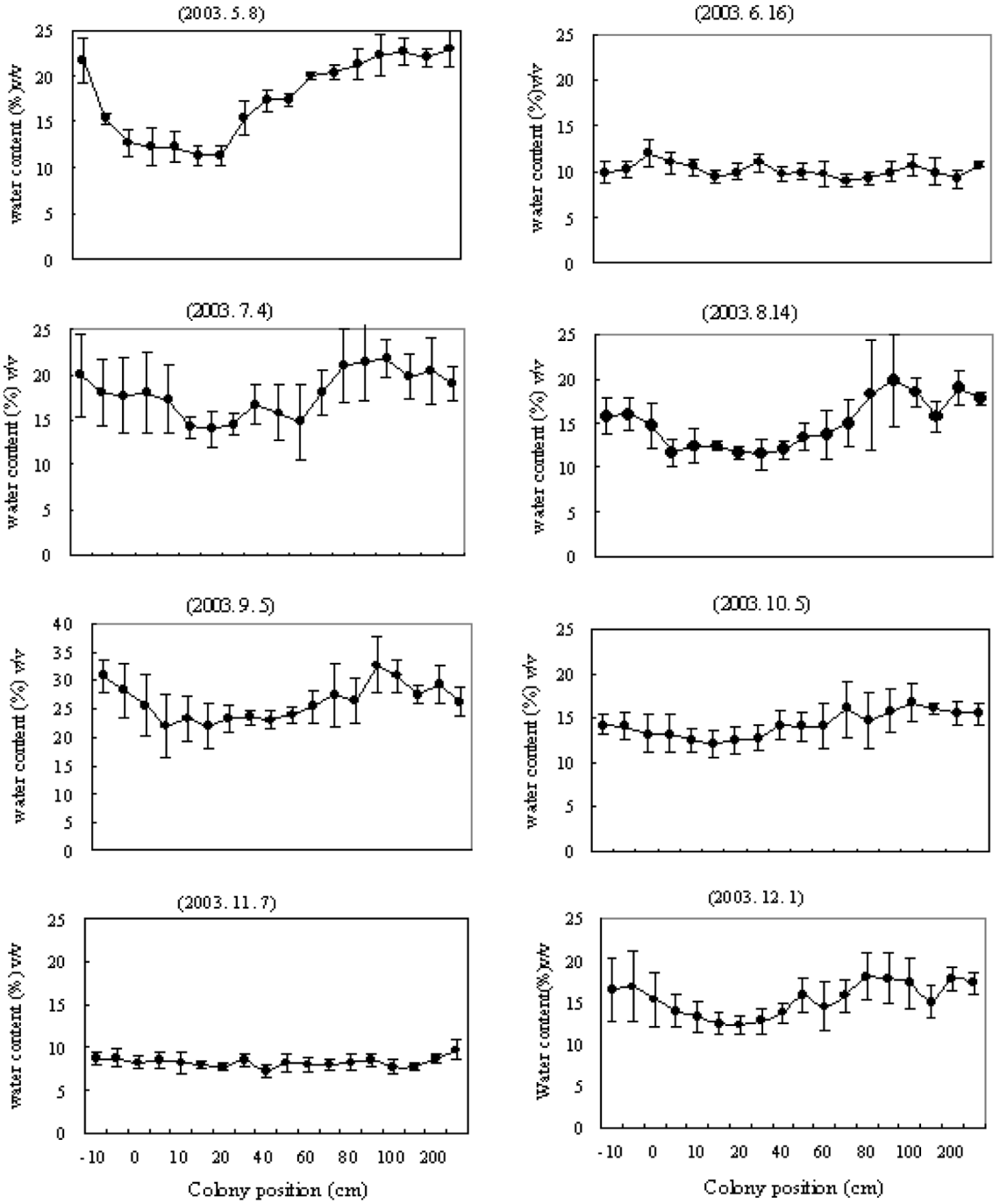

Fig. 7. Spatio-temporal water variation in *Tricholoma matsutake* soil colony positions from May to December 2003. Each point is the average of four replicates colonies. Error bars represent one standard deviation.

These changing patterns in ergosterol and water content in the *T. matsutake* colonies strongly support the hypothesis that active and dense *T. matsutake* colonies slowly absorb water and drain little, while the noncolonized soil and the decayed colonies absorb water little but drain more (Koo *et al*., 2003).

