

Original Article

Isolation of fungi from *Pasania edulis* trees attacked by *Platypus quercivorus*.HATA Kunihiko¹⁾ and SONE Koichi¹⁾

1) Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

Received Sep 18, 2009 / Accepted Dec 8, 2009

Summary

Fungi were isolated from wood chips taken from living or dead *Pasania edulis* trees previously attacked by the oak borer *Platypus quercivorus*. The pathogenic fungus *Raffaelea quercivora* was certainly isolated but in low frequencies. The most frequently isolated fungus was *Rhinoctadiella* sp., a dematiaceous hyphomycete. Isolation frequencies of the fungi considerably varied among trees. Presence or absence of beetle pores and wood discoloration in sample wood chips affected the isolation frequencies of the fungi.

Key Words: *Pasania edulis*, oak wilt, fungal isolation, *Raffaelea quercivora*, *Rhinoctadiella* sp.

Introduction

Recently, mass mortality of oaks and related broadleaf trees has come to be a serious problem of forest pathology in Japan. This mortality is closely associated with the attack of an ambrosia beetle *Platypus quercivorus* (Murayama) (Ito 2000, 2002). Based on intensive studies, the cause of the mortality has been ascribed to a hyphomycetous fungus *Raffaelea quercivora* Kubono et Ito, which has been isolated from trees attacked by *P. quercivorus* (Ito et al. 1998; Kubono and Ito 2002) and proved to be transported by the beetle (Kinuura 2002). *R. quercivora* proved to be the pathogen after inoculation tests, which caused typical symptoms of the mortality (Ito et al. 1998; Yamato et al. 2001). As an ambrosia beetle, *P. quercivorus* has mutualistic ambrosia fungi providing food for their hosts. They have been reported as yeast species including those of the genus *Candida*, while *R. quercivora* is suggested to have no nutritional roles for the beetle (Kinuura 2002). Thus fungi directly associated with *P. quercivorus* have been studied relatively in detail. However, although the growth and persistence of the pathogen and the yeasts should be affected by other fungi associated with their habitat, such fungi have not been well-studied.

In the prefectures along the Sea of Japan, *Quercus mongolica* Fisch. var. *grosseserrata* (Bl.) Rehd. et Wils. and *Quercus*

serrata Murr., especially the former, have been intensively damaged (Ito 2000, 2002). In contrast, various kinds of trees in Fagaceae and related families have been moderately damaged in Kyushu and Kii peninsula, (Ito 2000, 2002; Ito and Yamada 1998). Among such Fagaceous trees, *Pasania edulis* (Makino) Makino has been reported to suffer relatively conspicuous damages in southern Kyushu. In Kagoshima Pref., intensive studies on the life history of *P. quercivorus* on *P. edulis* have been conducted (Sone et al. 1998; Sone et al. 2000), but few cases of fungal isolation has been reported.

Thus, we tried to isolate fungi from *P. edulis* attacked by *P. quercivorus* in Sakurajima Island in Kagoshima Pref., in order to make a list of fungi associated with tissues of *P. edulis* attacked by *P. quercivorus* and examined the effects of several factors, which may affect fungal colonization, on the frequencies of the fungi.

Materials and Methods

Samples were collected from a stand on a col in the north-western slope of Sakurajima Island (ca. 350m above sea level) where evergreen broadleaf trees dominated. *P. edulis* was the dominant crown species occupying about 60% of the crown trees and *Persea thunbergii* (Sieb. et Zucc.) Kosterm. was the

second dominant, while few other species were present. Mass mortality of *P. edulis* started from 1998. By the time of the sampling, most of the *P. edulis* trees had been attacked by the beetle and many (about 70%) had been killed. *P. thunbergii* had also been attacked, but never killed.

