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Identification, Pathogenicity and Host Range of a Potential Bioherbicide, *Epicoccosorus nematosporus*, Causing Fingerprint Stem Blight on Water Chestnut, *Eleokaris kuroguwai*

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생물제초제로서의 올방개 지문무늬병균(*Epicoccosorus nematosporus*)의 동정, 병원성 및 기주범위

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ABSTRACT : *Epicoccosorus nematosporus* isolated from the infected tissues of water chestnut (*Eleokaris kuroguwai* Ohwi), a popular weed species in rice paddy fields, was firstly described in Korea, and the disease was named as fingerprint stem blight of water chestnut. Typical symptoms having a unique pattern of fingerprint-like lesions were formed on the stems, and the lesions rapidly enlarged until the stems were girdled, resulting in complete blight of the top plant parts. Conidia were hyaline, acute at each end, aseptate, and 44.5~72 μm in length. The fungus grew well at 28°C and produced spores most at 30~31°C. When water chestnut plants were inoculated with spore suspension (3.2×10^5 conidia/ml) of *E. nematosporus* isolates, all 17 isolates infected the plants and killed shoots at rates of 78.0~93.3% in 20 days in greenhouse conditions. The inoculated plants produced fewer (about one seventh) and lighter tubers than uninoculated healthy plants. The fungus was highly and specifically parasitic to water chestnut, but not to 31 common crops including rice and other 15 weed species. Therefore, we conclude that *E. nematosporus* may have a potential as a biological control agent of water chestnut in Korea.

***Key words* : water chestnut, *Eleokaris kuroguwai*, *Epicoccosorus nematosporus*, rice weed, pathogenicity, host range, fingerprint stem blight disease.**

Water chestnut, *Eleokaris kuroguwai* Ohwi, is distributed widely and has been a severe weed problem in rice production area in Korea (3, 8~11). It is a perennial sedge that propagates mainly by terminal tuber of rhizome (2, 7, 8, 10). Formation of the terminal tubers in soil and their irregular sprouting make it difficult to control. At present there is no way to control water chestnut effectively except hand weeding.

In the summer of 1990, naturally killed water chestnuts were observed at a rice field in Sangju, Korea (5). Infected water chestnut plants were blighted and killed during the growing season. Symptoms of the disease including a unique pattern of fingerprint-like lesions girdled the stems and increased in severity during the growing season. Thus, we named the disease as the water chestnut fingerprint stem blight disease (5, 6).

In the studies on biological control of weed with pathogens, there have been some notable examples; most weed species are hosts for many endemic pathogens (1, 3, 4, 17, 18, 19). Daniel *et al.* (4) suggested that these pathogens which can be as potential biocontrol agents must 1) be able to produce abundant and durable inoculum in artificial culture, 2) be genetically stable and specific for the target weed, and 3) be able to infect and kill the weed in environments of reasonably wide latitude. We have isolated 214 isolates of

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Epicoccosorus nematosporus from water chestnut from 22 regions in Korea during 1990 through 1992. The isolates varied somewhat in their spore size, colony color, and spore production. To use an organism as a biological control agent, selection of the promising isolate is most important (4, 15, 16, 20); it should be highly pathogenic to target organism(s) but not pathogenic to other crop plants. Promising biological control agents of pests performed best in areas where the target organisms were suppressed naturally (3).

The purpose of this research was to investigate the pathogenicity of *E. nematosporus* on water chestnut and to obtain information on the host range of the fungus.

MATERIALS AND METHODS

Collection of diseased plants. Infected water chestnut plants with typical symptoms (fingerprint-like lesions) were collected from rice fields of 22 counties in Korea during the summer of 1990 through 1992.

