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Isolation of *Cryphonectria parasitica* from Cankers on Chestnut Trees in Korea

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A total of 672 *Cryphonectria parasitica* was isolated from 2,536 blight lesions on chestnut twigs, which were collected from major chestnut plantations all over Korea. Isolation rates of each province ranged from 13.5% in Jeonbuk-do to 37.4% in Gyeongnam-do, with an average rate of 25.6%. The isolates were classified into six groups according to color and shape of colony on PDA: smooth margin (S), irregular margin (I), yellow to brown (Y), white (W), and white with yellow center (C). Among these groups, IY was the most abundant with an isolation rate of 65%. On the other hand, SW, SC, IW, and SY were quite rare, with isolation rates ranging from 1.5% to 5.8%. When the 672 isolates were inoculated on the chestnut twigs, 380 isolates (56.5%) caused lesions larger than the standard virulent isolate EP155-2, while 158 isolates (23.4%) caused smaller lesions than the standard hypovirulent isolate UEP-1. However, 87.4% of the isolates belonged to the virulent group and only 12.6% belonged to the hypovirulent group based on Bavendamm test. In the provinces of Jeonnam-do, Jeonbuk-do, and Gyeongnam-do, which have high density of chestnut trees, the rates of hypovirulent-like isolates were over 20%.

Keywords : Bavendamm test, chestnut blight, *Cryphonectria parasitica*, hypovirulent, phenol oxidase activity.

Chestnut blight, caused by *Cryphonectria parasitica*, is the most destructive disease of American and European chestnut trees. The pathogen *C. parasitica* penetrates through wounds on twigs, and diseased trees show typical swollen or sunken cankers, which are dark brown in color. Water sprouts usually emerge below the canker, which also become infected and eventually die (Roane et al., 1986). *C. parasitica* is native to East Asia and was introduced to North America and Europe about 100 years ago (Anagnostakis, 1987). To date, many plant pathologists have studied this fungus because of its hypovirulent isolates, which spontaneously heal the cankers on twigs (MacDonald and Fulbright, 1991), and as a model system for the study of transmissible hypovirulence (Roane et al., 1986). Since the isolation of hypovirulent strains from recovering cankers on chestnut twigs, several efforts have been made to control chestnut blight with hypovirulent *C. parasitica* isolates (Anagnostakis et al., 1986; Powell, 1995). During the last decade, *C. parasitica* was found both in Japan and China on native chestnut trees, which were known to be resistant to the pathogen (Milgroom et al., 1996). The report suggests the possibility that there may be a lot of chestnut trees infected by *C. parasitica* in Korea. However, there have been few research on chestnut blight caused by *C. parasitica* in Korea until 2-3 years ago (Joo et al., 2000a, b; Ju et al., 2000; Kang et al., 2000; Lee et al., 1999). This may be attributed to the fact that chestnut trees in Korea are resistant to this fungus even though blight lesion is quite common on chestnut twigs in the orchard. In this research, cankers on chestnut twigs were collected from all over Korea for the isolation of *C. parasitica*, and biological and pathological characteristics of the isolates were investigated.

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Materials and Methods

Isolation and identification of the pathogen. Bark tissues of chestnut trees with blight and canker symptoms on twigs were collected from chestnut plantations throughout Korea except Jeju-do. Each province had four to five sampling sites (plantations) and the sites were at least 15 km apart from each other. Bark tissues were collected from upper and lower margins of a blight lesion with a cork borer (ϕ 10 mm), and more than 100 bark discs were collected from each plantation. In the laboratory, the bark discs were surface-sterilized with flame after soaking for 10 seconds in absolute alcohol and then incubated on water agar (WA) at 25°C. Two to three days later, the actively growing hyphal tip was taken out and transferred onto potato dextrose agar (PDA) containing methionine and biotin (PDAmb: Difco PDA supplemented with 100 mg/L methionine and 1 mg/L biotin) and incubated at 25°C. Characteristics of the spores and fruiting bodies on the medium were observed under the microscope and compared with the description of Sivanesan and Holliday (1981) for identification.
Standard isolates of *C. parasitica*. EP155-2 and UEP-1 maintained at the Fungal Molecular Biology Laboratory of Jeonbuk National University were used as standard virulent and hypo-

virulent isolates, respectively, in this experiment. Pathological and biochemical characteristics of these isolates were compared with those of the isolates from cankers on chestnut trees.

