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Formation of Teleomorph of the White Root Rot Fungus, *Rosellinia necatrix*, and the Potential Role of its Ascospores as Inocula

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Stromata of the white root rot fungus, *Rosellinia necatrix*, were produced on diseased roots although they were reported to develop rarely in nature. Forty-two (42) out of 47 samples produced synnemata while 23 developed stromata. Forty-seven (47) isolates obtained from diseased root samples were divided into 24 mycelium compatibility groups (MCGs). Sixteen (16) out of 24 MCGs produced stromata. Single ascospore isolates from 10 stroma samples produced dsRNA-containing isolates from diseased tissue beneath stromata. The frequency of synnema production on axenic culture varied among isolates with different origin. The dsRNA was not transmitted vertically to the ascospore offspring despite the infection of various dsRNA in the parental isolates. The dsRNA was absent in 35 ascospore isolates in two stroma samples that originated from the isolates, in which dsRNA was not eliminated by hyphal tip isolation. Consequently, sexual reproduction in the white root rot fungus was suggested to produce propagules as a new infection source and to have the function to eliminate infectious factors such as mycoviruses.

Keywords **:** ascospore, *Rosellinia necatrix*, stromata, white root rot

Rosellinia necatrix Prilleux, the ascomycetous white root rot pathogen, causes destructive damage to numerous woody and herbaceous plants, especially to fruit trees, throughout the world (Ito and Nakamura, 1984; Khan, 1959; Sztejnberg and Madar, 1980; Watanabe, 1963). Diseased plants show yellowing, defoliation, wilting, and eventually death. Diseased roots typically have plumose mycelia on the surface and fan-shaped mycelial strands under the bark.

The causal fungus rarely produces teleomorph on diseased plants in nature (Francis, 1985; Teixeira de Sousa, 1991). Also, the production of its teleomorph on artificial media has not been reported yet. Some researchers (Hansen et al., 1937; Nakamura et al., 2000; Teixeira de Sousa, 1991) experimentally succeeded in the production of stromata on diseased roots and inoculated trees and observed the process of stroma formation. Stromata were developed beneath synnemata after subiculla were produced on the surface of trunks and roots.

Field observations on spatial distribution of diseased trees suggest that the white root rot fungus expands vegetatively through the soil from diseased roots to healthy roots (Itoi et al., 1964; Khan, 1959; Matsuo and Sakurai, 1954). The role of ascospores as propagules, however, remains unclear due presumably to the scarcity of teleomorph production. Ascospore isolates were only confirmed to be pathogenic to apple trees since previous reports mostly focused on taxonomy and identification of the white root rot fungus (Hansen et al., 1937; Teixeira de Sousa, 1991).

Matsumoto (1998) proposed a method to control white root rot using the hypovirulent factor, i.e. mycoviruses or double-stranded (ds) RNAs, which is known to reduce virulence in many plant pathogenic fungi (Anagnostakis, 1982; Nuss and Koltin, 1990; Rogers et al., 1986). Arakawa et al. (2002b) detected various dsRNA elements from isolates of the white root rot fungus in Japan and found a multisegment dsRNA as a hypovirulent factor (Arakawa et al., 2002a). Sexual reproduction functions as a mechanism to eliminate dsRNA associated with the hypovirulence in *Cryphonectria parasitica* (Murrill) Barr and other fungi (Buck, 1986; 1998). Such a mechanism has not been investigated in white root rot fungus.

This study demonstrated that stromata in the white root rot fungus were easily produced in the open air when diseased root samples were placed on the ground under the shade. It also compared the difference in virulence between single ascospore isolates and their presumed parents originating from vegetative hyphae in the tissues of diseased roots. Vertical transmission of dsRNA through sexual reproduction in the fungus was also investigated. The role of ascospores as propagules was discussed in terms of the life history of the white root rot fungus, *R. necatrix*.

