FULL PAPER

Takashi Osono

Endophytic and epiphytic phyllosphere fungi of red-osier dogwood (*Cornus stolonifera*) in British Columbia

Abstract Endophytic and epiphytic phyllosphere fungi associated with red-osier dogwood (*Cornus stolonifera*), a deciduous shrub, were examined in coastal British Columbia, Canada. Current-year shoots were divided into four types based on the absence or presence of inflorescence and secondary elongated shoots at the apex of primary shoots. Leaves on these shoots were then classified into six categories so as to examine the effect of flowering, secondary shoot elongation, and shoot order within current-year shoots on the occurrence of phyllosphere fungi. Species composition of fungi was markedly different between the interior and surface of leaves, whereas it was relatively similar among the six leaf categories in the interior or on the surface. Frequencies of the eight major species were not different between leaves on flowering and nonflowering shoots. The frequency of *Colletotrichum gloeosporioides* in the leaf interior was greater on leaves on the primary shoots that elongated the secondary shoots than on those that did not, and was greater on leaves on the primary shoots than on those on the secondary shoots. On the other hand, secondary shoot elongation and shoot order had no effect on the frequencies of *C. gloeosporioides* and the other seven epiphytes on leaf surfaces.

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

Introduction

Phyllosphere fungi include endophytes and epiphytes that colonize the interior and surface, respectively, of living leaves (Petrini 1991). The ecology of phyllosphere fungi on giant dogwood; [*Swida controversa* (Hemsley) Sojak, Cornaceae] has been reported in Japan, with reference to their distribution within the canopy (Osono and Mori 2004), seasonal and leaf age-dependent changes (Osono and Mori 2005), interactions with a pathogenic fungus (Osono et al. 2004b; Osono 2006), and occurrence and succession on decomposing leaves (Osono et al. 2004a; Osono 2005). Fungi associated with dogwood in North America have been extensively studied with reference to foliar diseases such as dogwood anthracnose, a widespread fungal disease caused by *Discula destructiva* Redlin (Redlin 1991; Margery et al. 1996). To my knowledge, however, there have been no reports on the phyllosphere fungi associated with healthy-looking leaves of dogwood in North America.

The purpose of the present study was to examine the endophytic and epiphytic phyllosphere fungi associated with red-osier dogwood (*Cornus stolonifera* Michx, syn. *C. sericea* L.) in coastal British Columbia, Canada. *Cornus stolonifera* is a deciduous shrub, 1 to 6 m tall, occurring in moist habitats and at forest edges. Preliminary observations indicated that some current-year shoots of *C. stolonifera* produced inflorescence and secondary elongated shoots at the apex of primary shoots developed from winter buds. The production of inflorescence and secondary shoots in the middle of the growing season often requires the translocation of assimilated photosynthates from older leaves on the current-year shoots to younger leaves (Akao et al. 1981; Whiley and Schaffer 1993; Hasegawa et al. 2003), which may cause changes in the nutrient content of older leaves and affect the colonization of the leaves by phyllosphere fungi. Furthermore, frequency of occurrence of phyllosphere fungi may be different between leaves on the primary shoots and the secondary elongated shoots within current-year shoots because of the difference in physical and chemical properties and/or leaf age (Osono and Mori 2004, 2005). Therefore, the further purpose of the present study was to evaluate the effect of flowering, secondary shoot elongation, and shoot order within current-year shoots on the occurrence of phyllosphere fungi.

T. Osono (✉)
Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, Victoria, Canada

Present address:
Laboratory of Forest Ecology, Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
Tel. +81-75-753-6079; Fax +81-75-753-6080
e-mail: fujijun@kais.kyoto-u.ac.jp

Materials and methods

Study site

Samples were collected at the vicinity of Pacific Forestry Centre, Canadian Forest Service, in Victoria, British Columbia, Canada (48°27′ N and 123°2′ W, 23 m a.s.l.). Mean annual temperature is 10.0°C, and mean annual precipitation was 834 mm over a 22-year period.

