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FULL PAPER

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Succession of microfungal flora on *Rodgersia podophylla* plants at the forest side of *Cryptomeria* plantation

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Abstract Microfungal flora on aerial parts of a forest floor plant, *Rodgersia podophylla*, were studied at the forest side of a Japanese cedar plantation. From May to October, *Nigrospora* spp. were dominantly isolated from leaves, whereas *Acremonium* spp. and *Fusarium* spp. were dominant on stems, suggesting that the aerial part of the plants about 1 m height can offer two different habitats for these dominant fungi. In September and October, we could easily discern different types of tissue on the withering leaves, i.e., brown (necrotic lesion), yellowish (border tissue between brown and green areas), and green (healthy tissue). *Nigrospora* spp. and *Pestalotiopsis* spp. were continuously isolated on the brown area as well as on green and yellowish areas. Pathogenicity of *N. sacchari* and *P. neglecta* on potted plant leaves was confirmed by inoculation. From these, the fungi of these two genera seemed to have changed from quiescent to pathogenic with leaf senescence. Sporulations by fungi of the two genera were recognized on overwintered stems. These fungi may overwinter in stems that are slow to decompose, and seem to go over to the leaves in the following spring. Thus, they could be candidates for parasites that may play an important role in decomposition of the plant.

Key words Habitat · Microfungi · *Rodgersia podophylla* · Succesion

Introduction

Fungal succession has been defined as "the sequential occupation of the same site by thalli either of different fungi or different associations of fungi" (Rayner and Todd 1979). In Japan, most research on fungal succession has been conducted with conifers, broad-leaved forest trees, or ericaceous woody plants (Aoki et al. 1990; Hata and Futai 1993; Hata et al. 1998; Okane et al. 1998; Osono 2002; Tokumasu 1998a,b; Tokumasu and Aoki 2002); that is, these studies were limited to the arboreal phyllosphere.

The forest floor plant *Rodgersia podophylla* A. Gray (Saxifragaceae) is a large perennial herb with a leaf composed of five microphylls (leaflets) that is distributed in Japan and Korea (Fig. 1A). In Japan, the plant indigenously grows during April to October in the mountains, often forming gregarious large populations (Ohwi and Kitagawa 1992). In the study site, *R. podophylla* buds at the beginning of May, and by June to July each of five microphylls expands up to 15–35 × 10–25 cm, and the stem (1–2 cm diameter) reaches 80–150 cm in height. Toward the end of August, many amorphous lesions with a brown center and yellowish circumference arise from everywhere on the leaf (Fig. 1B). Consequently, leaves are withered and fall onto the forest floor in late October. By the middle of December, the ground of the site becomes covered with snow, and in the next spring we could not find any remnants of the leaves, owing to complete decomposition, while overwintered stems were often visible, retaining their shapes.

The above-mentioned change in *R. podophylla* leaf tissue with various amorphous lesions strongly suggested the existence of colonized fungi. Wilson (2000) suggested that endophytic fungi may have been early successional saprophytes that evolved to colonize the plant material before it became senescent, and they may remain dormant until triggered by natural leaf senescence, abscission, or damage to grow and perhaps sporulate. Therefore, the fungi endophytically colonized in the plant leaf tissues may eventually turn pathogenic and may further contribute to early-stage decomposition of the plant leaves with amorphous necrotic lesions.

This research represents the first survey of the microfungal succession on *Rodgersia podophylla* plants. We aimed to characterize the fungi on aerial parts of the plant and discuss here the effects of plant aging and

