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Characteristics of Hypovirulent Strains of Chestnut Blight Fungus, *Cryphonectria parasitica*, Isolated in Korea

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Chestnut blight disease caused by *Cryphonectria parasitica* is widely distributed throughout chestnut tree plantations in Korea. We surveyed 65 sites located at 9 provinces in South Korea, and isolated 248 virulent and 3 hypovirulent strains of chestnut blight fungus. Hypovirulent strains had dsRNA virus in the cytoplasm, which is one of the typical characteristics of hypovirulent strains. In addition, they showed more characteristics of hypovirulent strains, i.e., suppressed conidiation, reduced pigmentation in colony color, and reduced phenol oxidase activity as well as reduced pathogenicity. Hypovirulent strains, KCPH-22, KCPH-135 and KCPH-136, had a genomic dsRNA band with the molecular weight of 12.7 kb, which is the L-dsRNA of CHV1. They also had a 2.7 kb defective dsRNA band. Single conidia isolated from hypovirulent strains were cultured and various phenotypes and absence of dsRNA bands were obtained from single conidial cultures, which means that hypovirulence transmission is unstable in asexual reproduction and variations in viral heredity by asexual reproduction. Biocontrol trial using hypovirulent strains was also carried out in the chestnut tree plantations, and canker expansion in the treated trees was stopped and healed by callus formation at the margin of the canker. These results show the potentials in successful biocontrol of chestnut blight if the vegetatively compatible hypovirulent strains could be directly used around the canker formed by compatible virulent strains.

KEYWORDS: **Biocontrol, Chestnut blight, *Cryphonectria parasitica*, dsRNA virus, Hypovirulent, Phenol oxidase activity, Viral heredity to conidia progeny**

Chestnut trees are economically important trees, whose fruits are delicious and contain many nutrients, and they are distributed all over the areas in Korea. Many tree seedlings were transplanted from the end of the 1960's to middle of the 1970's in Korea. Planted area was approximately 79,000 ha in 1999 and the total amount of production was estimated up to 10,000 ton. Total amount of exported fruits of chestnut tree from Korea to other countries was 27,029 (M/T) in 1999 (Korea Forest Service, 2004). Thus, chestnut production is making a great contribution to the increase of income in the forest area. However, losses in chestnut production by the chestnut blight fungus, *Cryphonectria parasitica*, were increased gradually because of the lacking of the proper disease management strategy. So far, *C. parasitica* was known to be a major pathogen of chestnut blight in Korea, even though many stem blights caused by the pathogens such as *Botryosphaeria*, *Cryptodiaporthe*, *Endothia*, *Diaporthe* and *Pseudovasella*, had been reported.

Since chestnut blight caused by *C. parasitica* was first reported in New York Zoological Garden in America in 1904, and destroyed chestnut forestry field of East America (Griffin *et al.*, 1983, 1991; Roane and Griffin, 1986), the pathogen has been spread to Europe and caused serious damage. In Korea, the disease was first reported as chestnut stem blight in 1925, and researches were limited in the field such as pathogens distribution, physiology, and ecology.

In 1964, hypovirulence was first discovered in Italy (Grente, 1965), and then this phenomenon was known to be associated with double stranded RNA in the cytoplasm of the pathogen (Day *et al.*, 1977). Virulent (V) strain was converted to hypovirulent (H) strain by the transmission of dsRNA virus in hypovirulent strain through hyphal anastomosis between V and H strains (Elliston, 1985; Fulbright, 1984). Based on this mechanism, hypovirulent strains were used to control blight in Europe and America, and successful control was reported by the introduction of hypovirulent strain on diseased part in Italy and France (Heiniger and Rigling, 1994). Hypovirulence altered colony morphology, reduced conidial formation, oxalate accumulation, laccase production and pigmentation. The main objectives of this study were to isolate the hypovirulent strains in Korea, to compare their cultural characteristics including phenol oxidase activity with virulent ones, to detect dsRNA virus and convert virulent strains into hypovirulent by hyphal fusion, to investigate hypovirus transmission to conidia progeny, and to try biocontrol on the chestnut trees in the field.