Isolation of pathogens. Diseased tissues were cut from the stems 2~3 mm in size and rinsed in 10% sodium hypochlorite for 1 min, in 70% ethyl alcohol for 1 min, rinsed quickly with sterile water, and placed on sterile filter paper to dry. The sections transferred to solid water agar media containing 200 μg/ml chloramphenicol, 88 μg/ml ampicillin and 70 μg/ml streptomycin. Hyphal tips were transferred aseptically to acidified potato dextrose agar (PDA, pH 5.5) and cultured for 5 days at 28°C in an incubator. The mycelium of each fungal isolate was preserved in a −70°C deep freezer in a Cryovial containing 1.5 ml of 40% glycerol : 10% skim milk=1 : 1 for future use.

Conidia production. To sporulate, aerial mycelia on 15-day-old culture media were removed with a spatula, and the culture media were then incubated for another 2 days at 28°C under 48 hr photoperiod. The illumination was supplied by three 20 W fluorescent lamps located 30 cm above the culture plates. After 2 days, conidia produced on the surface of plate were collected by washing them from the agar with 20 ml of distilled water and the conidial concentrations were determined with a hemacytometer under a light microscope.

Pathogenicity test. Water chestnut tubers were collected from a rice field in Sangju, Korea in October after harvesting of rice. Tubers were rinsed with tap water, dried for one day under shade and stored in air tight envelopes at 5°C in a refrigerator. For sprouting, the tubers were soaked in water, and incubated at 28°C for 7~10 days under the dark condition. The tubers were rinsed with tap water everyday to prevent bacterial contamination. Five germinated tubers were planted in an 1/5000-a Wagner pot filled with rice field soil After 20~30 days, seedlings grown up to 15 to 30 cm tall were thinned, remaining 100 stems per pot. Spore suspension of *E. nematosporus* (3.2×10^5 conidia/ml containing 0.5% dextrose in distilled water) was sprayed on the stems until runoff. The inoculated plants were moved to a greenhouse bench and covered with polyethylene film to simulate a dew chamber fitting to 90~95% relative humidity (RH) with an auto-humidifier and 28±3°C with an auto-thermometer. The polyethylene film was removed after 24 hr, and then the pots were placed on the greenhouse bench at 25~32°C. The progress of symptom development was observed and recorded for 60 days. In addition, the number of infected shoots, dead shoots, and regrown shoots, and the number and weight of underground tubers formed were recorded. The data were processed by SAS GLM procedure (IRRI) and analyzed by Duncan's multiple range test.

Host range test. To study the host range of the fungus, 31 common crops and 15 weed species (Table 3, 4) were inoculated with spores of *E. nematosporus* as described in the pathogenicity test. Plants used were grown in plastic pots (21×17 cm) containing fertilized upland soil ($N\text{-}P_2O_5\text{-}K_2O$=15-15-15 g/m^2). Ten seedlings were planted in each pot and grown to 3~5 leaf stages. Development of the symptoms on plants was observed 15 days after spray with conidial suspension of *E. nematosporus* isolate YCSJ-112. Uninoculated plants (sprayed with water) were used as controls.

RESULTS

Characters of the pathogen. A total of 214 isolates of *E. nematosporus* were obtained from 22 locations in Korea. The fungus was widely distributed and could be isolated from every location surveyed. All isolates appeared similar in their hyphal and conidial morphology. The fungus isolated from water chestnut in Korea was fitted to the previous description of *E. nematosporus* by Suzuki (14). The morphology of isolate YCSJ-112 from Sangju, Korea and isolate Nip-4 from Japan was almost identical (Table 1). Conidiomata appeared as scattered small black flecks, sized approximately 38~113×22~84 μm. Conidia were form-

Table 1. Comparison of morphological and cultural characters between Korean (YCSJ-112) and Japanese (Nip-4) isolates of *Epicoccosorus nematosporus* pathogenic to water chestnut