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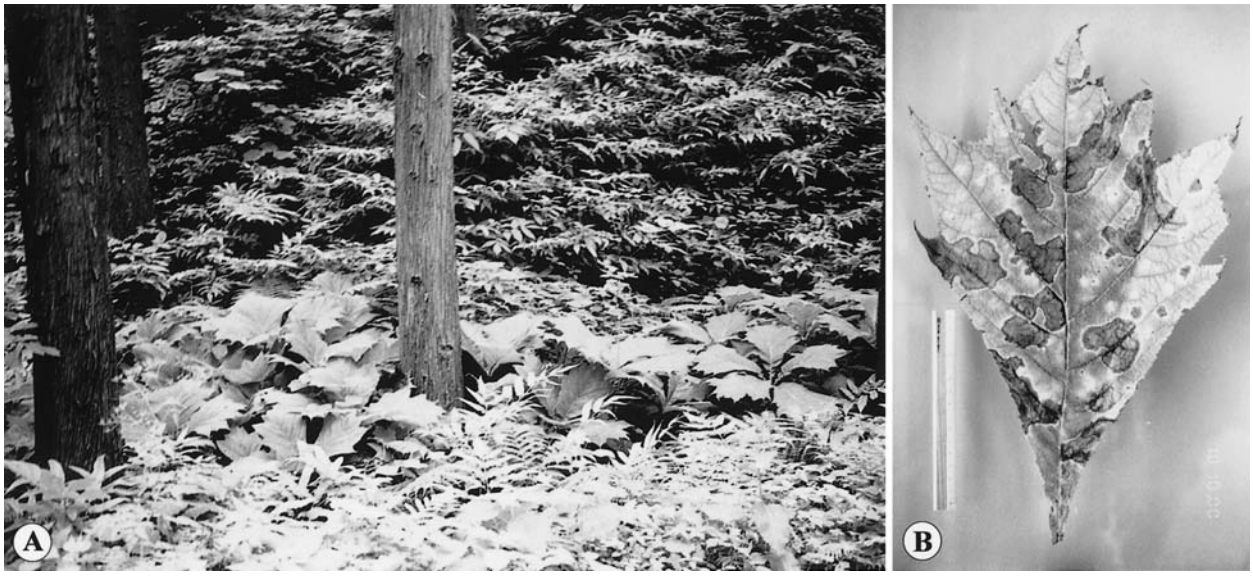

Fig. 1. **A** *Rodgersia podophylla* on forest floor. **B** Withering leaflet of *R. podophylla* with many amorphous lesions. Note: Brown area was surrounded by yellowish circumference. *Bar* **B** 15 cm

Table 1. Sampling dates and tissue segment numbers for isolation studies

Sampling year/date	Total number of leaf segments	Sampling year/date	Total number of Stem segments
2000 Aug. 13	–[a]	2002 May 5	18
2001 May 12	27	2002 May 13	18
2001 May 15	27	2002 May 22	18
2001 May 29	27	2002 June 5	18
2001 June 12	27	2002 June 19	18
2001 June 26	27	2002 July 18	18
2001 July 10	27	2002 July 31	18
2001 July 24	27	2002 Aug. 14	18
2001 Aug. 7	27	2002 Aug. 21	18
2001 Aug. 21	27	2002 Sept. 11	18
2001 Sept. 4	54	2002 Sept. 22	18
2001 Sept. 18	54	2002 Oct. 8	18
2001 Oct. 2	54	2002 Oct. 21	18
2001 Oct. 16	54	2003 Nov. 26	–[b]
2001 Oct. 30	27	2004 April 13	–[c]

[a] Sclerotia
[b] Fruit bodies
[c] Overwintered stems

withering phenomena of the leaf with lesions on fungal colonization.

Materials and methods

Study site and sampling

A relatively small area (50 × 200 m) with a large number of *Rodgersia podophylla* was selected as the research site, located at Kudoji, Hirosaki, Aomori Prefecture (140°25′ E, 40°31′ N) in Japan. The site is adjacent to a Japanese cedar [*Cryptomeria japonica* (Linn. fil.) D. Don] plantation. Three plants were brought back to the laboratory in each sampling time. The sampling dates are shown in Table 1.

Surface sterilization and media

The procedures for surface sterilization and isolation media are shown in Table 2. To isolate various microfungi from leaf segments, we conducted two different surface sterilization processes after samples were submerged in 80% ethanol (v/v). On LA and LB procedures, leaf segments were submerged for 5 s in 70% ethanol (v/v). On the LC procedure, leaf segments were rinsed in sterilized distilled water. The former method was considered more severe than the latter for surface sterilization effect. On SA and SB proce-

Table 2. Procedures for surface sterilization of tissue segments of *Rodgersia podophylla* and media for isolations

Organ segments	Sterilizing agent and time(s)			SDW	Medium	Isolation code
	Ethanol (80%)	Sodium hypochlorite (available Cl, 1%)	Ethanol (70%)			
Leaf	60		5		PSA	LA
	60		5		1/2 CMA[a]	LB
	60			180	PSA	LC
Stem	60	60		180	PSA	SA
	60	60		180	1/2 CMA	SB

SDW, sterilized distilled water; PSA, potato sucrose agar; CMA, cornmeal agar
[a]Tokumasu (1998a)

