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# Further Evidence that *Ramularia*-type Conidia *in vivo* Plays a Role as a Secondary Inoculum of *Mycosphaerella nawae*

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# 감나무 둥근무늬낙엽병균 *Mycosphaerella nawae* 분생포자의 2차 전염원으로서 역활

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**ABSTRACT : The characteristic *Ramularia* type conidia of *Mycosphaerella nawae* were formed on naturally infected leaves. Artificial inoculation with the conidia induced typical symptom on leaves, which was not distinguishable from those of ascospore infection, which has been considered as a primary inoculum source in nature. Also the morphology of the conidia produced on PDA was not different from those formed on artificially inoculated leaves or on naturally infected leaves at later stage of symptom development. Accordingly, we report the role of the conidia as a secondary inoculum of the circular leaf spot pathogen of persimmon for the first time.**

***Key words*** **: conidia, *Mycosphaerella nawae*, primary inoculum, secondary inoculum.**

The circular leaf spot disease of persimmon (*Diospyros kaki* Thunb. var. *domestica* Makino) occurrs nationwide where persimmon trees are cultivating. The disease has been considered as the most destructive in persimmon cultivation. Because it often causes severe epidemics in southern parts of Korea (6). This peculiar disease shows quite long interval from infection to symptom appearance, and the control of the disease is very difficult once after the symptom has appeared. In spite of importance of the disease, little knowledge on the disease development and the causal organism had been accumulated (6).

Effects of meteorological factors on activity of primary inoculum was reported in detail by Kang and Kwon (3, 5-11) as well as by Takuda and Hirosawa (13, 14). However, so far no reports on secondary inoculum of this pathogen are available. Ikata and Hitomi (2) observed conidia on PDA culture *in vitro* only once in 1929, but not from infected leaves showing typical symptom (4). The conidia of *Mycosphaerella nawae* were sporulated on artificial media after prolonged of incubation (6). We successfully demonstrated conidial sporulation on media and speculated that an important role of the asexial spores in nature (6, 8).

In this paper, we report the presence of the imperfect stage of *M. nawae*, by scanning electron microscopy followed by previous publication by the authors (11) that *in vitro* conidia were pathogenic to the host, which we for the first time revealed the role of *Ramularia*-type conidia *in vivo* playing as secondary inoculum involved in epidemiology of this disease.

## MATERIALS AND METHODS

**Artificial inoculation.** Artificial inoculation of conidial suspension on persimmon seedling was done on August 24, 1996 at the concentration of $12.3\times10^4$ spores per milliliter. The suspension was obtained by harvesting conidia from typical symptom developed by natural ascospore infection. We also exposed 2 year old seedling (pots 3×3 trees) to conidia released from diseased leaves with typical symptom. After inoculation, the plants were kept in isolation for two months.

**Preparation of leaf samples and *in vitro* colonies for scanning electron microscopy.** Leaves showing typical symptom were collected for microscopy to con-

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firm the conidial formation on persimmon leaves. Samples were collected before defoliation and after overwintering of the leaves infected either by naturally with ascospores or by artificially with conidia by natural infection of ascospore and by artificial infection of conidia. The infected leaf samples were cut into small pieces 5×5 mm with a surgical scalpel blades. The pieces were prefixed in soaking with 2.5% glutaraldehyde solution for 3 hr at 4°C room temperature. After rinsing with 0.1 M phosphate buffer (pH 7.4) for 3 times, specimens were postfixed with 2% $OsO_4$ (osmium tetroxide, Sigma, U. S.A) in soaking for 90 min at 4°C room temperature and it was rinsed 3 times with the same buffer solution (pH 7.4). The specimens were dehydrated through a series of ascending concentrations of ethanol, 20, 40, 60, 80, 90% for 30 min and ethanol 100% for 60 min, followed by isoamyl acetate+ethanol (1:1) mixture. After isoamyl acetate treatment for 30 min, the samples were dried by Hitachi HCP-2 critical point dryer and subjected to the gold coating by Eiko IB-2 ion coat.

## RESULTS

**Morphology of conidia.** The anamorphic characters of *M. nawae in vivo* on leaves developed typical symptoms from ascospore infection or conidial infection were observed under scanning electron microscope.

