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FULL PAPER

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Development and seasonal variations of *Lophodermium* populations on *Pinus thunbergii* needle litter

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Abstract A quantitative method was developed to describe *Lophodermium* (Rhytismataceae, Ascomycetes) populations on *Pinus thunbergii* needle litter, and seasonal patterns of population variation were examined based on this method. We focused on the formation of black zone lines across needles and the production of *Lophodermium* ascocarps on needle portions delimitated by zone lines. The study was carried out at a soil erosion control site on a mountain slope and at a coastal sand dune site. Most *Lophodermium* spp. on needle litter were identified as *L. pinastri* according to morphological characteristics of ascocarps and ascospores and DNA analysis. Zone lines were produced on needles when isolates of *L. pinastri* were in contact with other isolates of *L. pinastri* or with isolates of other species in dual culture tests. This observation provided a rationale to consider that individual colonies with ascocarps and delimitated by zone lines were occupied by a single *Lophodermium* isolate. Frequency of occurrence of *Lophodermium* colonies, total colony length, and mean colony number per needle were higher at the coastal sand dune site than at the soil erosion control site. Total colony length and mean colony number also varied with season. Mean colony length and mean ascocarp number per colony were not different between sites or seasons.

Key words Colony · Decomposition · *Lophodermium pinastri* · Pine · Zone line

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Introduction

Ecology of saprophytic microfungi on dead needles of conifers has been studied with reference to substrate succession (Kendrick and Burges 1962; Watson et al. 1974; Lehmann and Hudson 1977; Mitchell and Miller 1978; Soma and Saito 1979; Aoki et al. 1990, 1992; Ponge 1991; Tokumasu 1996, 1998; Tokumasu and Aoki 2002) and geographical distribution (van Maanen et al. 2000; Gourbiere et al. 2001; Iwamoto and Tokumasu 2001; Tokumasu 2001). In these studies, the abundance of microfungal species has been evaluated as a measure of frequency of occurrence that is calculated as the number of needles from which the species is isolated compared to the total number of needles tested, expressed as a percentage. This "qualitative" or "semiquantitative" description of fungal populations and communities is primarily associated with the inherent difficulties in quantitative description of fungal populations and communities, which makes it difficult to study the ecology of microfungi in the light of theories developed in general ecology.

Colonization of *Pinus thunbergii* needles by *Lophodermium* (Rhytismataceae, Ascomycetes) is a good system for studying the dynamics of fungal populations quantitatively because colonization is associated with the formation of black zone lines across the needle (Minter 1981) and because the production of fruiting bodies is helpful to identify fungi present in needle portions delimitated by zone lines by direct observation (Fig. 1). The system thus can be useful for the quantitative measurement of such parameters as the number of *Lophodermium* colonies per needle, length of the individual colony, and the number of ascocarps within the colony as well as frequency of occurrence. In this approach, it is assumed that a colony delimitated by zone lines was occupied by one individual fungal isolate and that the volume or length occupied by each colony reflected the mycelial abundance of the individual fungal isolate. This methodology has been utilized successfully in studies of population and community dynamics of wood decay fungi (Boddy 1992; Fukasawa et al. 2005) but rarely so in studies of litter-decomposing fungi.

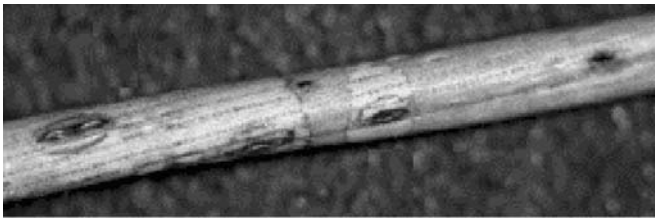

Fig. 1. *Lophodermium* colonies on *Pinus thunbergii* needle litter characterized by ascocarps and delimitated by *black zone lines*

