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FULL PAPER

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Seasonal and leaf age-dependent changes in occurrence of phyllosphere fungi of giant dogwood

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Abstract To examine the relative importance of leaf age and season on the occurrence of phyllosphere fungi, temporal patterns of epiphytic and endophytic phyllosphere fungi of giant dogwood (*Swida controversa*) were studied with reference to leaf emergence at first occurrence and in the middle of the growing season. A total of 15 and 44 species were isolated from the surface and interior of leaves, respectively. On the leaf surface, detection rate of fungi was consistently 100% and their frequencies increased during the growing season, whereas in the leaf interior, detection rate of fungi and their frequencies were low at leaf emergence and gradually increased during the growing season. Six epiphytic and two endophytic fungi were observed frequently. A white sterile mycelium was frequent only on the surface of newly emerged leaves in the first-order shoot in May. The other 7 species increased during the growing season. The frequencies of *Phomopsis* sp., *Pestalotiopsis* sp. 1, and *Trichoderma viride* were higher on the leaves of first-order shoots than those of higher-order shoots that emerged between July and September, suggesting the possible effects of leaf age on their occurrence. On the other hand, the frequencies of *Colletotrichum gloeosporioides*, *Clonostachys rosea*, *Cladosporium cladosporioides*, and *Phoma* sp. 1 were not different between the first- and higher-order shoots, suggesting the negligible effect of leaf age. The influence of phenological patterns of leaf emergence of deciduous trees on the diversity and composition of assemblages of phyllosphere fungi is discussed.

Key words Current-year shoot · Dogwood · Endophyte · Epiphyte · Season

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Introduction

Temporal dynamics of epiphytic and endophytic phyllosphere fungi on leaves of forest trees have been reported in evergreen and deciduous tree species (Petrini 1991; Carroll 1995; Dix and Webster 1995). Virtually no fungi were detected inside leaves just after leaf emergence, whereas fungi were readily detected on the surface of newly emerged leaves. Some phyllosphere fungi on evergreen leaves showed seasonal and leaf age-dependent variations (Ruscoe 1971; Cabral 1985; Wilson and Carroll 1994; Hata et al. 1998). Phyllosphere fungi on deciduous leaves increased over the growing season until leaf senescence, or increased initially and then decreased thereafter (Hogg and Hudson 1966; Wildman and Parkinson 1978; Breeze and Dix 1981; Sieber and Hugentobler 1987; Sahashi et al. 1999, 2000; Kaneko et al. 2003). In these studies of temporal patterns of phyllosphere fungi on deciduous trees, however, the age of leaves changed simultaneously with season, making it difficult to distinguish the relative importance of leaf age and season on the occurrence of phyllosphere fungi. More detailed studies are necessary that evaluate the temporal pattern of occurrence of fungi on deciduous leaves, separating the effect of leaf age and season.

Giant dogwood [*Swida controversa* (Hemsley) Sojak] is a deciduous tall tree of Cornaceae that is widely distributed in cool temperate regions of Japan, and some aspects of the phyllosphere mycobiota of this tree have already been studied (Osono et al. 2004a; Osono and Mori 2004; Osono 2005). Giant dogwood shows the flush + succeeding type of leaf emergence (Kikuzawa 1983) in which winter buds develop to first-order shoots in May, whose axillary buds sometimes elongate second-order shoots and produce new leaves in June. Such a shoot elongation pattern can give rise to higher-order shoots and new leaves under open conditions until September (Kodani and Togashi 1992, 1995). Therefore, current-year shoots of dogwood in the middle of the growing season include leaves of different age. Dogwood thus can be a suitable material to examine the seasonal and leaf age-dependent changes of phyllosphere fungi

as the effect of the leaf age can be separated from that of season by focusing on the phenological patterns of leaf emergence on current-year shoots.

In the present study, the temporal patterns of phyllosphere fungi on dogwood were analyzed with special reference to the flush + succeeding type of leaf emergence during the growing season from May to October. Fungi were isolated from the surface and interior of leaves by washing and surface sterilization methods, respectively. Frequencies of phyllosphere fungi were compared between shoot orders to examine the effects of leaf age. Based on the results, the influence of phenological patterns of leaf emergence of deciduous trees on the diversity and composition of assemblages of phyllosphere fungi is discussed.