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Materials and Methods

Isolation of fungal strains, and comparison in their cultural characteristics. *C. parasitica* was isolated from natural cankers on stem and twig infected with chestnut blight. Bark tissues were removed with sterilized cork borer (5 mm dia.) and placed on water agar (WA). After incubating for 2~3 days, mycelial plugs were removed from mycelial margin and then transferred on fresh PDA. The plates were kept at 25°C for 3 days. For cultural characteristics, radial mycelial growth on PDA were measured at 7 days after incubation (Anagnostakis and Aylor, 1984). For testing phenol oxidase activity, the strains were grown on modified Bavendamm's medium (0.5% tannic acid, 1.5% Difco malt extract, 2% Difco bacto agar, pH 4.5 adjusted with 1 N NaOH), and kept at 25°C. Mycelial growth and changes of colors were checked 4 days after incubation (Rigling *et al.*, 1989) (Lee *et al.*, 1991b).

Virulence of the isolated strains. For testing virulence of the isolated strains, excised stems were harvested from 3 different chestnut cultivars, i.e., Daebo (resistant), Eunki (moderate), and American (susceptible) chestnut. Stems were cut into 2.5 cm long sections, and the bark and wood tissue were separated using forceps. An agar plug (5 mm in diameter) was placed on the center of inner bark tissue section. Samples were then incubated in the dark at 25°C on moistened filter papers inside petri dishes. After 4 days, the zone containing brown, necrotic areas was measured, and the elliptical necrotic area was calculated (Lee *et al.*, 1992).

Detection of double stranded RNA(dsRNA) virus and hypovirulence conversion. DsRNA was extracted by modified methods (Day *et al.*, 1977). Mycelial disc (4mm) was placed on cellophane-covered PDAmb. Mycelium was harvested from cellophane-covered PDAmb after 4 days, frozen in liquid nitrogen, and then ground with mortar and pestle. Total nucleic acid was extracted with 6ml of a mixture of phenol: chloroform: isoamyl alcohol (25 : 24 : 1, v/v/v). The aqueous phage was adjusted to 15% ethanol and dsRNA was purified by chromatography on cellulose CF-11. To confirm the nature of the dsRNA with silver stain, gel was washed in 400 *ml* distilled water for 15 min, removed water, decolorized in pretreatment solution (80 *ml* methanol: 20 *ml* ethanol : 90 *ml* distilled water: 10 *ml* rinsing solution, v/v) with shaking for 10 min, and the remaining water was removed by aspirator. The dsRNA virus bands were visualized by Silver Stain Plus Kit (Bio-Rad) (Dodds, 1993). Hypovirulence conversion was performed by placing one plug of virulent strain (KCPV-19) 5 mm apart from one of a vegetatively compatible hypovirulent strain (KCPH-22) on fresh PDA, and incubating at 25~27°C for 3~7 days. Conversion of virulent strain into hypovirulent one was confirmed by changes of colony morphology and dsRNA analysis (Lee *et al.*, 1991a; Anagnostakis and Day, 1979).

Viral heredity to conidia progeny in hypovirulent strains. Single conidia were isolated from the culture of hypovirulent strains. Same phenotypes of single conidial cultures were grouped, and dsRNA banding patterns were analyzed for viral heredity in asexual reproduction (Anagnostakis *et al.*, 1998; Scibilia and Shain, 1989).

Biocontrol trial of chestnut blight by hypovirulence. After inoculation of the fungal mycelial plugs of virulent strain (KCPV-19), hypovirulent strain (KCPH-22) was inoculated at 4 weeks after inoculation. Chestnut blight canker was investigated and measured at 5 more weeks after inoculation. Fungi were re-isolated from the canker area, and dsRNA banding patterns were compared with those of original hypoviulent strains.