The purpose of the present study is to provide a basis for a quantitative method to describe *Lophodermium* populations on *P. thunbergii* needles and to examine the seasonal patterns of populations based on this method. First, we performed morphological observations of ascocarps and ascospores of *Lophodermium* on *P. thunbergii* needles and DNA analysis of the isolates to identify the species, as *Lophodermium* populations on pine needles in Japan may include several species (Hata et al. 1998). Second, dual culture tests were performed to determine if zone lines appeared on dead needles as a result of mycelial contact between *Lophodermium* isolates or between a *Lophodermium* isolate and an isolate of other fungi. Finally, we examined seasonal changes of populations on needle litter to evaluate the applicability and usefulness of the quantitative method. Study sites were located at a soil erosion control site on a mountain slope and a coastal sand dune, both common sites for *P. thunbergii* plantations in Japan.

Materials and methods

Study site

Samples were collected from two *P. thunbergii* forest stands – a mountain slope site and a coastal sand dune site – in Japan. The mountain slope site was located within a *P. thunbergii* plantation on a soil erosion control site at Mt. Tanakami, Shiga, Japan (34°45′ N, 135°56′ E, 400 m above sea level). The plantation was on a gentle, southeast-facing forest slope. Mean annual temperature over 21 years was 12.1°C and mean annual precipitation was 1509 mm at Shigaraki Weather Station about 10 km from the site. The coastal sand dune site was located within a natural forest of *P. thunbergii* at Arid Land Research Center of Tottori University, Tottori, Japan (35°32′ N, 134°12′ E, 7 m above sea level). Mean annual temperature over 29 years was 14.6°C and mean annual precipitation 1898 mm at Tottori Weather Station about 5 km from the site. The meteorological records during the study period are shown in Fig. 2.

A study site of 10 × 4 m was laid out at the most representative location of each site. There was no replication by site type. The plots were divided into ten subplots of 2 × 2 m. The overstory consisted only of *P. thunbergii* at both plots.

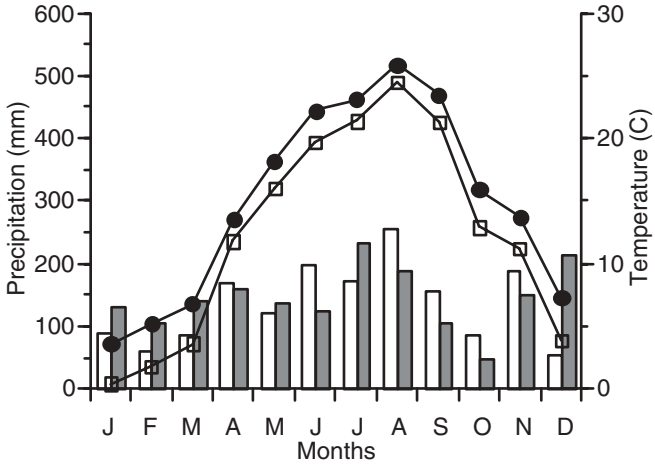

Fig. 2. Mean monthly temperature (*lines*) and monthly precipitation (*bars*) on the study sites during the study period. *Open boxes* and *open bars*, mountain slope site at Shiga; *filled circles* and *shaded bars*, coastal sand dune site at Tottori

Identification of *Lophodermium* species on *P. thunbergii* needle litter

Morphological observations were made on ascocarps and ascospores of *Lophodermium* on *P. thunbergii* needles. One hundred and six ascocarps from the mountain slope site and 92 ascocarps from the coastal sand dune site were used for the observations. Length and width of ascocarps, asci, and ascospores were recorded and compared with the description of Sakuyama (1993).

A molecular method was used to identify *Lophodermium* species. Fungi were isolated from *P. thunbergii* needle portions with *Lophodermium* ascocarps collected from the mountain slope and the coastal sand dune sites in May 2003. The surface sterilization method of Hata and Futai (1996) was used for the isolation. Consequently, 15 *Lophodermium*-like fungal isolates (7 isolates from the mountain slope site and 8 isolates from the coastal sand dune site) were obtained with reference to the culture description in Minter (1981). The DNA was extracted from mycelia of these isolates according to the methods of Gardes and Bruns (1993). The rDNA internal transcribed spacer (ITS) region was amplified using ITS-4 and ITS-5 primers (White et al. 1990). The polymerase chain reaction (PCR) products were digested with two enzymes, *Hinf* I and *Msp*I (Toyobo, Osaka, Japan), according to the method described in Matsuda and Hijii (1999), and the restriction fragment length polymorphism (RFLP) patterns of all isolates were obtained. The DNA base sequence of the ITS region of isolates, each isolate being from different RFLP types, was determined according to the method of Iwamoto et al. (2002). GenBank accession numbers for these data are AB247944, AB247945, AB247946, AB247947, AB247948, and AB247949. Sequenced isolates were compared with known species using BLAST searching.