Materials and methods

Study area

Samples were collected in a cool temperate deciduous forest dominated by Japanese beech (*Fagus crenata* Blume), in Ashiu Experimental Forest of Kyoto University (35°18′ N and 135°43′ E) about 40 km north of Kyoto, Japan. Details of the study area are described in Osono and Takeda (2001).

Seasonal patterns of shoot elongation and phyllosphere fungi

Five individual trees of dogwood were chosen along a forest road in the study area. Tree height was 8.5 m on average (range, 5.6–12.2 m), and mean diameter at breast height was 16.9 cm (9.4–27.1 cm). The five trees were not suppressed by neighbor trees and had two to three leaf layers within their crowns. The trees were located within an overall distance of 2 km.

In mid-April 2003, a total of five apical winter buds on the lowest leaf layers (2–3 m above the ground) were tagged per tree. At budbreak in early May, 7 to 11 leaves were expanded in the first-order shoots. Shoot elongation and leaf emergence were followed every month from early June to early October. The second-order shoots elongated in July and the third-order shoot in July and August. Several apical winter buds produced on current-year shoots developed new shoots in September. The number of leaves in each shoot was recorded, and one healthy looking, asymptomatic leaf was chosen in individual shoots that developed from the initial 25 winter buds. Two leaf discs were excised from each leaf with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. The discs were preserved in sterilized glass tubes (18 mm in diameter) and taken to the laboratory. One disc was surface sterilized and the other was subjected to a washing method. Fungal isolation was carried out within 6 h of sampling. The isolation of fungi was carried out from May to October. In November and December, most of the leaves were detached because of naturally occurring death and defoliation caused by zonate leaf blight (Osono et al. 2004b).

Fungal isolation

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 and Mori (2004).

Definition and data analysis

Detection rate was calculated as the number of leaf discs with the fungi divided by the total number of discs tested for each shoot each month, expressed as a percentage. Frequency of single species was calculated as the number of discs with the species divided by the total number of discs tested for each shoot each month, expressed as a percentage. The total number of leaf discs examined in each month was 11 to 25 in the first-order shoots, 8 to 11 in the second-order shoots, 5 to 7 in the third-order shoots, and 1 to 3 in the apical shoots. Frequencies of all species were summed to calculate sum of frequency for each shoot each month. When the frequency of a species on any shoot in any month was significantly higher ($P < 0.05$) than 0 by Fisher's exact probability test, the species was regarded as frequent and as a phyllosphere fungus.

A chi-square test was performed to examine the effect of season and shoot order on the frequency of phyllosphere fungi. The effect of shoot order represents the effect of leaf age. To examine the effect of season, the May + June, July + August, and September + October data were combined before the statistical analysis to satisfy the criteria for the chi-square test. The shoots collected in May and June were first-order shoots; those in June to October consisted of first- and higher-order (second-order, third-order, and apical) shoots. To examine the effect of shoot order, the frequency of phyllosphere fungi were compared between first- and higher-order shoots after the data of high-order shoots were combined to satisfy the criteria for the chi-square test. When the frequency of some phyllosphere fungi were low in first-order shoots in May and June, their frequencies in July to October were compared between first- and higher-order shoots.

Results

Detection rate and sum of frequency of fungi

A total of 15 and 44 species were isolated from the interior and surface of leaves, respectively (Tables 1, 2). Thirteen, 10, 10, and 3 species were isolated from the interior of leaf tissues of first-order, second-order, third-order, and apical shoots, respectively (Table 1). Thirty-five, 18, 18, and 3 species were isolated from the leaf surface, respectively (Table 2).

Table 1. Frequency (%) of fungi in the interior of leaves in first-order, second-order, third-order, and apical shoots

Fungus	First-order shoots (114)[a]	Second-order shoots (39)[a]	Third-order shoots (18)[a]	Apical shoots (4)[a]
Colletotrichum gloeosporioides	10	23	44	0
Phomopsis sp.	12	10	6	0
Aureobasidium sp. 1	11	0	0	0
Geniculosporium sp. 1	7	5	11	25
Xylaria sp. (anamorph)	5	5	17	0
Unidentified coelomycete species	7	13	0	0
Phoma sp. 1	3	10	6	0
Phyllosticta sp.	3	5	0	0
Pseudocercospora sp.	3	0	0	0
Unidentified ascomycete species	3	0	6	0
Arthrinium state of *Apiospora montagnei*	0	3	11	0
Nodulisporium sp.	1	0	0	25
Colletotrichum acutatum	0	0	6	25
Dark sterile mycelia	3	10	6	0
White sterile mycelia	2	10	6	0
Number of species	13	10	10	3

[a] Numbers in parentheses indicate the total number of leaves used for fungal isolation

Detection rate of fungi on the surface of dogwood leaves was 100% for every shoot every month (Fig. 1). In the interior of leaves, detection rate in first-order shoots was 0% to 4% in May and June and increased during July and August to reach 100% in September and October. Detection rate in second-order shoots was 9% in July and increased during August to reach 100% in September and October. Detection rate in third-order shoots was 60% in August and reached 100% in September and October. Detection rate in apical shoots was 67% in September and reached 100% in October.

