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FULL PAPER

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Colonization of Japanese beech leaves by phyllosphere fungi

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Abstract To examine the colonization of living leaves from buds and twigs by phyllosphere fungi of Japanese beech, the mycobiota were investigated on buds and twigs and on leaves covered with well-ventilated bags before budbreak. Ten phyllosphere fungi were isolated from rolled-up leaves within buds, bud scales, and twigs. However, frequencies of phyllosphere fungi on expanded leaves were reduced markedly when the buds were covered with well-ventilated bags before budbreak compared to the leaves that were not covered. This observation suggests that invasion of the fungi to the leaves from buds and twigs may be possible but is not the main route. Horizontal transmission may be common in endophytes and epiphytes of beech leaves. Phyllosphere mycobiota were then compared between sun and shade leaves. Of 13 species recorded as phyllosphere fungi, the frequencies of 2 species were lower and those of 3 species were higher in sun leaves than in shade leaves. Frequencies of the other 8 phyllosphere species were not different between sun and shade leaves. This result indicates that the colonization of leaves by some phyllosphere fungi was affected by the microenvironmental conditions on leaf surfaces.

Key words Bud · Endophyte · Epiphyte · *Fagus crenata* · Twig

Introduction

Phyllosphere fungi include those fungi that colonize the interior and surface of living leaves (Petrini 1991). Newly emerging leaves would be virgin, microbe-free habitat, and the majority of phyllosphere fungi of forest trees are transmitted horizontally (airborne spores or insect-dispersed spores) rather than vertically (buds and twigs or seeds) (Kinkel 1991; Petrini 1991). Some endophytic and epiphytic phyllosphere fungi have been demonstrated to infect by spores (Kinkel 1991; Osorio and Stephan 1991; Sahashi et al. 2000), but colonization from buds and twigs has also been suggested (Wildman and Parkinson 1979; Johnson and Whitney 1992; Toti et al. 1993; Sahashi et al. 1999). Manipulative experiments in which buds were covered with well-ventilated vinyl bags before budbreak may be useful to exclude the infection of airborne spores onto expanded leaves and to examine the invasion from buds and twigs.

Another aspect of colonization ecology of phyllosphere fungi relates to the microenvironmental conditions on leaf surfaces and physical, chemical, and phenological properties of leaves that affect the establishment of phyllosphere fungi on living leaves (Dix and Webster 1995). Sun and shade leaves are different in physical and chemical properties due to different light intensities (Kudo 1999) and hence may be different in their phyllosphere mycobiota. The differences in phyllosphere fungi between sun and shade leaves have, however, received little attention (Wilson et al. 1997; Wilson and Faeth 2001).

The purpose of the present study was to investigate the mycobiota on buds and twigs and on leaves covered with well-ventilated bags before budbreak to examine the fungal infection of living leaves from buds and twigs. A further aim was to compare the phyllosphere mycobiota between sun and shade leaves. Japanese beech (*Fagus crenata* Blume) was chosen as the material because it has already been used in studies evaluating phyllosphere mycobiota (Sahashi et al. 1999, 2000; Kaneko and Kakishima 2001; Osono 2002).

Materials and methods

Study area

Samples were collected in a cool temperate deciduous forest dominated by Japanese beech, in Ashiu Experimental

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Forest of Kyoto University (35°18′N and 135°43′E) about 40 km north of Kyoto, Japan. Details of the study site are described in Osono and Takeda (2001).

Collection of buds and twigs

Buds and twigs were collected from arbitrarily selected beech trees (about 20 m height) in the study site. Ten twigs were cut from 10 trees at about 5 m height on March 1998 and 4 twigs from 4 trees on November 1999. A total of 14 twigs were collected. From each twig, 5 buds and 5 bud-carrying twig segments (1 cm long) were taken, and a total of 70 buds and 70 twigs were used for surface sterilization method.

Leaves in ventilated bags

On April 2001 before budbreak, 6 twigs from 3 trees were selected at about 5 m height. Each twig was covered with a vinyl bag (20 × 15 cm) well ventilated with two pieces of membrane filter (Omnipore membrane filter; 0.2 μm mesh, 14.2 cm in diameter) on both sides. On August 2001, the 6 twigs covered with the vinyl bag and an additional 6 twigs that were not covered but were adjacent to the covered twigs were collected. The twigs were placed in paper bags and taken to the laboratory. From each twig 5 leaves were taken. Leaves in the well-ventilated bags were referred to as bag leaves and leaves outside the well-ventilated bags were referred to as control leaves. Two leaf disks were punched from each single leaf with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. One disk were used for a surface sterilization method and the other for a washing method. A total of 120 leaf disks were used for these methods.

