한국식물병리학회지 14(5) : 408-412(1998)
Korean J. Plant Pathol. 14(5) : 408-412(1998)

Microscopic Observation of the Pseudothecial Development of *Mycosphaerella nawae* on Persimmon Leaves Infected by Ascospore and Conidia

Jin-Hyeuk Kwon*, Soo-Woong Kang, Chang-Seuk Park[1] and Hee-Kyu Kim[1]
Gyeongnam Provincial Rural Development Administration, Chinju, 660-370, Korea
[1]College of of Agriculture, Gyeongsang National University, Chinju, 660-701, Korea

감나무 둥근무늬낙엽병균 *Mycosphaerella nawae*의 자낭포자 및 분생포자에 감염된 이병엽 상에서 위자낭각 형성과정 관찰

권진혁* · 강수웅 · 박창석[1] · 김희규[1]
경상남도농촌진흥원, [1]경상대학교 농과대학

ABSTRACT: In order to illustrate the role of conidia of *Mycosphaerella nawae* as a secondary inoculum in nature, pseudothecial development on persimmon leaves was investigated microscopically. The fungal ascospores have been believed as the primary or only inoculum source in nature, however, pseudothecia were readily formed on persimmon leaves infected naturally and artificially by conidia. The pseudothecia of *M. nawae* were found to form in the tissues of infected leaves while the leaves were still hanging on the trees. The size of pseudothecia were approximately 51.0~122.4×51.0~112.2 μm (82.8×72.5 μm in average), the shapes were spherical, ovoid or occidental pear type. The sizes of asci were approximately 30.6~61.2×8.2~10.2 μm (46.6×9.4 μm in average) and the shapes were cylinder or banana. The ascospores were mostly spindle type, and the sizes were 10.2~12.2×3.1~4.1 μm (11.4×3.2 μm in average)-like. The pseudothecial formation was initiated before defoliation and morphological characteristics of the pseudothecia, ascus and ascospores developed on the leaves were similar. Sequential development of the pseudothecia, ascus and ascospores on the infected leaves were fully illustrated in this study. Results indicated that conidia of *M. nawae* induce circular leaf spot of persimmon as much as ascospores, and might play an important role of the disease epidemics in nature.

***Key words*: ascospores, ascus, conidia, *Mycosphaerella nawae*, pseudothecia, secondary inoculum.**

Mycosphaerella nawae Hiura et Ikata belongs to the class Loculoascomycetes, producing the characteristic uniloculate pseudothecia, asci and ascospores. The only host is *Diospyros kaki* Thunb. var. *domestica* Makino, in which only the leaves are infected. According to Ikata and Hitomi (1), Kitajima (3), Takuda and Hirosawa (11, 12), this pathogen produces a small black fruiting bodies, in the back side of leaf spot from October to November, and then, become sclerotium-like before overwinter, which increases in the middle or late April of the following year. However, they have not histologically confirmed the initiation of pseudothecia development prior to defoliation before overwintering.

Epidemiology of this disease is little known even though some reported in Japan (1, 3, 11, 12). Since 1993, we have studied the monitoring of ascospore release as a primary inoculum (2, 6, 9, 10), environmental factors (5, 7) associated with ascospore release, pseudothecial development and the epidemiology of this disease, and also on fungicidal control in Korea (10). For the first time, Kwon (6, 8) investigated the imperfect stage of this pathogen in detail, and provided evidence that conidia are as infective as ascospores producing the typical circular leaf spot symptoms which is identifical to those by ascospore infection. Based on the stereoscopic observation of infected leaves, and on the dynamics of inoculum and macroscopic symptomatology, we further investigated to confirm the sequencial development of pseudothecia as a function of time. In order for the imperfect stage being involved in epidemiology to be significant, it is necessary to reveal the process of pseudothecia initiation. development to full maturity which leads to ascospore

*Corresponding author.

production in the following year.

Therefore, we report here the sequencial development of pseudothecia in infected leaf tissue before defoliation and before overwintering by ascospore and also by conidia produced *in vitro* via artificial inoculation. Also, the process of pseudothecia development in the infected foliages was confirmed by microscopic examination of the periodic samples.

MATERIALS AND METHODS

Monitoring of pseudothecia by natural infection. Ten leaf samples from severely infected orchard at Chinju in 1995 was examined at intervals of 10 days in detail and described in specimen preparation for microscopy, for pseudothecia development in mid April to the early August, 1996. Maturation rate was determined by counting thirty ascospores (Fig. 1).

Monitoring of pseudothecia by artificial inoculation of conidia. Conidia as inoculum were produced after incubation on PDA culture at 25°C for 90 days. Two year old persimmon trees grown in trees pots was sprayed with conidial suspension at the concentration of 2.23×10^5 spores/ml and move to humidity chamber for 24 hr and placed in greenhouse untill the typical symptom appears (Fig. 2). Leaf samples were collected immediately after defoliation and left in outdoor during the winter. At late April, pseudothecial development was inspected at intervals of 10 days. Maturation rate of pseudothecia was done as above.