Sum of frequency for all species isolated was consistently higher on the surface than in the interior (see Fig. 1). On the surface, sum of frequency in first-, second-, and third-order shoots was at a similar level (120%–170%) at leaf emergence. Sum of frequency increased to about 350% in September irrespective of the shoot order, and then decreased slightly in October. In the interior, sum of frequency increased from 0% to 4% in May and June to 175% in October in first-order shoots, from 9% in July to 175% in October in second-order shoots, and from 60% in August to 133% in October in third-order shoots. In apical shoots, sum of frequency on the surface and in the interior was between 67% and 167% in September and October.

Temporal patterns of phyllosphere fungi

Eight species were recorded as phyllosphere fungi (Fig. 2). Six species frequent on the surface were epiphytes and two species frequent in the interior were endophytes. These fungi were divided into three groups according to the occurrence patterns in relation to sampling season and shoot order (Fig. 2). Group I included a white sterile mycelium (here denoted as white sterile mycelium 1). This fungus was frequent on newly emerged leaves in first-order shoots in May and then disappeared thereafter. The frequency of this fungus was significantly higher in May–June than in the later months and significantly higher in first-order shoots than in higher-order shoots. The other seven species increased during the growing season, and their frequencies were significantly higher in July–August and (or) September–October than in May–June. These seven fungi were further divided into groups II and III according to the difference in frequency between the first-order and higher-order shoots during July to October. Group II included *Phomopsis* sp., *Pestalotiopsis* sp. 1, and *Trichoderma viride*, the frequencies of which were significantly higher in first-order shoots than in higher-order shoots during July to October. Group III included *Colletotrichum gloeosporioides*, *Clonostachys rosea*, *Cladosporium cladosporioides*, and *Phoma* sp. 1, whose frequencies were not significantly different between first- and higher-order shoots during July to October.

Discussion

The temporal patterns of occurrence of fungi were different between the surface and the interior of leaf tissues (see Fig. 1). Detection rate on the surface was consistently 100%, and sum of frequency increased during the growing season, indicating that the arrival of epiphytic fungi began soon after leaf emergence and occurred continuously and cumulatively over the growing season. In the leaf interior, detection rate and sum of frequency was low at leaf emergence and increased during the growing season. This result indicates that the infection of endophytic fungi was restricted to small areas inside of leaves and that the number of these small patches increased during the growing season. This mode of infection has been reported for *Rhabdocline parkeri* endophytic in Douglas-fir needles (Stone 1987). Infections by *R. parkeri* were confined to single epidermal cells, and the frequency of infection increased with needle

Table 2. Frequency (%) of fungi on the surface of leaves in first-order, second-order, third-order, and apical shoots