Characteristic *Ramularia* shaped conidia were observed from the leaves infected by ascospore of *M. nawae* (Fig. 1). Conidial scars on conidiogenous cell of thickened circular brown hyphe were visible, with pale colored conidia being blasted-out are as follows: The conidia and scars (Fig. 1A) were produced on typical lesions before defoliation, and conidia and scars (Fig. 1B, C) were also produced on defoliated leaves before overwintering. By artificial inoculation of *in vivo* conidial suspension of *M. nawae*, conidia were also produced (Fig. 2A). The conidia on conidiophores were formed solitarily or in chain emerging through the stomata of the leaf with lesions, and especially they were formed abundantly around stomata on the lesion which was identical to those by natural infection. Rarely, anamorphs were also observed from upper surface of infected leaf (Fig. 2B).

**Symptoms.** Typical symptoms caused by natural ascospore infection (Fig. 3A) was identical to those caused by spraying conidial suspension, and those (Fig. 3B) by conidial suspension harvested directly from diseased leaves previously infected by ascospore. Finally, 2-years-young trees were also artificially exposed during late

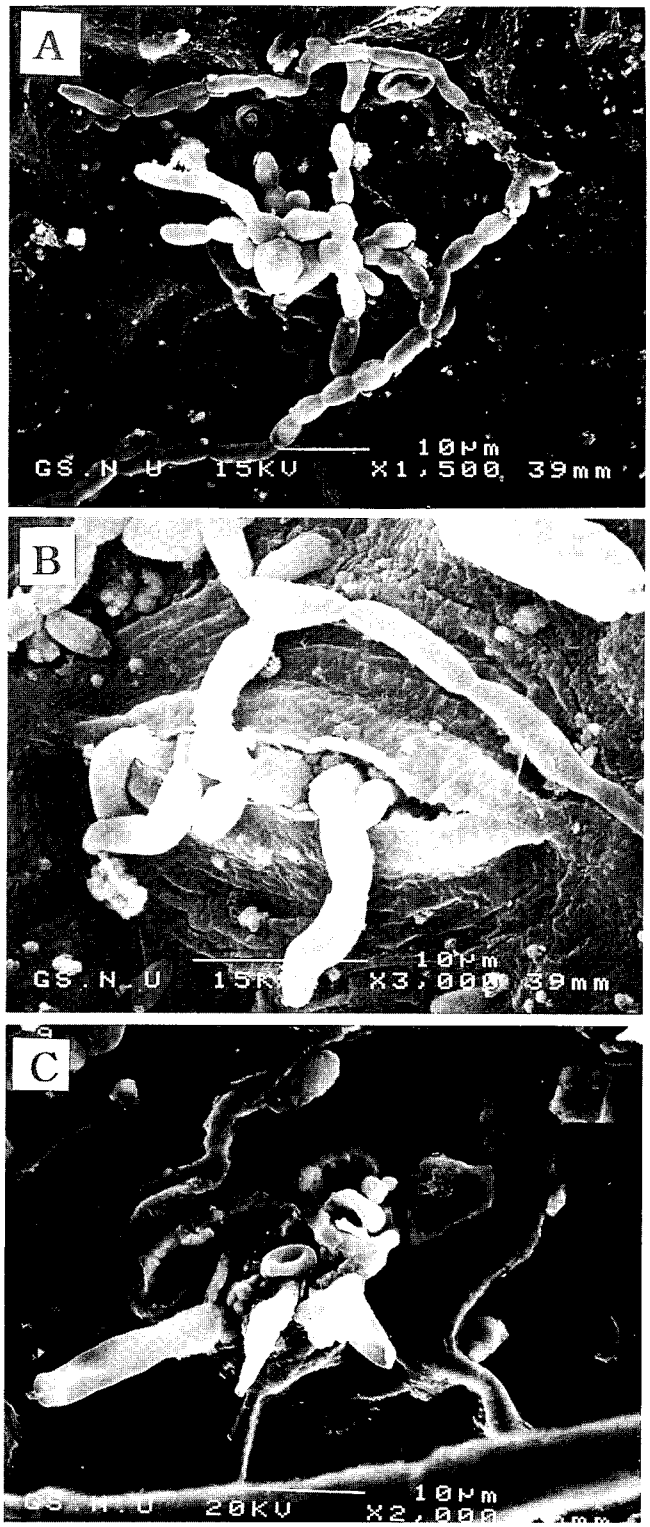


**Fig. 1.** Scanning electron microscopy of conidia emerging through stomata on the lesions that were induced by the naturally released ascospores of *Mycosphaerella nawae*. Conidia with scars and mycelium before defoliation(A) and after overwintered (B, C).