Categorization of current-year shoots and collection of leaves

In the study site, many-stemmed bushes of *C. stolonifera* (approximately 2 m in height) were distributed along creek banks. In September 2005, ten ramets were selected arbitrarily for the study. These ramets were adjacent to each other and suppressed by neighbor overstory trees.

Phenology of current-year shoots was observed preliminarily during the growing season of 2005. *Cornus stolonifera* elongated primary shoots from winter buds in March to April and produced numerous small flowers in dense terminal clusters (inflorescence) at the apex of some of these primary shoots. In the middle of the growing season, some flowering shoots expanded additional leaves on secondary shoots elongated from lateral buds at the apex of primary shoots. Some nonflowering primary shoots also produced additional leaves successively at their apex in the middle of the growing season. Therefore, current-year shoots could be divided into four types based on the combination of the absence/presence of inflorescence (F−/F+) and secondary elongated shoots (E−/E+) at the apex of primary shoots (Figs. 1, 2). The first type included those current-year shoots that had no inflorescence (denoted in this study as F−) and consisted of primary shoots only (denoted as E−); these shoots were denoted as F−E− (Fig. 1a). The second type (F+E−) included those current-year shoots that had inflorescence (F+) and consisted of the primary shoots only (E−) (Fig. 1b). The third type (F−E+) included those that had no inflorescence (F−) and expanded young leaves successively (E+) (Fig. 1c). The fourth type (F+E+) included those that had inflorescence (F+) and elongated one or two secondary shoots from the lateral buds at the apex of the primary shoots (E+) (Fig. 1d). Some of these secondary elongated shoots had inflorescence at their apex, but the presence of inflorescence on the secondary shoots was not taken into account in this study. The successive expansion of young leaves in F−E+ shoots was different in their origin from the leaf expansion on the secondary shoots in F+E+ shoots. In this study, however, I regarded F−E+ and F+E+ shoots as the same "treatment in natural experiment" in terms of the production of younger leaves later in the growing season than leaves on the primary shoots.

The ten ramets had a total of 489 current-year shoots, and 298 (81%) of these belonged to the F−E− type (Table 1). A chi-square test indicated the significantly greater number of F−E− shoots in this sample population. Most of these F−E− shoots were produced laterally on the previous-year shoots. Excluding these laterally produced shoots and only including shoots apically produced on previous-year shoots yielded a more evenly distributed sample population of current-year shoots, but the significantly greater number of F−E− shoots was still evident (see Table 1). In this study,