Fungus	First-order shoots (114)[a]	Second-order shoots (39)[a]	Third-order shoots (18)[a]	Apical shoots (4)[a]
White sterile mycelium 1	17	3	0	0
Cladosporium cladosporioides	43	51	53	50
Pestalotiopsis sp. 1	25	23	12	0
Trichoderma viride	15	10	12	0
Phoma sp. 1	46	79	53	75
Clonostachys rosea	11	36	29	25
Alternaria alternata	10	3	0	0
Phomopsis sp.	9	5	12	0
Epicoccum nigrum	7	0	0	0
Aureobasidium sp. 2	6	0	0	0
Arthrinium state of *Apiospora montagnei*	5	3	12	0
Coniothyrium sp.	4	0	0	0
Unidentified coelomycete species	7	8	12	0
Ascochyta sp.	4	0	0	0
Colletotrichum gloeosporioides	3	13	18	0
Pseudocercospora sp.	3	0	0	0
Lecanicillium psalliotae	3	0	0	0
Acremonium spp.	3	0	0	0
Penicillium glabrum	2	0	6	0
Penicillium citrinum	2	0	0	0
Pestalotiopsis sp. 3	1	0	6	0
Unidentified hyphomycete species	1	3	6	0
Lecanicillium lecanii	1	0	0	0
Ulocladium sp.	1	0	0	0
Pochonia suchlasporia	1	0	0	0
Septonema ochracea	1	0	0	0
Fusarium graminearum	1	0	0	0
Cladosporium tenuissimum	1	0	0	0
Trichoderma koningii	1	0	0	0
Cylindrocarpon state of *Nectria radicicola*	1	0	0	0
Volutella sp.	1	0	0	0
Nigrospora state of *Khuskia oryzae*	1	0	0	0
Fusarium solani	1	0	0	0
Trichoderma sp.	1	0	0	0
Monochaetia sp.	0	3	0	0
Acremonium kiliense	0	3	0	0
Trichoderma pseudokoningii	0	3	0	0
Fusarium sp.	0	3	0	0
Colletotrichum acutatum	0	3	6	0
Tritirachium oryzae	0	0	12	0
Geniculosporium sp. 1	0	0	6	0
Phyllosticta sp.	0	0	6	0
Dark sterile mycelia	0	5	6	0
White sterile mycelia	1	0	12	0
Number of species	35	18	18	3

[a] Numbers in parentheses indicate the total number of leaves used for fungal isolation

Table 3. Frequency (%) of fungi in group III[a] on leaves of giant dogwood and Japanese beech[b]

Fungus	Endophyte/epiphyte	Dogwood	Beech	Probability[c]
Colletotrichum gloeosporioides	Endophyte	41	0	***
Clonostachys rosea	Epiphyte	13	19	ns
Cladosporium cladosporioides	Epiphyte	59	33	***
Phoma sp. 1	Epiphyte	75	41	***

[a] For group III, see text
[b] Phyllosphere mycobiota of dogwood (Osono et al. 2004a) and beech (Osono 2002) were examined simultaneously at monthly intervals between May and December 1999
[c] The difference was tested using Fisher's exact probability test
*** $P < 0.001$; ns, not significant