Pathogenicity tests of *C. parasitica* isolates on the bark of chestnut twigs. Barks from chestnut twigs (3-5 cm thick and 100 cm long at 5 cm distance) were removed by using a cork borer (ϕ 5 mm), and each cork borer wound was inoculated with the same size of *C. parasitica* mycelial disc. Twigs inoculated with the isolates were wrapped with plastic film to prevent drying and incubated at 25°C for 5 days. After incubation, the bark of the twig was peeled off and the longest and the shortest diameters of each lesion on the inner surface of the bark were measured with a caliper to calculate the area of the lesion. The pathogenicity of the isolates was determined by comparing the lesion area with that of EP155-2 and UEP-1. All the twigs for inoculation of *C. parasitica* isolates were collected on the same day from three chestnut trees (variety unknown) growing side by side in the orchard to avoid variations in lesion size caused by variety and time of inoculation. In this experiment, there were three replications for each inoculation.

Phenol oxidase test (Bavendamm test). Mycelial discs (ϕ 5 mm) of *C. parasitica* isolates grown on PDA were transferred onto slightly modified Bavendamm's medium (Bavendamm, 1928). The medium contains 0.7% tannic acid, 1.5% Difco malt extract, and 2% Difco Bacto agar, and was adjusted using NaOH to pH 4.5. The tannic acid solution and malt extract agar suspension were autoclaved separately and mixed together right before dispensing into petri dishes. Plates were incubated at 30°C in the dark for 5 days. After incubation, discoloration of medium around each mycelial disc was compared with that of EP155-2 and UEP-1.

Results

Isolation and identification of the pathogen. Various fungi were isolated from blighted bark of chestnut twigs (Fig. 1A) in major chestnut plantations of Korea. Among the isolates, 672 were identified as *C. parasitica* by comparing mycological characteristics of the isolates on PDAmb with the description of Sivanesan and Holliday (1981) (Table 1). The isolates identified as *C. parasitica* were stored both in sterilized water at room temperature and in deep freezer (-70°C) after being suspended with 6% dimethyl sulfoxide solution for further experimentation.

Isolation rates of *C. parasitica*. Isolation rates of *C. parasitica* were generally high in the eastern provinces of Korea. Isolation rates were over 30% in Gangwon-do, Gyeongnam-do, and Jeonnam-do, with Gyeongnam-do showing the highest rate of 37.4% (Table 2). On the other hand, isolation rate was as low as 19.4% in Chungnam-do, which is one of major chestnut cultivation areas in the country. Jeonbuk-do had the lowest isolation rate (13.5%) among the eight provinces of Korea even though there were a lot of twig blighted chestnut trees in the sampling sites. Average isolation rate of the eight provinces was 25.6%.

Morphology of *C. parasitica* colony. Shape and color of *C. parasitica* colonies were variable and were divided into six groups (Fig. 1B): I = irregular margin, S = smooth margin, Y = yellow to brown, W = white, C = white with yellow center. In every province, IY was the most abundant group with a frequency of 65.2%. The next abundant group was IC (18.0%). However, other groups were rare, with frequencies ranging from 1.8% to 5.7% (Table 3). Standard isolates of each group showed the same mycological characteristics described in Table 1.

Pathogenicity test of *C. parasitica* isolates on the bark of chestnut twigs. In the bark inoculation tests, lesion sizes were quite variable and ranged from 11 cm^2 to 0.55 cm^2 (Fig. 1C). The standard virulent isolate EP155-2 and the standard hypovirulent isolate UEP-1 caused lesions of 3.79 cm^2 and 2.38 cm^2, respectively. Therefore, based on these two lesion sizes, the pathogenicity of *C. parasitica* isolates were divided into three groups: virulent (11.0-3.79 cm^2), moderately virulent (3.78-2.39 cm^2), and hypovirulent (2.38-0.55 cm^2). More than 80% of the isolates were virulent in Jeonbuk-do, Gyeongbuk-do, and Chungnam-do. On the other hand, the frequency of hypovirulent isolates was highest (48.4%) in Gangwon-do, and was much lower than those of virulent isolates in most areas. All over Korea, the frequencies of virulent, moderately virulent, and hypovirulent isolates were 57.7%, 20.4%, and 21.9%, respectively (Table 2).