Fig. 1. Current-year shoot of *Cornus stolonifera*. **a** Shoot without inflorescence (F−) and without secondary shoot elongation (E−) (denoted in the present study as F−E−). **b** Shoot with inflorescence (F+) and without secondary shoot elongation (E−) (F+E−). **c** Shoot without inflorescence (F−) and with secondary shoot elongation (E+) (F−E+). **d** Shoot with inflorescence (F+) and with secondary shoot elongation (E+) (F+E+). *Circles* indicate secondary elongated shoots (*E*); *arrows* indicate inflorescence (*F*)
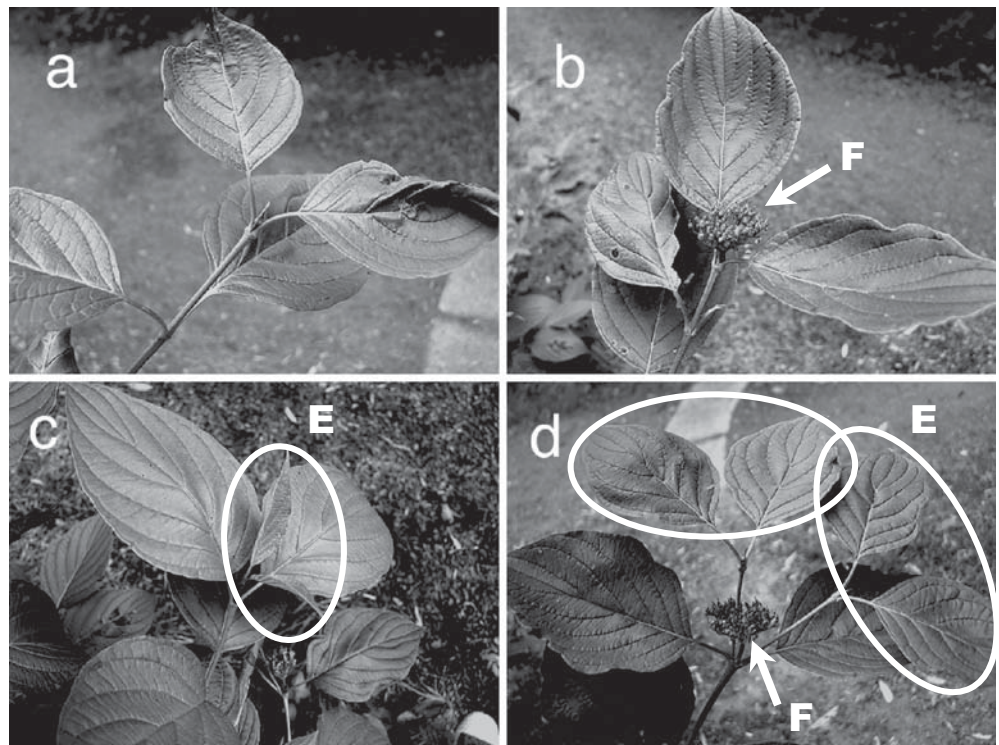
Table 1. Number of current-year shoot of *Cornus stolonifera* with or without inflorescence (F−/F+) and secondary shoot elongation (E−/E+) on 10 ramets

	F−E−	F−E+	F+E−	F+E+	Total	χ^2
Total number of current-year shoots	398 (81)	39 (8)	26 (5)	26 (5)	489 (100)	68.0***
Number of current-year shoot apically produced on previous-year shoots	46 (47)	26 (27)	10 (10)	15 (15)	97 (100)	4.3*

Numbers in parentheses indicate the relative proportions of total numbers
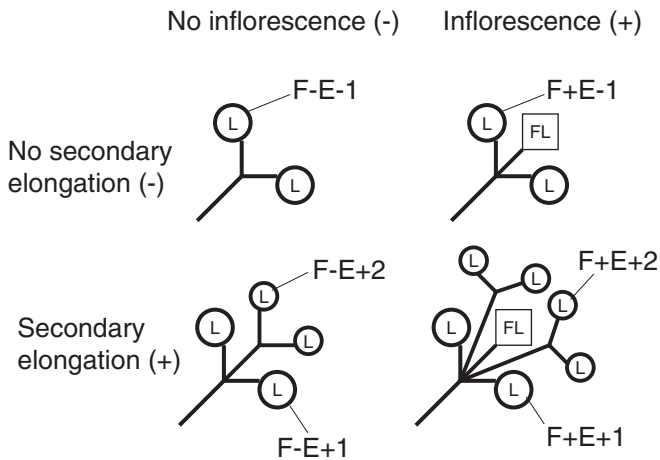The results of χ^2 test are shown: ***$P < 0.001$, *$P < 0.05$

Fig. 2. Schematic diagram of current-year shoot in four types and leaves in six categories. Current-year shoots can be divided into four types (F−E−, F+E−, F−E+, F+E+) based on the combination of the absence/presence of inflorescence (F−/F+) and secondary-elongated shoot (E−/E+). *L*, leaf; *FL*, inflorescence. F−E+ and F+E+ shoots were subdivided into primary and secondary shoots. Leaves on the primary shoots were denoted as F−E+1 and F+E+1, and leaves on the secondary shoots as F−E+2 and F+E+2. Leaves on F−E− and F+E− shoots were denoted as F−E−1 and F+E−1, respectively. Therefore, leaves on the current-year shoots fell into the following six categories: F−E−1, F−E+1, F−E+2, F+E−1, F+E+1, and F+E+2

I used these current-year shoots that were apically produced on previous-year shoots.

