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Identification of the Imperfect Stage of *Mycosphaerella nawae* Causing Circular Leaf Spot of Persimmon in Korea

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감나무 둥근무늬낙엽병균 *Mycosphaerella nawae*의 불완전 세대 동정

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ABSTRACT : Asexual spores of *Mycosphaerella nawae* were profusely produced on PDA after a prolonged incubation at 25°C for 90 days. When persimmon trees were artificially inoculated by the conidial suspension, typical symptoms of circular leaf spot of persimmon appeared on the leaves two month later. The imperfect stag of the fungus was identified as *Ramularia* sp. based on following morphological characteristics examined under a light microscope and a scanning electron microscope. Conidia were mostly ellipsoid, but occasionally cylindrical, elongated oval, taro, peanut or gourd shapes and measured as 12.2~32.6×6.1~10.2 μm. erect, hyaline, colorless-light brown. Conidia were formed solitarily or in chains on a medium and infected leaves. Conidiophore was erect, hyaline, colorless-light brown. and the size was 20.4~102.0×3.1~10.2 μm, respectively. In this paper, we firstly demonstratrated that asexual spores of *M. nawae* induced persimmon circular leaf spot in nature as well as sexual spores of the fungus. Therefore, it is hypothesized that the the imperfect stage of the fungus plays an important role in nature for epidemics as secondary inoculum.

Key words : **circular leaf spot, conidia, conidiophore, imperfect stage, *Mycosphaerella nawae*, persimmon, *Ramularia* sp., secondary inoculum.**

The circular leaf spot of persimmon (*Diospyros kaki* Thunb. var. *domestica* Makino) occurs nationwide and often causes severe epidermics in the southern part of Korea. However, mycological traits of the casual pathogen, *Mycosphaerella nawae* Hiura et. Ikata, is little known. The fungus belongs to the class *Loculoascomycetes* with fissitunicately discharging asci, producing ascospores which are septate and borne in unwalled locules (3). Most fungi in the genus *Mycosphaerella* readily produce conidia, but it is generally conceived that *M. nawae* does not produce conidia on media and in nature, although Ikata and Hitomi (2) observed conidial stage of the fungus on PDA culture only once in 1929. Thereafter, conidial production of the fungus and its role in nature remains ambiguous. However, we developed a mass sporulation method for conidial formation on PDA after a prolonged incubation at 25°C for 90 days (4).

The fungus has been known to overwinter as mycelia or mycelial mass within infected leave tissues and Pseudothecium generates at mid to late March and develops rapidly on mid April. Matured ascospores in the perithecium formed on infected leaves are known the primary inoculum source in the field (6, 7). However, the secondary inoculum source in the fields has not been clearly defined yet because Ikata and Hitomi reported that the conidia of the fungus obtained from culture did not induce typical symptoms on presimmon leaves when artificially inoculated (2, 3). In this study, we tried to demonstrate pathogenicity of conidia inducing typical of persimmon circular leaf spot and identified the imperfect stage of *M. nawae.*

MATERIAL AND MATHODS

Conidial production and inoculation. An isolate of *Mycosphaerella nawae* originated from a persimmon leaf showing typical symptoms of the circular leaf spot was collected in Chinju of Korea in 1994. The isolate was incubated at 25°C for 90 days in dark on potato

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dextrose agar (PDA) for conidial production. To confirm the pathogenicity, conidia produced on the medium were harvested with distilled water and filtered through four layers of cheeseclothes to remove mycelial fragments. The suspension was adjusted to 18.8×10^4/ml and sprayed onto 2-yr-old persimmon trees and kept outdoors for 2~3 months in isolation. Three persimmon trees were treated and experiment was repeated thrice.

Light and scanning electron microscopy of conidia. Morphological characteristics of conidia produce on the medium were directly examined under a light microscope at 300x. Naturally infected leaves collected from fields at late October were concomitantly examined under the microscope to examine the conidial development on infected leaves in the fields. The fungial colonis cultures on PDA for 90 days were cut into pieces ca. 5×5 mm in size with a surgical blade and fixed with 2.5% glutaraldehyde solution for 24 h at 4°C. After fixing the specimens were rinsed carefully with 0.1 M phospate buffer (pH 7.4) 2~3 times and soaked in 2% OsO_4 for 90 min at 90 min and rinsed carefully 2-3 times with the same buffer solution. They were dehydrated through a series of ethanol gradient (50, 75, 95 and 100%) after soaking for 40 min room temperature, and followed by isoamyl acetate for 40 min 2 times. After that, the samples were dried by Hitachi HCP-2 crytical point dryer and subjected to the gold coating by Eiko IB-2 ion coat. Specimens were examined by SEM (JSM-6400, Joel, Japen) under 1.500x or 3.000x.

