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FULL PAPER

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The seasonal occurrence of endophytic fungus, *Mycosphaerella buna*, in Japanese beech, *Fagus crenata*

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Abstract The seasonal occurrence of *Mycosphaerella buna* in leaves and contiguous organs of *Fagus crenata* was studied in a Japanese beech forest, Ibaraki, Japan, in 1998 and 1999. *Mycosphaerella buna* was not isolated from newly developed leaves in May, but it was isolated from asymptomatic leaves after June. The frequency of its occurrence gradually increased until leaffall. In contrast, *M. buna* was not isolated from overwintered buds, leaf petioles, or contiguous current-year twigs. The spermogonia and pseudothecia were observed in dead leaves after leaffall. The mature pseudothecia were found on dead leaves from May to July. The ascospores produced in the pseudothecia were suggested to infect newly developed leaves.

Key words Endophytic fungus · *Fagus crenata* · *Mycosphaerella buna* · Pseudothecia

Introduction

Many species of the genus *Mycosphaerella* are known to cause leaf spot on various trees, although some species are found only on the fallen leaves of deciduous trees (Ito and Kobayashi 1953; Katsuki 1965; Tomilin 1979; Katumoto 1983; Sivanesan 1983; Crous 1998; Vasilyeva 1998). *Mycosphaerella buna* R. Kaneko et Kakishima was described as a new species based on the pseudothecia found on overwintered fallen leaves of Japanese beech, *Fagus crenata* Blume, collected at Ogawa Forest Reserve, Kitaibaraki, Ibaraki Prefecture, Japan, and the fungus was also isolated from asymptomatic living leaves of *F. crenata* (Kaneko and Kakishima 2001). However, detailed information on the life cycle of this fungus is still lacking. For example, the route and timing of infection are not known.

To elucidate the life cycle of fungi with an endophytic stage, the seasonal occurrence of fungi on their host is basic information. In case of *F. crenata*, Sahashi et al. (1999) reported three dominant endophytic fungi in the Tohoku district of Japan and found that their occurrence differed among species in the organs of *F. crenata*.

Thus, we examined the seasonal occurrence of *M. buna* dwelling endophytically in *F. crenata* by isolation experiments of the fungus from asymptomatic living leaves, leaf petioles, and contiguous current-year twigs. We also observed the reproductive structures of this fungus on dead leaves. On the basis of these results, we discuss the life cycle of *M. buna*.

Materials and methods

Sampling

Samples of leaves and other organs of *F. crenata* were collected at Ogawa Forest Reserve (36°56′ N, 140°35′ E, 660 m above sea level), Kitaibaraki, Ibaraki Prefecture, Japan. The Ogawa Forest Reserve is an old-growth deciduous forest. The dominant canopy species in our experiment site are *F. crenata*, *Quercus crispula* Blume, and *Q. serrata* Murray (Masaki et al. 1992).

Nine beech trees (about 40 years old, 7–8 m in height, and 37–59 mm in breast height diameter, DBH) were used for sampling. Five twigs with asymptomatic leaves were excised from a tree at about 2 m above ground at intervals of approximately 40 days from May to October in 1998 and 1999. Thus, a total of 45 twigs with asymptomatic leaves were used for samples at each sampling time. Leaves from the samples of 1998 and leaves, leaf petioles, and contiguous

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current-year twigs from the samples of 1999 were used for isolation of fungi. Five twigs with dormant buds were excised from each of the same nine beeches (a total of 45 buds) on April 12, 1998 and April 25, 1999. The samples were stored in a refrigerator and were used for isolation within 48 h.

Dead leaves still attached to twigs were collected using four vinyl mesh bags and placed near the base of each tree on November 17, 1997 and November 11, 1998. A total of 20 leaves were collected from the four bags (5 leaves per bag) at the end of November of the same year and from April to October of the next year.

Isolation

For the isolation of *M. buna* from leaves, 7-mm-diameter disks were taken from four parts (top, middle, center, and base) of an asymptomatic living leaf with a cork borer (Fig. 1). A total of 45 leaf disks for each leaf part (1 leaf per twig) were used for isolation in 1998. In 1999, a total of 45 leaf disks from only the middle part of leaves were used because there were no significant differences in the frequency of occurrence of *M. buna* among leaf parts in 1998. For isolation from leaf petioles and current-year twigs, a 7-mm-long leaf petiole segment adjoining the sample leaf and a 7-mm-long current-year twig segment were cut from each sample twig. A total of 45 leaf petiole segments and current-year twig segments were used, respectively. For isolation from dormant buds, buds were separated into three parts: scales, young leaves, and young twigs in the scales (Fig. 1).