Formation of zone lines

Dual culture tests were performed to see if zone lines appeared on needles as a result of mycelial contact between different *Lophodermium pinastri* (Schrad.) Chevall. isolates. The four isolates of *L. pinastri* (Lp1, Lp2, Lp3, and Lp4), already used in DNA sequencing, were used for the test. Inocula of the isolates were cut out from the margin of the mycelia on the previously inoculated petri dishes on 2% malt-extracted agar [MEA; malt extract 2% and agar 2% (w/v)] with a sterile cork borer (6 mm in diameter). Inocula from each of ten pairs of four isolates, including the pairs of the same isolate, were placed on the surface of 2% MEA in petri dishes (9 cm in diameter) at a distance of approximately 4 cm. Dead needles of *P. thunbergii* (2–3 cm in length) were autoclaved at 120°C for 20 min and were placed between the inocula. Three plates were prepared for each pair. The plates were incubated at 25°C in darkness and observed at a given interval. The formation of zone lines was observed under a binocular microscope with a magnification of 40×.

Other dual culture tests were performed to see if zone lines also appeared as a result of mycelial contact between a *L. pinastri* isolate and an isolate of another species. One *L. pinastri* isolate (Lp4) and three isolates (denoted as O1, O3, and O4) of unidentified species obtained from *P. thunbergii* needle litter with the surface sterilization method were used for the test according to the method described above. Five plates were prepared for each of the three pairs.

Sample collection for seasonal observation

Needle litter of *P. thunbergii* was collected from litter layers in the mountain slope site and the coastal sand dune site. Sampling took place four times: in May, August, and November 2003 and in February 2004. On each sampling occasion, litter layer material was collected from 20 subplots on two study sites using a 15×15 cm quadrat. Ten needles then were arbitrarily extracted from each quadrat, and a total of 200 needles were used for observation of *Lophodermium* colonies in each month at two study sites as described next.

Measurement of *Lophodermium* colonies

In the present study, we defined a colony of *Lophodermium* as bearing ascocarps and surrounded by black zone lines. Length of individual needles was recorded, and the following measurements were made under a binocular microscope with a magnification of 40×: number of colonies per needle, length of individual colonies, and number of ascocarps within a single colony. The data from ten needles in each quadrat were pooled and used as a replicate. Frequency of occurrence was calculated as a percentage of the ten needles from each quadrat that had *Lophodermium* colonies. Total colony length on needles was calculated as a sum of the length of individual colonies and expressed as a percentage of total needle length. Mean values were calculated for number of colonies per needle, length of individual colony, and number of ascocarps per colony.

Statistical analysis

Two-way analyses of variance were performed to evaluate differences in frequency of occurrence, total colony length, mean colony number per needle, mean colony length, and mean ascocarp number per colony, using site (mountain slope site, coastal sand dune site) and season (May, August, November, February) as independent variables. When comparing frequency of occurrence and total colony length, the arcsine transformation was used because the data were in the form of proportions. Spearman's rank correlation coefficients were calculated for the relationship between mean colony length and ascocarp number per colony.