Character[a]	YCSJ-112	Nip-4
Conidia		
Length	45~72 μm	46~81.7 μm
Widths	2.5~4.8 μm	3.9~5.5 μm
Color	Hyaline	Hyaline
Septum	Aseptate	Aseptate
Nuclear	1	1
Shape	Lunate at each end like a nematode	Lunate at each end like a nematode
Conidiophore		
Size	35~67 μm	42.5~79.7 μm
Septum	1~2 septate	1~2 septate
Shape	Short palisade cylindrical, ampulliform to dolliform	Short palisade cylindrical, ampulliform to dolliform
Appressoria		
Size (ø)	25~35 μm	not reported
Color	Chocolate brown	not reported
Shape	Clavate, circular irregular	not reported
Mecelia		
Width	8~10 μm	8~10 μm
Color	Hyaline	Hyaline
Septum	Septate	Septate
Mat color on PDA	Blackish gray	Blackish gray

[a] The morphological and cultural characters of the fungus were observed after artificial inoculation with both isolates (YCSJ-112 & Nip-4). Conidial suspension (3.6×10^5 conidia/ml) collected from PDA medium was sprayed onto 20-day-old seedlings and inoculated plants were incubated for 48 hr in a dew chamber (RH 90~95) at 25~30°C and then placed on a greenhouse bed. Appressoria were observed under the microscope 24 hr after inoculation.

ed on short palisade conidiophores which were formed on conidiogenous cells, single-nucleate, hyaline, 1-celled with lunate ends like a nematode, and range in size from 44.5~72×2.8~4.8 μm (Fig. 1-A). Conidial masses appeared light pink and sporulated abundantly under fluorescent light for 24 hr after removing aerial mycelia. Size of the conidia formed on PDA was a little smaller than that formed on natural lesions (36~65× 2.8~4.6 μm) (Fig. 1-B). Conidiophores were infrequently formed, 1~2 septate, cylindrical, unbranched, palisade, and formed from the upper cells of the conidiomata. Conidiogenous cells were enteroblastic, integrated, dark brown or black, and ampulliform to dolliform. Appressoria were formed abundantly, colored chocolate brown, shaped clavate or circular with the edge usually complete and sometimes irregular, and often becoming complex and forming long closely branched chains (Fig. 1-C). On PDA colonies appeared greyish black to black with dense mycelium. Mycelium was septate and composed of hyaline to pale brown, and small dense felty colonies occurred sometimes (Fig. 1-D).

Disease symptoms. Symptoms first appeared on stems of water chestnut 4 to 5 days after inoculation in greenhouse conditions (Fig. 1-G, H, I, J) Dark brown lesions, initially pinpoint to 0.1~0.3 mm in diameter, were enlarged with time to become elliptical water-soaked and 3 to 5 mm in diameter and fused with each other within the next 6 to 7 days. The lesions became reddish brown, and thereon the concentric dense growths of tiny black clusters were formed like a pattern of fingerprint. The lesions which rapidly elongated and expanded around the stem, and frequently girdled the stem completely within the next 10 to 15 days. The girdled stems of water chestnut ultimately died back from the upper part of the lesions (Fig. 1-F).

Pathogenicity. All 17 isolates of *E. nematosporus* developed high incidences of the fingerprint blight disease on water chestnut seedlings, of which the symptoms were similar to those observed in field conditions, and thus were confirmed their pathogenicity on water chestnut (Table 2). The infected shoots were ultimately