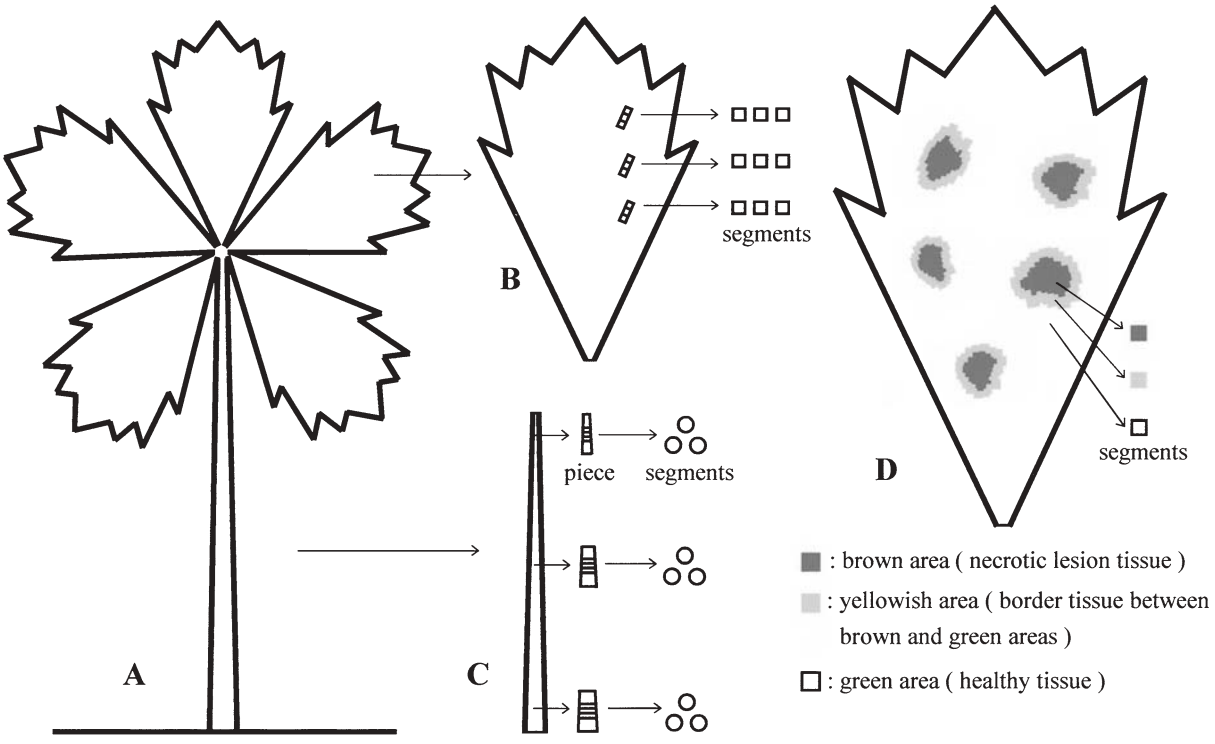

Fig. 2. Illustration of *Rodgersia podophylla* aerial parts and segments for isolation. **A** *R. podophylla* aerial parts. **B** Leaf segments. **C** Stem segments. **D** Three types of segments on withering leaflet

dures, stem segments were sterilized by sodium hypochlorite (available Cl, 1%) of osmotic agent to suppress contamination caused by emerging bacteria from hairs on stems. In addition, half-strength cornmeal agar (Tokumasu 1998a) was used because its low sugar content suppresses overgrowth of fast-growing microfungi.

All segments were air dried for 30 min on a clean bench. After drying, segments were placed on media (three segments per plate) and incubated at 20°C in darkness for 3–4 weeks.

Isolation from leaf segments

Leaf segments approximately 5 × 5 mm were cut from the side of the midrib region (Fig. 2B,D). A total of 27 or 54 segments (9 or 18 segments per microphyll) were obtained from three plants from one sampling time (see Table 1). From May to August and Otober 30, 2001, each of 9 segments was prepared for LA, LB, and LC; during September to October, each of 18 segments were prepared for LA, LB, and LC.

