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FULL PAPER

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Isolation of endophytic fungi from leaves of *Pasania edulis* and their within-leaf distributions

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Abstract Endophytic fungi were isolated from leaves of *Pasania edulis*, one of the most important trees of the warm temperate forests in southern Kyushu, by the surface sterilization method using H_2O_2 as a sterilizing agent. From a tree in the Experimental Nursery of Kagoshima University, located at the city of Kagoshima, *Phyllosticta* sp. and *Colletotrichum* spp. were frequently isolated. From a stand in a laurel forest in Mt. Takakuma, an ascomycetous fungus (Ascomycete sp. 1) and *Phomopsis* sp. were frequently isolated. *Phyllosticta* sp. was isolated more frequently from petiole segments and leaf segments with midrib and *Phomopsis* sp. from petiole segments and leaf-base segments with midrib than other segments. *Colletotrichum* spp. were isolated less frequently from petioles and Ascomycete sp. 1 from petiole segments and leaf-base segments with midrib than other segments. As possible causes of such biases in within-leaf distributions of the endophytes, differences in infection modes and negative interactions of major endophytes within leaves are suggested.

Key words Endophytic fungi · *Pasania edulis* · Within-leaf distribution

Introduction

Endophytic fungi, defined as fungi inhabiting inside of healthy plant tissues, are now considered as ubiquitous symbionts of plants from their surprisingly common detection from many plant species (Petrini 1986). Studies of endophytic fungi in tree leaves have been carried out for many host species since the latter half of the 1970s, when their significance as such common symbionts and possible mutualists of plants was recognized (Carroll et al. 1977; Bernstein and Carroll 1977; Carroll and Carroll 1978). Forest tree endophytes have been studied mainly in cool temperate forests of Europe and North America, but forest tree endophyte studies in other regions of the world, e.g., warm temperate to tropical forests, are less explored. In Japan, studies of endophytic fungi in tree leaves have gradually accumulated. For example, *Pinus* spp. (Hata and Futai 1996; Hata et al. 1998), ericaceous plants (Okane et al. 1998), and *Fagus crenata* (Sahashi et al. 1999) have been studied for the endophytes living inside them. However, endophytes have not been well studied in most warm temperate forest trees. In the present study, we carried out isolation of endophytic fungi from leaves of *Pasania edulis* Makino to survey their assemblages and within-leaf distribution.

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Materials and methods

Location of the sampling sites and their vegetation

Two stands, one each in the Experimental Nursery and in Takakuma Experimental Forest of Kagoshima University, were used as sampling sites. The Experimental Nursery is located at the main campus of Kagoshima University in the central region of Kagoshima City (~3 m above sea level). Here several *Pasania edulis* trees were growing, and one of them was used for sampling. Around the sample tree, various tree species such as *Persea thunbergii* (Sieb. et Zucc.) Kosterm., *Prunus zippeliana* Miq., *Quercus gilva* Bl., *Q. myrsinaefolia* Bl., *Cryptomeria japonica* (L. fil.) D. Don., and *Celtis sinensis* Pers. were densely planted, and in the understory, seedlings of the aforementioned trees, ferns, *Ardisia crenata* Sims, *Achyranthes bidentata* Blume var. *japonica* Miq., and *Ficus erecta* Thunb. were growing.

Takakuma Experimental Forest is located at Mt. Takakuma in Tarumizu City. In the sampling stand (~550 m above sea level), *Pasania edulis* was dominant and accounted for about 40% of the dominant crown trees. The other dominant crown tree species were *Persea thunbergii*, *Castanopsis cuspidata* (Thunb.) Schottky var. *sieboldii* (Makino) Nakai, *Neolitsea sericea* (Bl.) Koidz., *Quercus*

acuta Thunb., and *Q. salicina* Bl. The canopy was closed. In the understory, *Cleyera japonica* Thunb., *Symplocos lucida* Sieb. et Zucc., *Camelia japonica* L., and *Illicium anisatum* L. were dominant.

Isolation and cultural methods

To see the outline of endophytic mycobiota of *Pasania edulis* leaves, endophytes were isolated from 25 1-year-old leaf samples collected from a tree of *P. edulis* in the Experimental Nursery on May 22, 2000. Following this preliminary survey, endophytes of *P. edulis* leaves were isolated from a stand in Takakuma, where this tree species is dominant. On September 1, 2000, 25 current-year leaves were collected from five trees (5 leaves from each tree). On October 25, 2000, 25 current-year and 25 1-year-old leaves were collected from five different trees (5 leaves of each age from each tree).