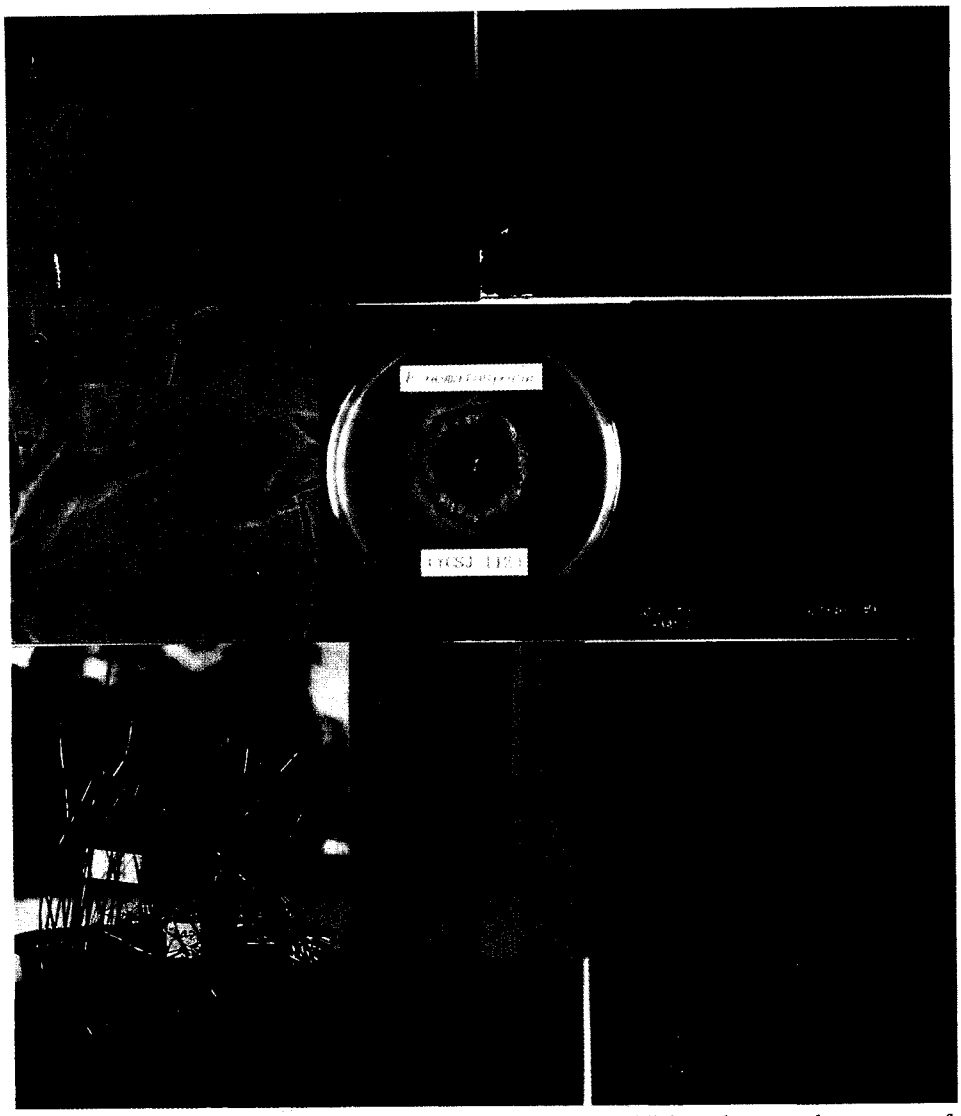

Fig. 1. Pathgenicity and morphological characters of the fingerprint stem blight pathogen and symptoms of the disease. A, Conidia naturally occurring on the stem; B, Conidia on PDA medium; C, Appressoria; D, Mycelial mat formed on PDA medium; E, Underground tuber formation after spraying conidial suspension of isolate YCSJ-112; left, treated with conidia; right, untreated control; F, Artificially inoculated with conidial suspension of *E. nematosporus* isolate YCSJ-112 under greenhouse condition; G, Symptoms of initial pinpoint within 4~5 days after inoculation; H, Water soaked lesion girdling the stem within 10 days; I, Early symptom of fingerprint within 10~15 days; J, Infected plants showing symptoms of the fingerprint stem blight disease (bars=20 μm).

killed in 10 to 20 days. The infection rate (weeding efficiency) of the fungal isolates varied from 78.0% to 99.3%, among which YCSJ-112 was the most virulent isolate. Three isolates, YCPE-9, YCCA-6 and YCSJ-112, were also very virulent isolates, showing weeding efficiencies of 90.4%, 97.5% and 99.3%, respectively. All 17 isolates suppressed the formation of underground tubers, and the number of tubers was 4.3~13.0/pot, while it was 83.7/pot in the uninoculated control (Table 2), indicating that averagely the inoculated plants produced one seventh tubers compared to the uninoculated healthy plants. Fewer tubers were formed as the infection rate increased, and the infection rate and the underground tuber formation were negatively cor-