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Materials and Methods

Production of stromata on diseased roots and inoculated twigs. Stroma production on diseased roots was induced as described previously (Nakamura et al., 2000). Samples of diseased roots (1.5-5.0 cm diameter, 5-30 cm long) were collected from Japanese pear and *Chloranthus glaber* in Chiba and from Japanese pear and grapevine in Hiroshima from February 1999 to November 2000. A total of 47 samples were collected, and each sample included 1-10 fragments of diseased roots detached from a single tree (Table 1). Fragments of diseased roots were placed in a hole on the ground surface in the shade of trees and covered with rice straw for each sample, and incubated for 7-20 months. Fragments of diseased roots were observed by the naked eyes or under a stereomicroscope to check the presence of synnema and stromata in November 1999 to September 2001 after incubation.

Four samples with stromata that produced naturally on diseased trees were also collected from nonagricultural lands in April-May 2000 (Table 2). Isolates used for stroma production on inoculated mulberry twigs are listed in Table 3. Stromata were also obtained from inoculated twigs. Six isolates were used for inoculation of fragments of sterilized mulberry twigs (9-11 cm long, 0.8-1.4 cm diameter) in plastic bags (Table 3). The inoculated twig fragments

Table 1. Stroma production on diseased root samples and isolates of *Rosellinia necatrix* from the samples used in this study

Sample No.	Origin[a]	Production of stromata	Isolates[b]	
			MCG	dsRNA
CA1	P/C	–(0/8/9)[c]	93	–
CA2	P/C	–(0/5/6)	93	–
CA3	P/C	–(0/0/6)	93	–
YD	P/C	–(0/3/5)	94	+(IV)
Y3	P/C	+(1/1/1)	95	–
Y4	P/C	–(0/0/1)	96	–
YB	P/C	–(0/1/1)	97	–
YA	P/C	+(3/6/6)	97	–
B10	P/C	–(0/2/2)	99	–
E6	P/C	+(4/7/7)	99	–
E5	P/C	+(6/7/7)	100	–
M10	P/C	–(0/1/1)	101	–
M12	P/C	+(7/10/10)	101	–
C-16-1	P/H	–(0/1/2)	139	+(III)
X-16-2	P/H	–(0/0/3)	139	–
D-13	P/H	–(0/0/1)	139	–
F-17	P/H	–(0/1/2)	139	–
F-18	P/H	+(1/3/3)	139	–
F-19	P/H	–(0/3/3)	139	–
G-12	P/H	+(1/3/3)	139	+(II)
G-16	P/H	+(1/2/2)	139	+(III)
G-18	P/H	+(3/3/3)	139	–
G-24	P/H	–(0/1/1)	139	–
H-13	P/H	+(3/3/3)	139	–
H-14	P/H	–(0/2/2)	139	–
I-22	P/H	+(1/2/2)	139	–
U-1	P/H	–(0/2/3)	139	–
U-6	P/H	+(1/2/2)	139	–
C-18	P/H	+(3/3/3)	140	–
D-3	P/H	+(1/3/3)	141	–
F-2-1	P/H	–(0/2/3)	142	–
I-20	P/H	–(0/2/2)	142	–
F-2-2	P/H	–(0/3/3)	150	+(IV)
F-5	P/H	–(0/3/3)	150	–
U-2	P/H	+(2/3/3)	151	–
D-7	P/H	+(1/1/1)	310	–
J-11	P/H	+(1/3/3)	311	–
I-4	G/H	+(2/3/3)	136	+(IV)
I-5	G/H	+(1/3/3)	136	+(IV)
3-9	G/H	–(0/2/2)	138	–
1-2	G/H	–(0/3/3)	152	–
3-3	G/H	–(0/3/3)	152	+(V)
3-1	G/H	+(3/3/3)	153	–
3-5	G/H	+(1/2/2)	155	–
S2-1	C/C	+(2/5/5)	276	–
S2-2	C/C	+(2/2/2)	277	–
S4	C/C	–(0/2/2)	278	–

[a] P/C, Japanese pear in Chiba; P/H, Pear in Hiroshima; G/H, Grapevine in Hiroshima; C/C, *Chloranthus glaber* in Chiba.
[b] Mycelial compatibility group of isolates derived from diseased tissue and presence of dsRNA in the isolates. Type of dsRNA in parenthesis was determined on the basis of electrophoretic patterns according to Arakawa et al. (2002).
[c] No. of root fragments with stromata; No. of root fragments with synnemata; No. of root fragments treated.