Isolation from stem segments

A total of 18 stem segments (9 segments per stem) were obtained from two plants from one sampling time. Three pieces approximately 3 cm long were cut out from the top, middle, and lower portion of the stem. After surface sterilization, circular segments approximately 2 mm were prepared from each piece (Fig. 2C). In one

Table 3. Microfungal succession on leaf segments of *Rodgersia podophylla* in 2001

Fungi	May	June	July	Aug.	Sept.	Oct.	Oct. 30	Probability
	[a]Healthy →				Withering →		Withered	
Acremonium sp. 1	18.5							ns
Chaetomium spp.	1.2	1.9						ns
Epicoccum purpurascens	1.2		1.9		0.9			ns
Phoma sp. 1	1.2				0.9			ns
Pestalotiopsis spp.	4.9	7.4	9.3	3.7	7.4	7.4		ns
Nigrospora spp.	1.2	7.4	11.1	24	21.3	24	18.5	**
Collectotrichum gloeosporioides		1.9	5.6	1.9	5.6	0.9		ns
Alternaria alternata			1.9					ns
Cladosporium cladosporioides			1.9					ns
Mucor sp.			3.7		8.3			ns
Arthrinium phaeospermum					0.9			ns
Botrytis cinerea						0.9		ns
Xylaria spp. (anamorph)					3.7	6.5		ns
Phialophora sp.						0.9		ns
Total number of leaf segments	81	54	54	54	108	108	27	

Colonization frequency (%) = (number of segments from which the fungus was detected/total number of segments, respectively) × 100
Sterile mycelium taxa were omitted from this table
[a] Leaf tissue conditions
** $P < 0.01$; ns, nonsignificant

sampling time, each 9 segments were prepared for SA and SB.

Isolation from withered leaf segments

From September to October, the withered leaf (Fig. 1B) segments were obtained from three tissue types, a brown area (necrotic lesion tissue), yellowish area (border between brown and green areas), and green area (healthy tissue) (see Fig. 2D). For each tissue type, a total of 18 segments were obtained, in which 6 segments each were prepared for LA, LB, and LC.

Direct isolations of fungi found on the plant

Fungi on the plant were directly isolated from their reproductive structures visible to the naked eye. We found sclerotia on fallen leaves on August 13, 2000, and minute fruit bodies of a basidiomycete on withered leaves on November 26, 2003 (see Table 1), from which we carried out isolations.

Inoculation experiment with isolates from withering leaves

To investigate the pathogenicity of the fungi isolated from leaves, inoculation experiments were conducted on leaves of potted plants of *R. podophylla. Nigrospora sacchari, Pestalotiopsis neglecta*, and *Colletotrichum gloeosporioides* were selected as test fungi. Before inoculation, leaves were wounded by a sterilized needle. A few drops of conidial suspensions of *N. sacchari* (approx. 6.3×10^4 conidia/ml), *P. neglecta* (approx. 1.3×10^5 conidia/ml), and *Colletotrichum gloeosporioides* (approx. 6.3×10^4 conidia/ml) obtained from potato sucrose agar (PSA) culture were applied onto wounded parts of leaves with small pipettes. Inoculated leaves were covered with plastic bags to maintain the moist condition. Next day, plastic bags were removed. Sterilized distilled water was used instead of spore suspensions for the control plant.

Isolation from overwintered stem

On April 13, 2004, overwintered stems were brought into the laboratory (see Table 1). The samples were rinsed in running water and cut into approximately 5-cm pieces. The pieces were prepared for SA, placed on a total of five plates (2 pieces per plate), and incubated at 20°C/12 h BL-B : D for 3 weeks.

Analyses of data

To compare the number of individual isolated microfungal taxa among LA, LB, and LC on leaves, and SA and SB on stems, Fisher's protected least significant differences (LSD) test was used to detect significant differences among isolation procedures. The occurrence of individual microfungi was evaluated by the colonization frequency calculated by the following equation: colonization frequency (%) = (number of segments from which the fungus was isolated/total number of segments investigated) × 100 (Okane et al. 1998).