F−E+ and F+E+ shoots were subdivided into the primary and secondary elongated shoots. Leaves on the primary shoots were denoted as F−E+1 and F+E+1 and leaves on the secondary shoots as F−E+2 and F+E+2. Leaves on F−E− and F+E− shoots were denoted as F−E−1 and F+E−1, respectively, because they were on the primary shoots.

In September 2005, 1 current-year shoot was arbitrarily selected for each of four types and harvested from each of ten ramets, and a total of 40 current-year shoots were sampled from the ten ramets. The shoots were placed in paper bags and taken to the laboratory. Two leaves were selected for each of the six categories, and a total of 120 leaves were harvested for the investigation.

Two leaf disks were punched out from each single leaf with a sterile cork borer (6 mm in diameter) from the central part of the leaves, avoiding the primary vein. The disks were used for fungal isolation, one disk for a surface sterilization method and the other for a washing method, as described next. Fungal isolation was carried out within 24 h of sampling.

Fungal isolation

A surface sterilization method (Hata 1997) and a modified washing method (Tokumasu 1980) were used according to the method described in Osono and Takeda (2001). Fungi isolated with the surface sterilization method were regarded as inhabitants of the leaf interior, whereas those isolated only with the washing method were regarded as inhabitants of the leaf surface (Osono and Mori 2004, 2005). Frequency of individual species was calculated as the number of leaf disks from which the species were grown divided by the total number of disks tested for each leaf category (20 disks), expressed as a percentage. Fungal species isolated from all the six leaf categories were regarded as frequent species, and the other species were regarded as infrequent species.

Statistical analysis

Sørensen's quotient of similarity (*QS*) was calculated to compare the similarity of fungal assemblages of leaf interior and leaf surface by the following equation:

$$QS = 2a/(2a + b + c)$$

where *a* is the number of common species and *b* and *c* are the number of species specific to the interior and the surface, respectively (Osono and Mori 2004).

Occurrence patterns of fungal species within the habitats of a single leaf (i.e., interior or surface) and within the six leaf categories were classified using cluster analysis (Osono and Mori 2004). Cluster analysis results in a hierarchical dendrogram showing species linkages in a criterion similarity (Pearson's correlation coefficient). In the present study, the group-average method was used.

The distribution of frequent species within current-year shoots was analyzed in terms of the flowering, the secondary shoot elongation, and the order of shoots. A chi-square test was performed for frequencies of fungi because data were in the form of proportions. The effect of flowering was determined by comparing the frequency of fungi between (F−E−1) + (F−E+1) and (F+E−1) + (F+E+1). The effect of secondary shoot elongation was determined by comparing the frequency of fungi between (F−E−1) + (F+E−1) and (F−E+1) + (F+E+1). The effect of shoot order was determined by comparing the frequency of fungi between (F−E+1) + (F+E+1) and (F−E+2) + (F+E+2).

Table 2. Frequency (%) of fungi in the interior of *Cornus stolonifera* leaves

Fungus	F–E–1	F–E+1	F–E+2	F+E–1	F+E+1	F+E+2
Frequent species						
*Colletotrichum gloeosporioides**	20	60	40	35	45	20
Infrequent species						
Ascomycete species	0	5	0	0	0	0
Aureobasidium sp.	0	5	0	0	0	0
Cladosporium sp.1	0	0	0	0	5	0
Cladosporium sp.2	0	0	0	0	5	0
Coelomycete species 1	15	10	0	15	15	0
Coelomycete species 2	0	0	0	5	0	5
Coelomycete species 3	0	0	0	5	0	0
Epicoccum nigrum	0	0	5	0	0	0
Hyphomycete species	0	0	0	0	5	0
Phomopsis sp.	0	0	0	0	5	5
Septoria sp.	0	0	0	0	0	5
Stenella sp.	5	0	0	0	0	0
White sterile mycelia	10	0	0	5	5	5
Number of species	4	4	2	5	7	5