Endophytes were isolated by a surface sterilization method using hydrogen peroxide (H_2O_2) as the sterilizing agent, which is basically the same procedure as reported in Hata et al. (1998). Sample leaves were dipped in 70% ethanol for 1 min to wet the surface, surface-sterilized for 15 min in a solution of 15% H_2O_2, dipped again for 1 min in 70% ethanol, and then rinsed in sterilized distilled water. From the surface-sterilized leaves, segments approximately 2 mm × 2 mm were aseptically cut with a sterile scalpel. For the samples from the Experimental Nursery, ten segments (petiole segment, basal, central, and distal segments with midrib, with lateral vein, and without vein), and for the samples from Mt. Takakuma, five segments (petiole segment, basal and distal segments with midrib, and those without vein) were separated from each leaf as shown in Fig. 1. In the latter case, segment separation was simplified following the result of the former survey. The segments were then placed on 2% malt extract (Difco) agar medium in a 9-cm-diameter plate and incubated at room temperature (samples from the Nursery) or at 20°C (samples from Takakuma) for more than 1 month. The fungi growing out of the segments during the incubation period were recorded as endophytic fungi.

Data analysis

The isolation frequency (IF) of a single endophyte taxon was calculated by the following formula:

$$IF = (N_i/N_t) \times 100(\%)$$

where N_i and N_t are the number of segments from which the fungus was isolated and the total number of segments examined, respectively. Isolation frequencies of endophytes were compared using the chi-square test or Fisher's exact probability test.

Results

Table 1 shows endophytic fungi isolated from *Pasania edulis* leaves and their isolation frequencies. In 1-year-old

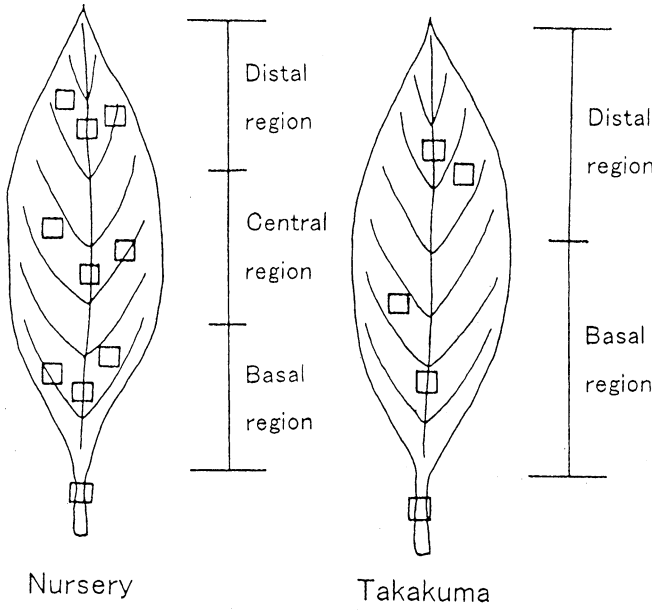

Fig. 1. Leaf segments examined in the present study. *Nursery*, samples from the Experimental Nursery: 10 segments ~2 × 2 mm (petiole segment, segments from basal, central, and distal regions with midrib, with lateral vein, and without vein) were separated from each sample leaf. *Takakuma*, samples from Mt. Takakuma: 5 segments ~2 × 2 mm (petiole segment, segments from basal and distal regions with midrib, and those without vein) were separated from each sample leaf

Table 1. Endophytes isolated from *Pasania edulis* leaves and their isolation frequencies with the result of the comparison of isolation frequencies among leaf segments

Sampling site	Nursery	Takakuma		
Sampling month	May	September	October	
Leaf age	1-year	0-year	0-year	1-year
Detected fungi	Isolation frequency (%)			
Phyllosticta sp.	56.0***	10.4	4.8	10.4
Colletotrichum spp.	22.0	8.8	5.6	9.6
Ascomycete sp. 1	0	36.8	34.4	48.0***
Phomopsis sp.	0	5.6	24.0*	25.6**
Apiognomonia sp.	0	8.8	0	0
Penicillium sp.	0	0.8	0.8	0
Coelomycete sp. 1	0	0	0.8	0
Hyphomycete sp. 1	0	0	0	2.4
Hyphomycete sp. 2	0	0	3.2	0.8
Hyphomycete sp. 3	0	0	0.8	0
Sterile sp. 1	0.4	0	4.0	0
Sterile sp. 2	3.2	0	1.6	5.6
Sterile sp. 3	1.6	0	0	0
Sterile sp. 4	4.0	0	5.6	8.0
Sterile sp. 5	0	3.2	2.4	3.2
Sterile sp. 6	0	3.2	1.6	1.6
Sterile sp. 7	0	0	2.4	0
Sterile sp. 8	0	0	0.8	0
Sterile sp. 9	0	0	2.4	2.4
Sterile sp. 10	0	0	0.8	0
Sterile sp. 11	0	0	0.8	1.6