Collection of sun and shade leaves

Sun and shade leaves were collected from 3 trees in September 2001. A total of 24 twigs (12 carrying sun leaves and 12 carrying shade leaves) were cut at about 2 m height. Sun leaves were located at the periphery of canopy and shade leaves were located near the main trunk and suppressed by leaves in the upper canopy. The twigs were placed in paper bags and taken to the laboratory. From each twig 5 leaves were taken, and 2 leaf disks were punched from each single leaf with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. One disk was used for a surface sterilization method and the other for a washing method. A total of 240 leaf disks was used for these methods.

The leaves were then oven-dried at 40°C for 1 week. Leaf mass and leaf area were measured, and leaf mass area (LMA) was calculated. The leaves were then combined and ground in a laboratory mill to pass a 0.5-mm screen. Nitrogen contents were estimated by automatic gas chromatography (NC analyzer; Sumitomo, Osaka, Japan). Polyphenol was extracted with 50% methanol (v/v) at 75°C for 60 min, and its content was estimated with the Folin–Ciocalteau method (Waterman and Mole 1994).

Fungal isolation

For the isolation of fungi, a surface sterilization method (Kinkel and Andrews 1988) and a modified washing method (Harley and Waid 1955) were used according to the methods described in Osono (2002). Isolation was carried out within 6 h of sampling.

For surface sterilization, samples were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface-sterilized for 1 min (living leaves), 3 min (buds), or 5 min (twigs) in a solution of 15% hydrogen peroxide (v/v) and submerged again for 1 min in 70% ethanol. The samples were rinsed with sterilized distilled water, transferred to sterile filter paper in Petri dishes (9 cm in diameter), and dried for 24 h after plating to suppress vigorous bacterial growth (Widden and Parkinson 1973). Each bud was then cut longitudinally and separated into outer bud scales and young rolled-up leaves within it. The samples were placed on 9-cm Petri dishes containing LCA (Miura and Kudo 1970), two (living leaves) or 5 samples (rolled-up leaves, bud scales, and twigs) per plate. LCA contains glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v). LCA was used because its low glucose content suppresses overgrowth of fast-growing species and because LCA effectively induces sporulation and is useful for identification (Osono and Takeda 1999).

For modified washing, leaf disks were washed in a sterile test tube using a vertical type shaker at 2000 rpm for 1.5 min to isolate fungi growing actively on the surface. The disks were washed serially in two changes of 0.005% Aerosol-OT (di-2-ethylhexyl sodium sulfosuccinate) solution (w/v) and rinsed with sterile distilled water four times. The washed disks were treated in the same manner as used in the plating-out procedure of the surface-sterilized leaves.

The plates were incubated at 20°C in darkness and observed at 3 days and at 2, 4, and 8 weeks (Osono and Takeda 1999). Any fungal hyphae or spores appearing on the plates were isolated onto fresh LCA plates and incubated and identified.

Definition and data reduction

Frequency of occurrence of a species was calculated as a percentage of the number of samples with the species to the total number of samples tested. When the frequency of a species on any leaf type was significantly ($P < 0.05$) higher than zero by Fisher's exact probability test, the species was regarded as frequent. Fisher's exact probability test was used when comparing the frequency of a species between sun leaves and shade leaves and between bag leaves and control leaves. The chi-square test was used when comparing the frequency of a species among rolled-up leaves, bud scales, and twigs.

Table 1. Frequency (%) of fungi isolated from rolled-up leaves, bud scales, and twigs of *Fagus crenata*

Fungus	Rolled-up leaves	Bud scales	Twigs	Probability
Phyllosphere fungi (frequent)				
Phomopsis sp.	1	19	97	***
Ascochyta sp.	13	54	29	***
Geniculosporium sp. 1	0	0	7	**
Phoma sp. 1	3	9	1	ns
Discula sp.	1	6	3	ns
Phyllosphere fungi (infrequent)				
Xylaria sp. (anamorph)	0	3	1	ns
Arthrinium sp.	0	0	3	ns
Pestalotiopsis sp. 3	0	0	3	ns
Alternaria alternata	0	0	1	ns
Nodulisporium sp. 3	0	0	1	ns
Other fungi				
Verticillium sp.	3	6	0	ns
Phoma sp. 2	3	0	1	ns
Coelomycete sp.	0	1	0	ns
Arthrinium phaeospermum	0	0	1	ns
Colletotrichum gloeosporioides	0	0	1	ns
Nigrospora sphaerica	0	0	1	ns
Arthroconidial fungus	0	0	1	ns
White sterile mycelium	0	0	1	ns