On March 2001, 6 mature trees of *P. edulis*, which had been attacked by the beetle in the previous year (in 2000) or in one more year earlier (in 1999), were selected from those comprising the crown layer, to collect samples (Table 1). Four of the selected trees had been killed and the other two were alive. Wood chips were collected directly from the trunks (0.5-1.5m above ground) of these trees standing in the field using a hand drill sterilized before each sampling by 70% ethanol. For each selected tree, a hole was made and wood chips obtained from the hole were separately collected and carried back to the laboratory in a polyethylene bag. Wood chips (which had not been torn into

small pieces but kept to be a long chip) were selected according to the presence / absence of the brownish discoloration of wood caused along with the infection of the pathogen and the pores of the beetle gallery, to give sample codes (Table 1). From each sample wood chip, 5 semicircular pieces of about 1.5 cm diameter and 1 mm thick were cut out by sterile scalpel and then surface-sterilized by 15% hydrogen peroxide for 1 min and placed on water agar plates (20g agar in 1 liter distilled water). Plates were incubated at 20 °C for 1 month and fungi growing out of the chips were recorded, isolated and identified. The use of water agar here was for the purpose of slowing the growth of fast-growing fungi which may exist and hinder the slow-growing fungi.

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

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

where N_i and N_t are the number of wood chips from which the fungus was isolated and the total number of chips examined, respectively. In order to examine among-tree variations in IF, IF of each fungus was compared between that of chips in trees from which the fungus was isolated and that of chips in trees from which the fungus was not isolated using Fisher's exact probability test, because the numbers of chips from which the fungi were isolated in a single tree were generally too small to conduct chi-square test, which is a normal statistical method to apply for this kind of case. In order to examine the effect of factors such as tree survival, attack year, beetle pore and discoloration, IFs of the fungi were compared between chips from dead trees and survived trees, between chips from trees attacked in 2000 and 1999, between chips with and without beetle pores and between chips with and without discoloration, using Fisher's exact probability test.

Table 1. Synopsis of the sample wood chips.

Tree Code	Attack Year	Tree Survival	Sample Code	Beetle Pore	Discoloration	Nos. of Chips
E9A1	1999	Alive	1-1a	+	+	5
			1-1b	-	-	5
			1-1c	-	+	5
			1-2a	-	+	5
			1-2b	-	-	5
E9D1	1999	Dead	2-pa	+	-	5
			2-pb	-	+	5
			2-1a	+	+	5
			2-1b	-	-	5
			2-2a	-	+	5
			2-2b	-	-	5
E9D2	1999	Dead	3-pa	+	+	5
			3-pb	-	+	5
			3-pc	-	-	5
			3-ua	-	+	5
			3-ub	-	-	5
			3-da	+	+	5
			3-db	+	-	5
			3-dc	-	-	5
E0A1	2000	Alive	4-pa	+	+	5
			4-pb	+	-	5
			4-1	-	-	5
			4-2	-	-	5
E0D1	2000	Dead	5-1	+	+	5
			5-2	+	-	5
			5-3	-	+	5
E0D2	2000	Dead	6-pa	+	+	5
			6-pb	-	-	5
			6-2a	+	+	5
			6-2b	-	-	5
			6-3	-	-	5

Table 2. List of fungi isolated from the wood chips examined and their isolation frequencies.

Fungi	Frequency
<i>Rhinocladiella</i> sp.	12.3 ^a (19/155) ^b
<i>Phomopsis</i> sp.	6.5 (10/155)
<i>Raffaelea quercivora</i>	2.6 (4/155)
<i>Fusarium</i> sp.	2.6 (4/155)
<i>Cryptosporiopsis</i> sp.	2.6 (4/155)
Sterile WA1	1.9 (3/155)
Aphyllophorales sp. 1	1.3 (2/155)
<i>Trichoderma</i> sp.	0.6 (1/155)
unidentified	1.3 (2/155)

a: Isolation frequency (%) defined in the text.

b: Number of wood chips colonized by the fungus / Total number of wood chips examined

Results

Table 2 shows the list of fungi isolated from the sample wood chips and their frequencies. *R. quercivora* was certainly isolated from *P. edulis* trees attacked by *P. quercivorus*, but the frequency was only 2.6%. The fungus most frequently isolated from the wood chips was *Rhinochadiella* sp., followed by *Phomopsis* sp. Other fungi, including *Fusarium* sp., *Cryptosporiopsis* sp., a white sterile fungus “Sterile WA1”, “Aphylllophorales sp. 1”, a species of Aphylllophorales, which formed tiny fruiting bodies in test tubes, and *Trichoderma* sp. were rather rare isolates.