Phenol oxidase activity (Bavendamm test) of *C. parasitica*. Degree of discoloration of the medium around the colony was graded into three levels according to the discoloration degrees of the standard virulent isolate EP155-2 and the standard hypovirulent isolate UEP-1. Level 3 was at least the same or darker than EP155-2, level 2 was the same as UEP-1, and level 1 was lighter than UEP-1 (Fig. 1D). In this test, 87.1% of all the isolates belonged to level 3, and only 12.9% belonged to levels 1 and 2 (Table 2).

Comparison of pathogenicity test on bark and Bavendamm test of *C. parasitica*. Results of these two tests were not closely matched (Fig. 2). Only 31% of hypovirulent (levels 1 and 2) isolates in the Bavendamm test caused smaller lesions than UEP-1 on the bark of chestnut twigs, while 79.5% of virulent (level 3) isolates caused larger lesions than UEP-1 (Table 4). On the other hand, only 18.4% of hypovirulent isolates in the pathogenicity test on the bark had relatively lighter discoloration than UEP-1, and as much as 88.6% of virulent and moderately virulent isolates in the pathogenicity test had relatively darker discoloration than EP155-1.

Comparison of Bavendamm test and colony type of *C. parasitica*. Some relationships were observed between the

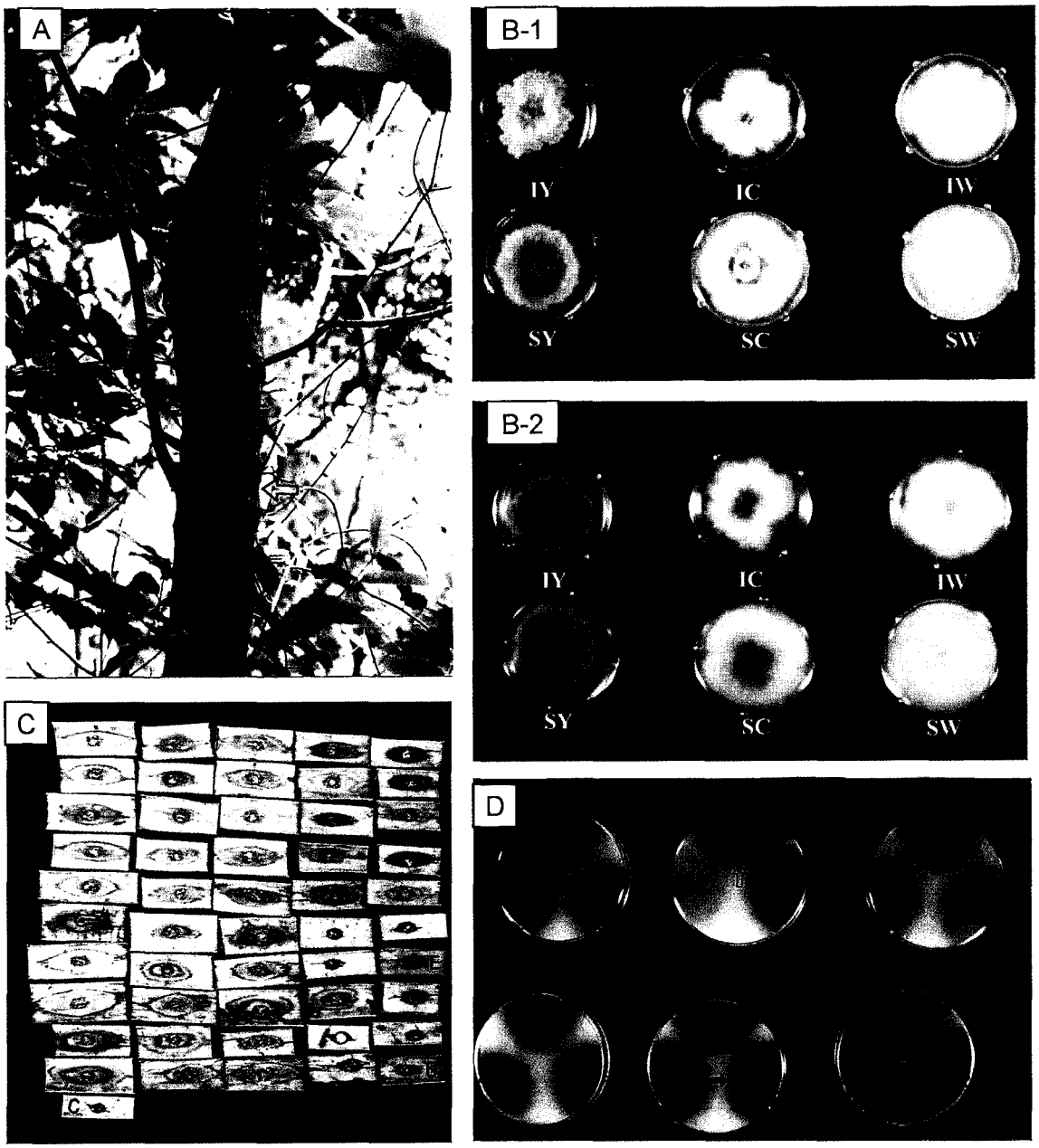

Fig. 1. Typical symptoms, colony morphology, pathogenicity, and phenol oxidase activity of *Cryphonectria parasitica*. **A**: typical swollen (solid arrow) and sunken (open arrow) canker symptoms on naturally infected chestnut twig. **B**: six types of *C. parasitica* colony on PDA (**B-1**: upper surface of colony, **B-2**: lower surface of colony, Y: yellow to brown, W: white, C: white with yellow center, I: irregular margin, S: smooth margin). **C**: lesions on inner bark surface of chestnut twigs by inoculation of *C. parasitica* isolates (E: standard virulent isolate EP155-2, U: standard hypovirulent isolate UEP-1, C: agar plug control). **D**: phenol oxidae activities of *C. parasitica* isolates on Bavendamm medium (solid arrow: EP155-2, open arrow: UEP-1).

two tests. Level 3 isolates in the Bavendamm test were 71.8% IY, 16% IC, and 1.2-5.2% IW, SY, SW, and SC. On the other hand, the most dominant colony type of levels 1 and 2 in the Bavendamm test were IC (27.1%) and SW (25%). The isolates of other colony types occupied 1.3-17% (Fig. 3).