RESULTS

Anamorphic characteristics of *Mycosphaerella nawae*. Morphological charcteristics of the fungus examined under a light microscope and a SEM are summarized in Table 1 in comparison with closely related fungi. The fungus produced conidia singly or in chains.

Young hyphae on PDA medium (Fig. 1A) were septated, 3.1~8.2 μm thick with averages 5.3 μm, colorless to light olive and growing mycelial tips stretched under a prosperous condition and old hyphae were characteristically articulated (Fig. 1B). The conidia were mostly none or rarely 1~3 septated, developed solitary or in chains (Fig. 1C) and the shapes were mostly ellipsoid, occasionally long ellipsoid, peanut shell types, inverted brown, gourd and cylinder shape (Fig. 1D). They were also colorless or light brown. Their sizes were 12.2~32.6×6.1~10.2 μm (avg. 21.0×9.4 μm). The types of germinatiom were mono-polar or bi-polar (Fig. 1E). The conidia were *Ramularia* shaped characteristically. The conidiophores were erect, hyaline and short, colorless or light brown (Fig. 1F). Their sizes were 20.4~102.0×3.1~10.2 μm (avg. 49.2×5.1 μm). The septa were mostly non-septated and occasionally uni-septated, conidia were also observed from the leaf symptoms by ascospore infection.

From symptom leaf lesion reproduced by artificial inoculation of either ascospore, the conidia developed (Fig. 1G) and conidial scars on conidiogenous cell of thickened circular brown hyphe were visible conspicuously (Fig.

Table 1. Comparison of anamorphic characteristics of *Mycosphaerella nawae* between present isolate, *Ramularia rubella, Pseudocercosporella capsellae* and Ikata's description of *M. nawae*

Characters	Present isolate[a]	*R. rubella* (8)	*P. capsellae* (5)	*M. nawae*(2)[b]
Conidium				
chain of conidia	present	–	–	–
shape	ellipsoid, obovoid, cylinder, peanut, bottle gourd, clostridial	obovoid, smooth, thin-walled, hyaline	cylinder, obclavate	clostridial cylinder
color	colorless~light brown	–	colorless	colorless~light olive
size	12.2~32.6×6.1~10.2 μm	15.0~27.3×7.3~10.0 μm	20~50×2~3.5 μm	15~30×1.5~3
septa1	0~3	1	1~5	1~3
Conidiophore				
scars	circular brown*	circular black	truncate unthickened	–
shape	erect, hyaline, short	stroma, thin-walled hyaline, fan-like,	cylinder~ampulliform	–
color	colorless~light brown	–	colorless	colorless~light olive
size	20.4~102.0×3.1~10.2 μm	27.5~95.0×2.5~5.5 μm	5~15×2~4 μm	15~33×2~3 μm
septa	0~1			0~1
Type of germination	mono-polar, bi-polar	–	–	none

[a]Conidia were confirmed on PDA culture and infected leaf by authors.
[b]Conidia have not been confirmed from infected leaf, since 1929.
*Refer to Fig. 1G

