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Phytophthora citricola, a Causal Agent of Jujube (*Zizyphus jujuba*) Fruit Rot

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대추 역병균인 *Phytophthora citricola*의 동정

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ABSTRACT: *Phytophthora* rot on jujube fruit has occurred at several cultivation areas in Kyung-buk and Kyung-nam provinces. Symptoms consisted of brownish to reddish rot on fruits resulting in early drop or mummification. The causal fungus isolated from infected fruits and adjacent leaf stalks was identified as *Phytophthora citricola*, which has never been reported in Korea. Sporangia were semi-papillate, noncaducous and highly variable in shapes. Plerotic oospores with paragynous antheridia were abundant in single cultures. Sporangia of two isolates were measured as 38-76×20-40 μm and averaged 51.4×27.0 and 55.6×36.0 μm. Oogonia were ranged from 26 to 36 μm and averaged 31.3 and 32.0 μm. Colony pattern was slightly radiated with sparse aerial mycelia on common media. Minimum, optimum and maximum temperatures for mycelial growth were recorded at 7, 25, and 32°C, respectively. Among tested media, 10%V8A was the best and 25°C was better than 15°C for oospore formation of the fungus. The jujube isolates of *P. citricola* were readily differentiated from other closely related species in the genus, namely; *P. nicotianae, P. citrophthora, P. cactorum, P. capsici*, and *P. palmivora* on the basis of PCR-RFLP of r-DNA. The fungus showed strong pathogenicty to jujube, apple, pear, orange, persimmon and eggplant, and relatively weak to citron, tomato, pepper and cucumber. In this study, *P. citrocola* is firstly identified and jujube fruit rot caused by the fungus is recorded as a new disease in Korea.

***Key words*: jujube fruit rot, identification, PCR-RFLP of rDNA, *Phytophthora citricola*.**

Jujube is one of the commonly cultivated fruit trees in Korea because its fruits are being utilized for tea, beverage and oriental medicine. Anthracnose is known as the most serious disease on the fruit deteriorating quality and yield (8). However, it was found from our survey that *Phytophthora* fruit rot also damaged to the cultivation in some fields. The *Phytophthora* rot on jujube fruit was firstly observed at Changryung of Kyungnam province in 1993 and the disease was recorded in the book 'Compendium of Fruit Tree Diseases with Color Plates' published by National Institute of Agricultural Science and Technology, RDA, Korea (8). However, the causal pathogen was not specifically described and identified in the book.

In 1997 and 1998, the *Phytophthora* disease was observed in several fields at Chilgok and Kyungsan of Kyungbuk province and Taegu. The causal pathogen of *Phytophthora* was constantly isolated from jujube fruits and leaf stalks showing brownish to reddish rot. The fungus was identified and its pathogenicity to jujube and other fruits was examined in comparison with *P. cactorum* and *P. cambivora*, which are known major pathogens attacking fruit trees in Korea (5). To investigate the genetic differentiation of the jujube isolates from closely related species in the genus, ribosomal DNA of the fungi was analyzed by PCR-RFLP.

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MATERIALS AND METHODS

Isolation and identification of the pathogen. Jujube fruits and leaf stalks showing brownish to reddish rot were collected from several fields at Chilgok, Kyungsan and Taegu in 1997 and 1998. Both a *Phytophthora* semi-

selective medium and water agar were used to isolate the causal pathogen. The semi-selective medium consisted of corn meal agar (CMA, 17 g/l) was supplemented with pimaricin 10 ppm, rifampicin 10 ppm, ampicillin 100 ppm and PCNB 50 ppm. Among the *Phytophthora* collections, two representative isolates designated as P-97101 and P-9813 were used throughout this study.

To investigate morphological characteristics of the fungus, isolates were cultured on 10%V8 agar for 3~4 days and sporulated as described by Jee *et al.* (4, 5). Effect of temperature on mycelial growth of the jujube isolates was examined on corn meal agar (CMA) in dark at 2~5°C intervals from 5 to 35°C. The two isolates were cultured for 2 weeks in dark on various cultural media as 10%V8 juice agar (V8A), potato dextrose agar (PDA), CMA and oat meal agar (OMA) to examine colony patterns and oospore formation at 15 and 25°C. Oospore numbers formed on the media were counted by a microsyringe technique developed by Ko *et al.* (4, 6).