Comparison of pathogenicity test on the bark and colony type of *C. parasitica.* In general, the isolates which caused lesions larger than the standard hypovirulent isolate UEP-1 had colony type of irregular margin (IY = 49.7%, IC = 14.1%) rather than smooth margin. The frequencies of IY and IC in the isolate group that caused lesions smaller than

Table 1. Comparison of morphological characteristics of *Cryphonectria parasitica* isolated from chestnut blight with previous description

Characteristic	Fungus isolated	*C. parasitica*[a]
Conidia		
Shape	bacilliform	bacilliform
Color	hyaline	hyaline
size (μm)	4-5×2.5	3-5×1.5
Pycnidia		
Shape	globose	globose
Color	yellowish brown	yellow to yellowish brown
Ostiloe	yes	yes
size (μm)	100-300	300
Stroma		
Shape	erumpent	erumpent
Color	yellow	yellow to yellowish brown
width (mm)	0.9-3	3
height (mm)	2.5	2.5
Ascospore		
Shape	ellipsoid	elliptic
Color	hyaline	hyaline
Septum	1	1
size (μm)	10-12×5-6	7-12×3-5.5
Ascus		
Shape	cylindrical	cylindrical
Color	hyaline	hyaline
size (μm)	30-55×10-12	32-55×7-8.5
Perithecium		
Shape	globose to flask	globose to depressed globose
Color	brown to black	black blown to black
size (μm)	250-300 (width)	400 (width)
Colony on PDA		
Shape	concentric	–
Margin	irregular~regular	–
Color	white~yellow	–

[a] Sivanesan, A. and Holliday, P. 1981. *Cryphonectria parasitica.* CMI Description of Pathogenic Fungi and Bacteria No. 704, 2 p.

UEP-1 remained at 15.3% and 3.9%, respectively (Fig. 3).

Discussion

The chestnut blight fungus *C. parasitica* was isolated from 25.6% of cankers on chestnut twigs collected all over the country. The result supports the assumption that chestnut trees cultivated in Korea are not resistant to this pathogen. Disease occurrence rate was not related to plantation size because isolation rate was not always high in huge chestnut plantations. For example, isolation rates in Gyeongnam-do and Chungnam-do, which are the first and second largest chestnut cultivation areas, were 37.4% and 19.4%, respectively. In this research, even though the variety of sampling sites was not confirmed, the differences in isolation rates might be the result of factors such as environmental, especially weather conditions and edaphic factors, and the variety of the chestnut trees, rather than the size of cultivation area (Huang et al., 1996: Joo et al., 2000b; Lee et al., 1999, 2000).