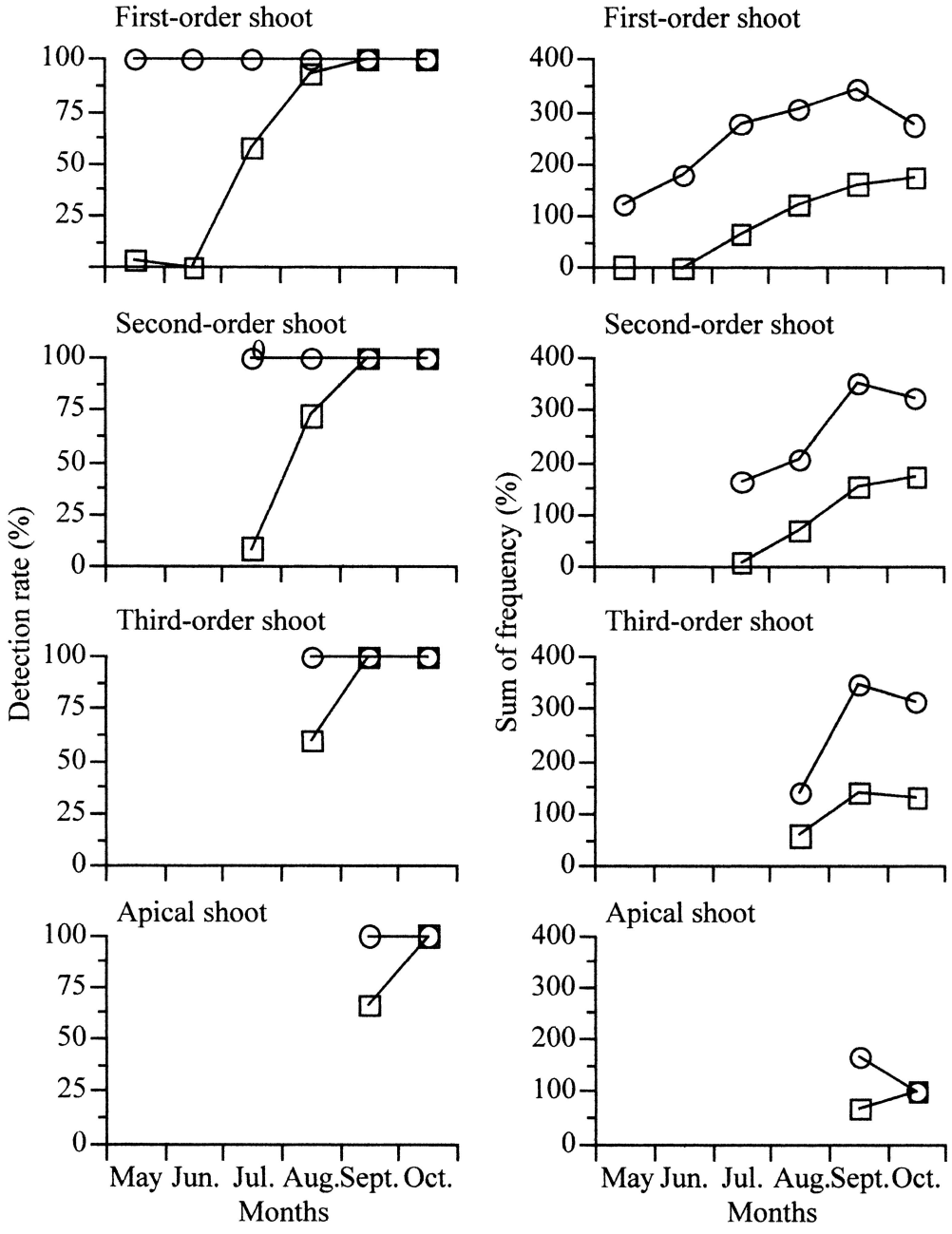

Fig. 1. Temporal changes in detection rate (*left*) and sum of frequency of fungi (*right*) on leaves in first-order, second-order, third-order, and apical shoots. ○, surface; □, interior

age. Detection rate in the interior of newly emerged leaves was higher in higher-order shoots than in first-order shoots (see Fig. 1), suggesting that endophytic fungi had higher potential to infect leaves that emerged later during the growing season.

Eight species were recorded as phyllosphere fungi (see Fig. 2). Seven of the 8 species except the white sterile mycelium 1 were common to the previous investigations (Osono et al. 2004a; Osono and Mori 2004). The short period of occurrence of the white sterile mycelium 1 in May suggests that the fungus infected from dormant fungal structures in bud scales or bark tissues. Alternatively, production of airborne spores of this fungus may be especially high in May. A similar pattern of occurrence was reported on *Discula* sp. endophytic in leaves of Japanese beech. Spores of this fungus were released for a very short time in late May that corresponded to the emergence of new leaves (Sahashi et al. 1999, 2000). Another explanation may be that the fungus was excluded from leaf surfaces by competition with other phyllosphere fungi or by microenvironments on the leaf surfaces unsuitable for the fungus.

The frequencies of the other seven fungi increased during the growing season to reach a maximum between August and October (see Fig. 2). This pattern of occurrence has been reported for many phyllosphere fungi (Hogg and Hudson 1966; Wildman and Parkinson 1978; Breeze and Dix 1981; Sieber and Hugentobler 1987; Sahashi et al. 1999, 2000; Kaneko et al. 2003). These phyllosphere fungi behaved in two different ways, depending on the effect of shoot order (i.e., leaf age). The higher frequencies of group