DNA isolation. The two jujube isolates and closely related five species of *Phytophthora* were used. All isolates were obtained from the culture collections in the Division of Plant Pathology, National Institute of Agricultural Science and Technology, RDA, Korea (3). *P. nicotianae* (P-9501 and P-9660), *P. citrophthora* (SP-13), *P. cactorum* (Pb-9), *P. capsici* (Pa-11) and *P. palmivora* (P-9601) were originally isolated from eggplant, sesame, yuzu, yuzu soil, apple, pepper and areca palm, respectively.

Isolation procedures of total DNA were basically followed by Lee and Taylor (7). Young mycelia grown in clarified 10%V8 juice broth for 2 days were rinsed twice with distilled water prior to isolation of DNA as follows: 1) Mycelia in 400 μl of DNA extraction solution (3% SDS, 50 mM EDTA, 50 mM Tris-HCl pH 7.2 and 1% 2-mercaptoethanol) were macerated with a glass rod and incubated at 65°C for one hour. 2) 400 μl of phenol : chloroform (v/v, 1 : 1) was added to the tube, vortexed and centrifuged at 13,000×g for 10 min. 3) After collecting supernatant (aqueous phase) to a new 1.5 ml tube, 20 μl of 3 M sodium acetate and 0.54 volume (220 μl) of isoprophanol were added and centrifuged at 12,000× g for 5 min. to precipitate DNA. 4) The pellet was rinsed twice with 70% ethanol, vacuum dried ca. 5 min. and dissolved in 50 μl of TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA). 5) RNase 1.0 μl was added to the total DNA extract and reacted for 30 min. at 37°C.

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Primers used in this study were designed by White *et al.* (11) for the amplification of internal transcribed spacers (ITS) including 5.8S ribosomal DNA in the nuclear DNA repetitive unit. The primers consisted of ITS1 : 5'-TCCGTAGGTGAACCTGCGG-3', ITS4: 5'-TCCTCCGCTTATTGATATGC-3', NS1 : 5'-GTAGTCATATGCTTGTCTC-3', NS8 : 5'-TCCGCAGGTTCACCTACGGA-3'. Each template DNA 100ng prepared as above was added in the reaction mixture [1X buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% triton X-100), each dNTP 0.1 mM, each primer 1 pM, MgCl 1.5 mM, Tag polymerase 2 units (promega)]. The thermal cycles were performed 35 times with profile of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min. The first denaturation and the last extension time were extended to 4 min and 8 min, respectively. The success of amplification was monitored by analyzing 5 μl of the reactant on 1% agarose gel electrophoresis.

PCR-amplified rDNA regions of each isolate was digested separately with two restriction enzymes, *Hae*III and *Afa*I, according to the manufacturer's (Takara, Japan) instructions. The digested fragments were separated by 3% MetaPhor agarose (FMC Bioproducts) with TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA).

Pathogenicity test. Pathogenicty of the jujujbe isolate P-9813 to fruits of apple, pear, orange, citron, persimon, tomato, pepper, eggplant and cucumber was examined in comparison with *P. cactorum* Pb-9 and *P. cambivora* Pb-6, which were originated from apple fruits by Jee *et al.* (5). An agar disk of the isolate grown on 10%V8A for 5 days was inserted to a wound of each fruit made by a cork borer (7 mm in diameter). After sealing up the wound with a tape, fruits were incubated at 22°C for 5 days. Each of three fruits was used for the test and the rot degree was mean of the replicates. Pathogenicity of P-9813 to jujube fruit was tested without wound as follows. About 30 ml of the zoospore suspension (ca. 10^4 zoospore/ml) of the fungus prepared as Jee *at al.* (5) was sprayed on thirty jujube fruits contained in a moisture box. The box was incubated at 25°C and the number of infected fruits wase recorded 5 days after inoculation. The same number of the fruits was non-inoculated as control, and the fungus was re-isolated from rotten fruits to confirm Koch's roles.

