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Factors Affecting Sporulation, Germination, and Appressoria Formation of *Epicoccosorus nematosporus* as a Mycoherbicide Under Controlled Environments

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To develop *Epicoccosorus nematosporus* as a mycoherbicide of *Eleocharis kuroguwai*, the optimum temperature and humidity for sporulation of the pathogen were studied. Conidial production was most abundant at 28°C with RH 60%, which yielded 661 mg in 9 cm Petri dish. Light intensity of 3,000 up to 7,500 lux was effective in stimulating conidial production of *E. nematosporus* on oatmeal agar. Light intensity affected sporulation more significantly than temperature. In the pot test, at least 12 h of dew period at 20°C and 25°C was required to achieve satisfactory conidial germination and appressorial formation. Few were killed at 8 h of dew period regardless of temperature. Sixteen hours of a single dew treatment immediately after inoculation killed more plants than did two or three repetitive dew treatments of 8-12 h.

Keywords **:** dew period, *Eleocharis kuroguwai, Epicoccosorus nematosporus*, relative humidity, temperature.

Water chestnut (*Eleocharis kuroguwai* Ohwi) is one of the major rice weed species in Korea, affecting 19% of paddy fields (Kim and Kwon, 1985; Kim et al., 1977), as well as in Japan (Suzuki, 1991). It is a perennial sedge that propagates mainly by terminal tuber or rhizome, and grows and flowers in rice paddies from August to October (Kim and Kwon, 1985; Kim et al., 1977). Formation of abundant (average of 146 tubers/m^2) underground terminal tubers and their irregular sprouting habits make it difficult to control this weed even with the use of herbicides. In a survey of 22 rice fields in Korea, dead water chestnut plants were observed. The causal fungus was isolated from the lesions, and was identified and reported (Hong et al., 1996). This disease, which was named as fingerprint stem blight disease (FSBD) of water chestnut by the first author, has never been reported before and was detected due to its peculiar symptom.

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The importance of environmental conditions for mycoherbicides to incite infections (primary infection) has been recognized since the early stages of mycoherbicide research (Tebeest et al., 1978; TeBeest and Templeton, 1985). The effects of dew and temperature on the number of primary infections have been reported in many mycoherbicide field tests (Elwakil et al., 1990; Kirkpatrick et al., 1982; Makowski et al., 1990; TeeBeest and Templeton, 1985; Walker, 1981). Under unfavorable environmental conditions, the number of initial infections by mycoherbicides is low, the effectiveness of which is based on the dispersal and secondary infections of these pathogens. Effectiveness has a functional relationship with the increase in pathogen population. This study was carried out to determine the interrelation among the host, environment, and pathogen, and how these affect sporulation, conidial germination, and appressoria formation.

Materials and Methods

Sporulation from aged mycelia. To evaluate the age of mycelial mat influencing sporulation, cultures were fully grown at 10 days on 9 cm Petri dish at 28°C in the dark. Aerial mycelia were removed at 3, 6, 12, and 20 days after the mycelia had fully grown. Cultures were stored under 4,500 lux of fluorescent light for 48 h in the environmental chamber, and temperature and relative humidity were maintained at 28±1°C and 60±10%, respectively. Each trial was replicated five times.

Effect of temperature and relative humidity on sporulation. The fungus was grown on oatmeal agar for 5 days and was placed under 4,500 lux light intensity in the growth chamber. Treatments were 6×4 factorial design temperatures (15, 20, 25, 28, 30, 33°C) and RH (30, 60, 80, 100%). Fresh conidial mucilage was collected with mineral oil coated spatula, preventing the mucilage from sticking to the spatula, and weighed.

Effects of temperature and light intensity on sporulation. The fungus was grown on oatmeal agar for 5 days and was placed in the growth chamber. Treatments were 3×4 factorial design temperatures (20, 25, 30°C) and light intensity (1,500; 3,000; 4,500; 7,500 lux). Relative humidity was fixed at RH 60±10%. Fresh weight of conidial mucilage was examined as described above.