*Significant effects of secondary shoot elongation ($P < 0.05$) and shoot order ($P < 0.05$) were found, whereas the effect of flowering was not significant ($P > 0.05$)

Table 3. Frequency (%) of fungi on the surface of *Cornus stolonifera* leaves

Fungus	F–E–1	F–E+1	F–E+2	F+E–1	F+E+1	F+E+2
Frequent species						
Alternaria sp.*	15	10	30	15	40	10
*Epicoccum nigrum**	45	40	25	40	45	75
*Cladosporium cladosporioides**	45	10	30	35	40	45
*Cladosporium herbarum**	25	25	35	40	45	40
*Colletotrichum gloeosporioides**	30	15	30	15	10	20
Fusarium sp.*	5	15	10	10	20	5
*Lecanicillium psalliotae**	25	20	15	10	25	10
*Sclerotinia sclerotiorum**	20	20	20	10	35	20
Infrequent species						
Acremonium kiliense	0	0	10	0	0	0
Aureobasidium sp.	15	5	0	15	0	0
Botrytis sp.	10	10	10	0	15	10
Cladosporium sphaerospermum	0	0	10	0	0	0
Cladosporium sp. 1	0	5	0	0	0	0
Cladosporium sp. 2	0	0	5	5	0	5
Cladosporium sp. 3	0	0	0	5	0	0
Coelomycete sp. 3	15	30	35	5	10	0
Coelomycete sp. 4	5	5	0	0	0	0
Coelomycete sp. 5	0	0	0	5	0	0
Paecilomyces farinosus	0	5	5	20	10	5
Penicillium brevicompactum	5	0	5	0	0	5
Penicillium citrinum	0	20	10	10	5	5
Penicillium miczynskii	0	5	0	0	5	0
Penicillium thomii	0	0	0	5	0	10
Penicillium sp.	0	0	0	0	0	5
Phomopsis sp.	5	0	25	20	15	5
White sterile mycelia	5	0	0	0	0	0
Number of species	15	16	17	17	14	16

*No significant effects of flowering, secondary shoot elongation, and shoot order were found ($P > 0.05$)

Results

Phyllosphere fungal assemblage

Fungi were isolated from the interior of 72 of 120 (60%) leaf disks examined, 0 to 2 species (mean, 0.6 species) per disk, and from the surface of 120 of 120 (100%) leaf disks,

1 to 5 species (mean, 2.8 species) per disk. A total of 32 species were isolated: 14 species from the interior (Table 2), 26 species from the surface (Table 3), and 8 species being common on both habitats. The Sørensen's *QS* for interior and surface fungal assemblage was 0.40.

One endophytic and eight epiphytic fungi were regarded as frequent species (Tables 2, 3). *Colletotrichum gloeospo-*

rioides with its teleomorph *Glomerella cingulata* was the most frequent species in the interior (Table 2). *Epicoccum nigrum* was the most frequent species on the surface, followed by *Cladosporium herbarum*, *C. cladosporioides*, *Sclerotinia sclerotiorum*, *Alternaria* sp., *C. gloeosporioides*, *Lecanicillium psalliotae*, and *Fusarium* sp. (Table 3).

Effect of flowering, secondary shoot elongation, and shoot order

Two to 7 species were isolated from the interior and 14 to 17 species from the surface of leaves in the six categories (see Tables 2, 3). The number of species was consistently greater on the surface than in the interior in each leaf category but was at similar values among the leaf categories in the interior or on the surface. Species composition was markedly different between the interior and surface of leaves, whereas it was relatively similar among the six leaf categories in the interior or on the surface (Fig. 3).