RESULTS

Isolation and identification the pathogen. The causal fungus of jujube fruit rot was readily isolated from freshly infected inner tissues of infected fruits and leaf stalks

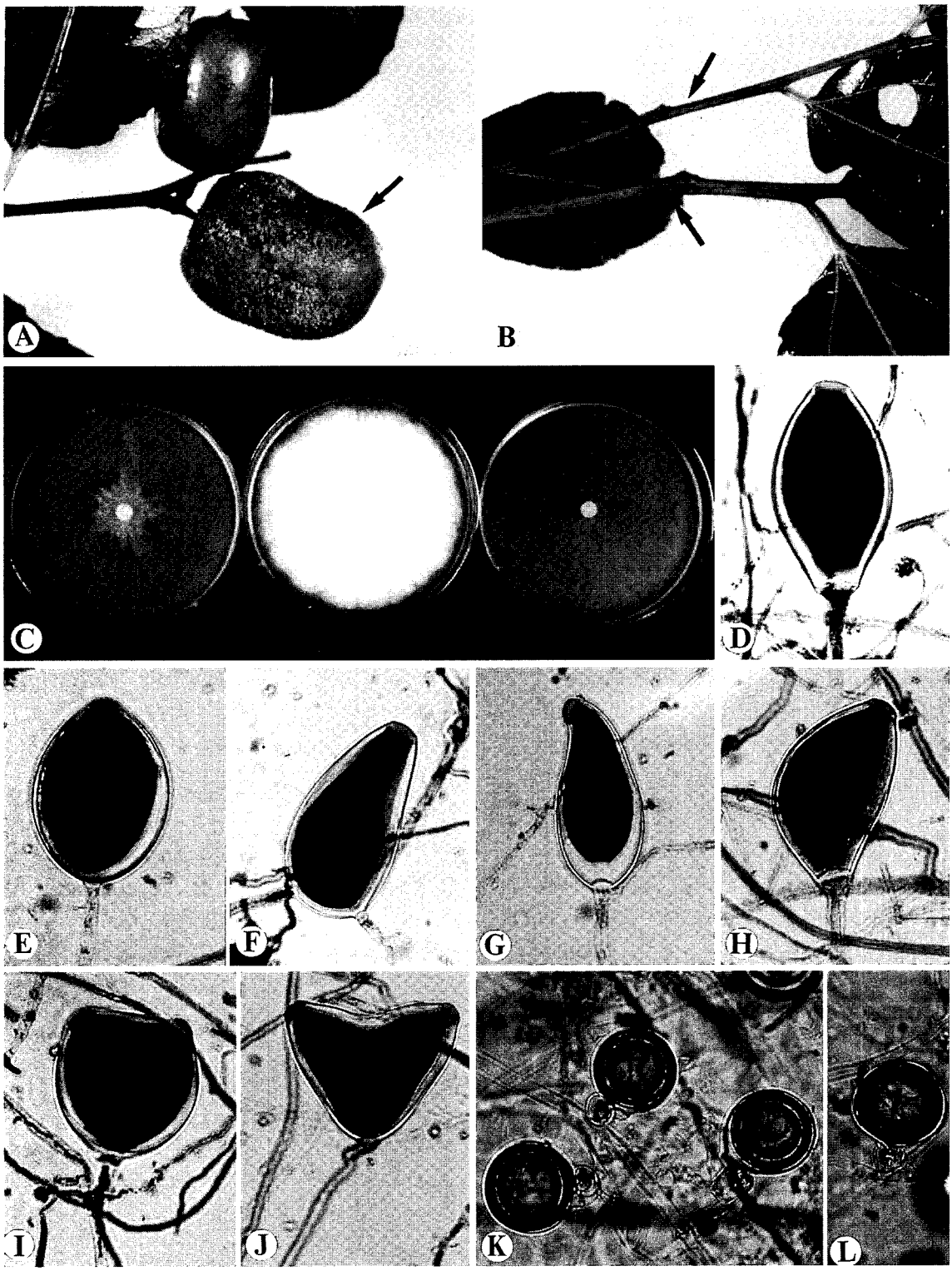

Fig. 1. Symptoms of the jujube fruit rot caused by *Phytophthora* sp. and morphological features of the fungus. Typical symptoms on fruits (A) and leaf stalks (B), colony patterns (C) on 10%V8A (left), PDA (center) and CMA (right), various shapes of sporangia (D~J), and oospores with paragynous (K) and amphigynous antheridia (L).

showing brownish to reddish spots (Fig. 1-A, B). The fungus was more effectively obtained from the *Phytophthora* semi-selective medium than the water agar. Occasionally, abundant sporangia and oospores were form-

ed on the fruit surface and the infected fruits dried up or dropped prematurely in the fields. Since all isolates collected from jujube produced sporangia and aseptate, coarse and stiffly branched mycelia (5~8 μm in width), they belonged to the genus *Phytophthora*.