Table 2. Production of stromata by *Rosellinia necatrix* on inoculated mulberry twigs

Isolate No.	MCG	Origin[a]	dsRNA	Production of stromata
w96	82	P/S	+	+(1/14/14)[e]
w98	80	P/S	+	+(2/14/14)
w112	85	P/S	+	–(0/20/20)
w118[b]	86	P/S	+	–(0/18/18)
w153[c,d]	95	P/C	–	–(0/14/14)
w422[e]	158	P/M	+	–(0/20/20)

[a] P/S, pear in Saga; P/C, Pear in Chiba; P/M, Pear in Mie.
[b] Synnema production poor in axenic culture.
[c] Synnema production abundant in axenic culture (unpublished).
[d] Isolated from diseased root sample Y3 in Table 1.
[e] No. of twig fragments with stromata; No. of twig fragments with synnemata; No. of twig fragments placed in a pot.

Table 3. List of isolates of vegetative origin (infected tissue) and of ascospore origin from six different stroma samples of *Rosellinia necatrix*

Sample No.	Host plant	Locality	Origin of stroma	Isolate[a] from	
				infected tissue (parent)	single ascospores (offsprings)
KA1	unknown	Akita	N[b]	w536	w540, w541, w542
KM1-1	*Quercus* sp.	Chiba	N	w490	w491, w493, w494
KN-1	unknown	Hyogo	N	w427	w443, w447, w450
M12	Japanese pear	Chiba	A[c]	w233	w611, w612, w614
N1	unknown	Ibaraki	N	w503	w511, w513, w515
Y3	Japanese pear	Chiba	A	w153	w462, w464, w467

[a] All isolates belonged to different mycelial compatibility groups.
[b] Natural material from non-agricultural land.
[c] Produced artificially on diseased root, according to Nakamura et al. (2000).

were incubated for 2 months in plastic bags and then placed in a pot (25 cm diameter, 30 cm deep) containing a small amount of commercial soil (Kanuma soil) and covered with rice straw. The twig fragments were incubated for five months in the shade of trees, and then the stromata production was observed as above.

Isolates of *R. necatrix* from vegetative hyphae and ascospores. Six pairs of isolates of vegetative origin (infected tissue, referred to as parents) and of ascospore origin were used to determine genetic diversity in offspring through sexual reproduction (Table 3). Pure cultures were obtained from fan-shaped mycelia or from small pieces of infected sapwood and, in case of samples with stromata in non-agricultural lands, from small pieces of sapwood beneath stromata. Mycelia or sapwood pieces were placed on 2% water agar (WA) containing 200 ppm of streptomycin sulphate and incubated at 23°C in the dark for a week. A piece of hypha was transferred from a growing colony onto potato-dextrose agar (PDA). Identification of isolates was based on the presence of pear-shaped swellings adjacent to the septa in the hyphae. A piece of bark with stromata was detached, rinsed with tap water, and then placed overnight in a Petri dish containing a piece of heavily moistened filter paper. A mass of ascospores glued in slime exuding from the apex of stromata was picked up with a needle and then streaked on WA or one-fifth strength PDA containing 200 ppm streptomycin sulphate. Isolation plates were incubated at 23°C in the dark for a few days. A single germinating ascospore was transferred onto PDA. Several single ascospore isolates were obtained from a stroma for each sample.

Mycelial compatibility groups. Parents and their offspring were paired in all possible combinations on 9-cm-diameter oatmeal agar (OA) plates to determine mycelial compatibility groups (MCGs). Inoculum blocks (3×3×3 mm) were cut from the margin of growing colonies. Six blocks were placed along the periphery with same interval (ca. 2 cm) and one in the center (Fig. 1) and incubated at 23°C in the dark. Plates were checked for the presence of demarcation lines after 3 weeks. Isolates growing together without a demarcation line were considered to belong to the same MCG.