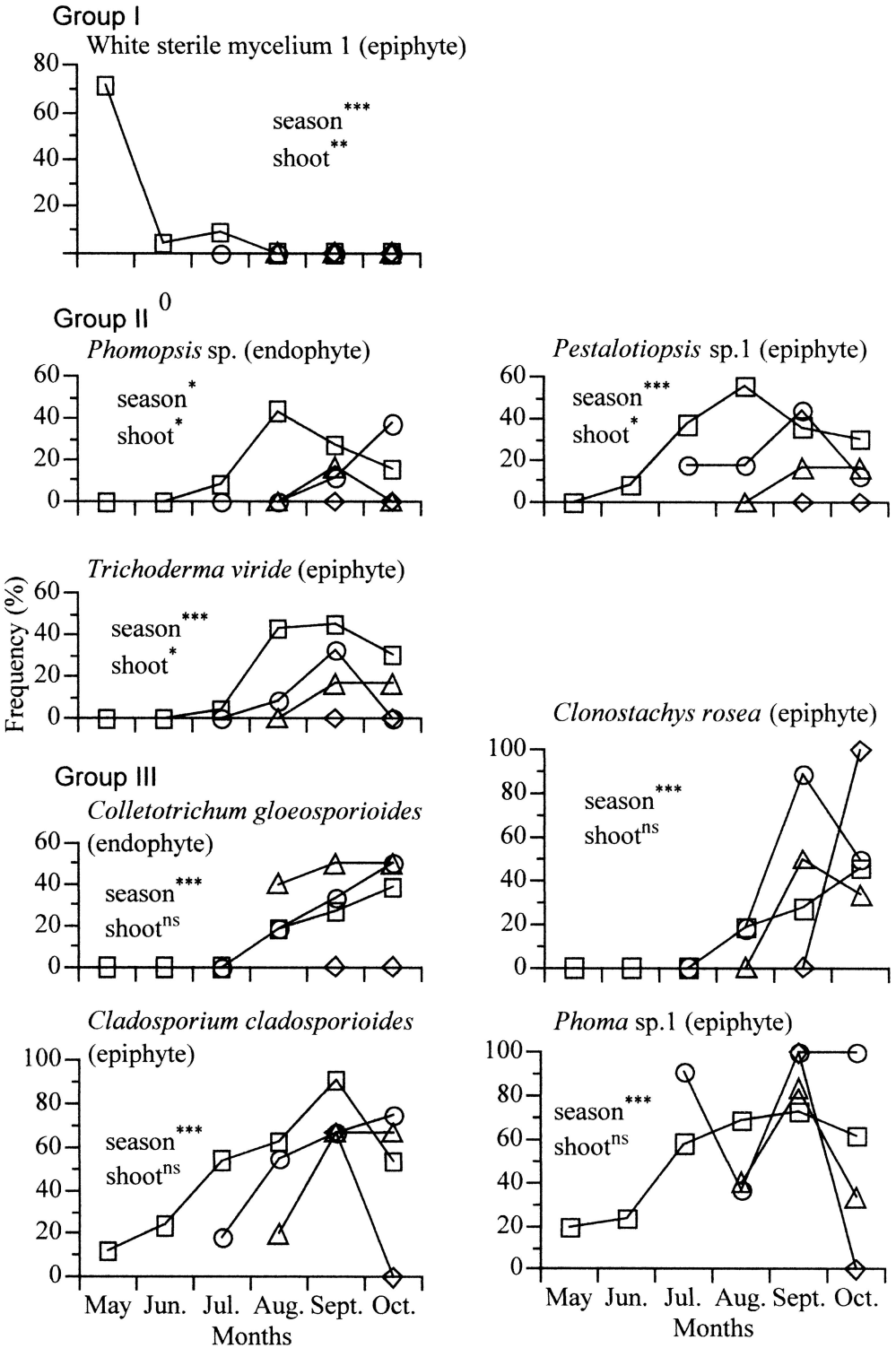

Fig. 2. Temporal changes in the frequency of phyllosphere fungi. The phyllosphere fungi were divided into three groups (I, II, III) according to the occurrence patterns in relation to season and shoot order (see text). The differences in frequencies among seasons (May + June vs. July + August vs. September + October) and between shoot orders (first- vs. higher-order shoots) were tested using a chi-square test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; *ns*, not significant; □, first-order shoots; ○, second-order shoots; △, third-order shoots; ◇, apical shoots

II fungi in first-order shoot than in higher-order shoots suggest that their colonization was proportional to leaf age and/or that their potential to colonize leaves was lower later in the growing season. On the other hand, the frequencies of group III fungi were not different between first- and higher-order shoots, suggesting the negligible effect of leaf age and the high potential to colonize leaves over the growing season.

The higher potential of group III fungi to colonize the phyllosphere between July and October suggests the higher chance for these fungi to colonize leaves of deciduous trees that expand additional leaves later in the growing season, such as dogwood, than those of other trees that expand leaves in spring, after which no more new leaves appear, such as beech (Kikuzawa 1983). In fact, the frequencies of three fungi in group III over the growing season were

higher in dogwood than in beech (Table 3). Other factors such as host specificity may affect the frequencies, but this difference is partly attributable to the difference in the pattern of leaf emergence between dogwood and beech. This suggestion is, however, based only on the 1-year observation of a limited number of hosts and, to our knowledge, no previous studies of temporal patterns of epiphytic and endophytic phyllosphere fungi have considered the phenology of leaf emergence of deciduous trees. Thus, further studies are needed that compare the temporal patterns of phyllosphere fungi on trees with different types of leaf emergence.

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