When comparing the colonization frequencies of isolates among microfungal taxa on leaves or stems by Tukey's test, the microfungal taxa with significant differences from infrequent isolates were regarded as dominant. Tukey's test was performed as follows: the data matrices containing microfungal taxa (columns) × colonization frequency of each month (rows) shown on Table 3 or Table 4 were used for corresponding comparison to detect dominance on

Table 4. Microfungal succession on stem segments of *Rodgersia podophylla* in 2002

Fungi	May	June	July	Aug.	Sept.	Oct.	Probability
	[a]Healthy →					Withered	
Phoma spp.	3.7		5.6	2.8	38.9	2.8	ns
Fusarium spp.		11.1	5.6	22.2	38.9	55.5	**
Phomopsis spp.		7.7	13.9	13.9	5.6		ns
Alternaria alternata			5.6				ns
Rhizoctonia spp.			22.2	11.1	5.6		ns
Acremonium spp.			19.4	30.5	24.9	47.2	*
Colletotrichum gloeosporioides			8.3	11.1	5.6		ns
Cylindrocarpon destructans				2.8			ns
Clonostachys rosea				2.8			ns
Geniculosporium sp.				2.8			ns
Coelomycete 1				8.3			ns
Pestalotiopsis neglecta				5.6	2.7		ns
Trichoderma viride						2.8	ns
Xylaria sp. 1 (anamorph)						5.6	ns
Coelomycete 2						2.8	ns
Total number of stem segments	54	36	36	36	36	36	

Colonization frequency (%) = (number of segments from which the fungus was detected/total number of segments respectively) × 100
Sterile mycelium taxa were omitted from this table
[a]Stem tissue conditions
* $P < 0.05$; ** $P < 0.01$; ns, nonsignificant

leaves or stems, respectively. For Tukey's test, percent data for recovery of individual microfungal taxa on leaves and stems were transformed to arcsine-square root values before analyses (Mazzola et al. 2001).

Analyses were performed using Microsoft Excel 2000 software. The statistical analyses used in Yanai (1998) were followed.

Results

Microfungal flora observed on aerial parts of *R. podophylla*

No significant differences were detected by LSD among isolation procedures in isolated microfungal taxa from leaves or stems, respectively. Therefore, the colonization frequency (%) was calculated from the integrated number of isolated microfungal taxa from each isolation procedures in leaves or stems.

Appendix A shows 43 microfungal taxa isolated from aerial parts of the plant in this study: 1 basidiomycete, 1 zygomycete, 2 ascomycetes, 36 mitosporic fungi, and 3 mycelia sterilia. Members of the genera *Phoma*, *Acremonium*, and *Fusarium* were distinguished by morphological and cultural characteristics, i.e., conidia, conidiogenus cells, hyphal pigments, growth rates, colony surface texture and margin shape, and so on. *Rhizoctonia solani* 1 was isolated from sclerotia on fallen leaves. A total of 23 and 25 microfungal taxa were isolated from leaves and stems, respectively. Among them, a minute basidiomycete *Flagelloscypha* sp., discovered on blighted leaves, is worth noting because the species of *Flagelloscypha* had been previously reported only once in this country (Tanaka 1999).

Microfungal succession on leaves

Table 3 shows the change in colonization frequencies of the microfungi isolated from leaves in 2001. Among the fungi isolated from the leaves, *Nigrosora* spp., *Colletotrichum gloeosporioides*, and *Pestalotiopsis* spp. were continuously isolated over 5 months. In these three genera, *Nigrospora* spp. were most continuously isolated, over 6 months (1.2%–24%), *C. gloeosporioides* was isolated over 5 months (0.9%–5.6%), and *Pestalotiopsis* spp. were isolated over 6 months (3.7%–9.3%). Other fungi except *Chaetomium* spp. and *Xylaria* spp. were only infrequently isolated (less than 2 months). *Acremonium* sp. 1 was isolated in high frequency, but only from young leaf segments in May (18.5%).

The dominance of *Nigrospora* spp. ($P < 0.01$) was detected by Tukey's test on isolated microfungal taxa on leaves.

Microfungal succession on stems

Table 4 shows the changes in colonization frequencies of the microfungi isolated from stems in 2002. Among the fungi isolated from the stems, *Fusarium* spp. were continuously isolated over 5 months (5.6%–55.5%). In May, when the plant began budding and started to grow, only *Phoma* spp. were isolated at low percentage (3.6%). *Acremonium* spp. showed relatively high colonization frequencies (19.4%–47.2%) from July to October. *Phomopsis* spp. were isolated from June to September (5.6%–13.9%), but not in October when the stem was withering. In October, after the stem withered, the fungi isolated included *Acremonium* spp. (47.2%), *Fusarium* spp. (55.5%), *Trichoderma viride* (2.8%), *Xylaria* sp. 1 (5.6%), and coelomycete 2 (2.8%).