Table 2. Pathogenicity (weeding efficiency) of *Epicoccosorus nematosporus* isolates on water chestnut and their inhibition of tuber formation[a]

Isolate	Sampling site	Weeding efficiency (%)[b]	Tuber formation (No./pot)[c]
YCPC-2	Pocheon	83.0 ab	7.2 a[e]
YCYC1-5	Echeon	83.8 ab	10.6 a
YCYP-1	Yangpyeong	81.2 a	9.0 a
YCNY-3	Namyangju	78.0 a	12.7 a
YCPE-9	Poeun	97.5 bc	5.8 a
YCCA-6	Cheonan	90.4 abc	6.8 a
YCKJJ-10	Kongju	86.2 abc	11.9 a
YCJSJ-6	Jangsoo	83.7 ab	5.8 a
YCKJ-7	Kimjae	89.6 abc	8.6 a
YCJSS-16	Changseong	87.0 abc	8.2 a
YCYA-1	Youngam	81.4 a	7.3 a
YCSJ-112	Sangju	99.3 c	4.3 a
YCYD-5	Youngdeok	87.8 abc	9.5 a
YCMY-135	Milyang	85.0 abc	6.3 a
YCCY-10	Chinyang	83.6 ab	7.4 a
YCCN-12	Changnyeon	81.5 a	13.0 a
YCCW-10	Cheolwon	84.7 abc	10.7 a
Control[d]		0	83.7 b

[a] Water chestnut shoots (25 days old) were inoculated with 20 ml per pot of conidial suspension containing 3.2×10^5 conidia/ml and 0.5% dextrose in water. Plants were thinned to 100 stems per pot before applying the spore suspension.

[b] Weeding efficiency (relative number of dead plants) was examined 30 days after inoculation.

[c] The underground tuber formation was examined 2 months after inoculation.

[d] Untreated check sprayed with water.

[e] Data are means of 3 replications; each replicate is the average of 5 values. Means followed by the same letters in each column are not significantly different (p= 0.05) by Duncan's multiple range test.

related (r= − 0.973). Also underground tubers became lighter by the fungal infection (Fig. 1-E). There were no differences among isolates in the number and weight of underground tuber formation.

Host range. All tested crop plants and weeds except water chestnut appeared to be non-hosts of *E. nematosporus*, as no diseases occurred in the plants. There were no differences in overall plant size, leaf number and plant vigor between the inoculated test plants and the corresponding control plants in 15 days (Table 3, 4). In contrast, water chestnut became heavily infected; evidence of infection was apparent within 6

Table 3. Response of annual crops to the inoculation of *Epicoccosorus nematosporus* (YCSJ-112) isolated from water chestnut (*E. kuroguwai*) in greenhouse conditions[a]

Test plant	Symptoms produced[b]	Plant growth[c]
Rice (*Oryzae sativa*)	−	+
Barley (*Hordeum vulgare*)	−	+
Millet (*Setaria italica*)	−	+
Millet (*Panicum miliaceum*)	−	+
Indian-millet (*Sorghum bicolor*)	−	+
Buckwheat (*Fagopyrum esculentum*)	−	+
Soybean (*Glycine max*)	−	+
Peanut (*Arachis hypogaea*)	−	+
Kidney bean (*Phaseolus vulgaris*)	−	+
Sesame (*Sesamum indicum*)	−	+
Perilla (*Perilla frutescens* var. *japonica*)	−	+
Sweet potato (*Ipomea batatas*)	−	+
Red bean (*Phaseolus angularis*)	−	+
Green pea (*Phaseolus radiatus*)	−	+
Tobacco (*Nicotiana tabacum*)	−	+
Rape seed (*Brassica campestris* subsp. *napus*)	−	+
Chinese radish (*Raphanus sativus*)	−	+
Chinese cabbage (*Brassica campestris* var. *capitata*)	−	+
Onion (*Allium cepa*)	−	+
Welsh onion (*Allium fistulosum*)	−	+
Crown daisy (*Chrysanthemum coronarium* var. *spatiosum*)	−	+
Garlic (*Allium sativum*)	−	+
Celery (*Apium graveolens*)	−	+
Carrot (*Daucus carota*)	−	+
Cucumber (*Cucumis sativus*)	−	+
Pumpkin (*Cucurbita moschata*)	−	+
Melon (*Cucumis melo*)	−	+
Watermelon (*Citrullus vulgaris*)	−	+
Egg-plant (*Solanum melogena*)	−	+
Tomato (*Lycopersicon esculentum*)	−	+
Red pepper (*Capsicum annuum*)	−	+
Water chestnut (*Eleokaris kuroguwai*)	+	−