Results and Discussion

Isolation of fungal strains, and comparison in their cultural characteristics. Two hundreds and fifty one strains of chestnut blight fungus were isolated from natu-

Table 1. Strains of *Cryphonectria parasitica* isolated from chestnut trees and used in this study

Strain No.[1)]	Region	Virulence/ Hypovirulence
KCPV-9	Haengjung, Juan, Suncheon, Chonnam	Virulent
KCPV-19	Doshin, Euidang, Gongju, Chungnam	Virulent
KCPH-22	Buchon, Hwagae, Hadong, Kyungnam	Hypovirulent
KCPH-135	Junggum, Hwagae, Hadong, Kyungnam	Hypovirulent
KCPH-136	Junggum, Hwagae, Hadong, Kyungnam	Hypovirulent

[1)]KCP : Korean strains of *C. parasitica*.

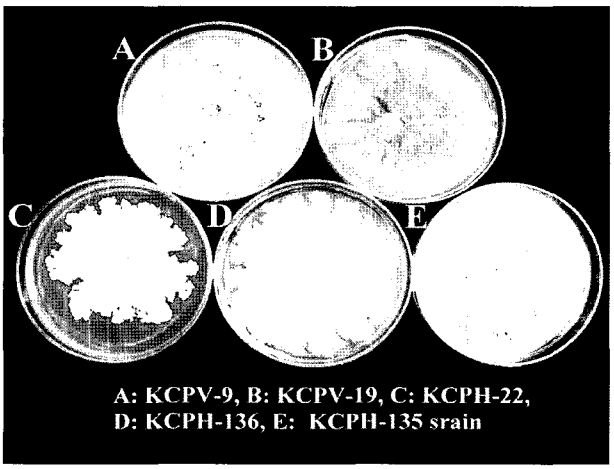


Fig. 1. Colony morphology of virulent (A and B) and hypovirulent (C, D, and E) strains of chestnut blight fungus, *Cryphonectria parasitica*, on PDA medium.

ral cankers on stem and twig infected with chestnut blight. Among these isolates, KCP-9 and KCP-19 were selected as representatives of virulent strains, while KCP-22, KCP-135, and KCP-136 were selected as those of hypovirulent strains based on cultural characteristics, pathogenicity, and the presence of dsRNA virus (Table 1). Hypovirulent strains showed abnormal morphology, reduced conidiation, and reduced mycelial growth as compared with normal virulent strains on culture medium (Fig. 1). Color reactions on Bavendamm media, which indicate phenol oxidase activity, showed that virulent strains (V) turned this medium dark brown, while hypovirulent stains (H) produced lighter yellow to brown (Table 2).

Table 2. Comparison of virulent and hypovirulent strains of *Cryphonectria parasitica* in mycelial growth and phenol oxidase activity

Strain No.	Mycelial growth[1)]	Color reaction[2)]
KCPV-9	+++	++
KCPV-19	+++	++
KCPH-22	+	–
KCPH-135	+	–
KCPH-136	+	–

[1)] +++ : good growth, ++ : fairly good growth, + : poor growth.
[2)] ++ : dark brown, – : yellowish brown.