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

In this study, phyllosphere denotes the interior and surface of living leaves. Fungi isolated from living leaves were categorized into three groups: endophytes, epiphytes, and others. Endophytes were frequent species isolated from the phyllosphere by the surface sterilization method. Epiphytes were frequent species isolated from the phyllosphere by the washing method.

Fungi isolated from rolled-up leaves, bud scales, and twigs were first categorized into two groups: phyllosphere fungi and other fungi. Phyllosphere fungi included those fungi recorded as phyllosphere fungi of beech leaves according to Sahashi et al. (1999, 2000), Kaneko and Kakishima (2001), Osono (2002), and the result of the present study. Second, the phyllosphere fungi were divided into two groups: frequent species, which were frequent in either rolled-up leaves, bud scales, or twigs, and infrequent species that were frequent in none of these organs.

Results

Phyllosphere fungi on rolled-up leaves, bud scales, and twigs

Six, 7, and 16 fungal species were isolated from rolled-up leaves, bud scales, and twigs, respectively (Table 1). As frequencies of fungi were similar between the two sampling occasions, the results are combined and presented in Table 1. Phyllosphere fungi as a group accounted for 76%, 93%, and 94%, respectively, of the sum of frequency of all fungi isolated from these organs. Of 10 phyllosphere fungi isolated, 5 species were frequent on these organs. *Phomopsis* sp. was isolated from 97% of twigs examined and was also frequent on bud scales. *Ascochyta* sp. was frequent on bud scales, twigs, and rolled-up leaves. *Geniculosporium* sp. 1 was frequent in twigs. *Phoma* sp. 1 and *Discula* sp. were frequent in bud scales. An anamorphic state of *Xylaria* sp., *Arthrinium* sp., *Pestalotiopsis* sp. 3, *Alternaria alternata*, and *Nodulisporium* sp. 3 were isolated from rolled-up leaves, bud scales, or twigs with frequencies less than or equal to 3%.

Table 2. Frequency (%) of phyllosphere fungi isolated from leaves covered with well-ventilated bag (bag leaves) and leaves without coverage (control leaves) of *Fagus crenata*

Fungus	Bag leaves	Control leaves	Probability
Endophytes			
Xylaria sp. (anamorph)	0	40	***
Geniculosporium sp. 1	0	33	***
Epiphytes			
Ascochyta sp.	3	67	***
Pestalotiopsis sp. 1	3	27	*
Tritirachium oryzae	50	0	***

* $P < 0.05$; *** $P < 0.001$

Phyllosphere fungi on leaves covered with well-ventilated bag

No fungus was isolated from the interior of bag leaves, whereas 8 species were isolated from the interior of control leaves. Nine species were isolated from the surface of bag leaves and 31 from the surface of control leaves. Five species were recorded as phyllosphere fungi (Table 2). *Xylaria* sp. (anamorph), *Geniculosporium* sp. 1, *Ascochyta* sp., and *Pestalotiopsis* sp. 1, frequent in control leaves, were isolated from bag leaves with a frequency less than or equal to 3%. On the contrary, *Tritirachium oryzae* was isolated frequently and exclusively from bag leaves with a frequency of 50%.

Leaf mass area (LMA) and concentrations of nitrogen and total polyphenol of sun and shade leaves of beech are shown in Table 3. Sun leaves had higher LMA, lower concentration of nitrogen, and higher concentration of total polyphenol than shade leaves.

Seventeen and 12 species were isolated from the interior of sun and shade leaves, respectively. Twenty-nine and 34 species were isolated from the surface of sun and shade leaves, respectively. Thirteen species were recorded as phyllosphere fungi (Table 4). Three species frequent in the interior were regarded as endophytes and 11 species frequent on the surface were regarded as epiphytes. *Ascochyta* sp. was frequent on both habitats. The frequencies of *Ascochyta* sp. and *Arthrinium* sp. (anamorph of *Apiospora montagnei* Sacc.) were significantly lower in sun leaves than in shade leaves. The frequencies of *Pestalotiopsis* sp. 1, *Cladosporium cladosporioides*, and *A. alternata* were significantly higher in sun leaves than in shade leaves. The frequencies of the other 8 phyllosphere species were not significantly different between sun and shade leaves.