Effect of temperature and dew period on conidial germination and appressoria formation. The fungus was grown on oatmeal agar for 5 days and was placed in the growth chamber. Treatments were 4×5 factorial design temperatures (15, 20, 25, 30°C) and dew period (4, 8, 12, 16, 20 h). Fresh weight of conidial mucilage was examined as described above.

Preparation of conidial inoculum. *E. nematosporus* isolate YCSJ-112 was used. Aerial mycelia on 15-day-old PDA culture were removed with a spatula, and were kept in the growth chamber at 28°C for 48 h under a 4,500 lux fluorescent lamp. Conidia produced on PDA were collected by washing with 20 ml of distilled water. The conidial concentration in water was adjusted by a hemacytometer prior to use.

Preparation of water chestnut seedlings. Water chestnut tubers were collected from rice paddy fields in October. Tubers 10-15 mm in diameter were rinsed with tap water, dried for 1 day in the shade, and stored at 5°C in the refrigerator in plastic envelopes (Clean Bag, Clean Rap Co. Korea) until use.

For sprouting, the tubers were soaked in tap water, and then incubated at 28°C for 7-10 days under dark conditions. During the incubation, the tubers were rinsed with tap water everyday to prevent bacterial concentration.

Ten uniformly germinated tubers were selected and transplanted in 1/5,000 Wagnor pot filled with clay loam soil. The soil was fertilized ($N\text{-}P_2O_5\text{-}K_2O$ = 15-15-15 g/m^2) before planting. When the seedlings have grown up to 15-30 cm high after 20-30 days, the shoots were thinned equally to 50-100 stems per pot. Conidial suspension at concentration of 10^5 conidia/ml containing 0.5%(w/w) dextrose was sprayed onto stems until runoff with a mist sprayer (Model, DS-121D-6 Dasol Sci. Co. Korea, pressure: 0.1/cm^2). The inoculated plants were kept in dew chambers (Model, DS-53C, Dasol Sci. Co., Korea) with various temperatures and dew periods. After the treatments, pots were moved to a greenhouse bench at 28±3°C. Symptom development was recovered including number of infected, dead, and regrown shoots, and number and weight of underground tubers.

Measuring the conidial germination and appressorial formation. Thirty-day-old water chestnut seedlings were inoculated by spraying conidial suspension of 6.0×10^5 conidia/ml until runoff. After keeping them in dew chamber for 24 h, the shoots were cut into 1 cm long and placed in small vials with 2 ml of distilled water, and were vigorously shaken for 1 minute. Number of conidia, germ tube, and appressorial formation were counted under a light microscope (×100) using a hemacytometer. Ten shoots were counted for each treatment, five times replicated.

Effect of temperature and dew period on conidial germination and appressoria formation. The fungus was grown on oatmeal agar for 5 days and placed in the growth chamber. Treatments were 4×5 factorial design temperatures (15, 20, 25, 30°C) and dew period (4, 8, 12, 16, 20 h).

Results and Discussion

Sporulation from aged mycelia. There were significant differences in sporulation according to age of mycelia. *E. nematosporus* sporulated the most as soon as it reached the Petri dish edge and 12-20 days after. The most effective time for sporulation was after full growth (Table 1), followed by 3 and 6 days after.

Table 1. Effect of culture age on the sporulation of *Epicoccosorus nematosporus*

Age of culture[a] (Days after full growth)	Fresh weight of conidia mucilage (mg/petridish)[b]
0	440.8 a
3	385.5 b
6	227.5 c
12	46.1 d
20	15.0 d

[a] *E. nematosporus* fully grown on oatmeal agar at 28°C at 10 days.
[b] Values are the mean of five plates and three repetitions. Data were analyzed statistically and means were separated by Duncan's new multiple range test for significance at $p = 0.05$.