Thus, for sustainable *T. matsutake* production we suggest that the front edge of *T. matsutake* soil colony be managed intensively in terms of irrigation and *T. matsutake* ectomycorrhizal root development, because the colony front is the only live and growing body.

Generally, water content of *T. matsutake* soil colony changed depending on the rainfall, but the active colony portion was affected much less than the noncolony and the decayed portion of the colony. Water contents in the ahead and the 90 cm back (estimated to be 6 to 8 years old) were more variable, ranging from 7 to 32%, compared to the water contents in the 10 to 30 cm back (1 to

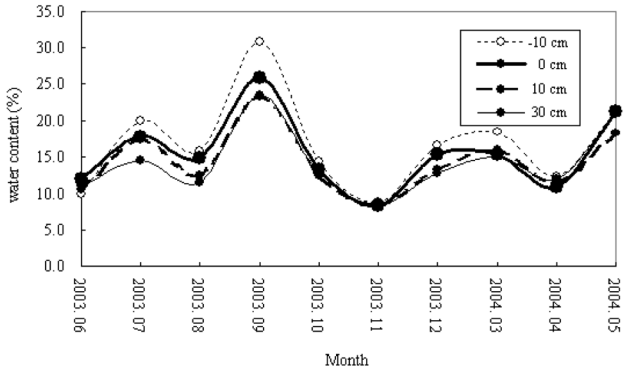

Fig. 8. Monthly water content changes in *Tricholoma matsutake* colony by positions at Ssanggok valley in the Sogri Mt. National Park during June 2003 to May 2004.

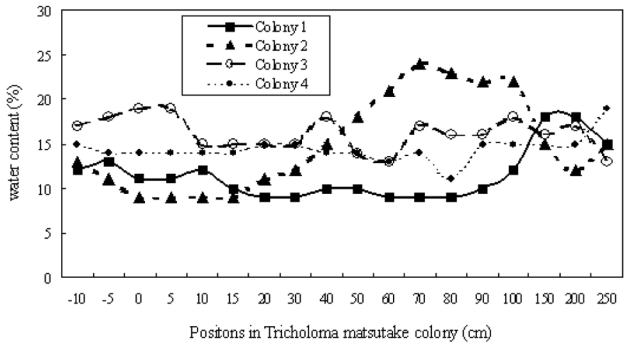

Fig. 9. Soil water content variation of four colony locations by distance from the colony front edge in *Tricholoma matsutake* soil colony.

2 years old colony), which ranged from 8 to 23% (Fig. 7). When it rained in September, the water content differed by colony points, with 16% at colony front and 32% at the ahead and 90 cm back (Fig. 7). In contrast, in the dry period in November, all the soil water contents ranged from 7 to 8% (Fig. 7, Fig. 8). The water content change patterns are quite similar to Hur *et al.* (2004), which showed that water content changed from 9.1 to 11.2% at the physiologically active *T. matsutake* hyphal zone, and from 11.7 to 19.5% at the noncolony during August to October.

On the other hand, soil water contents were highly variable among the four locations during the season (Fig. 9). The soil water within *T. matsutake* colony was lower at the front edge than at the other points. These variable water contents may be due to the heterogeneous forest soils which may have varied by microtopography, slope, soil texture, soil organic matter array, coarse rock debris, etc.

Soil temperature. Soil temperature in the *T. matsutake* colony ranged from 20.4 to 21.1°C on 31 August, and gradually decreased to 11.5 to 12.0°C on the 28th October in 2005 (data from Koo *et al.*, 2007). The 19.0°C, soil temperature that is known to form *T. matsutake* primordia (Owaga, 1991) arrived around mid September. Generally, the temperatures in *T. matsutake* colonies were from 0.2 to 0.4°C higher than in the ahead of colony. This slightly higher temperature may mean that *T. matsutake* mycorrhizae and hyphae are actively respiring and consuming water in the soil.

The front edge of *T. matsutake* colony is a specific point for high priority research. The most active front edge of the circular *T. matsutake* colony is only about 20 cm wide, and we suggest that this active part should be managed most significantly in terms of irrigation and *T. matsutake* ectomycorrhizal development. Understanding this narrow active zone in *T. matsutake* colony can improve irrigation efficiency and vegetation control effect. This understanding may increase the effectiveness of managing *T. matsutake* production.

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