Virulence test. Inoculation test for the isolates followed the method of Uetake et al. (2001). Three mycelial discs were placed on OA in a thick Petri dish (9 cm diameter, 2 cm thick) and

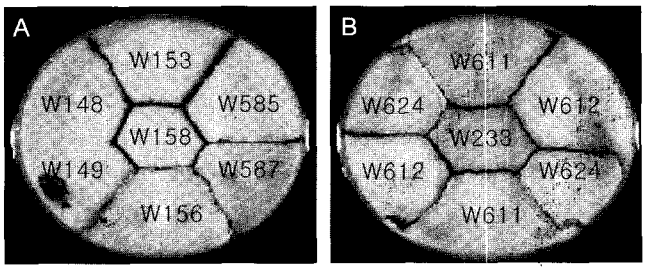

Fig. 1. Mycelial incompatibility in isolates of *Rosellinia necatrix* 4 weeks after incubation at 23°C in the dark on oatmeal agar. (A) mycelial incompatibility in five isolates namely, w148, w149, w153, w156 and w158, from pear and two isolates, w585 and w587, from *Chloranthus glaber*. There was no demarcation line between isolate w148 from diseased root sample CA1 and isolate w149 from sample CA2, and the isolates belong to the same mycelial compatibility group; (B) mycelial incompatibility between isolates from single ascospores in a stroma (w611, w612 and w624) and an isolate from infected tissue beneath the stroma (w233).

incubated for 10 days under light at 25°C. Eight pieces of autoclaved mulberry twigs (1.5-2.0 cm long, 1 cm diameter) were then placed on each culture plate. Cultures were incubated for 3 weeks until mycelia covered the whole mulberry twigs. Two seedlings of *Lupinus luteus* were grown in Kanuma soil in a plastic pot (5 cm wide, 15 cm long, 10 cm deep) in a greenhouse at 25°C for 3 weeks. Upper soil (ca. 2 cm deep) in the pot was removed. Then, an inoculum piece of mulberry twig was placed in contact with the hypocotyl and covered with soil to the original depth. Eight plants in four pots were used for each isolate and grown under the conditions described above. Symptoms on inoculated plants were observed daily from 1 to 2 weeks after inoculation, and virulence of each isolates was estimated using a disease index (DI) of 0-2: DI 0, showing no symptom; DI 1, plants wilted; DI 2, plants killed.

Detection of dsRNA. The dsRNA was detected according to the method of Arakawa et al. (2002b). Each isolate was grown on cellulose membrane overlaid on PDA plates (5 cm diameter) for a week. The cellulose membrane was stripped with mycelia from PDA plates and ground in liquid nitrogen with a mortar and

Table 4. Virulence of isolates of *Rosellinia necatrix* derived from vegetative origin (infected tissue) and of ascospore origin from six different stroma samples to *Lupinus luteus*

Stroma sample No.	Isolate No.	Origin of isolates[a]	Disease index[b]	Stroma sample No.	Isolate No.	Origin of isolates	Disease index
KA1	w536	V	1.5	M12	w233	V	1.25
	w540	A	1		w611	A	0.38**
	w541	A	1.63		w612	A	2*
	w542	A	1.13		w614	A	1.88
KM1-1	w490	V	2	N1	w503	V	1.88
	w491	A	2		w511	A	2
	w493	A	1.88		w513	A	1.88
	w494	A	1.88		w515	A	2
KN1-1	w427	V	2	Y3	w153	V	2
	w433	A	0		w462	A	2
	w447	A	2		w464	A	1.63
	w450	A	0		w467	A	1.63

[a] A, Isolated from a single ascospore; V, Isolated from infected tissue

[b] Average of two experiments with eight replicate plants per experiment. Disease index was rated two weeks after inoculation: 0, showing no symptom; 1, plants wilted; 2, plants killed.

pestle. The ground material (100 mg) was transferred to a 1.5 ml microcentrifuge tube, suspended in 750 µl of extraction buffer (0.1 M Tris-HCl, 0.1 M NaCl, 1% SDS, 0.1% 2-mercaptoethanol, pH 8.0) and centrifuged at 15,000×*g* for 10 minutes. The supernatant was collected, mixed with an equal volume of PCI solution (TE-saturated phenol : chloroform : isoamylalchol = 25 : 24 : 1, v/v) in a new tube and centrifuged at 10,000×*g* for 10 minutes. The PCI treatment was repeated twice. Aqueous phase was mixed with 40 µl of 5 M NaCl and 600 µl of 2-propanol in a new tube and incubated at 4°C for an hour. Total nucleic acids were collected by centrifugation at 20,000×*g* for 15 minutes at 4°C, and the pellet was dissolved in 150 µl of nuclease reaction buffer (50 units of DNase [DNase I, Takara Biochem., Ohtsu], 20 units of S1 nuclease [Takara Biochem., Ohtsu], 30 mM sodium acetate, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM $ZnSO_4$). DNA and single-stranded RNA in the solution were digested after several hours. After subsequent PCI treatment, 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol were added to the tube, and the sample was precipitated by centrifugation at 20,000×*g* for 15 minutes at 4°C. The pellet was dissolved in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C as a dsRNA preparation. The preparations were electrophoresed on 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and dsRNA fragments were visualized by staining with ethidium bromide.