The isolated *C. parasitica* showed prominent characteristics on PDA. Generally, isolates of the yellow-colored colony had strong virulence while isolates of the white-colored colony showed hypovirulence (Anagnostakis et al., 1986). Marginal characteristics of the colony were related to the virulence of the isolate. Isolates of irregular-margined colony were more virulent than the isolates of smooth-margined colony. These results were considered logical because the standard virulent isolate EP155-1 had irregular-margined yellow colony while the standard hypovirulent isolate UEP-1 had somewhat irregular-margined but white colony on PDA. In the isolation study, the IY-type isolate, which seemed to be the most virulent, was dominant among *C. parasitica* in Korea with an isolation rate of 65.2%. On

Table 2. Occurrence of *Cryphonectria parasitica* in blighted twigs of chestnut trees and relative rates of three virulence levels of *C. parasitica* based on the inoculation test on chestnut twigs and by phenol oxidase activity assay

Province	Lesions examined (number)	*C. parasitica* isolated (number/%)	Twig inoculation test (number/%)[a]			Phenol oxidase activity (number/%)[b]		
			Virulent	Moderately virulent	Hypovirulent	3	2	1
Gangwon	295	64 (21.7)	12 (18.8)	21 (32.8)	31 (48.4)	56 (87.5)	8 (12.5)	0 (0)
Kyonggi	302	101 (33.4)	46 (45.5)	27 (26.7)	28 (27.7)	92 (91.1)	8 (7.9)	1 (1.0)
Gyeongnam	420	157 (37.4)	105 (66.9)	32 (20.4)	20 (12.7)	124 (79.0)	30 (19.1)	3 (1.9)
Gyeongbuk	218	44 (20.2)	36 (81.8)	4 (9.1)	4 (9.1)	40 (90.9)	4 (9.1)	0 (0)
Jeonnam	282	90 (31.9)	41 (45.6)	24 (26.7)	25 (27.8)	71 (78.9)	18 (20.8)	1 (1.1)
Jeonbuk	252	34 (13.5)	30 (88.2)	4 (11.8)	0 (0)	27 (79.4)	4 (11.8)	3 (8.8)
Chungnam	356	69 (19.4)	58 (84.1)	1 (1.4)	10 (14.5)	65 (94.2)	3 (4.3)	1 (1.4)
Chungbuk	411	113 (27.5)	60 (53.1)	24 (21.2)	29 (25.7)	110 (97.3)	3 (2.7)	0 (0)
Total	2536	672 (26.5)	388 (57.7)	137 (20.4)	147 (21.9)	585 (87.1)	78 (11.6)	9 (1.3)

[a] Lesion size: virulent ≥ standard virulent isolate (EP155-2) > Moderately virulent > standard hypovirulent isolate (UEP-1) ≥ hypovirulent.
[b] Discoloration in Bavendamm test: 3 (not lighter than EP155-2), 2 (same as UEP-1), 1 (lighter than UEP-1).

Table 3. Relative frequencies of colony types of *Cryphonectria parasitica* isolates on potato dextrose agar

Province	*C. parasitica* isolates	Colony types on potato dextrose agar [a,b]					
		IY	SY	IC	SC	IW	SW
Gangwon	64	49 (76.6)	0 (0)	11 (17.2)	1 (1.6)	1 (1.6)	2 (3.1)
Kyonggi	101	66 (65.3)	2 (2.0)	21 (20.8)	3 (3.0)	7 (6.9)	2 (2.0)
Gyeongnam	157	95 (60.5)	0 (0)	36 (22.9)	4 (2.5)	15 (9.6)	7 (4.5)
Gyeongbuk	44	32 (72.7)	0 (0)	2 (4.5)	6 (13.6)	0 (0)	4 (9.1)
Jeonnam	90	48 (53.3)	5 (5.6)	11 (12.2)	12 (13.3)	2 (2.2)	12 (13.3)
Jeonbuk	34	22 (64.7)	3 (8.8)	2 (5.9)	2 (5.9)	0 (0)	5 (14.7)
Chungnam	69	43 (62.3)	1 (1.4)	14 (20.3)	6 (8.7)	0 (0)	5 (7.2)
Chungbuk	113	83 (73.5)	1 (0.9)	24 (21.2)	4 (3.5)	0 (0)	1 (0.9)
Total	672	438 (65.2)	12 (1.8)	121 (18.0)	38 (5.7)	25 (3.7)	38 (5.7)

[a] I: irregular margin, S: smooth margin, Y: yellow to brown, C: white with yellow center, W: white.
[b] numbers in parenthesis mean occurrence ratio (%) in each province.