Inspection of pseudothecia in the infected leaf samples before defoliation during September. Typical symptomatic leaf samples from either ascospore infection or *in vivo* conidial inoculation at June, 1996 were collected before defoliation at 20th of September, 1996, and were subjected to microscopic examination for the pseudothecial initiation and their developmental process in the infected leaf tissue (Fig. 3).

Specimen preparation for microscopy. Those samples, collected periodically from September 1995 before defoliation to January 1996 after overwintering, were observed by light microscopy (Nikon, Japan) at 600× magnification. The infected leaf samples or specimens 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 2 hr at 4°C room temperature. After rinsing with 0.1 M phosphate buffer (pH 7.4) for 3~6 times, specimens were postfixed with 2% OsO_4 (osmium tetroxide, Sigma, U.S.A) in soak-

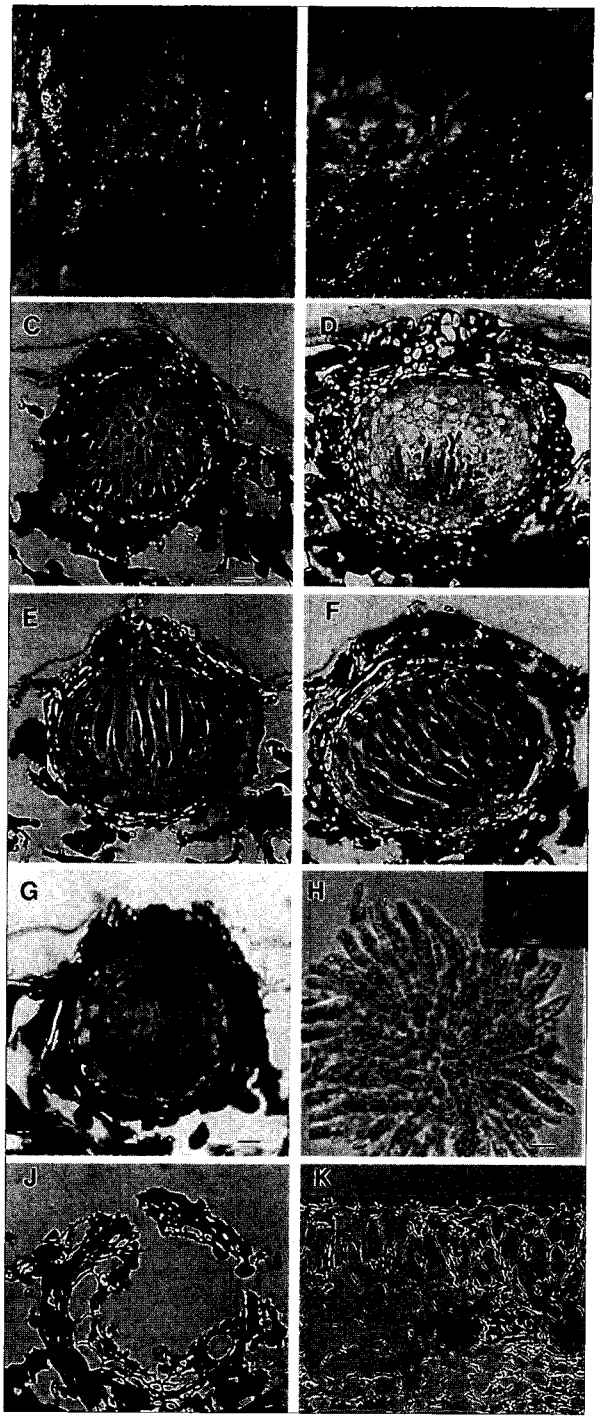

Fig. 1. Sequencial development of pseudothecia and asci of *Mycosphaerella nawae* in a naturally infected leaf tissue. Macroscopic view of pseudothecia (A) on the upper surface of lesion and pseudothecia (B) on the lower surface of lesion formed on the overwintered leaf by dissecting microscopy; Light microscopy of immature pseudothecium (C) in mid April. Ascus (D) developed in hymenial layer; E, F, G. Series of maturation of pseudothecia from late April through May; H, Mature asci; I, Ascospore; Vaccant pseudothecium (J) from late July to early August; K, Healthy tissue; Bars indicate 10 μm.