[a] Host plants at the cotyledon to 3~5 leaf stages of growth (15 to 20 days old) were sprayed with conidial suspension (3.6×10^5 conidia/ml) containing 0.5% dextrose until run-off. The inoculated plants were incubated for 24 hr in a dew chamber (RH 90~95%) at 25~28°C and then placed on a greenhouse bed. Data were collected 10 days after inoculation.

[b] + : symptoms produced and heavily infected, − : no symptom developed.

[c] + : normal growth, − : abnormal growth caused by *E. nematosporus* infection.

Table 4. Response of annual and perennial weeds to the inoculation of *E. nematosporus* (YCSJ-112) isolated from water chestnut in greenhouse conditions[a]

Test plant	Symptoms produced[b]	Plant growth[c]
Bulrush (*Scirpus hotarui*)	−	+
Autumn rush (*Fimbristylis miliacea*)	−	+
Umbrella-sedge (*Cyperus difformis*)	−	+
Rice flatsedge (*Cyperus iria*)	−	+
Flatsedge (*Cyperus serotinus*)	−	+
Spikerush (*Eleokaris congesta* subsp. *japonica*)	−	+
Green kyllinge (*Kyllinga brevifolius* var. *leiolepis*)	−	+
Pickerelweed (*Monochoria vaginalis*)	−	+
Pickerelweed (*Monochoria korsakowii*)	−	+
Arrowhead (*Sagittaria trifolia*)	−	+
Bog pondweed (*Potamogeton distinctus*)	−	+
Arrowhead (*Sagittaria pygmaea*)	−	+
Water plantain (*Alisma canaliculatum*)	−	+
Indian jointvetch (*Aeschinomene indica*)	−	+
Barnyard grass (*Echinochloa crus-galli*)	−	+
Water chestnut (*Eleokaris kuroguwai*)	+	−

[a] Host plants (15 to 20 days old) were sprayed with conidial suspension (3.6×10^5 conidia/ml) containing 0.5% dextrose until run-off. The inoculated plants were incubated 24 hr in a dew chamber (RH 90~95%) at 25~28°C and then placed on a greenhouse bed. Data were collected 15 days after inoculation.

[b] + : symptoms produced and heavily infected, − : no symptom developed.

[c] + : normal growth, − : abnormal growth caused by *E. nematosporus* infection.

days on stems as greyish brown lesions which were pinpoint to 1~5 mm in diameter, indicating that water chestnut was the only host of the fungus among the tested plants.

DISCUSSION

The genus *Epicoccosorus* has only one species, *E. nematosporus*, which was first reported from Japan in 1991 by Suzuki (14). In Korea, we first report that the casual agent of the fingerprint stem blight on water chestnut in nature was identified as *E. nematosporus*. Furthermore, we first report the significant natural incidence of this disease on water chestnut in a large scale. Suzuki (14) has divided the fungus into three groups according to the size of conidia. Based on our experiments, the Korean isolates could not be differentiated by the morphological characters and collecting sites. Also, Korean and Japanese isolates were similar in many aspects such as hyphal and conidial morphology and cultural characteristics. However, in a pathogenicity test, the Korean isolates were usually more pathogenic to water chestnut than Japanese isolates (unpublished data), and there were no significant differences in pathogenicity among the Korean isolates. When conidial suspensions of 17 fungal isolates were sprayed on water chestnut shoots in greenhouse tests, all of the isolates were able to kill the seedlings within 10~15 days, suggesting that Korean *E. nematosporus* isolates may be endemic pathogens attacking water chestnut in natural conditions. The highly frequent occurrence of *E. nematosporus* in various geographical areas in Korea suggests that the fungus may be distributed nationwide and probably adapted well in rice fields in Korea.