Appendix A. Frequency (%) of microfungi isolated from *Rodgersia podophylla* aerial parts

Fungus	Leaves	Stems
Flagelloscypha sp.	–[a]	
Mucor sp.	2.3	
Chaetomium cochliodes	0.2	
Chaetomium globosum	0.2	
Epicoccum purpurascens	0.6	
Nigrospora oryzae	5.3	
Nigrospora sphaerica	3.5	
Nigrospora sacchari	7	
Arthrinium phaeospermum	0.2	
Botrytis cinerea	0.2	
Phialophora sp.	0.2	
Geniculosporium sp.	0.2	
Clonostachys rosea	0.2	
Pestalotiopsis glandicola	0.2	
Pestalotiopsis neglecta	6.3	1.3
Cladosporium cladosporioides	0.2	0.4
Alternaria alternata	0.2	1.3
Colletotrichum gloeosporioides	2.5	2.6
Xylaria sp. 1 (anamorph)	2.1	1.3
Xylaria sp. 2 (anamorph)	0.2	
Trichoderma viride		0.4
Phoma sp. 1	0.4	
Phoma sp. 2		0.9
Phoma sp. 3		1.3
Phoma sp. 4		0.9
Phoma sp. 5		5.6
Acremonium sp. 1	3.1	
Acremonium sp. 2		3
Acremonium sp. 3		12
Acremonium sp. 4		1.3
Acremonium sp. 5		0.9
Acremonium sp. 6		2.1
Fusarium sp. 1		17.5
Fusarium sp. 2		1.3
Fusarium sp. 3		2.1
Phomopsis sp. 1		5.6
Phomopsis sp. 2		0.4
Rhizoctonia solani 1	–[a]	
Rhizoctonia solani 2		1.7
Rhizoctonia solani 3		5.1
Cylindrocarpon destructans		0.4
Coelomycete 1		1.3
Coelomycete 2		0.4
Number of species	23	25

Colonization frequency (%) = (number of segments from which the fungus was detected/total number of segments) × 100
Total number was 486 leaf and 234 stem segments
Sterile mycelium taxon were omitted from this table
[a] The fungus was isolated from thalli on the leaf in nature

Among isolated microfungal taxa from leaves, *Acremonium* spp. ($P < 0.05$) and *Fusarium* spp. ($P < 0.01$) were shown to be dominant in frequencies over others.

Microfungal succession on withered leaves

Figure 3 shows fungal succession on three different tissue types in withering leaves. On this figure, five genera were selected from the isolated fungi as representative, because they were frequently isolated in this period.

From brown areas (necrotic lesion), *Nigrospora, Pestalotiopsis*, and *Xylaria* were continuously isolated over 8 weeks. Especially, *Nigrospora* spp. were isolated at the highest colonization frequency (11.6%–16.7%). *Mucor* sp. was isolated only in September. From yellowish areas (border tissue between brown and green areas), *Nigrospora* spp. and *Pestalotiopsis* spp. were continuously isolated (8 weeks and 6 weeks, respectively). *Nigrospora* spp. showed high colonization frequencies of 16.7%–38.9%. *Mucor* sp., *C. gloeosporioides*, and *Xylaria* spp. were only once or intermittently isolated. From green areas (healthy tissue), *Nigrospora* (16.7%–38.9%) and *Pestalotiopsis* (5.6%–16.7%) were continuously isolated over 8 weeks. *Xylaria* spp. were continuously isolated for 6 weeks from Sept. 18 (5.6%).

Pathogenicity of selected isolates from withered leaves

For *N. sacchari* and *P. neglecta*, inoculated parts of leaves discolored yellowish after 1 week and lesions with a brown center and yellowish circumference were apparent on the leaves in 1 month (Fig. 4A,B). The same fungi were recovered from the lesions with isolation procedure LA (shown in Table 2). With *C. gloeosporioides*, no such lesions appeared in 1 month.

Fungi detected from overwintered stem

In 15 days of incubation, several fungi were observed growing out of the stem pieces (Fig. 5A): these included *Nigrospora oryzae*, *N. sphaerica*. (Fig. 5B), *N. sacchari*, *Pestalotiopsis neglecta* (Fig. 5C), *Epicoccum purpurascens*, *Fusarium* sp., and *Mucor* sp. It was noted that some *Nigrospora* species and *P. neglecta* were found on all five Petri dishes.