Results and discussion

Identification of *Lophodermium* species

Ascocarps of *Lophodermium* measured 0.5–1.87 × 0.25–0.75 mm at the mountain slope site and 0.63–1.7 × 0.33–0.63 mm at the coastal sand dune site, with means of 1.18×0.49 mm and 1.02×0.48 mm, respectively. Length of asci and ascospores measured 87.5–142.5 µm and 62.5–130 µm, respectively, on the mountain slope site, and 105–125 µm and 92.5–100 µm, respectively, on the coastal sand dune site. Morphological observations indicated that these fungi mostly belonged to *L. pinastri* but included a few ascocarps of other species such as *L. conigenus* (Brunaud) Hilitzer. The homology analysis of DNA sequence of 15 test isolates with reference to the GenBank database indicated that all but 1 isolate showed a similarity to *L. pinastri.* One isolate originating from the sand dune site showed a similarity to *L. conigenus*. These results indicate that *Lophodermium* spp. on *P. thunbergii* needles in the study sites mostly consisted of *L. pinastri* but possibly included other species with low frequency. In the present study, thus, we did not distinguish species within *Lophodermium* and referred to the populations only as *Lophodermium* for the sake of simplicity.

Zone line formation and colony development

In the dual culture tests, black zone lines appeared on needles when an isolate of *L. pinastri* was in contact with any other isolate of *L. pinastri* or isolates of three other species (Fig. 3). No zone lines appeared when the same isolate was used for the test. These results suggest that (i) black zone lines on needles were produced as a result of mycelial contact between an isolate of *Lophodermium* and a different isolate, (ii) colonies delimitated by black zone lines were occupied by individual fungal isolates of *Lophodermium* or other species, and (iii) the volume or length occupied by each colony reflected the abundance of the individual fungal isolate. In the present study, therefore,

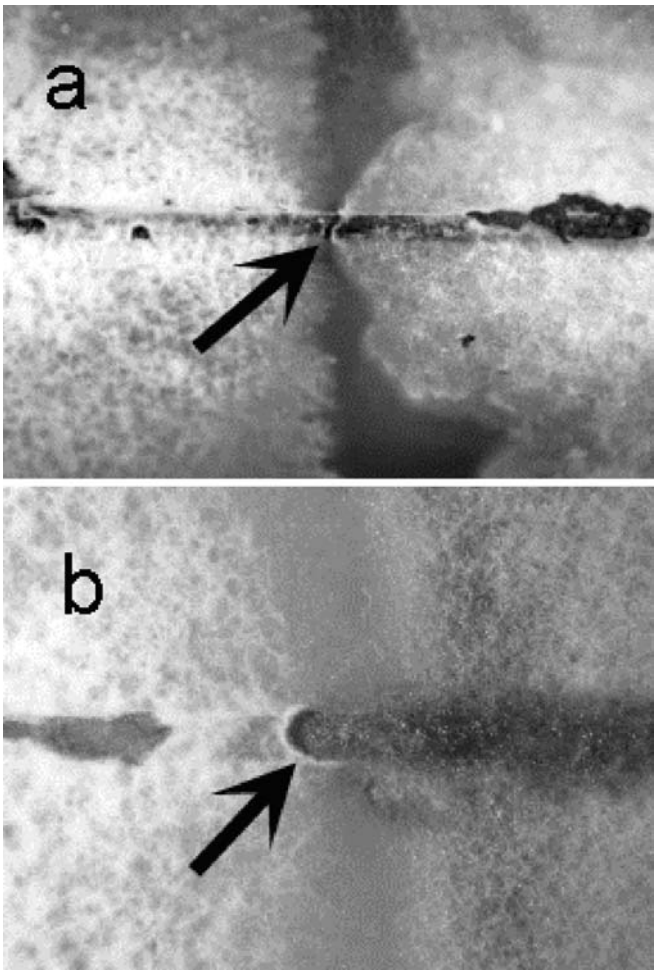

Fig. 3. Zone lines (*arrows*) appeared on needles in dual culture tests. **a** Zone line between two colonies of *Lophodermium pinastri* [Lp1 (*left*) and Lp2 (*right*)]. **b** Zone line between *L. pinastri* (Lp4, *left*) and unidentified species (O4, *right*)

colonies of *Lophodermium* were defined as the needle portion with their ascocarps and delimitated by black zone lines, as shown in Fig. 1.