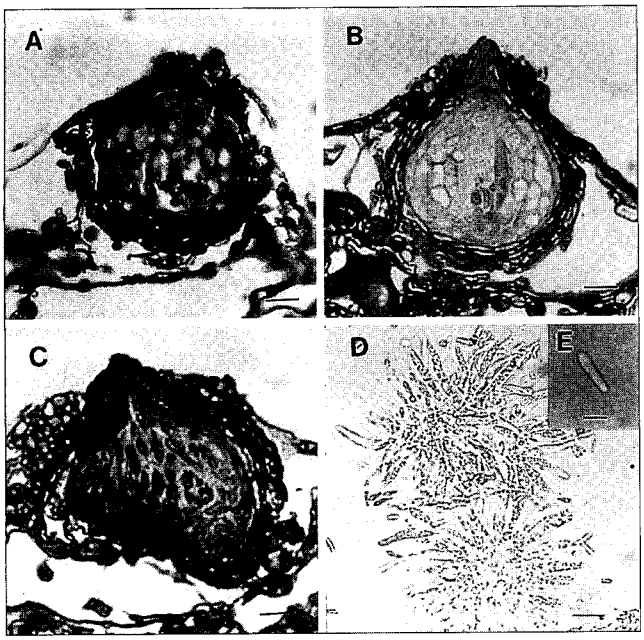

Fig. 2. Sequential development of pseudothecium of *Mycosphaerella nawae* in the infected leaf tissue after overwintering, induced by artificial spray inoculation of conidia obtained from PDA media for 90 days incubation. A, Immature pseudothecium; B, C, Mature pseudothecium; D, Asci; E, Ascospore; Bars indicate 10 μm.

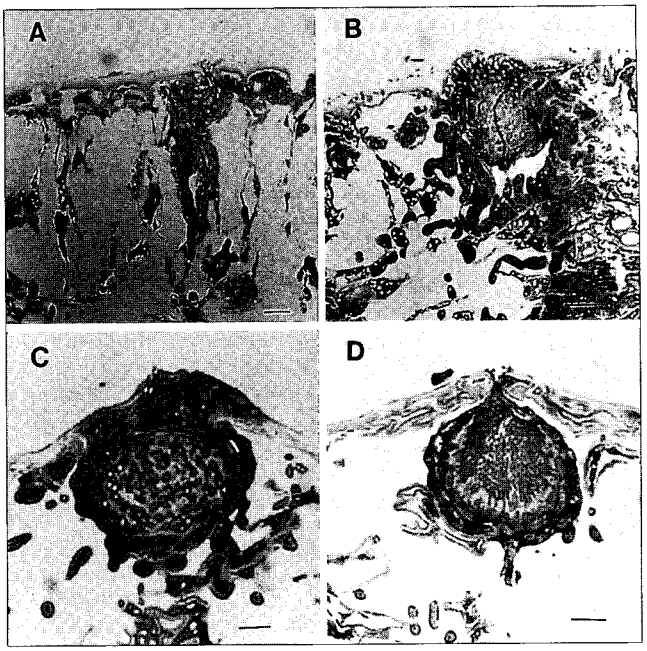

Fig. 3. Sequencial development of pseudothecium of *Mycosphaerella nawae* during September in the naturally infected leaf tissue induced by either ascospore or conidia before defoliation. A, Initiation of pseudothecium; B, Distruption of parenchyma cells and pseudothecium initiation; C, Immature pseudothecium in the leaf tissue infected by ascospore; D, Premature pseudothecium in the leaf tissue infected by in vivo conidia; Bars indicate 10 μm.

ing 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 60, 80, 90 and 100% for 20 min room temperature, replaced propylen oxide 2 times for 10 min, followed by propylen oxide-epon (1 : 1) mixture for 2~3 hr. The samples were made using a Ultramicrotome (REICHERT-JUNG). After polymerization for 24 hr at 60°C oven, the formated tissue was semithin sectioned to 0.35 μm, stained with toluidine blue and after ultrathin section 70 nm, double stained with uranyl acetate for 20 min, lead catrated for 10 min, fixed slide glass, observed by light microscopy. This method made it possible to clearly observe development of pseudothecia.

RESULTS

Developmental process of pseudothecium by natural infection. Defoliated infected leaf tissue was observed for the fruiting body formation as shown in Fig. 1.

Fruiting bodies of *M. nawae* were formed mostly on the lower side of infected leaf lesion and recognized as small black round granules (pseudothecia) erumpent in subcuticular layer of the lesion (Fig. 1B) and rarely on the upper surface of lesion (Fig. 1A) before overwinter.