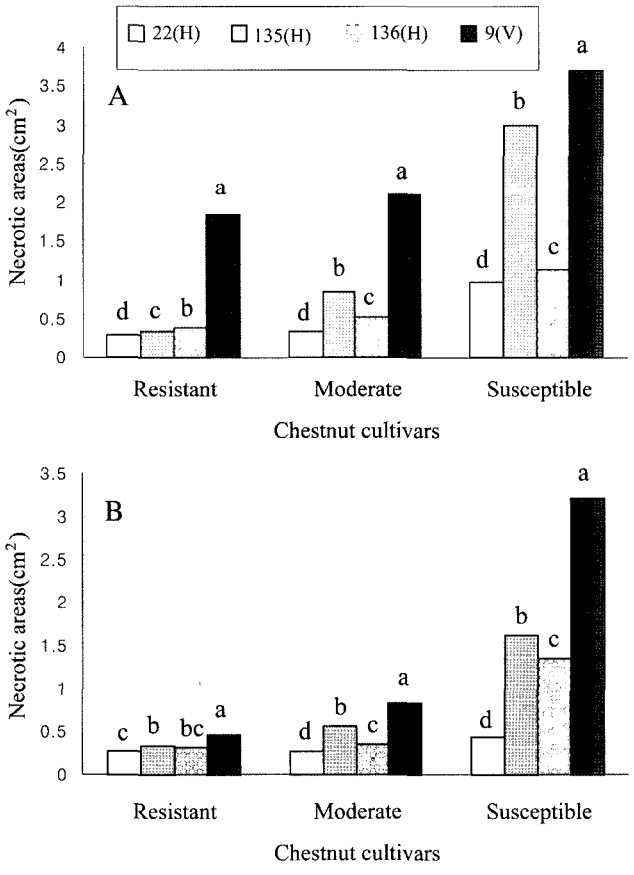
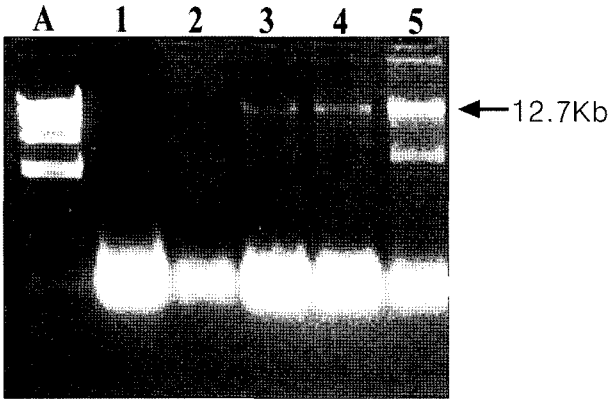


Fig. 2. Virulence test of virulent (V) and hypovirulent (H) strains of *Cryphonectria parasitica* on (A) bark tissue and (B) wood tissue excised from resistant (Daebo), moderate (Eunki), and susceptible (*Castanea dentata*) cultivars. The same letter on the bar within each cultivar is not significantly (p = 0.05) different by Duncan multiple range test.

Virulence of the isolated strains. Necrotic areas on both bark and wood tissues, which indicate the virulence of strain, were relatively small by the inoculation of hypovirulent strains (KCPH-22, KCPH-135, and KCPH-136) as compared to those by virulent strain (KCPV-9). In addition, the necrotic areas on the bark/wood tissues were generally increased depending on the susceptibility of the cultivars. Eunki, a susceptible cultivar, and American chestnut (*Castanea dentata*), a highly susceptible species, showed the distinct differences in virulence between hypovirulent and virulent strains by the artificial inoculation (Fig. 2A, 2B). The results of the virulence test on the bark and wood tissues had shown the same tendancy, but the results on bark tissue are more clear than these results on wood tissue in discriminating hypovirulent from virulent strains.

Detection of double stranded RNA(dsRNA) virus and hypovirulence conversion. Hypovirulent strains, KCPH-22, KCPH-135 and KCPH-136, had a genomic dsRNA band in the cytoplasm with the molecular weight of 12.7kb, and a defective dsRNA band with the molecular weight of 2.7 kb (Fig. 3). Conclusively, three hypovirulent strains examined had *Cryphonectria Hypovirus* 1 (CHV1) in the cytoplasm. Hypovirulence was successfully converted by cross pairing of a virulent strain (KCPV-19) with a vegetatively compatible hypovirulent



Lane A: λ marker, Lane 1: KCPV-9, Lane 2: KCPV-19, Lane 3: KCPH-22, Lane 4: KCPH-136, Lane 5: KCPH-135 srain

Fig. 3. DsRNA banding patterns of *Cryphonectria parasitica*. Lanes 1 and 2 : virulent strains, Lanes 3~5 : hypovirulent strains.