Fig. 1 shows the isolation frequencies of the fungi for each tree. In general, isolation frequencies of the fungi considerably

varied among trees. Species except for *Rhinochadiella* sp. and *R. quercivora* were isolated only from single trees. These biases in fungal isolation among trees proved to be highly significant (IFs of chips in trees from which the fungus was isolated were significantly higher than those of chips in trees from which the fungus was not isolated) except for Aphylllophorales sp.1 and *Trichoderma* sp., which were extremely low in IF, isolated only from 1 or 2 chips (Table 3). Effects of tree survival and attack year were shown to be significant in *Phomopsis* sp. and *Fusarium* sp. for tree survival and *Rhinochadiella* sp., *Phomopsis* sp. and *Fusarium* sp. for attack year, but the effects were much lower than among-tree variations judging from significance level, especially for *Phomopsis* sp. and *Fusarium* sp. (Table 3).

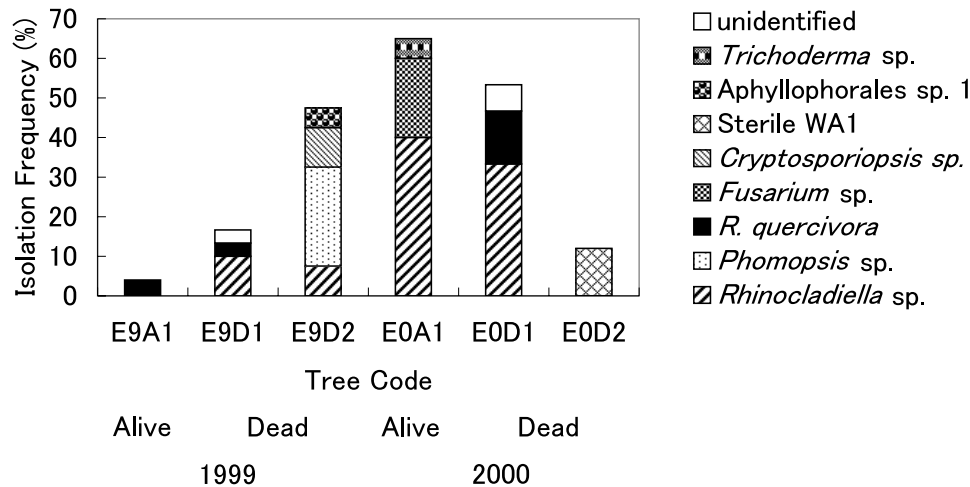


Fig. 1. Isolation frequencies of the fungi obtained from wood chips from each sample tree.

Table 3. Effect of several factors on isolation frequencies of the fungi, shown as P values of Fisher's exact probability test.

Fungi	Tree ^a	Survival ^b	Attack year ^c	Beetle pore ^d	Discoloration ^e
<i>Rhinochadiella</i> sp.	0.0018 ⁺⁺	0.19	0.0059 ⁺⁺	2.1 X 10 ⁻⁵⁺⁺⁺	1
<i>Phomopsis</i> sp.	5.2 X 10 ⁻⁷⁺⁺⁺	0.035 ⁺	0.0071 ⁺⁺	0.51	0.0076 ⁺⁺
<i>Raffaelea quercivora</i>	0.040 ⁺	1	0.30	0.021 ⁺	0.62
<i>Fusarium</i> sp.	0.00021 ⁺⁺⁺	0.0064 ⁺⁺	0.021 ⁺	0.021 ⁺	0.12
<i>Cryptosporiopsis</i> sp.	0.0040 ⁺⁺	0.32	0.16	1	0.62
Sterile WA1	0.0038 ⁺⁺	0.56	0.056	0.62	0.61
Aphylllophorales sp. 1	0.065	1	0.52	0.52	0.23
<i>Trichoderma</i> sp.	0.13	0.29	1	0.15	0.48

a: among-tree variations in IF shown as differences between trees from which the fungus was isolated and trees from which the fungus was not isolated.

b: effect of survival or death of the host tree on IF shown as differences between survived trees and dead trees.

c: effect of attack year of the host tree on IF shown as differences between trees attacked in 1999 and 2000.

d: effect of the presence of beetle pore on sample wood chips on IF shown as differences between wood chips with and without beetle pore.

e: effect of the discoloration of sample wood chips on IF shown as differences between wood chips with and without discoloration.