*, **, *** Isolation frequencies among leaf segments significantly different by chi-square test at 0.05, 0.01, and 0.001 levels, respectively

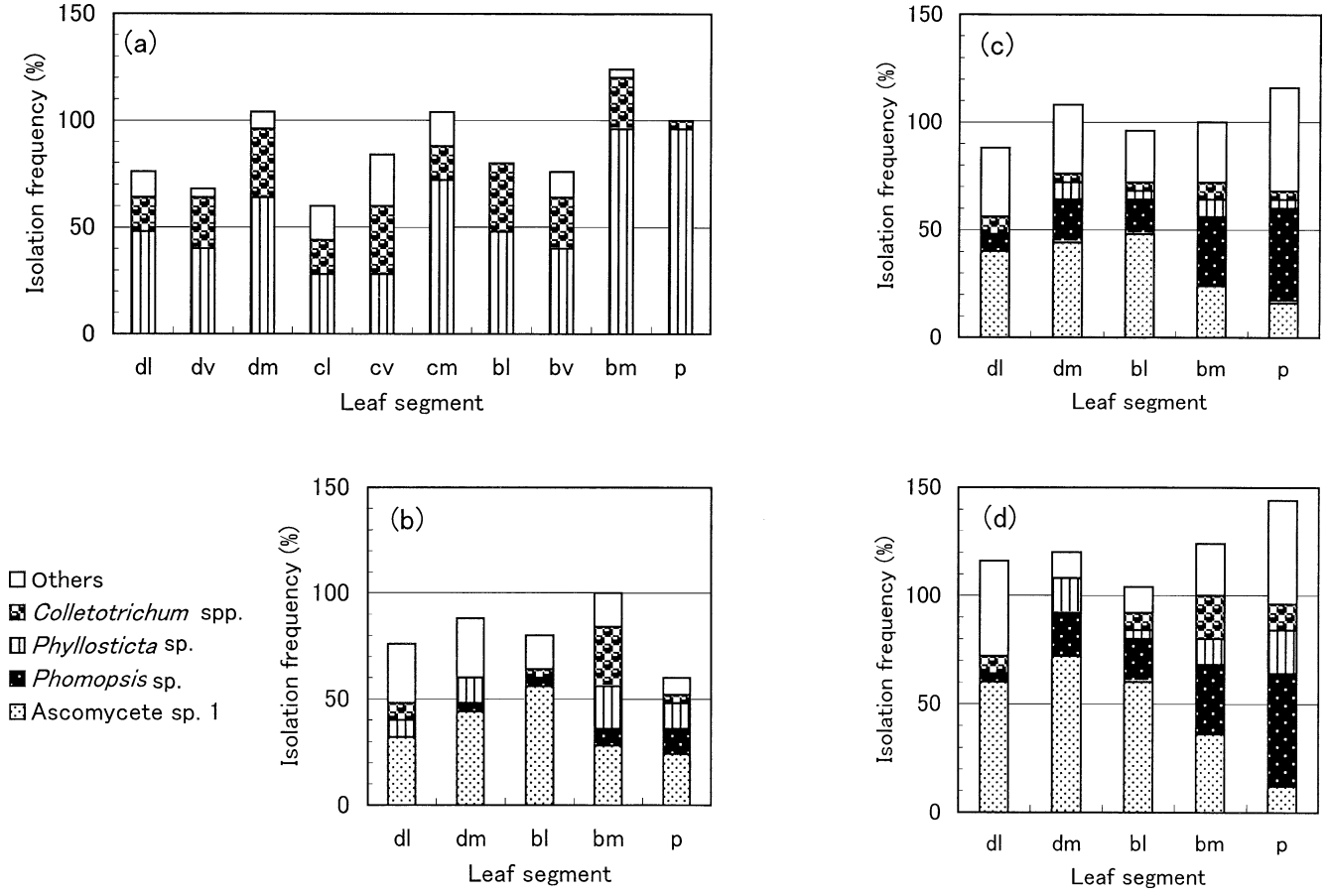

Fig. 2. Isolation frequencies of the dominant endophytes for respective leaf segments for (**a**) 1-year-old leaf samples from the nursery site on May 22, 2000; (**b**) current-year leaf samples from Takakuma site on Sept. 1, 2000; (**c**) current-year leaf samples from Takakuma site on Oct. 25, 2000; (**d**) 1-year-old leaf samples from Takakuma site on Oct. 25, 2000. *dl*, distal segment without vein; *dv*, distal segment with lateral vein; *dm*, distal segment with midrib; *cl*, central segment without vein; *cv*, central segment with lateral vein; *cm*, central segment with midrib; *bl*, basal segment without vein; *bv*, basal segment with lateral vein; *bm*, basal segment with midrib

leaves sampled from the Experimental Nursery, *Phyllosticta* sp. was most frequently isolated. *Colletotrichum* spp. (*Colletotrichum gloeosporioides* is the dominant member, and a few other species including *C. acutatum* occur in low frequencies) were also isolated in high frequency. In current-year leaves sampled from Mt. Takakuma on September 1, 2000, an unidentified ascomycete species (Ascomycete sp. 1) was most frequently isolated. In current-year and 1-year-old leaves sampled from Mt. Takakuma on October 25, Ascomycete sp. 1 was most frequently isolated, and *Phomopsis* sp. was second in isolation frequency in both leaf ages. Thus, Ascomycete sp. 1, *Phomopsis* sp., *Phyllosticta* sp., and *Colletotrichum* spp. were considered to be major endophytes of *Pasania edulis* leaves. Other isolates included *Apiognomonia* sp., *Penicillium* sp., an unidentified Coelomycete species, three unidentified Hyphomycete species, and 11 sterile isolates.

Figure 2 shows the isolation frequencies of the four major colonizers for each leaf parts, i.e., within-leaf distribution patterns, for (a) 1-year-old leaf samples from the nursery site on May 22, (b) current-year leaf samples from Takakuma site on September 1, (c) current-year leaf samples from Takakuma site on October 25, and (d) 1-year-old leaf samples from Takakuma site on October 25. Some of these endophytes showed characteristic within-leaf distribution patterns, which resulted in significant differences in isolation frequencies among leaf segments (see Table 1).