The procedures for surface sterilization are shown in Table 1. After surface sterilization, fungi were isolated with the methods previously reported (Kaneko and Kakishima 2001).

The frequency of occurrence (OF) of *M. buna* was calculated using the following formula:

$$OF\ (\%) = Ni/Nt \times 100$$

where Ni is the number of segments from which *M. buna* was isolated and Nt is the total number of segments investigated.

A statistical analysis of the OFs of *M. buna* isolated from each part (top, middle, center, or base) of leaves was made using one-factor ANOVA, following the normality test and Bartlett's test. The normality test was used to examine normality of the data and Bartlett's test was used to examine the equality of variances of the OFs among the four parts. One-factor ANOVA was used to examine equality of means of the OFs among the four parts (Yanai 1998).

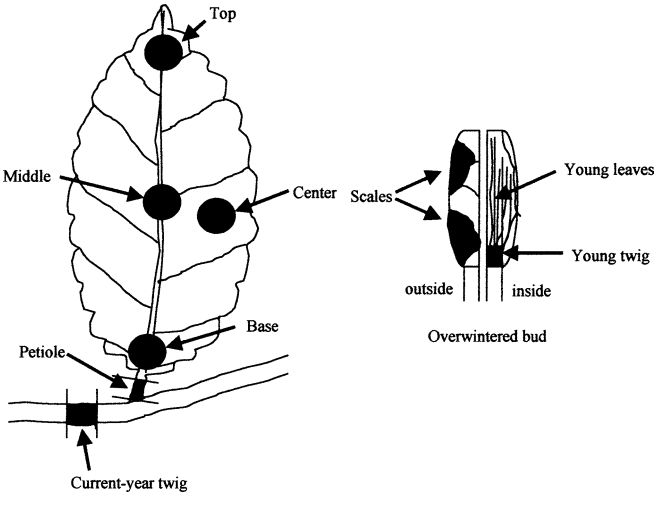

Fig. 1. Illustration of organs of *Fagus crenata*; *black areas* indicate parts used for isolation of *Mycosphaerella buna*

Morphological observation of spermogonia and pseudothecia of *M. buna* in dead leaves

Twenty dead leaves collected as described were used for morphological observations. Vertical sections of spermogonia and pseudothecia were prepared with a microtome equipped with a freezing unit or by hand. These sections were stained with lacto-fucshin or lacto-phenol for light microscopic observations. Germination of ascospores was observed to examine their maturity. The ascospores were cultured on 1% malt extract (Nissui) agar medium (MA) at 20°C in the darkness, and their germination rate was calculated after 24 h.

The frequency of occurrence (PF) of mature pseudothecia was calculated using the following formula:

$$PF\ (\%) = Pi/Pt \times 100$$

where Pi is the number of dead leaves having mature pseudothecia and Pt is the total number of dead leaves investigated.

Table 1. Procedures for surface sterilization of organs of *Fagus crenata*

Organs	Sterilizing agent and time(s)		
	Ethanol (70%)	Sodium hypochlorite Available Cl (1%)	Ethanol (70%)
Buds (rolled-up leaves, their bases)	30	60	30
Bud scales	60	120	30
Leaf disks just after unfolding	30	60	30
Leaf disks from leaves in growing season	60	120	30
Petiole segments	60	180	60
Twig segments	60	180	60

Results

Seasonal occurrence of *M. buna* in asymptomatic living leaves

In 1998, *M. buna* was not isolated from any part of the leaves on May 4, but the fungus occurred in all four leaf parts from June to October (Fig. 2). The OFs of the fungus in the middle and central parts of leaves gradually increased from June (13%, 18%) to October (58%, 40%), whereas the OFs in the top and basal parts of leaves were variable. The OF of the fungus in the top part decreased from June (16%) to July (9%), then increased in October (18%), whereas that in the basal part increased from June (27%) to July (49%), then decreased in September (22%) and increased again in October (33%).