+: P<0.05 ++: P<0.01 +++P<0.001

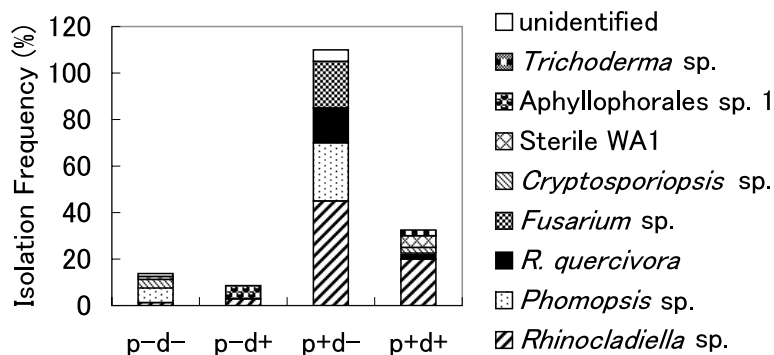


Fig. 2. Isolation frequencies of the fungi obtained from wood chips with or without beetle pores and wood discoloration. p-d-: wood chips with neither beetle pores nor discoloration. p-d+: wood chips without beetle pores but with discoloration. p+d-: wood chips with beetle pores but without discoloration. p+d+: wood chips with both beetle pores and discoloration.

Fig. 2 shows the isolation frequencies of the fungi isolated from wood chips with or without beetle pores and wood discoloration. Fungi in total were isolated more frequently from segments with beetle pores than without those, and from segments without discoloration than from those with discoloration, concentrating on chips with pores and without discoloration. For each fungus, *Rhinocladiaella* sp., *R. quercivora*, and *Fusarium* sp. were significantly more frequently isolated from chips with beetle pores than those without pores, while *Phomopsis* sp. was isolated more frequently from segments without discoloration than those with discoloration (Table 3).

Discussion

R. quercivora was surely isolated from *P. edulis* trees attacked by *P. quercivorus*. However, the isolation frequency of *R. quercivora* was rather low in the present study. There may be two possibilities for this low isolation frequency. One is the method used. Surface sterilization by 15% hydrogen peroxide for 1 min might be too rigorous to isolate *R. quercivora* from wood chips, or the medium used, water agar, might be too poor for some fungi including *R. quercivora*. The other is the sampling season; *R. quercivora* may not be easy to survive until next spring. Such difficulty in isolation of *R. quercivora* from trees killed some time ago has also been known in other cases (Ito et al., 1998). However, *R. quercivora* was isolated both from trees attacked in 2000 and 1999, with no significant difference in IF, which shows that *R. quercivora* can persist in the trees at least nearly 2 years.

Rhinocladiaella sp., which was isolated most frequently in the present study, is a dematiaceous hyphomycete species which

formed slow-growing black colonies on culture. They formed *Cladosporium*-like conidia in addition to *Rhinocladiaella*-type conidia. In Ito et al. (1998), *Phialophora* sp. is reported as dominant dematiaceous fungi isolated from trees attacked by *P. quercivorus*. Among such dematiaceous fungi, species of *Fonsecaea* form conidia of *Rhinocladiaella*-, *Cladosporium*- and *Phialophora*-type in addition to *Fonsecaea*-type (Larone 1995). Thus, present "*Rhinocladiaella* sp." and "*Phialophora* sp." of Ito et al. (1998) might be the same or related species belonging to the genus *Fonsecaea* or similar fungi. Anyway, these dematiaceous fungi are likely to be the cause of black color of the old gallery wall of *P. quercivorus* judging from the high isolation frequencies and concentration on chips with beetle pores.

Conspicuous variations in isolation frequencies of the fungi among trees suggests that fungal colonization of beetle-attacked trees is considerably variable. The factors determining such variations may include survival and vitality of trees and insects, attack year and season, and the member of fungi originally associated with the host tree or beetles before attack. In the present study, however, although tree survival and attack year on fungal isolation frequencies showed statistically significant effects on some fungi, their effects were much lower than those of among-tree variations, which suggests that such effects were only as results of large variations in IF among tree individuals. Concentration of 6 of 8 fungi on single trees supports such discussion.