Results

Production of stromata on diseased roots and inoculated twigs. Forty-two (42) out of 47 samples produced synnemata, while 23 developed stromata (Table 1). The number of stromata per root fragment ranged from one to several hundreds. Presence of synnemata did not always lead to stroma production although stromata were always produced on root fragments with synnemata. Stromata were observed within one year after treatment in most cases. It took 20 months for a sample from Japanese pear (G-12) and two samples from grapevine (1-4 and 3-5, Table 1) to produce stromata. Neither host plant species nor date of treatment affected stromata production. Stromata produced on 16 samples included numerous ascospores, but asci were immature in the rest of seven samples and had no ascospore.

Forty-seven (47) isolates from diseased tissue in 47 root samples were divided into 24 MCGs. Sixteen (16) out of 24 MCGs produced stromata. Fifteen (15) samples from Japanese pear in the same orchard were colonized by MCG 139, while other MCGs consisted of one to three isolates. Stroma production in MCG 139 was recognized in 7 out of 15 root samples. Stroma was not observed in root samples colonized by four MCGs namely, MCG 93, 142, 150, and 152.

Stromata were produced on fragments of mulberry twigs inoculated with two isolates, i.e. w96 and w98, out of six although synnemata were produced on all twig fragments inoculated with the six isolates (Table 3). Isolate w96 and w98 produced stromata on only 1 and 2 of 14 twig fragments, respectively. The number of stromata produced was very low, i.e. three stromata were produced on a twig fragment inoculated with isolate w96 and four were produced on two twig fragments inoculated with isolate w98. Isolate w153 from diseased tissue of root sample Y3

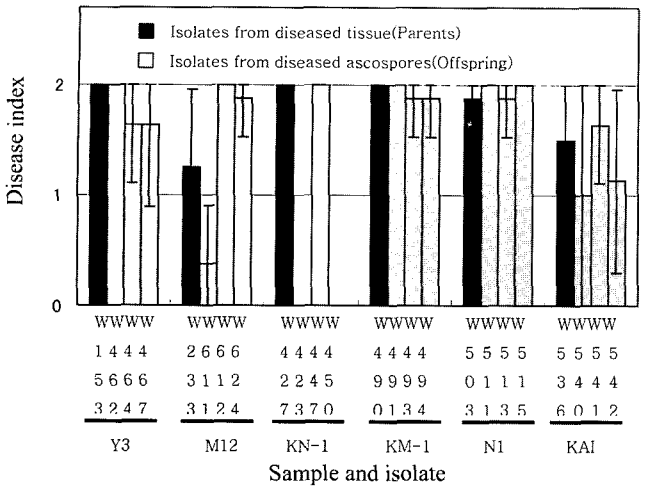

Fig. 2. Virulence of isolates of *Rosellinia necatrix* derived from diseased tissue and single ascospores from the stroma to *Lupinus luteus*. Inoculation test was made with eight replicates, and disease index(DI) was rated 2 weeks after inoculation: DI 0, showing no symptom; DI 1, plants wilted; DI 2, plants killed. Bars represent standard deviation.

which developed stromata failed to produce stromata on the twig fragments when inoculated.

Comparison in mycelial compatibility groups and virulence between isolates from diseased tissue and from single ascospores. Pairing of isolates from vegetative hyphae with three single ascospore isolates revealed that four isolates from each stroma sample belonged to different MCGs (Fig. 1B).