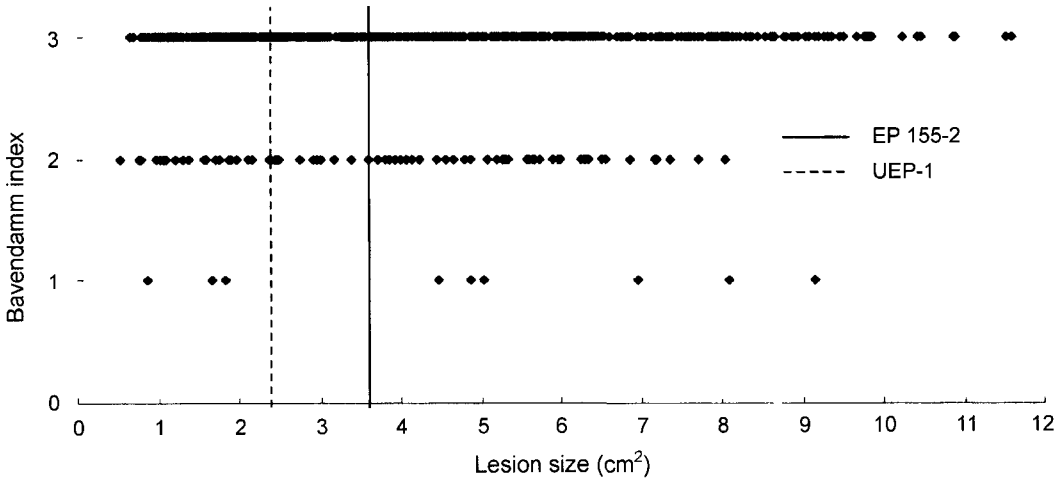

Fig. 2. Relationships of Bavendamm index and lesion size on chestnut twigs (Bavendamm index: 3 = virulent; 2 & 1 = hypovirulent-like).

Table 4. Comparison of phenol oxidase activity and virulence on chestnut twigs

Phenol oxidase activity[a]	Twig inoculation test		Total
	Virulent	Hypovirulent	
3	465	120	585
2 & 1	60	27	87
Total	525	147	672

[a] 3: more virulent than standard virulent isolate EP155-2.
2 and 1: same or less virulent than standard hypovirulent isolate UEP-1.

the other hand, the isolation rate of white-colored colony, regardless of margin shape, was as low as 9.4%. Therefore, based on the isolation rate and colony shape, most of *C. parasitica* causing blight on chestnut twigs must have been of the irregular-margined yellow colony.

The standard virulent isolate EP155-2 caused smaller lesion in this experiment compared with previous reports (Chen and Nuss, 1999; Huang et al., 1996). This can be attributed to the differences in inoculation methods and resistance of chestnut varieties used in the experiment. In this experiment, lesion size was measured 5 days after inoculation. However, Chen and Nuss (1999), who reported bigger lesion than that of this experiment, measured the lesion 10 days after inoculation. In addition, oriental chestnut trees are known to be resistant to *C. parasitica*, but American chestnut is rather sensitive to the pathogen. This could be the reason why EP155-2 did not cause large lesions on twigs of chestnut trees inoculated in this experiment.In the inoculation test on chestnut twigs for pathogenicity evaluation, more than half of the total 672 isolates caused lesions larger than EP155-2, while 22.2% caused lesions smaller than UEP-1. These results mean that 21.9% of the isolates are hypovirulent. However, results of the pathogenicity test did not show similar trends with that