The results of tests of normality showed that the P value of top, middle, center, and base leaf parts was 0.27, 0.15, 0.27, and 0.27, respectively, and thus the OF of the fungus from each part was regarded as a variable following nomal distribution. The result of Bartlett's test showed that the variances were not significantly heterogenous among the OFs from the four parts of living leaves (0.16, $P > 0.05$). According to these results, one-factor ANOVA was used to examine the difference among them. The result showed that there was no significant difference among the OFs of *M. buna* from the four parts of the leaves (0.18, $P > 0.05$).

Therefore, the OFs of the fungus in 1999 were examined concentrating on the middle part of leaves. Seasonal occurrence of the fungus on the middle part of the leaves in 1998 and 1999 is shown in Fig. 3. In both years, *M. buna* was not isolated from leaves in May, but the fungus occurred in June or July, and the OFs of the fungus gradually increased and reached 58% and 52% in October 1998 and 1999, respectively.

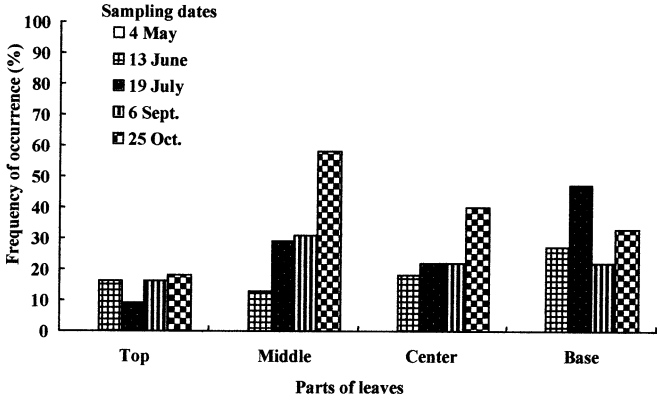

Fig. 2. Seasonal occurrence of *Mycosphaerella buna* in four parts of asymptomatic living leaves of *Fagus crenata* in 1998

Occurrence of *M. buna* in dead leaves

Spermogonia with spermatia were observed very often on dead leaves just after leaffall in November. However, they were rarely found on dead leaves in March and April.

In 1998, the asci formed in pseudothecia of *M. buna* on overwintered dead leaves collected on April 12 were still immature and ascospores were not found in their asci. Mature ascospores in pseudothecia were found on dead leaves collected from May to July, and the OF was highest on May 4 (100%) and then gradually decreased (Fig. 4). Ascospores were not observed in pseudothecia in the dead leaves after September. In 1999, the asci on the dead leaves collected on April 25 were still immature. Mature ascospores were found from May to July, and the OF was highest on May 18 (70%) and then decreased gradually (Fig. 4). Ascospores were not observed in pseudothecia after August. The germination rate of ascospores calculated in every sampling time was more than 90% from May to July.

Occurrence of *M. buna* in other organs

Mycosphaerella buna was not isolated from bud scales, young leaves, or young twigs in the scales in overwintered buds collected in 1998 and 1999. *Mycosphaerella buna* was also not isolated from either leaf petioles or current-year twigs collected from May to October, although the fungus was detected in the contiguous asymptomatic leaves collected simultaneously.