In spring, erumpent pseudothecia become semi-erumpent as they mature after overwintering (Fig. 1C) as of April. Solitary pseudothecia were scatterd in lesion and their shape varies from globose, ovoid, flask and pear shaped. Ascostroma was blakish brown with 2~3 pseudoparenchyma layers. The size was 51.0~122.4 μm high with the average of 82.8 μm and 51.0~112.2 μm wide with the average of 72.5 μm (Fig. 1E, F, G). Asci were differentiated in the bottom of pseudothecium as of early May (Fig. 1D). Thereafter, pseudothecium proceeds to full maturity. The morphology of asci was cylinder or banana shaped. Ascus was colorless and its end was rounded a little with granula body in the beginning and later disappeared as the ascospore developed. There were 8 ascospores in each ascus. The size was 30.6~61.2×8.2~10.2 μm with average of 46.6×9.4 μm (Fig. 1H). The ascospore was hyaline, spindle shaped and two celled with unequally septated with constrictions. Both ends were tapering. The size was 10.2~12.2×3.1~4.1 μm with average of 11.4×3.2 μm (Fig. 1I). Empty pseu-

dothecium (Fig. 1J) was observed in late July to early August after releasing ascospores.

Developmental process of pseudothecia by artificial inoculation of conidia. As shown in Fig. 2, pseudothecia on the leaves by spraying conidial suspension were observed the mature form from late April (Fig. 2A), and also, observed typical hymenial layer of asci (Fig. 2B, C, D) and ascospore released (Fig. 2E). Morphology of pseudothecia, ascus and ascospore was identical to those produced by natural ascospore infection.

Pseudothecia formation in infected leaves before defoliation. As shown in Fig. 3, we were able to confirm the pseudothecial initials being developed (Fig. 3A), and proceeds to prematured pseudothecia without ascus development (Fig. 3B, C, D) from the leaf lesions before defoliation on 20th of September. Immature pseudothecium on the leaf tissue infected by ascospore (Fig. 3C), and premature pseudothecium on the leaf tissue infected by exposing to *in vivo* conidia (Fig. 3D).

DISCUSSION

So far, population dynamics of primary inoculum of *M. nawae* was substantially influenced by the environmental conditions of each year in Korea (2, 4-10). Takuda and Hirosawa (11, 12) reported that this fungus overwintered as mycelial state and perithecium initiates in following spring from mid to late March, increased abruptly in April, and reached to full maturity in May producing ascus and ascospores. However, our result indicated that this pathogen overwintered as prematured pseudothecia, and in the following spring, the pseudothecia were matured in mid to late April to early May (Fig. 1 and Fig. 2) in Korea. Such a differences in timing for initiation of pseudothecia development might be due to the difference of climatic conditions between two countries.

Morphological characters of pseudothecia, ascus and ascospore were similar to those reported by Ikata and Hitomi (1) and Kitajima (3). Earlier reports in Japan (1, 3, 11, 12) emphasized that ascospores are primary inoculum from infected leaves and no conidia are produced *in vivo*. Ikata and Hitomi (1) had observed the conidia *in vitro* only once, and the conidia have not been confirmed thereafter in Japan.

We (6) have previously reported that *Ramularia*-like conidia of this pathogen were as infective as ascospore, resulting in identical symptom being developed upon inoculation of either inoculum. When the young persimmon trees were inoculated with conidial suspension in early April, and were maintained in greenhouse, the identical macroscopic symptom developed in late May (6). We also exposed young trees to *in vivo* conidial inoculum at mid August, when no ascospore are available as inoculum in the persimmon orchard environment in Korea, and confirmed that the symptom developed in late September.

In this paper, we provided substantial evidence by illustrating the developmental process of pseudothecia in the inoculated leaf tissue induced by conidia as a sole source of inoculum. Therefore, based on our previous result (6) and further evidence provided in this paper, we would conclude that conidia serve as a secondary inoculum in the epidemiology of circular leaf spot of persimmon.

요 약

감나무 둥근무늬낙엽병균(*Mycosphaerella nawae*)의 자낭포자 및 분생포자에 의해 감염된 이병엽에 위자낭각형성 과정을 해부학적으로 관찰하였다. 관찰은 야외에서 자연발병한 이병엽과 PDA 배지상에서 형성된 분생포자를 인공접종하여 발병한 이병엽 그리고 자연상태에서 분생포자에 의해 2차 감염되어 발병한 이병엽을 조사하였다. 이병엽 월동후, 병반상에서 형성한 위자낭각의 크기는 51.0~122.4×51.0~112.2 μm(평균 82.8×72.5 μm)이었으며 모양은 구형, 난형 및 서양배형 등이고, 자낭의 크기는 30.6~61.2×8.2~10.2 μm(평균 46.6×9.4 μm)이었으며 모양은 원통형 및 바나나형 등이었다. 자낭포자의 크기는 10.2~12.2×3.1~4.1 μm(평균 11.4×3.2 μm)이었고 모양은 방추형이었다. 위자낭각, 자낭, 자낭포자의 형태, 크기 등을 조사한 결과 자연발병과 인공접종에 의해 발병한 이병엽간에 차이가 없었다. 또, 자연발병 및 인공접종에 의한 발병에 의해서도 병반부의 조직중에 낙엽전 및 월동전에 이미 위자낭각이 형성되었다.

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(Received September 14, 1998)