Discussion

The habitats of plants associated with fungi may be distinguished as phyllosphere and rhizosphere, according to aerial and subterranean plant parts, respectively (Fokkema and Schippers 1986). Petrini (1986, 1996) suggested a few dominant endophytic taxa occur within a single plant species and that these dominant fungi often showed specificity to one or a few taxonomically related host species. Blodgett et al. (2000) reported that *Alternaria* spp. were most common in leaves, petioles, stems, and roots of the herbaceous plant *Amaranthus hybridus*. In this study, we examined two different tissues, leaf and stem, on the plant aerial part. Isolation data in the present study suggested different dominant microfungi between leaves and stems, although the years of the isolations were different. The dominant microfungal genera were *Nigrospora* on leaves and *Acremonium* and *Fusarium* on stems. In other words, this result suggested the aerial part of *R. podophylla* plant at about 1 m height provided two different specific habitats for these dominant genera.

In our inoculation experiment, the pathogenicity of *N. sacchari* and *P. neglecta* was demonstrated on potted plant

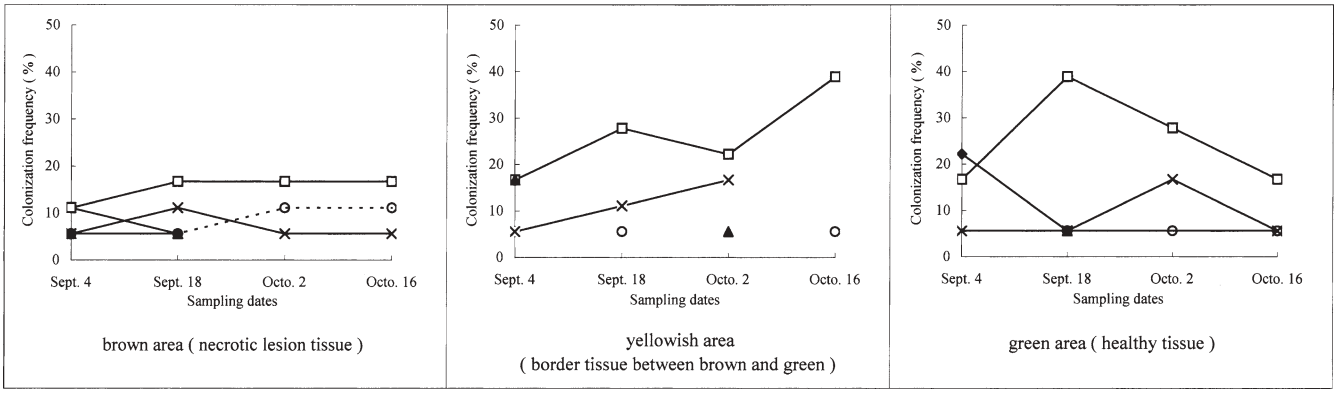

Fig. 3. Microfungal succession on withering leaves of *Rodgersia podophylla* in 2001: *Nigrospora* spp. (□), *Pestalotiopsis* spp. (×), *Xylaria* spp. (○), *Colletotrichum gloeosporioides* (▲), *Mucor* sp. (◆). Colonization frequency (%) = (number of segments from which the fungus was detected/total of 18 segments examined) × 100

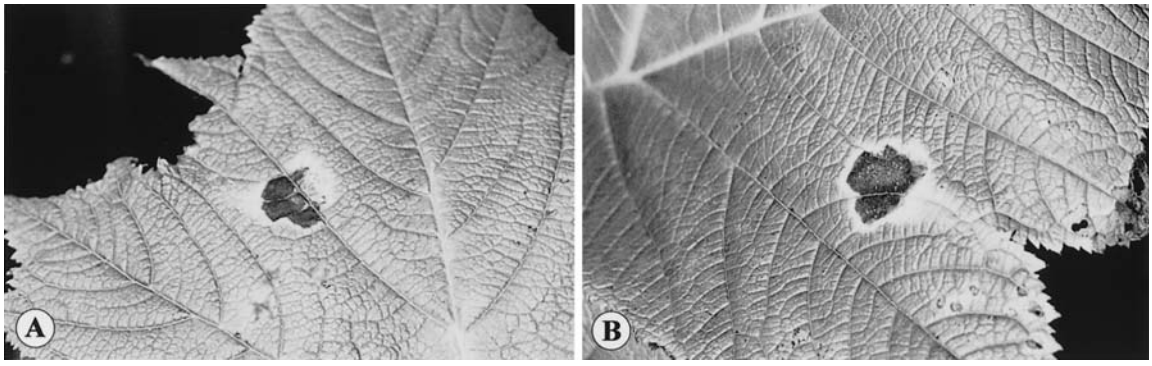

Fig. 4. Lesions on leaves of *Rodgersia podophylla*, produced by isolates of *Nigrospora sacchari* (**A**) and *Pestalotiopsis neglecta* (**B**). Note the discolorations or tissue around the lesion