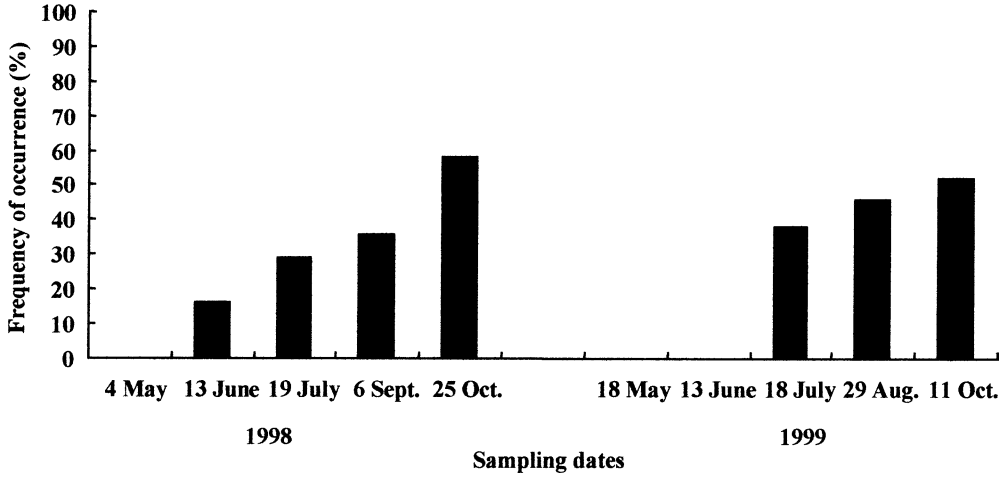

Fig. 3. Seasonal occurrence of *Mycosphaerella buna* in the middle part of asymptomatic living leaves of *Fagus crenata* in 1998 and 1999

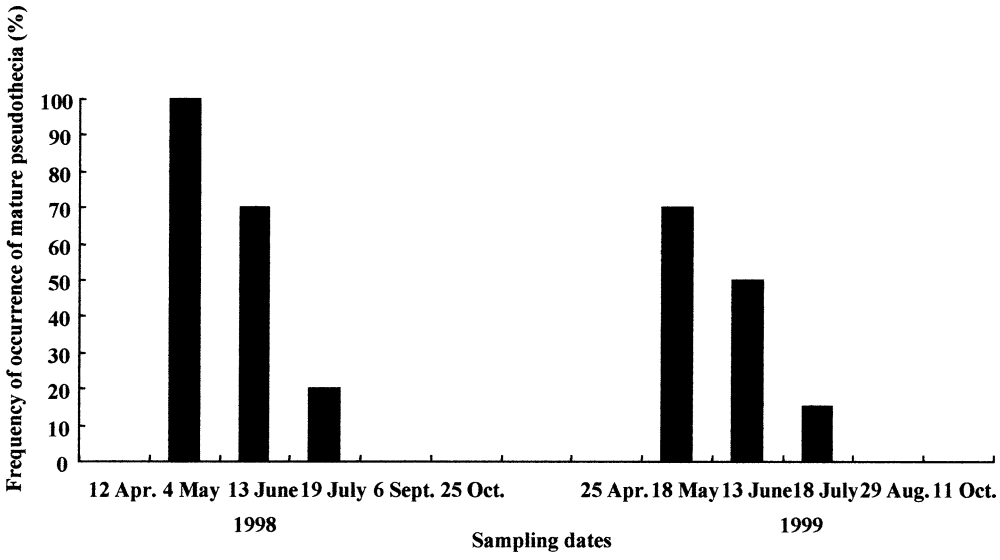

Fig. 4. Seasonal occurrence of mature pseudothecia of *Mycosphaerella buna* in overwintered dead leaves in 1998 and 1999

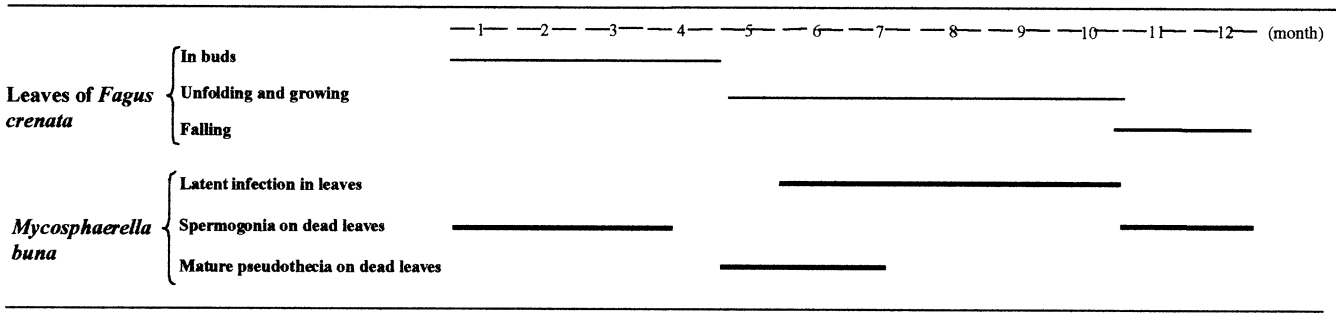

Fig. 5. Diagram of the relationships of the phenological events of *Mycosphaerella buna* and *Fagus crenata*