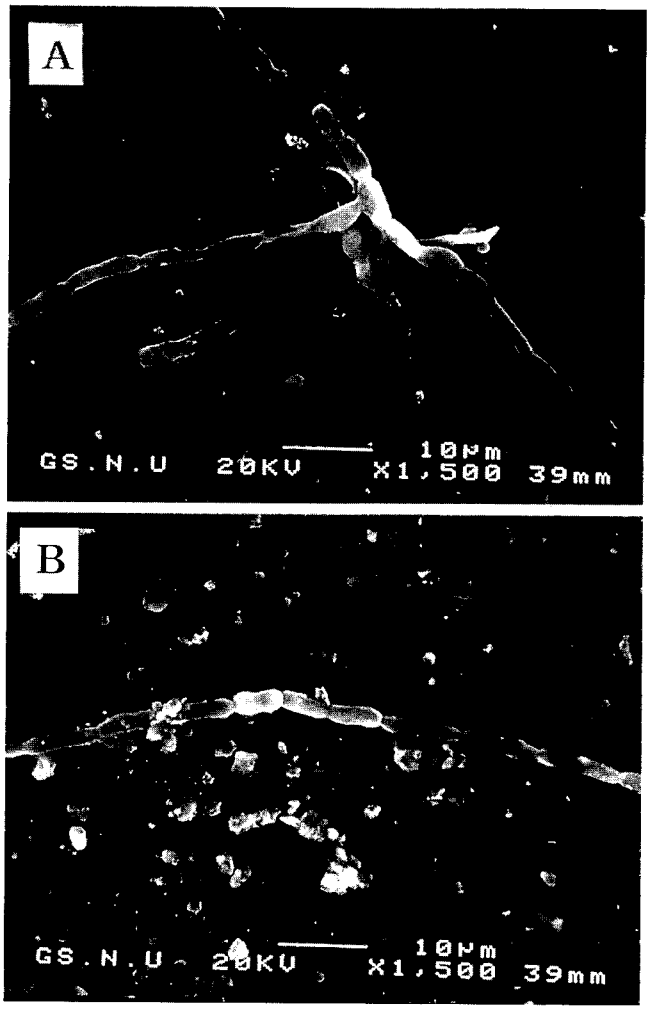


**Fig. 2.** Scanning electron microscopy of conidia of *Mycosphaerella nawae*. A : Conida emerging from stomata of the lesions induced by previous artificial inoculation of conidial suspension. B : Upper surface of leaf infected with conidia.

August to the diseased seedlings with typical symptoms as a inoculum source of conidia, as shown in Fig. 2. It is the time period that conidia are only available source of inoculum (Fig. 1A).

In conclusion, we were able to confirm the secondary infection by imperfect stage of *M. nawae* (Fig. 3C). Furthermore, there was no difference in pathogenicity between ascospore and conidia.