The existence of beetle pores showed significantly positive effects on the isolation frequencies of *Rhinocladiaella* sp., *R. quercivora* and *Fusarium* sp. This suggests that these fungi are mainly associated with the gallery wall of beetles and not colonized within wood tissues deeply. In the case of *R. quercivora*,

which usually invades into wood tissues and cause discoloration, the result may indicate that it can invade into wood tissues but the ability of persistence there is limited, although the possibility may also exist that the apparent effect of beetle pores is only as a result of biased sampling because we collected samples from standing trees without looking at the inside of the trunk. On the other hand, discoloration of the wood chips showed negative effect on fungal isolation frequency, especially on *Phomopsis* sp. This may imply that discolored wood tissues are not suitable for the colonization of some fungi including *Phomopsis* sp., which has been reported as endophytic fungus also in *P. edulis* (Hata et al. 2002).

Acknowledgements

The authors thank Dr. H. Masuya, Forestry & Forest Products Research Institute, for his help in the identification of *R. quercivora*. We are also grateful to Mr. Yoshikazu Sato and the members of the Kagoshima Prefecture Forest Experiment Station for their general help in this work.

Reference

- Hata K, Atari R, Sone K (2002) Isolation of endophytic fungi from leaves of *Pasania edulis* and their within-leaf distributions. *Mycoscience* 43: 369-373
- Ito S (2000) Microbe-insect interactions threatening forest ecosystems. In: Futai K, Hijii N (eds) *Forest microbe ecology*. Asakura Shoten, Tokyo, pp 257-269 (in Japanese)*
- Ito S (2002) Fungi associated with oak wilt and the mechanisms of the mortality. *Shinrin-Kagaku* 35: 35-40 (in Japanese)*
- Ito S, Kubono T, Sahashi N, Yamada T (1998) Associated fungi with the mass mortality of oak trees. *J Jpn For Soc* 80: 170-175 (in Japanese with English summary)
- Ito S, Yamada T (1998) Distribution and spread of the mass mortality of oak trees. *J Jpn For Soc* 80: 229-232 (in Japanese)
- Kinuura H (2002) Relative dominance of the mold fungus, *Raffaelea* sp., in the mycangium and proventriculus in relation to adult stages of the oak platypodid beetle, *Platypus quercivorus* (Coleoptera; Platypodidae). *J For Res* 7: 7-12
- Kubono S, Ito S (2002) *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 225-260
- Larone DH (1995) *Medically important fungi - a guide to identification*. 3rd. edition Springer-verlag
- Sone K, Mori T, Ide M (1998) Life history of the oak borer, *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae). *Appl Entomol Zool* 33: 67-75
- Sone K, Uto K, Fukuyama S, Nagano T (2000) Effects of attack time on the development and reproduction of the oak borer, *Platypus quercivorus* (Murayama). *Jap J Appl Entomol Zool* 44: 189-196
- Yamato M, Yamada T, Suzuki K (2001) Wilt of oaks - Blockage of xylem sap ascent. *Bull Tokyo Univ For* 106: 69-76 (in Japanese with English summary)

* The titles are approximate translations of the original Japanese title by the authors of this paper.

カシノナガクイムシに加害されたマテバシイからの菌の分離

畑 邦彦¹⁾・曾根 晃一¹⁾

1) 鹿児島大学農学部生物環境学科

要 旨

カシノナガクイムシに加害されたマテバシイより採取した木片から菌の分離を試みた。病原菌*Raffaelea quercivora*は分離されたが、分離頻度は低かった。最も高頻度で分離された菌は黒色糸状不完全菌*Rhinoctadiella* sp.であった。菌の分離頻度は宿主個体間で大きく異なっていた。一方、試料木片におけるカシノナガクイムシの坑道の存在及び材の変色の有無は菌の分離頻度に影響を与えた。

キーワード：マテバシイ，ナラ・カシ・シイ類集団枯損，分離，*Raffaelea quercivora*，*Rhinoctadiella* sp.