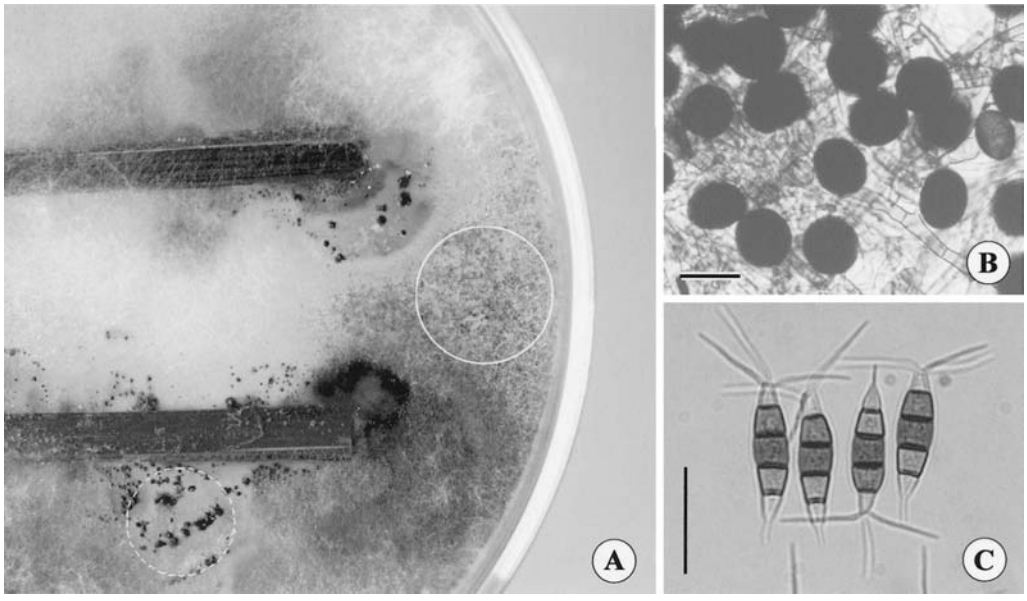

Fig. 5. *Nigrospora sphaerica* (*N*) and *Pestalotiopsis neglecta* (*P*), isolated from overwintered *Rodgersia podophylla* stems. **A** Colonies of N (*inside circle delineated with solid line*) and P (*inside circle delineated with dotted line*). **B, C** Conidia of *Nigrospora sphaerica and Pestalotiopsis neglecta*. *Bars* **B,C** 20 µm

leaves (see Fig. 4). Species of *Nigrospora* were reported to be endophytic fungi (Hata et al. 1998; Okane et al. 1998; Cannon and Simmons 2002) or causal agent of decaying on banana leaves (Meredith 1962). *Pestalotiopsis* was known as pathogenic and endophytic fungi (Hata et al. 1998; Suto and Kobayashi 1993; Okane et al. 1998). Wilson (2000) reported that endophytic fungi are early successional saprophytes and that their dormant phases turn pathogenic by host senescence. On *R. podophylla* leaves, species of *Nigrospora* and *Pestalotiopis* may be early colonizers, which turn pathogenic by leaf senescence. Probably, they could be main candidates for parasites that may play roles as early leaf decomposers of the plant in our study site.

Species of *Nigrospora* and *Pestalotiopis* were also detected from overwintered stems of *R. podophylla* (see Fig. 5). The withered leaf and stem were in contact with each other before snowfall in the preceding year. These fungi might have moved from leaves to stems for overwintering, and may go over to a new plant in the next season. Thus, the plant decomposition phenomena of *R. podophylla* could affect the life cycle of the fungi on leaves and stems of the same plant.

This is the first systematic study on microfungal flora and succession for herbaceous forest floor plants in this country. Further studies are needed with other plant species to elucidate microfungal flora on forest floor plants in this area.

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