None of the frequent species showed significant differences in frequencies between leaves on flowering and nonflowering shoots (Tables 2, 3). The frequency of *C. gloeosporioides* in the interior was greater on leaves on the primary shoots that elongated the secondary shoots than on those that did not (Table 2). No significant effect of secondary shoot elongation was found on the frequencies of *C. gloeosporioides* and the other seven epiphytes on leaf surfaces (Table 3). The frequency of *C. gloeosporioides* in the interior was significantly ($P < 0.05$) greater on leaves on the primary shoots than on those on the secondary shoots (Table 2). No significant effect of shoot order was found on the frequencies of *C. gloeosporioides* and the other seven epiphytes on leaf surfaces (Table 3).

Discussion

In the present study, eight fungal species were regarded as major phyllosphere fungi of *C. stolonifera*, and *C. gloeosporioides* was isolated both in the interior and on the surface of leaves. Osono and Mori (2004) reported *C. gloeosporioides* as a frequent species both in the interior and on the surface of *S. controversa* leaves in Japan. In contrast, *C. gloeosporioides* was rarely detected on other hosts in south-

ern Vancouver Island. For example, the isolation frequency of this species was only 3% on leaves of *Acer macrophylla* (Sieber and Dorworth 1994), and this species was absent from leaves of *Alnus rubra* and *Rubus* spp. (Sieber et al. 1991; Shamoun and Sieber 2000). The frequent occurrence of species in *Epicoccum*, *Cladosporium*, *Alternaria*, and *Sclerotinia sclerotiorum* was consistent with the result of *S. controversa* (Osono and Mori 2004, 2005; Osono et al. 2004a). The Sørensen's QS in *C. stolonifera* (0.40) is at a similar level to those observed for phyllosphere fungal assemblages on *S. controversa* leaves (0.26–0.38; Osono and Mori 2004, 2005).

The frequency of *C. gloeosporioides* in the leaf interior was greater on leaves on the primary shoots that elongated the secondary shoots than on those that did not. One of the possible explanations for this difference is the change of nutrient content within leaf tissues as a consequence of secondary shoot elongation. Translocation of nutrients from older leaves to the younger parts of current-year shoots (Whiley and Schaffer 1993) can change nutrient content in older leaves, making leaves more susceptible to the interior colonization by *C. gloeosporioides*. In contrast, secondary shoot elongation had no significant effect on the epiphytes, suggesting that the nutritional changes associated with secondary shoot elongation was less effective on epiphytes than on endophytes. Similarly, no significant difference in the frequencies of phyllosphere fungi between flowering and nonflowering shoots may imply the negligible effect of the possible changes in leaf nutrients as a result of inflorescence production on phyllosphere fungi.

The frequency of *C. gloeosporioides* in the leaf interior was greater on leaves on the primary shoots than on those on the secondary shoots. Osono and Mori (2004) reported that the difference in leaf mass per area and concentrations of nitrogen and polyphenols between leaves on the primary and secondary shoots had a potential effect on the occurrence of phyllosphere fungi. Alternatively, the shoot order also can affect phyllosphere fungi when their colonization is proportional to leaf age and/or the inoculum potential of phyllosphere fungi changed seasonally (Osono and Mori 2005). However, the latter explanation does not seem to be applicable here as the shoot order had no effect on the frequency of *C. gloeosporioides* on leaf surfaces. On the other hand, the negligible effect of shoot order on epiphytes may be because the possible nutritional difference between the primary and the secondary shoots can be less effective on epiphytes than on endophytes, or because the colonization of leaves by epiphytes was not associated with leaf age (Osono and Mori 2004, 2005).