Discussion

A diagram of the relationships of the phenological events of *M. buna* and *F. crenata* is shown in Fig. 5. *Mycosphaerella buna* was not isolated from new leaves just after unfolding in May, but the fungus occurred in leaves after June. The OF of *M. buna* gradually increased until leaffall during growing season. Therefore, it was clarified that *M. buna* continued to exist latently within asymptomatic sound leaf tissues of *F. crenata* until leaffall. Similar seasonal changes have been reported in *Apiognomonia errabunda* (Rob.) Höhn. isolated from the healthy leaves of *F. sylvatica* L. (Sieber and Hugentobler 1987), *Discula quercina* (Westend.) Arx from the leaves of *Quercus garryana* Dougl. (Wilson and Carroll 1994) and a sterile fungus that seems to belong to the genus *Ascochyta* from the leaves of *F. crenata* (Sahashi et al. 1999).

Spermogonia were observed on dead leaves just after leaffall. However, mature pseudothecia were only observed on overwintered dead leaves from May to July, especially with a peak in May. In May, the leaves of *F. cerenata* newly unfolded. These results showed that the periods when leaves newly developed coincided with the periods when pseudothecia matured on overwintered dead leaves. Therefore, the ascospores produced in the pseudothecia were suggested to infect newly developed leaves. Such relationships between hosts and fungi have been reported in two species of *Mycosphaerella* that cause leaf diseases, *M. acanthopanacis* Sydow et Hara on *Kalopanax pictus* (Thunb.) Nakai (Sakamoto 1994) and *M. chaenomelis* Suto on *Chaenomeles sinensis* (Thouin) Koehne (Suto 1999) and *Gnomonia errabunda* C.P. Robin on *F. sylvatica* (Hogg and Hudson 1966). However, *M. buna* was not isolated from new leaves just after unfolding in May, although its ascospores had already matured on overwintered dead leaves. A similar time lag between fungal infection in newly developed leaves and maturity of the ascospores in fallen leaves has been reported (Hata 1996; Hogg 1966). Hogg (1966) suggested that there would be abundant spores on the leaves in May, but little, if any, mycelial growth within the leaves.

Mycosphaerella buna occurred in asymptomatic leaves of *F. crenata* without any significant difference among the four leaf parts (top, middle, center, base). It was reported that some endophytic fungi were isolated at high frequency from a specific part (Carroll et al. 1977; Carroll and Carroll 1978; Sieber and Hugentobler 1987; Dobranic et al. 1995; Hata 1996). *Diaporthe eres* Nitschke and *Bisporella* sp. occurred most frequently in the basal part and the central

parts, respectively, of leaves of *F. sylvatica* (Sieber and Hugentobler 1987). A species of *Phialocephala* occurred mainly in the basal parts of needles of *Pinus densiflora* Sieb. & Zucc. (Hata 1996). However, *A. errabunda* was isolated from all leaf parts of *F. sylvatica* (Sieber and Hugentobler 1987). The differences of these occurrences may be caused by the differences in the infection process of the fungi into the leaves, as suggested by Sieber and Hugentobler (1987), Hata (1996), and Sahashi et al. (1999).

The frequencies of dead leaves with mature pseudothecia in 1999 were comparatively lower than those in 1998. One possible reason is an extraordinary drought occurred from November 1998 to February 1999. The rate of precipitation in this season to that in average year was 1%, 23%, and 43% in November, January, and February, respectively, of the value in the nearest observation site of the Meteorological Agency (Mito Local Meteorological Observatory 1998–1999). The delay of the occurrence of *M. buna* in asymptomatic leaves in 1999 may have been caused by this reduction of mature pseudothecia of the fungus in the dead leaves.

In conclusion, the life cycle of *M. buna* in *F. crenata* indicated that the fungus infects living leaves by ascospores in pseudothcia produced on fallen leaves and exists in leaves until leaffall, then produces spermogonia on the fallen leaves immediately after falling, and matures as ascospores in pseudothecia on overwintered fallen leaves at the time when new leaves unfold.

Further studies should be undertaken to clarify the infection process of *M. buna* into leaves and also to clarify the function of the fungus in leaf tissues.

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