Compared to the conidia formed on natural lesions of water chestnut stem, those formed on PDA and treated with fluorescent light appeared smaller with less lunate ends, and obtuse (Fig. 1-A, B). Environmental factors including nutritional conditions can affect the morphology of spores (12, 13). Artificial culture conditions may have influenced the size and shape of conidia in our study. However, when the spore suspension of artificially cultured *E. nematosporus* was inoculated on water chestnut shoots, they were readily infected, showing symptoms as observed in fields. This suggests that the pathogenicity of the fungus may not vary depending on environmental factors, especially in rice paddy fields where moisture content is high. Symptom development was rapid, initiating in four days and caus-

ed death of the plants within 10~15 days after adjacent lesions were coalesced to girdle the stem. Consequently, the growth of the underground tubers was significantly affected by the infection, which may inhibit propagation of the weed. Considering these aspects, this fungus may be a potential biocontrol agent of water chestnut that can be used in Korea.

All tested crop plants except for water chestnut were immune to infection by *E. nematosporus* when inoculated with the conidial suspension. Only water chestnut became heavily infected by the pathogen. This result coincides with the study of Suzuki (14) who demonstrated that no symptom was developed in all of 111 plant species including weeds, but that some plants in genera *Eleokaris* and *Scirpus* produced weak disease symptoms. The result obtained in his and our studies indicates that *E. nematosporus* seemed specifically pathogenic to water chestnut.

Although additional studies must be conducted to give full confidence to *E. nematosporus* as a biological control agent of water chestnut, our study provides some definite advantages for *E. nematosporus* as a biological control agent based on the following aspects. *E. nematosporus* occurring naturally in rice fields in Korea 1) is highly pathogenic and specific to water chestnut, 2) affects underground tubers as well as stems, and 3) easily produces spores in mass.

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요 약

한국의 수도 재배 지대에서 문제가 되는 다년생 논잡초 올방개에 자연상태에서 기주특이적으로 기생하여 올방개 줄기를 고사시키는 병원균인 *Epicoccosorus nematosporus*를 우리나라에서 처음으로 분리, 동정하였으며, 이 병을 올방개 지문무늬병(fingerprint stem blight disease of water chestnut)으로 명명하였다. 분생포자는 분생자층 위에서 형성이 되며 무격막, 단핵이며, 44.5~72 μm 크기의 양끝이 뾰족한 선충 모양이다. 갈색의 병반상에서 흑색의 분생자층은 발달하여 특이한 지문무늬를 형성하는 것이 특징이다. 이 병균은 전국의 올방개가 자라는 논에서 분포하며 매년 발생한다. 온실 접종시 초기의 병징은 접종후 4~5일만에 바늘끝 모양의 회흑색의 반점으로 나타나며 시간이 경과하면서 병반은 급속도로 진전하여 접종후 10~15일 정도 후 약 10 mm 내외의 갈색병반이 형성이 되어 올방개 줄기를 감싸고 그 줄기 전체는 고사하게 된다. 분생포자 현탁액(3.2×10^5 conidia/ml)을 온실 조건하에서 분무접종 하였을 때 20일 이내에 약 78.0~93.3% 정도의 고사율을 나타내었다. 올방개 지문무늬병균의 분생포자 현탁액(3.2×10^5 conidia/ml)을 온실 조건하에서 벼 등 31개 작물과 피 등 15개 잡초의 유묘에 접종하였을 때 올방개를 제외한 어떤 식물에도 병원성이 없었다.

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