Cornus stolonifera has a wide range of geographic distribution in the coastal, montane, and boreal forests of Canada. Further research directions include the potential antagonistic effects of phyllosphere fungi on pathogenic fungi, the study of phyllosphere fungi associated with *C. stolonifera* from other locations of coastal forest and from other forest regions of Canada, the study of phyllosphere fungi associated with other dogwood tree species in North America, and the functional associations of phyllosphere fungi with host plants.

Fig. 3. Dendrogram of fungal associations on live leaves by the group-average method with Pearson's correlation coefficient based on the frequency of individual species
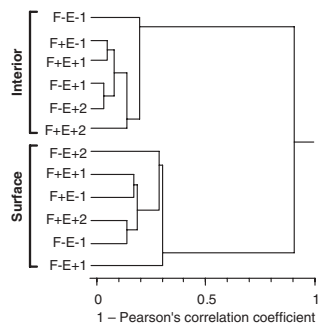
References

Akao S, Tsukahara S, Hisada H, Ono S (1981) Contribution of photosynthetic assimilates to development of flower and spring flush in *Citrus unshiu*. J Jpn Soc Hortic Sci 50:1–9

Hasegawa S, Koba K, Tayasu I, Takeda H, Haga H (2003) Carbon autonomy of reproductive shoots of Siberian alder (*Alnus hirsuta* var. *sibirica*). J Plant Res 116:183–188

Hata K (1997) Collection, detection, and isolation of fungi: endophytes (in Japanese). Trans Mycol Soc Jpn 38:110–114

Margery DL, Hibben CR, Britton KO, Windham MT, Redin SC (1996) Dogwood anthracnose: understanding a disease new to North America. Plant Dis 80:349–358

Osono T (2005) Colonization and succession of fungi during decomposition of *Swida controversa* leaf litter. Mycologia 97:589–597

Osono T (2006) Fungi associated with *Swida controversa* leaves infested by zonate leaf blight and their antagonistic effects on the pathogenic fungus (in Japanese). Appl For Sci 15:7–12

Osono T, Mori A (2004) Distribution of phyllosphere fungi within the canopy of giant dogwood. Mycoscience 45:161–168

Osono T, Mori A (2005) Seasonal and leaf age-dependent changes in occurrence of phyllosphere fungi of giant dogwood. Mycoscience 46:273–279

Osono T, Takeda H (2001) Organic chemical and nutrient dynamics in decomposing beech leaf litter in relation to fungal ingrowth and succession during three year decomposition processes in a cool temperate deciduous forest in Japan. Ecol Res 16:649–670

Osono T, Bhatta BK, Takeda H (2004a) Phyllosphere fungi on living and decomposing leaves of giant dogwood. Mycoscience 45:35–41

Osono T, Mori A, Koide K (2004b) Defoliation of giant dogwood (*Swida controversa*) caused by zonate leaf blight (in Japanese). Appl For Sci 13:161–164

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (eds) Microbial ecology of leaves. Springer, New York, pp 179–197

Redlin SC (1991) *Discula destructiva* sp. nov., cause of dogwood anthracnose. Mycologia 83:633–642

Shamoun SF, Sieber TN (2000) Colonisation of leaves and twigs of *Rubus parviflorus* and *R. spectabilis* by endophytic fungi in a reforestation site in British Columbia. Mycol Res 104:841–845

Sieber TN, Dorworth CE (1994) An ecological study about assemblages of endophytic fungi in *Acer macrophyllum* in British Columbia: in search of candidate mycoherbicides. Can J Bot 72:1397–1402

Sieber TN, Sieber-Canavesi F, Dorworth CE (1991) Endophytic fungi of red alder (*Alnus rubra*) leaves and twigs in British Columbia. Can J Bot 69:407–411

Tokumasu S (1980) Observations on the fungal flora on pine needle litter. In: Biseibutsu no Seitai, vol 7 (in Japanese). Gakkai Shuppan Center, Tokyo, pp 129–144

Whiley AW, Schaffer B (1993) Carbon-14 photosynthate partitioning in avocado trees as influenced by shoot development. Hortscience 28:850–852