The fungus grew well on common media as 10%V 8A, PDA, CMA and OMA and showed slightly radiated colony patterns with sparse aerial mycelia (Fig. 1-C). Morphological characteristics of the fungus are summarized in Table 1. The fungus produced semi-papillate (inconspicuously papillate) sporangia rarely on agar but readily in water. The semi-papillate sporangia were highly variable in shapes as ovoid, limoniform, obpyriform, clavate, distorted as slightly flattened or skewed in one side, or occasionally bifurcated with two apices (Fig. 1-D~J). All isolates were homothallic since abundant oospores were formed by single isolates on 10%V8A. Oospores filled up oogonia as a plerotic type and antheridia were single celled and paragynous (Fig. 1-K). However, amphigynous antheridia were also observed infrequently (Fig. 1-L). The sporangia were ranged as 38-76×20-40 (av. 51.4×27.0 and 55.5×36.0) μm and oogonia were 26-36 (av. 31.3 and 32.0) μm. Oospores were measured as 20-32 and averaged 24 and 25.2 μm (Table 2).

Minimum, optimum and maximum temperatures for mycelial growth were recorded at 7, 23~27, and 32°C, respectively (Fig. 2). Number of oospores produced by the fungus at 25°C was higher than those at 15°C in all tested media. 10%V8A was the most effective for oospore formation among the media. While the fungus produced more than 1.2×10^5 oospores/ml of 10%V8 at 25 °C, it produced less than 9.0×10^3 oospores/ml of PDA and CMA. OMA was also effective to induce oospores, however, the numbers were less than those of 10%V 8A (Table 3).

PCR-RFLP of ribosomal DNA. The primers, ITS1 and ITS4 successfully amplified the small subunit and ITS regions of rDNA of all isolates used in this study. The amplified small subunit and ITS regions of the *Phy-*

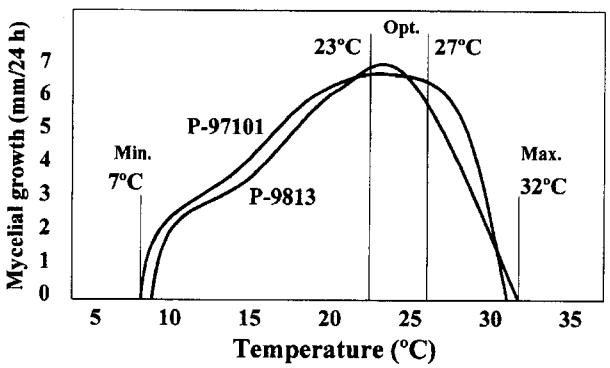

Fig. 2. Effect of temperature on mycelial growth of the jujube isolates of *Phytophthora* sp.

Table 1. Morphological characteristics of the *Phytophthora* sp. caused jujube fruit rot

Structure	Characteristics of the investigated isolate (P-9813)
Sporangia	Readily formed in water, semi-papillate, noncaducous, ovoid, obclavate, skewed, bifurcate
Sporangiophores	Long, slender, irregular
Chlamydospores	Not observed
Colony pattern	Slightly radiate, sparse aerial mycelia
Sexuality	Homothallic, abundant oospores
Oogonium	Spherical, smooth, thin walled
Oospore	Plerotic, colorless
Antheridium	Mostly paragynous but rarely amphigynous

Table 3. Effect of temperature and medium on oospore formation of *Phytophthora* sp. caused fruit rot of jujube

Medium	Number of oospores produced/ml of medium ($\times10^3$)			
	P-97101		P-9813	
	15°C	25°C	15°C	25°C
10%V8A	122.8±13.2	145.6±17.3	110.0±15.2	140.0±12.3
PDA	0.4±0.1	8.6±1.4	1.5±1.0	6.5±2.0
CMA	0.2±0.2	4.8±0.9	3.3±1.2	6.6±2.4
OMA	107.5±11.4	118.4±15.6	88.8±11.1	132.4±16.4

Table 2. Sizes of reproduction structures of the jujube isolates and *Phytophthora citricola*