Virulence of four isolates from a stroma sample was similar to each other in four stroma samples, Y3, KM-1, N1 and KA1, although results were inconsistent in samples Y3 and KA1 (Fig. 2). On the other hand, stroma samples M12 and KN-1 showed variation in their virulence. In sample M12, virulence of an ascospore isolate w611 was lower than that of parental isolate w233 from diseased tissue, but two other ascospore isolates, w612 and w624, were more virulent than isolate w233. Two ascospore isolates (w423 and w450) from the sample KN-1 were not pathogenic, and one ascospore isolate (w447) was as virulent as the parental isolate (Fig. 2).

Detection of dsRNA in single ascospore isolates. Single ascospore isolates from ten stroma samples, which produced dsRNA-containing isolates from diseased tissue beneath stromata, were examined for the presence of dsRNA (Table 5). DsRNA was not detected from all single ascospore isolates although various types of dsRNA were present in the isolates from diseased tissue.

Discussion

Stromata of the white root rot fungus, *R. necatrix*, were produced easily on diseased roots although its stromata were reported to rarely develop (Francis, 1985; Teixeira de Sousa and Whalley, 1991). Several stroma samples were collected from diseased trees in forestland in various areas of Japan (data not shown), and thus stromata appear to develop easily in forestlands. On the other hand, stromata could not be found in orchards of Japanese pear, grapevine, loquat, and apple. In this study, the white root rot fungus occurring in orchards was found to have the ability for sexual reproduction. Consequently, stroma in orchards probably does not depend on immediate removal of diseased plants from the field and on longer time for development, as mentioned previously (Francis, 1985; Teixeira de Sousa, 1991; Nakamura et al., 2000). Stroma production was reported to require approximately 2 years after infection (Francis, 1985; Teixeira de Sousa and Whalley, 1991;

Table 5. Stroma samples of *Rosellinia necatrix* used and detection of dsRNA in single ascospore isolates

Sample No.	Host plant	Locality	Stroma production[a]	Type of dsRNA in isolates from diseased tissue[b]	Presence of dsRNA in single ascospore isolates[c]
G12-3	Pear	Hiroshima	AR	II	?/16
G16-2	Pear	Hiroshima	AR	III	?/16
1-4-2	Grapevine	Hiroshima	AR	IV	0/9
1-5-2	Grapevine	Hiroshima	AR	IV	0/9
96-1	Pear	Saga	AT	V	0/21
98-1	Pear	Saga	AT	IV	0/15
KM1-1	*Quercus* sp.	Chiba	N	IV	0/11
MC1-1	unknown	Chiba	N	V	0/5
KB1-1	unknown	Ibaraki	N	II	0/3
KK1-1	*Dendropanax trifidus*	Tokyo	N	IV	0/10

[a] AR, production artificially on diseased roots; AT, production artificially on inoculated twigs; N, production naturally on a diseased tree.
[b] Based on electrophoretic patterns, according to Arakawa et al. (2002).
[c] No. isolates containing dsRNA/No. isolates tested.

Nakamura et al., 2000), and it was supported by results of this study which showed that diseased root samples treated in winter and spring produced stromata the following summer.

Easiness of stroma production in the white root rot fungus was not related to host plant species, dates of treatment, and presence of dsRNA although dsRNA in other ascomycetous fungi was known to reduce sexual reproduction (Nuss and Koltin, 1990). MCG, however, could have possibly affected stroma production since stroma did not develop on plural root samples colonized by four MCGs. MCG is determined by mycelial incompatibility, which shows pigmented lines of demarcation or by sparse zones between different mycelia and is used for investigation of genetic variation among isolates (Nalim et al., 1995; Worrall, 1997). All isolates belonging to different MCGs are, therefore, considered to differ genetically. The frequency of synnema production on axenic culture varied among isolates with different origin (unpublished data). Similarly, frequency of stroma production differed among isolates belonging to different MCGs. Variation on easiness of stroma production might be attributed to the properties of MCGs.

Stromata on two root samples from *Chloranthus glaber*, which were treated in August 2000, were not produced in the same year, while those on samples treated in winter and spring were produced the following summer (data not shown). Stroma production seemed to depend on incubation of samples in the rainy season, mainly June in Japan. In preliminary experiments, stromata did not develop on several diseased root samples placed on the ground without rice straw (data not shown). Long moisture period was considered to be important for stroma production (Teixeira de Sousa and Whalley, 1991).