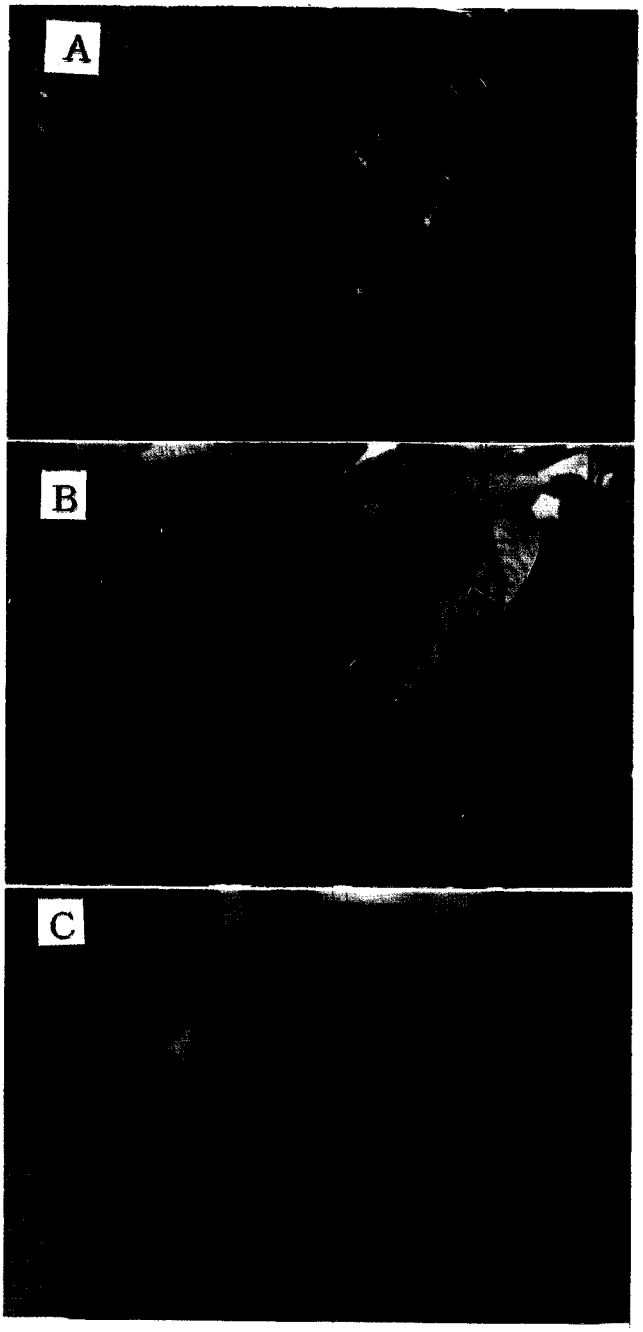


**Fig. 3.** Various symptoms of circular leaf spot disease of persimmon. A : Typical symptom, B : Symptoms induced by conidial suspension obtained from diseased leaves, C : Symptoms induced by secondary inoculum of conidia released from diseased leaves.

## DISCUSSION

*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 (4). 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). We identified this fungus as *Ramularia* sp. based on morphological characteristics, and also on other literatures describing the genera *Pseu-*

*docercosporella* and *Ramularia* (1, 12, 15).

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 and (Fig. 1A, B). Also there are many other characters, that could distinguish present isolate from *Pseudocercosporella* : 1) hyphae, conidiophores and conidia colourless to pale. 2) conidial formation singly or in chains are those criteria as *Ramularia* (1).

The conidia and conidiophores were developed through the stomata on the lesions, and especially they were profusely formed around stomata and occasionally on the upper surface rarely. Two-year-old trees were also artificially exposed during the late October for two month to the diseased nurseries with typical symptoms (Fig. 3A. B), when the conidia are the sole source of inoculum available at that time of the year and succesful 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* (Fig. 3B, C).

*M. nawae* produces ascospores in uniloculate pseudothecia, as a role source of primary inoculum. So far, the role of imperfect stage in disease epidemiology has not been reported. We have identified and illustrated the imperfect stage *in vitro* and also *in vivo* on the lesion, being involved in disease cycle. This is the futher evidence, we believe, that the conidia play a role of secondary inoculum.

## 요 약

감나무 둥근무늬낙엽병균(*Mycosphaerella nawae*)의 이병엽상에서 형성된 분생포자 특징을 주사전자현미경으로 관찰하였다. 분생포자는 단생 또는 연쇄상이었다. 형태적인 특징과 병원성으로 보아 자낭포자에 감염된 이병엽으로부터 분생포자 현탁액을 만들어 인공접종 결과 자연상태에서 발병된 병반과 동일하였고, 병반상에 형성된 분생포자의 형태는 자낭포자에 의한 자연감염된 것과 분생포자 인공접종 후 형성된 것과는 전혀 차이가 없었다. 따라서 감나무 둥근무늬낙엽병 발생에 2차 전염원으로서 분생포자의 역활이 입증되었다.

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