Structures	Jujube isolate		*P. citricola*	
	P-97101	P-9813	Erwin & Ribeiro[a]	CMI Descriptions[b]
Sporangium				
Length	(38-) 51.4 (-76)	(48-) 55.5(-64)	(30-) 47 (-75)	57 (-100)
Breadth	(20-) 27.0 (-34)	(28-) 36.0 (-40)	(21-) 34 (-44)	33 (-40)
L/B ratio	1.9	1.5	1.4	1.7
Oogonium	(26-) 31.3 (-34)	(28-) 32.0 (-36)	(18-) 26 (-35)	40 (-58)
Oospore	(20-) 24.0 (-30)	(22-) 25.2 (-32)	(16-) 22 (-30)	–

[a]Erwin, D. C. and Ribeiro, O. K. 1996. *Phytophthora Diseases Worldwide*. APS press.
[b]Waterhouse, G. M. and Waterston, J. M. 1966. *Phytophthora citricola*. CMI Descriptions of Pathogenic Fungi and Bacteria. No. 114.

tophthora species were about 2,600 bp (data not shown). RFLP analysis of the amplified fragments showed that the two jujube isolates of P-97101 and P-9813 were identical but distinct from other species as *P. nicotianae, P. citrophthora P. cactorum, P. capsici* and *P. palmivora* (Fig. 3). When the amplified fragments were digested with *Hae*III or *Afa*I, electrophoretic patterns of each species presented unique bands, which were readily distinguishable from each other species.

Pathogenicity. The jujube isolate P-9813 caused rot on all tested fruits, namely; jujube, apple, pear, orange, citron, persimon, tomato, pepper, eggplant and cucumber (Table 4). However, *P. cactorum* Pb-9 did not infect tomato and cucumber, and *P. cambivora* Pb-6 did not cause rot on tomato, eggplant and cucumber. Jujube isolate P-9813 showed stronger pathogenicity to apple, pear, orange and citron than *P. cactorurm* and *P. cambivora* (Table 4). Among the fruits, jujube, apple, pear, orange, persimmon and eggplant were relatively susceptible to the jujube isolate of *Phytophthora* compare to other fruits as citron, tomato, pepper and cucumber.

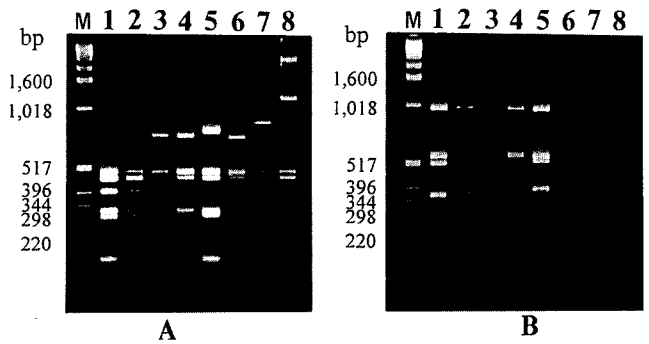

Fig. 3. Restriction patterns of the small subunit and ITS regions of *Phytophthora* spp. digested with *Hae*III (A) and *Afa*I (B). Lane 1; Jujube isolate P-97101, 2; Jujube isolate P-9813, 3; *P. nicotianae* P-9501, 4; *P. nicotianae* P-9660, 5; *P. citrophthora* SP-13, 6; *P. cactorum* Pb-9, 7; *P. capsici* Pa-11, 8; *P. palmivora* P-9601.

Table 4. Pathogenicity of jujube isolate of *Phytophthora* to fruits of trees and vegetables in comparison with apple isolates of *P. cactorum* and *P. cambivora*

Inoculated fruit	Degree of fruit rot			
	Jujube isolate (P-9813)	*P. cactorum* (Pb-9)	*P. cambivora* (Pb-6)	Untreated control
Apple	3.5[a]	3.0	3.0	0
Pear	3.5	3.0	2.5	0
Orange	4.0	3.5	1.0	0
Citron	2.5	2.5	2.0	0
Persimon	3.5	3.5	3.5	0
Tomato	2.0	0	0	0
Pepper	2.0	3.0	1.0	0
Eggplant	4.0	4.0	0	0
Cucumber	1.0	0	0	0
Jujube[b]	3.0 (100)[c]	–	–	0

[a]Mean of three fruits inoculated through wound: 0; no 1; weak 2; moderate 3; severe 4; complete rot.
[b]Thirty jujube fruits were inoculated with zoospore suspension (ca. 10^3/ml) without wound.
[c]Infection rate (%) of 30 jujube fruits.