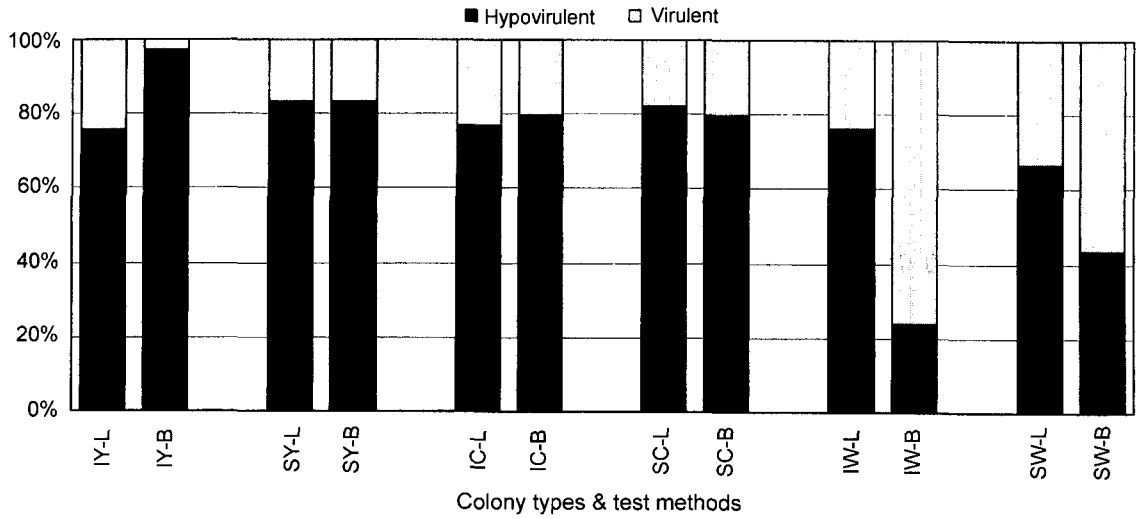

Fig. 3. Relative ratio of virulent and hypovirulent isolates confirmed by the lesion size on chestnut twigs by inoculation (L) and Bavendamm test (B) in each colony type of *Cryphonectria parasitica* (Y: yellow to brown, W: white, C: white with yellow center, I: irregular margin, S: smooth margin).

of the phenol oxidase test (Bavendamm test) while in some isolates, the results were exactly the opposite. This means that some virulent isolates of the pathogenicity test on the bark belonged to hypovirulent groups (levels 1 and 2) in the Bavendamm test, while some hypovirulent isolates of the pathogenicity test on the bark belonged to virulent group (level 3) in the Bavendamm test. In addition, morphological characteristics of colony did not coincide with the result of the pathogenicity test or the Bavendamm test. Therefore, none of the above test methods seemed to be a determinative tool to define the virulence of *C. parasitica*. During the successive cultures of *C. parasitica* isolates, marginal shape of colony of some isolates changed to some extent. This phenomenon was more common for isolates of smooth-margined colony than that of the irregular-margined colony. This instability in the morphology of some isolates may cause discrepancy in the results of tests related to the morphology of the isolates.Several factors in the host-parasite-environment complex may affect the result of the inoculation tests on chestnut twigs. In addition, the differences in chestnut variety or even among the trees of the same variety may affect the lesion size. However, the Bavendamm test only evaluated the activity of the phenol oxidase and the test environment was controlled constantly. The property of the medium used in the test was also always constant. This means that the result of the Bavendamm test is more stable and reproducible than that of the inoculation test. However, the inoculation test is much closer to natural conditions and produces more direct results. It has been reported that some hypovirulent *C. parasitica* isolates caused discoloration of the medium in the Bavendamm test (Rigling et al., 1989). Therefore, the twig inoculation test appeared to be the best method so far to check the pathogenicity of *C. parasitica* isolate, but there should be replications to minimize experimental error and to increase the reliability of the results. Also, the results of the Bavendamm test could serve as supplemental evidences to determine the pathogenicity of *C. parasitica*. Finally, detection of ds-RNA from the isolate suggests that the isolate is a hypovirulent one.Results of this study show that 27 isolates of *C. parasitica* from chestnut trees seemed to be hypovirulent both in the inoculation and Bavendamm tests. The possibilities of these isolates as hypovirulent ones are very high. Detection of dsRNA not only from these isolates but also from the isolates suspected as hypovirulent either in the inoculation or Bavendamm tests may increase the number of hypovirulent *C. parasitica* isolates in Korea.

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