In the samples from the nursery, *Phyllosticta* sp. was isolated from petiole segments and segments with midrib significantly more frequently than segments with lateral vein and segments without vein ($P < 0.001$ by Fisher's exact probability test). *Colletotrichum* spp. were isolated from petiole segments significantly less frequently than other segments ($P < 0.05$ by Fisher's exact probability test). Thus, significant biases in within-leaf distribution were observed for these endophytes in the nursery. However, no significant differences in isolation frequency were observed between segments with lateral vein and segments without vein, and between distal and central segments for all the six fungal taxa isolated from the nursery (Fisher's exact probability test). Thus, in the samples from Takakuma, segments with lateral vein and central segments were omitted from the survey.

In Takakuma samples, Ascomycete sp. 1 tended to be isolated less frequently from petiole segments and basal segments with midrib than from other leaf segments ($P < 0.05$ by Fisher's exact probability test in 1-year-old leaves on October 25, 2000). On the contrary, *Phomopsis* sp. was isolated from petiole segments and basal segments with midrib significantly more frequently than other leaf segments on samples on October 25 ($P < 0.01$ by Fisher's exact probability test for both leaf ages).

Current-year and 1-year-old leaves sampled on October 25 were similar in mycobiota (18 species from current-year and 12 from 1-year-old leaves, 11 in common; see Table 1). Endophytes were isolated without significant difference in isolation frequencies between both age classes (Fisher's exact probability test), except for Ascomycete sp. 1, which were isolated more frequently from 1-year-old leaves ($P < 0.05$). Current-year leaves sampled in September and October were also similar in mycobiota (8 species from September samples and 18 from October samples, 7 in common; see Table 1). In this case, isolation frequencies of 3 species (*Phomopsis* sp., Sterile sp. 4, and *Apiognomonia* sp.) were significantly different ($P < 0.001$ for *Phomopsis* sp. and *Apiognomonia* sp., and $P < 0.05$ for Sterile sp. 4 by Fisher's exact probability test). Samples from the nursery and those from Takakuma showed apparent differences in endophytic mycobiota. Seven species, the 4 major endophytes, *Apiognomonia* sp., Sterile sp. 5, and Sterile sp. 6, showed significant differences in isolation frequencies (in the case of the 4 major endophytes, $P < 0.001$). Ascomycete sp. 1 and *Phomopsis* sp., major endophytes from Takakuma samples, were not isolated from the nursery, whereas *Phyllosticta* sp. and *Colletotrichum* spp. were much less frequently isolated from Takakuma.