Seasonal changes of *Lophodermium* colonization

Mean frequency of occurrence of *Lophodermium* ranged from 37% to 65% (Fig. 4a) and was significantly higher in the coastal sand dune site than in the mountain slope site (Table 1). Mean total colony length accounted for 23% to 51% of needle length (Fig. 4b) and was significantly higher in the coastal sand dune site than in the mountain slope site and significantly different between seasons (Table 1). Mean colony number per needle ranged from 2.0 to 7.0 (Fig. 4c) and was significantly higher in the coastal sand dune site than in the mountain slope site and significantly different between seasons (Table 1). The interaction of site and season was also significant for colony number per needle (Table 1). Mean colony length ranged from 11 to 22 mm (Fig. 4d) and was not significantly different between sites or seasons (Table 1). Mean ascocarp number per colony ranged from 2.6 to 3.4 (Fig. 4e) and was not significantly different between sites or seasons (see Table 1).

Table 1. Results of two-way analyses of variance evaluating the difference in *Lophodermium* colony parameters between sites and seasons

	F value		
	Site	Season	Site × season
Frequency of occurrence	6.5*	1.8 ns	1.7 ns
Total colony length	16.0***	3.3*	1.6 ns
Mean colony number per needle	8.8**	5.0**	2.9*
Mean colony length	2.7 ns	1.0 ns	0.4 ns
Mean ascocarp number per colony	0.2 ns	0.7 ns	1.0 ns

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, nonsignificant

The high frequency of occurrence and total colony length of *Lophodermium* on needles indicate that this fungus was a major taxon in fungal communities; this was more true in the coastal sand dune site than in the mountain slope site. The frequent occurrence of *Lophodermium* in recently dead needles already has been reported in previous studies (Kendrick and Burges 1962; Soma and Saito 1979; Tokumasu 1996, 1998). The results of the present study explicitly show the quantitative dominance of this fungus in terms of the amount of resource (needle) it occupied. It should be borne in mind, however, that the proportion of needle length occupied by *Lophodermium* was underestimated because *Lophodermium* colonies without ascocarps but with *Leptostroma* conidiomata were not taken into consideration in the present study (van Maanen et al. 2000). The determination of seasonal effects on total colony length and colony number per needle, which was not detected on frequency of occurrence, suggests that the quantitative method adopted in the present study is more sensitive to describe population dynamics of *Lophodermium* on *P. thunbergii* needles than the qualitative method.

The higher frequency of occurrence, total colony length, and colony number per needle in the coastal sand dune site than in the mountain slope site indicate a geographical difference in the level of *Lophodermium* colonization. The difference in colonization level is primarily ascribed to the higher colony number per needle in the coastal sand dune site, as the mean colony length is not different between the sites (Fig. 4d). The higher mean temperature and precipitation in the coastal sand dune site (see Fig. 1) would favor the colonization of needles by *Lophodermium*. van Maanen et al. (2000) also reported effects of temperature and precipitation on the frequency of *L. pinastri* on *P. sylvestris* needles in altitudinal transects.

Seasonal variations in the total colony length and colony number of *Lophodermium* are more obvious in the coastal sand dune site than in the mountain slope site, especially between May and August. Tokumasu (1998) reported a decrease in frequency of *Lophodermium* on *P. densiflora* needle litter during summer. The seasonal difference also can be partly ascribed to the unusual litterfall in the coastal sand dune site in June 2003 caused by strong winds associated with a typhoon, which resulted in an increased number