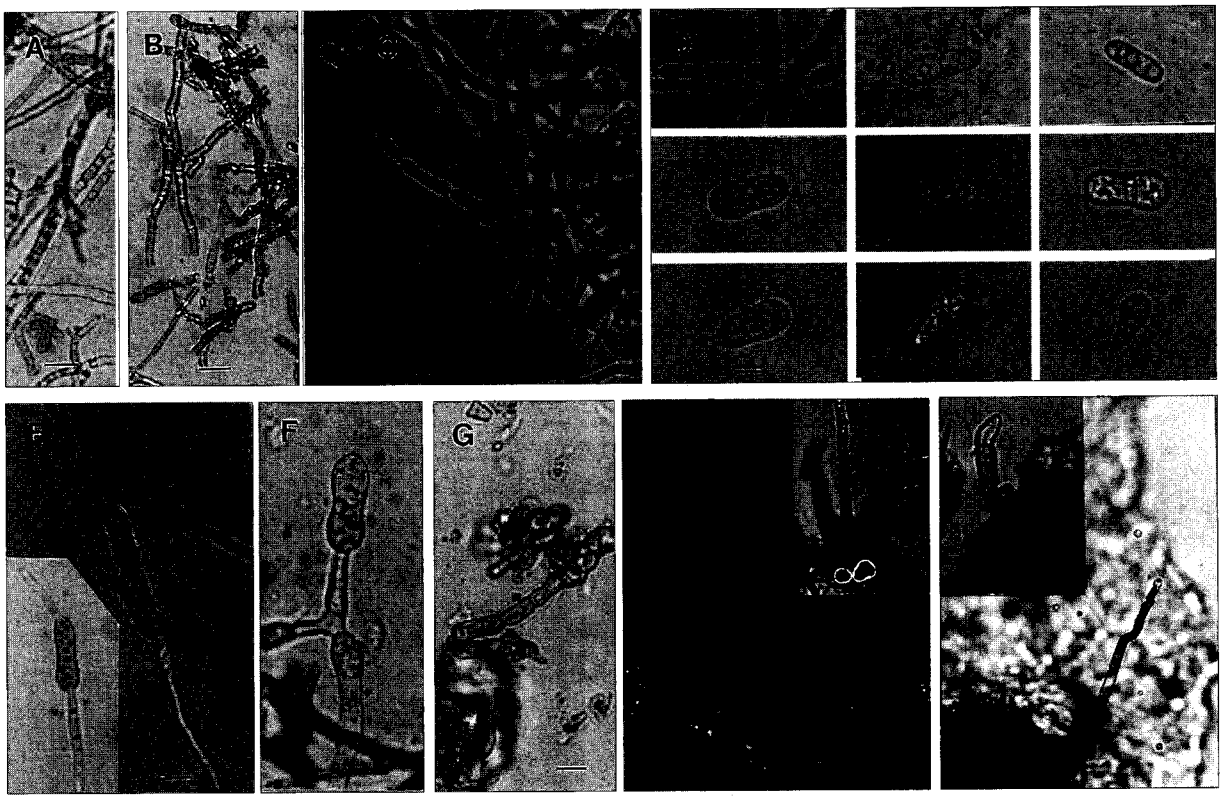

Fig. 1. Anamorphic characteristics of *Mycosphaerella nawae* grown on PDA medium for 90 days or from natually infected leaves. A : Newly growing mycelium, B: Old mycelium (over 30 days), C : Chain of conidia formed on PDA (catenate), D : Various forms of conidia, E : Germinating conidial on PDA medium. F : Conidiophore and conidia, G, H : Conidia and conidial scars formed in infected leaves naturally. Bars indicate 10 μm.

1H middle), with pale-colored conidia being blasted-out (Fig. 1H left, right) are as follows.

Symptom development. Such anamorphic characters *in vivo* were also confirmed *in vitro* PDA medium incubated for 90 days at 25°C (Fig. 2). there were no difference in macroscopic symptom among those.

Typical symptom by natural ascospore infection (Fig. 3A) was identical to that (Fig. 3B) resulted by spraying conidial suspension produced in PDA medium.

In conclusion, we were able to confirm the secondary infection by imperfect stage of *M. nawae*. Furthermore, there were no difference in pathogenicity between ascospore and conidia.

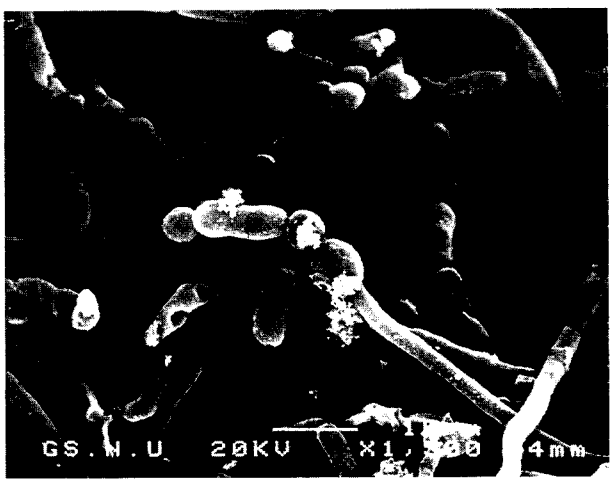

Fig. 2. Scanning electron microscopy of conidia and mycelium of *Mycosphaerella nawae* produced on PDA medium for 90 days of incubation.