Discussion

In the present study, four endophyte members were dominant. Species of *Phyllosticta* are sometimes reported as major endophytes of tree leaves. In Japan, Okane et al. (1998) have reported a species of *Guignardia* (anamorph: *Phyllosticta*) as a dominant endophyte of *Rhododendron* leaves. *Colletotrichum* species are commonly known as foliar pathogens, but some of them, especially *C. gloeosporioides*, have sometimes been reported as leaf endophytes (Okane et al. 1998). *Phomopsis* species have also been reported as endophytes. Especially, a species of *Phomopsis* has been reported as a major endophyte of Japanese beech leaves and twigs (Sahashi et al. 1999), which is worthy of note because Japanese beech (*Fagus crenata*) and *Pasania edulis* both belong to the tree family Fagaceae. These three fungal genera might be considered as genera that include common endophytes, possibly with relatively wide host ranges. If more host-specific endophytes are to be looked for, Ascomycete sp. 1 might be a candidate. The occurrence of *Apiognomonia* sp. is also interesting, because *Apiognomonia* and their anamorph *Discula* species are known as one of the major endophytes of Fagaceae (Sahashi et al. 1999).

Patterns of endophyte distribution within plant organs have widely been reported (Carroll 1995). In the present study, clear patterns of within-leaf distributions were observed for major endophytes. Isolation frequencies of *Phyllosticta* sp. in the nursery site tended to be higher in petiole segments and segments with midrib. In Takakuma, *Phomopsis* sp. showed a similar tendency, that the isolation frequencies were higher in petioles and basal segments with midrib. This pattern of within-leaf distribution may suggest the infection routes of these species as from twigs by the mycelium.

On the contrary, *Colletotrichum* spp. were less frequently isolated from petiole segments than other segments in the nursery. Ascomycete sp. 1 in Takakuma tended to be isolated less frequently from petiole segments and basal segments with midrib than other segments. This pattern may suggest the infection process of these fungi from spores on leaf surfaces. Thus, within-leaf distribution of these major endophytes may be determined by their infection modes.

Another possible explanation of the within-leaf distribution patterns of these endophytes is the interactions of these endophytes within leaves. On both sites, two major endophytes were isolated with inverse distribution tendencies; one seems to prefer the leaf base while the other seem to avoid the leaf base. This observation may be attributed to the competitive interactions of the endophytes within leaves.

In the case of Japanese beech, endophytic fungus *Phomopsis* sp. was isolated more frequently from petiole segments than leaf segments (Sahashi et al. 1999). In that case, *Phomopsis* sp. was isolated nearly 100% from twig segments, and thus the fungus seems to infect leaves from twigs through petioles. On the other hand, isolation frequency of *Phomopsis* sp. in beech leaf segments decreased while that of other endophytes increased in autumn. This finding suggests that these endophytes interact negatively each other. In endophytes of pine needles, Hata et al. (1998) reported that *Leptostroma* anamorph of *Lophodermium pinastri* complex was isolated from the middle region of needles more frequently than the basal region whereas *Phialocephala* sp. was isolated from the middle region less frequently than the basal region. In that case, both *Leptostroma* and *Phialocephala* sp. showed such distribution patterns even in the absence of the other endophyte species. Thus, their within-needle distribution patterns seemed to be determined mainly by their infection routes. However, negative interactions between endophytes might also be present, because isolation frequencies of *Phialocephala* sp. gradually decreased with needle age, just the contrary to *Leptostroma*, which gradually increased with needle age.

The small difference in endophytic mycobiota was observed between September samples and October samples, and between current-year and 1-year-old leaves in October in the Takakuma site. On the other hand, a conspicuous difference in endophytic mycobiota was observed between

samples from the nursery and those from Takakuma. This result might be attributed to the difference in circumstances of these two sites. In the present study, however, the experimental design was not planned to study such differences according to sampling location. So, other factors, such as sampling season, might also have affected the results. Further studies for *Pasania edulis* endophytes will be required to answer such questions.

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