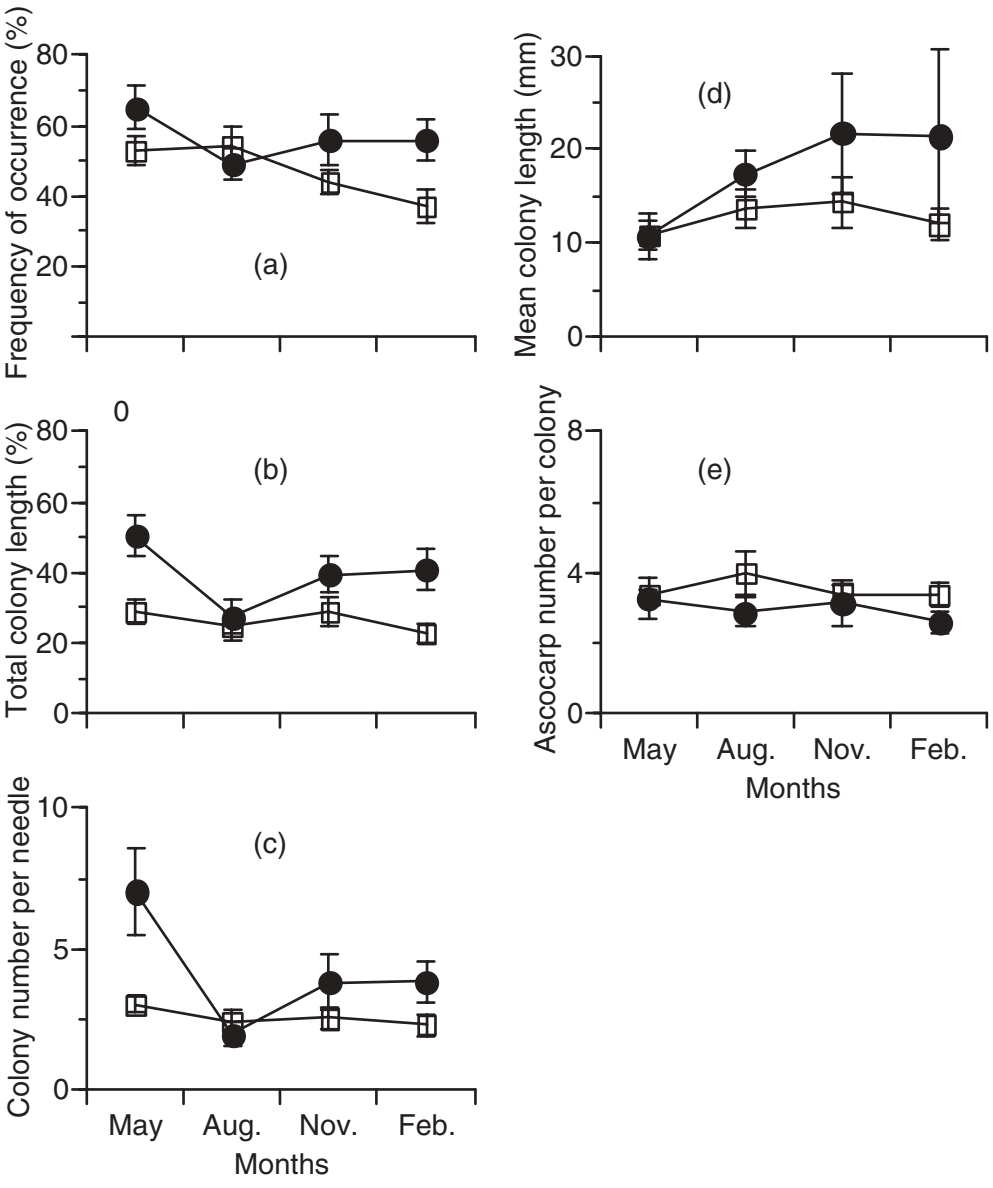

Fig. 4. Seasonal changes of frequency of occurrence (%) (**a**), total colony length (% total needle length) (**b**), mean colony number per needle (**c**), mean colony length (mm) (**d**), and mean ascocarp number per colony (**e**). *Open boxes*, mountain slope site; *filled circles*, coastal sand dune site. *Bars* indicate standard errors ($n = 10$)

of freshly fallen needles with premature *Lophodermium* ascocarps in the litter layer.

Mean colony length and ascocarp number per colony were relatively constant among seasons in the mountain slope site, whereas they were more variable seasonally in the coastal sand dune site, although the differences were not significant. The latter probably reflects the increased number of freshly fallen needles with immature *Lophodermium* colonies as a result of strong winds, as discussed earlier. Further studies are needed regarding the factors influencing the variation of colony size of *Lophodermium* populations and its consequences on their reproduction.

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