Stromata developed on approximately half of 15 root samples colonized by MCG 139, and stromata on a sample from Japanese pear and two samples from grapevine developed in the second year after treatment. Also, only a few stromata were produced on mulberry twigs inoculated. Stroma production in the white root rot fungus was presumably affected by slight differences in field conditions such as temperature and physical and nutrient conditions other than moisture as described previously (Teixeira de Sousa and Whalley, 1991). Development of stromata always followed appearance of synnemata on both diseased roots and inoculated twigs (Hansen et al., 1937; Teixeira de Sousa and Whalley, 1991; Nakamura et al., 2000). However, presence of synnemata did not always induce development of stromata. Conidia on synnemata were considered to be involved in mating events since conidia never developed into mycelia (Nakamura et al., 2000). Stroma production in the white root rot fungus might need mating events to succeed. Isolate w153 from diseased root sample Y3, which produced stromata, failed to produce stromata on the twig fragments. The conditions or mating events as mentioned above is suggested to have some effects on the stroma production of isolate w153 since inoculated twigs were placed in a pot, not on the ground.

Most of the single ascospore isolates in six stroma samples were pathogenic to *Lupinus luteus*, which was reported to be suitable to estimate virulence in the white root rot fungus (Uetake et al., 2001). The result revealed that ascospores of the white root rot fungus possibly played a role as propagules. Three single ascospore isolates and their parental isolate in each stroma sample belonged to different MCGs although interaction between single ascospore isolates from a stroma in another *Rosellinia* species, *R. desmazieresii*, showed mycelial compatibility (Sharland and Rayner, 1988). Single ascopsore isolates from the same stroma had variation of virulence. Also, two of three single ascospore isolates in stroma sample M12 had higher virulence compared with their parental isolate. Stroma production in orchards is suggested to lead to the spread of strains with higher virulence.

Ascospores of the white root rot fungus are known to have low ability of germination (Khan 1959). Hansen et al. (1937) were barely successful in germinating ascospores by means of lactic acid treatment. However, ascospores from stroma samples produced in this study germinated easily on WA and PDA without any treatments. Percentage of ascospore germination on WA was 20-45% in 48 hours (data not shown). On the contrary, percentage of ascospore germination in each of two stroma samples, MC1-1 and KB1-1, which were collected in the field and supposed to be very old, was extremely low, less than 1%. Ascospores in stromata within a few years after development are considered to germinate easily in nature as well as on media.

DsRNA was not transmitted vertically to ascospore offspring despite the infection of various dsRNA in parental isolates. DsRNA was absent in 35 ascospore isolates in two stroma samples that originated from the isolates, in which dsRNA was not eliminated by hyphal tip isolation. In other ascomycetous fungi, dsRNA was transmitted to ascospore offspring although the frequency of transmission was generally low (Buck, 1986, 1998). The mechanism that dsRNA was entirely absent in ascospore offspring of the white root rot fungus is not clear. More work is needed to understand the lack of dsRNA in the ascospore offsprings.

The frequency of dsRNA presence in the isolates collected from non-agricultural lands was lower compared with that in the isolates collected from agricultural lands (Arakawa et al., 2002b). Appearance of dsRNA-free strains is presumably promoted in non-agricultural land, especially

in forests, because of high stroma production. Arakawa et al. (2002a) demonstrated that a multisegment dsRNA in an isolate collected from Japanese pear orchard was effective to reduce virulence in white root rot fungus. Similar multisegment dsRNA was detected in the isolate from diseased root sample G-16, but its ascospore offspring from stroma sample G16-1 did not contain the dsRNA. If the dsRNA is used for biocontrol of the white root rot disease, stroma development in the orchards must be prevented because sexual reproduction has the function to eliminate the dsRNA from the fungal strain. In other words, sexual reproduction can cure the fungus from hypovirulence. Immediate removal of diseased plants in orchards is very important for the success of biocontrol using hypovirulent factor.

In this study, sexual reproduction in the white root rot fungus were suggested to produce propagules as new infection source and to have the function to eliminate infectious factors such as mycoviruses. To investigate more precisely about the roles of ascospores in the white root rot fungus, the way of ascospore dispersal and population structure in both orchards and forests needs to be further studied.

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