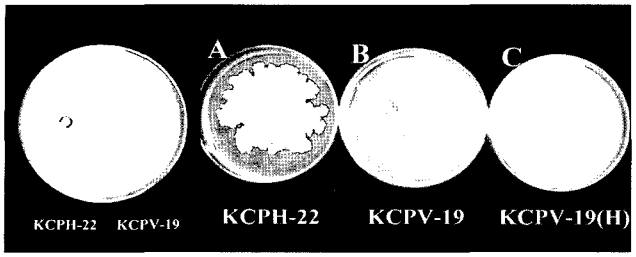
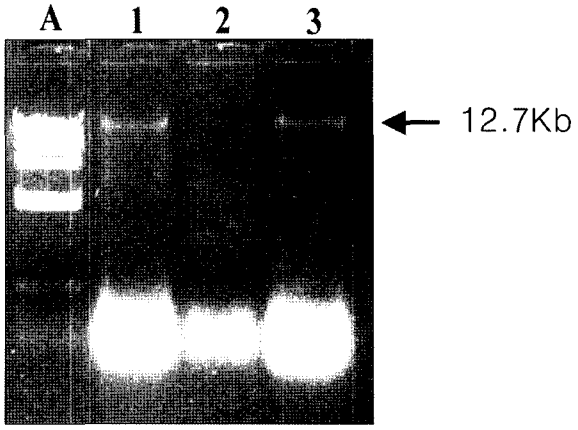


Fig. 4. Colony morphology of hypovirulent, virulent, and converted hypovirulent strains on PDA medium. A: KCPH-22 (hypovirulent), B: KCPV-19 (virulent), C: KCPV-19(H) (converted hypovirulent).

strain (KCPH-22). Colony morphology of virulent strain was changed into those of hypovirulent by the transmission of dsRNA virus, and then the conversion was finally confirmed by the presence of dsRNA virus band through electrophoresis (Fig. 4 and 5).

Viral heredity to conidia progeny in hypovirulent strains. Various phenotypes and the absence of dsRNA bands were obtained from single conidial cultures of hypovirulent strain, which means that hypovirulence transmission is unstable in asexual reproduction (Fig. 6A, 6B, 6C). Single conidial isolates from three hypovirulent



Lane A: λ marker, Lane 1: KCPH-22, Lane 2: KCPV-19, Lane 3: KCPV-19(H) strain

Fig. 5. DsRNA banding patterns of hypovirulent, virulent, and converted hypovirulent strains of *Cryphonectria parasitica*. Lane 1: KCPH-22 (hypovirulent strain), Lane 2: KCPV-19 (virulent strain), Lane 3: KCPV-19 (H) (converted hypovirulent strain).

strains, KCPH-22, KCPH-135 and KCPH 136, were consistently yield five distinct phenotypes by both colony color and morphology: A type (colony with white color

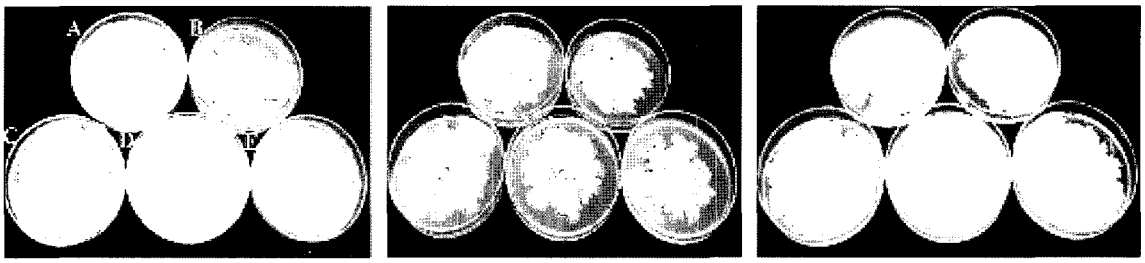


Fig. 6. Diversity in colony morphology of single conidial isolates from three hypovirulent strains. I: KCPH-22, II: KCPH-135, III: KCPH-136.