DISCUSSION

The anamorphic culture of *Mycosphaerella nawae* and infected persimmon leaves forming asexual reproduction structures were sent to IMI for identification of the imperfect stage of the fungus. Dr. J. C. David of the institute tenatively identified as *Pseudocercosprella* sp. without conviction since the asexual stage of persimmon

Fig. 3. Various symptoms of circular leaf spot disease of persimmon. A : Typical symptom, B : Symptom induced by conidia inoculation produced in PDA medium.

isolates has never been described and identified. However, we identified this fungus as *Ramularia* sp. based on morphological characteristics, we observed, and also on other literatures describing the genera *Pseudocercosporella* (5) and *Ramularia* (8). *Mycosphaerella* spp. produce abundant conidia in nature. However, conidia of *M. nawae* have been noticed only once on PDA culture (2) and it is not reported that conidia are produced on leaf lesion so far since 1929 (3). For the first time, in this paper, we provided evidence that the imperfect stage of *M. nawae* plays a role in epidemiology of disease based on results of inoculation tests (Fig. 1~3).

The mycelial growth of *M. nawae* were extremely slow *in vitro* on PDA medium and conidiation was abundant in 90 days incubation at 25°C, which is extremely prolonged period compared to the other fungal pathogen. Light microscopy revealed that conidia were mostly ellipsoid, occasionally long ellipsoid, peanut shell type, inverted oval, fusiform and cylinder shape (Fig. 1). The morphology of conidia by scanning electron microscopy was almost same regardless of their sources, i.e. *in vitro* culture, and *in vivo* leaf lesion of natural ascospore infection, or that of artificial inoculation of conidia.

Therefore the conidial scars on conidigenous cells were conspicuous, thickened circular brown (Fig. 1G). Also there are many other characters, we observed, that could distinguish present isolates from *Pseudocercosporella* : 1) hyphae, conidiophores and conidia colourless to pale. 2) conidial formation singly or in chains are those criteria as *Ramularia* (1).

Another distinct trait of this isolate is the morphology of conidia which is, mostly solitary, ellipsoid-ovoid to subglobose and only 0~1 septate. Long, multiseptate conidia in *Ramularia* are always catenate (1). Most of all, conidia of *Pseudocercosporella* are long and more of *Cercosporella* type. Yoshikawa and Yokoyama (9) described the diagnostic features of *Thedgonia, Cercospora* and *Septotrullula*. These were distinguishable from our sample that the first two genera polyblastic and acropetal conidia as well as the morphology of them. The possibility of this specimen being *Septotrullula* could be ruled out by septated nature of conidium.

Two-year-old trees were also artificially exposed during the late August for one month to the diseased nurseries with typical symptoms (Fig. 3B) and successful infection was confirmed within a month and half by Koch's postulates. Thus, we were able to prove the secondary infection by imperfect stage of *M. nawae.*

요 약

PDA 배지상에서 감나무 둥근무늬낙엽병균(*Mycosphaerella nawae*)의 분생포자의 특성을 조사하였다. 분생포자는 PDA 배지상에 25°C에서 90일간 배양후 다량 형성되었다. 분생포자 형성은 단생 또는 연쇄상으로 모양은 타원형, 원통형, 토란형, 땅콩형, 조롱박형 등으로 다양하였으며, 분생포자 크기는 12.2~32.6×6.1~10.2 μm이며, 분생자병은 직립, 반투명, 무색 또는 담갈색으로 크기는 20.2~102.0×3.1~10.2 μm였다. 이런 형태적인 특징으로 보아 본 균의 무성세대는 *Ramularia* sp.로 동정되었다. PDA 배지상에서 형성된 분생포자를 인공접종 하여 발병을 확인하였고, 또한 그 병징은 자낭포자에 자연감염에 의하여 발병된 병징과 형태적인 차이는 없었다.

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