DISCUSSION

Since the jujube isolates were homothallic and produced semi-papillate sporangia and paragynous antheridia, they belonged to *Phytophthora* group III according to Stamps *et al.* (9). The differentiation of semi-papillate sporangia from papillate and non-papillate is difficult to interpret, however, apical thickening of the semi-papillate is shallower than papillate (3~5 μm) and exit pore is narrower (5~7 μm) than non-papillate (ca. 12 μm) as shown in Fig. 1 (1, 2, 9). All investigated morphological characteristics of the present isolates matched well with *P. citricola* in the *Phytophthora* group III described by different authors (1-3, 9, 10). The fungus can be readily differentiated from other closely related species in the genus by its semi-papillate, non-caducous and highly variable shapes of sporangia, homothallism and paragynous antheridia (1, 9). The result of PCR-RFLP of rDNA further confirmed the genetic difference of the jujube isolates of *P. citricola* from other species as *P. nicotianae, P. citrophthora, P. cactorum, P. capsici* and *P. palmivora* (Fig. 3). Averaged sizes of sporangia and oospores of the present isolates were slightly bigger than those of described by Erwin and Ribeiro (1), but the sizes fell into the category of *P. citricola* (1, 9, 10).

Further RFLP analysis of the amplified fragments of the small subunit and ITS regions of rDNA resulted that each species of *Phytophthora* showed unique band patterns, which made it clear that *Phytophthora* species has its own genetic structure distinguishable from each other. Since identification of *Phytophthora* species is difficult because of the morphological differentiations are so small and overlapping features exist among species (1, 9), PCR-RFLP of rDNA is a useful tool to differentiate and evaluate the relatedness of species in the taxonomy of *Phytophthora* judging from the results obtained in this study.

P. citricola has been reported worldwide, causing rots on fruits and roots of a number of fruit trees, ornamental plants and vegetables (1, 3, 10). However, the fungus has

never been reported in Korea previously. Pathological importances as host range and distributions of the fungus remain to be investigated. Since jujube isolates of *Phytophthora* showed strong pathogenicity to fruits of trees and vegetables, it is suspected that the fungus infect not only jujube but many other important plants cultivated in the country. Contrarily, it is also conceivable that not only *P. citricola* but other species in the genus may cause rot on jujube fruits.

요 약

*Phytophthora*속 균에 의한 대추역병이 경남과 경북의 일부 재배지역에서 발생되고 있다. 병징은 과일에 적갈색의 반점이 생기면서 조기 낙과되거나 미이라처럼 마르기도 하는데, 간혹 잎자루나 어린가지에도 적갈색 병반이 나타나기도 한다. 분리된 역병균은 국내 미기록 종인 *P. citricola*로 동정되었는데 균학적 특성을 요약하면 아래와 같다. 유주자낭은 반돌출형의 돌기를 가지고 있으며 비탈락성으로 매우 다양한 형태를 나타낸다. 자웅동주균으로 단균주가 다량의 난포자를 형성하는데 난포자는 충만형이며 대부분은 측착형인 장정기를 부착하고 있으나 간혹 저착형인 것도 있었다. 유주자낭의 크기는 38-76×20-34(평균 51.4×26..9) μm이며, 난포자는 24~36 (평균 29.9±3.0) μm였다. 균총은 약간 방사선 형태로 나타나며 기중균사는 잘 형성되지 않았는데 균사 생육 최저, 최적, 최고 온도는 각각 7, 23-27, 32°C로 조사되었다. ribosomal DNA의 PCR-RFLP 분석 결과, 공시한 역병균은 종별로 서로 구분되는 밴드 형태를 보였는데, 대추역병균인 *P. citricola*는 *P. nicotianae, P. citrophthora, P. cactorum, P. capsici, P. palmivora* 등과 확실히 구분되었다. 대추역병균은 대추, 사과, 배, 복숭아, 오렌지, 가지 등에는 강한 병원성을 감귤, 고추, 토마토, 오이 등에는 다소 약한 병원성을 나타내었다. 본 연구로 국내에서는 *P. citricola*가 처음으로 동정되었으며 본 병원균에 의한 대추역병은 처음으로 기록되는 바이다.

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