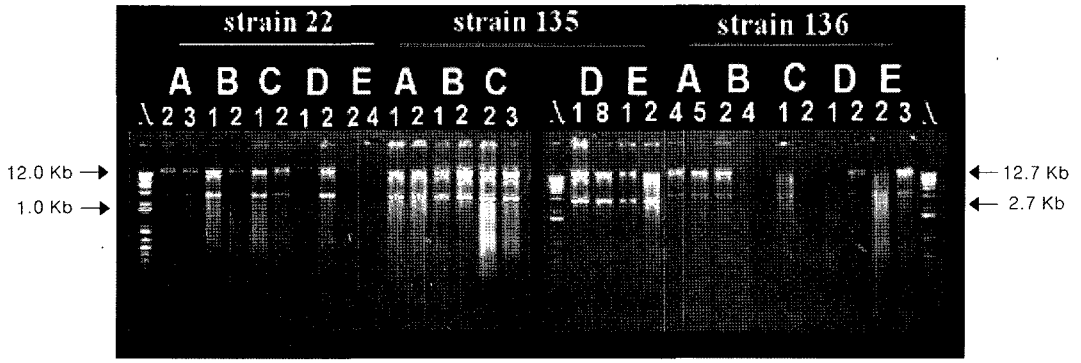


Fig. 7. DsRNA banding patterns of single conidial cultures isolated from 3 hypovirulent strains (KCPH-22, 135, and 136). Single conidial isolates were grouped into five different cultural phenotypes (A, B, C, D, E), and dsRNA banding patterns of two single conidial isolates for each phenotype were representatively compared.

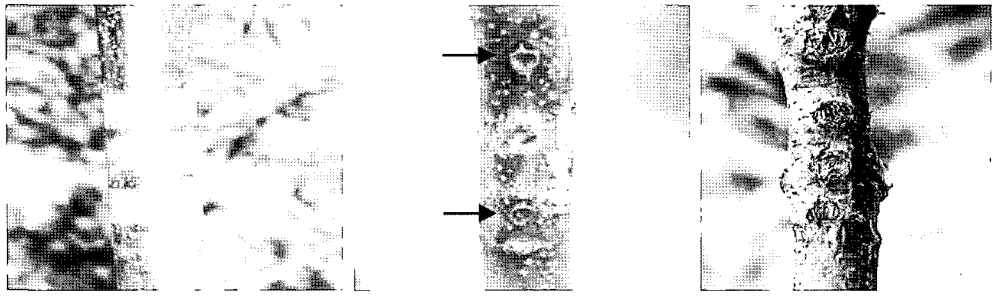


Fig. 8. Biocontrol trial of chestnut blight in the field experiment. Hypovirulent strain (KCPH-22) was inoculated (arrows) at 4 weeks after inoculation of virulent strain. Canker expansion was stopped and healed by callus formation with 5 more weeks after inoculation of hypovirulent strain.

and smooth margin), B type (colony with white color and typical morphology), C type (colony with white color and irregular margins), D type (colony looks like virulent strains), E type (colony with yellow color and irregular margins). DsRNA virus was stably detected from the conidia progeny of only one hypovirulent strain (KCPH-135), but was not stable from the other two strains (Fig. 7).

Biocontrol trial of chestnut blight by hypovirulence. Inoculation of virulent strain (KCPV-19) caused canker with brown discoloration on wood tissue, and produced sometimes yellowish brown pycnidia protruding from bark tissue. In contrast, inoculation of hypovirulent strain (KCPH-22) at the margin of cankers, which were artificially formed by inoculating mycelial plugs of virulent strain, stopped the expansion of canker formation, and healed cankers by developing callus on the bark tissue at the margin of canker (Fig. 8). Healing of cankers can be explained by the transmission of dsRNA virus from the hypovirulent strain to the virulent strain, and finally converted into hypovirulent. This hypothesis was proved by the detection of dsRNA virus from 3 bark tissues collected randomly at the margin of cankers. These results show the potentials in successful biocontrol of chestnut blight if the vegetatively compatible hypovirulent strains could be directly used around the canker formed by compatible virulent strains.

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