Discussion

Phyllosphere fungi colonized rolled-up leaves, bud scales, and twigs of beech, but their invasion to expanded leaves was reduced when buds were covered with vinyl bags before budbreak. These observations indicate the fungal invasion from buds and twigs may be possible but that airborne spores or insect-dispersed spores may play a more important role for infection. This finding is consistent with the results of Bayman et al. (1998), who reported horizontal transmission was more important than vertical transmission of xylariaceous endophytes of trees. Sahashi et al. (2000) reported an endophytic *Discula* sp. infected beech leaves with spores discharged from litter. It should be noted, however, that buds and twigs may also be important for infection to petioles, as Sahashi et al. (1999) reported high isolation frequency of *Phomopsis* sp. on petioles might result from hyphal invasion from adjacent twigs. *Tritirachium oryzae*, not detected in rolled-up leaves, bud scales, and twigs, colonized successfully on the surface of leaves covered with bags. These results suggest that the fungus was latent in rolled-up leaves, bud scales, or twigs but was not detected with the isolation method used in the present study and that the successful colonization of leaves by the fungus relied on the reduced immigration of spores of other phyllosphere species.

Sun and shade leaves of beech differed in LMA and concentrations of nitrogen and total polyphenol, which is consistent with previous reports (reviewed in Kudo 1999). Marked difference in LMA reflects the higher sunlight intensity on the surface of sun leaves than on shade leaves; this may also cause severe desiccation on the surface of sun leaves. *Ascochyta* sp. was reduced on the surface and failed to colonize in the interior of sun leaves, suggesting that its hyaline hyphae were sensitive to high sunlight intensity and severe desiccation on the surface of sun leaves. Conversely, the dematiaceous species *C. cladosporioides* and *A. alternata* were resistant to severe sunlight and desiccation, and survived on the surface of sun leaves due to the dark pigmentation (melanization) of the hyphal wall (Butler and Day 1998). Hyphal tips of *C. cladosporioides* and *A. alternata* also showed high tolerance to desiccation (Park 1982). These properties may provide increased competitive-

Table 3. Leaf mass area (LMA) and concentrations of nitrogen and total polyphenol of sun and shade leaves of *Fagus crenata*

Property	Sun leaves	Shade leaves	Probability
LMA (mg/cm^2)	11.0 (0.4)	4.7 (0.2)	***
Nitrogen (%)	1.6 (0.1)	1.9 (0.1)	***
Total polyphenol (%)	8.1 (0.2)	4.7 (0.6)	***

Standard errors in parentheses
*** $P < 0.001$

Table 4. Frequency (%) of phyllosphere fungi isolated from sun and shade leaves of *Fagus crenata*

Fungus	Sun leaves	Shade leaves	Probability
Endophytes			
Geniculosporium sp. 1	40	45	ns
Xylaria sp. (anamorph)	23	20	ns
Ascochyta sp.	18	40	**
Epiphytes			
Pestalotiopsis sp. 1	72	48	**
Cladosporium cladosporioides	63	43	*
Alternaria alternata	10	0	*
Clonostachys rosea	40	38	ns
Phoma sp. 1	10	17	ns
Trichoderma viride	12	13	ns
Coniothyrium sp.	7	12	ns
Cladosporium tenuissimum	2	8	ns
Pestalotiopsis sp. 2	8	2	ns
Ascochyta sp.	0	22	***
Arthrinium sp.	3	13	*

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

ness relative to other fungi on sun leaves and hence frequencies of these species. Difference in chemical properties between sun and shade leaves, induced by different light intensities, may also indirectly influence the availability of nutrients and competitive interactions between phyllosphere fungi (Dix and Webster 1995).

Our results indicated horizontal transmission may be common in endophytes and epiphytes of beech leaves, but that the pathway of fungal infection may be different between lamina and petioles. The present study also demonstrated the possible effect of microenvironmental conditions on the colonization of phyllosphere fungi on beech leaves. Leaf properties such as LMA and physical and chemical properties are highly influenced by, and change simultaneously with, microenvironments (Kudo 1999). Further studies are thus required to distinguish the effect of microenvironments from that of leaf traits on the colonization by phyllosphere fungi of living leaves of forest trees.

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