Effect of temperature and relative humidity on sporulation. Conidial production was most abundant at 28°C with RH 60%, followed by 30°C with RH 60% (Table 2). *Epicoccosorus nematosporus* did not produce conidium at RH 100% regardless of temperature. Temperatures of 28-30°C were optimal, but few were produced below 15°C and above 33°C. The temperature curve of a reproductive process of a microorganism is usually similar to that of vegetative growth; that is, the optimum is usually nearer to the maximum than to the minimum (Alderman and Nutter, 1994). It is assumed that growth increases with rise in temperature above the minimum up to the optimum value; usually, low temperature limits reproduction (Hannusch and Boland, 1996). The influence of air temperature on sporulation was similar to the results of Aragaki and Hine (1963). Range of temperature for sporulation is almost always narrower than that permitting mycelial growth. Yarwood (1959) reported that the range of relative humidity

Table 2. Effect of various temperatures and relative humidies on the sporulation of *Epicoccosorus nematosporus* under light illumination[a]

Temp. (°C)	Fresh weight of conidial mucilage (mg/petridish)				
	RH 30	60	80	100%	LSD (0.05)
15	6.8	5.8	0.3	0.0	1.4[b]
20	23.2	43.1	14.2	0.0	6.3
25	22.1	87.0	130.9	0.0	73.4
28	80.0	666.1	291.8	0.0	67.0
30	93.0	384.0	264.7	0.0	58.5
33	32.6	119.4	104.4	0.0	5.5
LSD(0.05)	59.9	66.7	25.8	–	

[a] Light intensity of 4,500 lux was treated.
[b] Values are not significantly different at $p \leq 0.05$ according to least square difference test.

Table 3. Effect of temperatures and light intensities on the sporulation of *Epicoccosorus nematosporus*

Light intensity (lux)	Fresh weight of conidial mucilage (mg/petridish)			
	20°C	25°C	30°C	LSD (0.05)
1,500	46.8	50.2	48.6	9.9[a]
3,000	362.2	384.6	410.4	61.5
4,500	481.2	464.6	468.2	54.0
7,500	494.8	492.6	477.6	98.1
LSD(0.05)	48.1	54.8	79.7	

[a] Values are not significantly different at $p \leq 0.05$ according to least squares difference test.

permitting sporulation may be very narrow, e.g., RH 90-100% and 98-100% for sporalation of *Peronospora destructor* and *Bremia lactucae*, respectively. Nevertheless, very damp conditions may not be the best for the final stages of spore formation.

Effect of temperature and light intensity on sporulation. Light intensity of over 3,000 lux affected sporulation more significantly than temperature (Table 3). Results of the study showed that optimum temperatures for mycelial growth and for conidial germination of the fungus were consistent. Penetration of plant tissues by many plant pathogens occurs soon after formation of infection structures such as appressoria. There are several guides regarding these early stages of pathogenesis. The effects of visible light and other radiations on growth and development of fungi have been reviewed by Mark et al. (1965) and Lythgoe (1961). The most striking effect of visible light is on the initiation and development of spores for a number of species; some species must have light for sporulation, if not, sporulation is completely inhibited by continuous darkness.

Optimal temperature and surface wetness period for germ tube and appressoria formation. At dew periods of 12 h at temperature between 20°C and 30°C, more than 80% of conidia germinated and 60% of them formed appressoria (Tables 4, 5). Dew period appeared to be more important than temperature. At least 12 h of dew period was critical for satisfactory conidial germination and appressorial formation. For dew period of less than 4 h, few conidia germinated in all temperatures. Many pathogenic fungi require a period of free moisture for spore germination and successful infection. Wastie (1972) concluded that moisture is the most important requirement for germination and infection by conidia of *C. gloeosporioides*. Others (Mark et al., 1965) have shown that several species of *Colletotrichum* penetrate their hosts within 9-12 h after inoculation. Daniel (1973) and Skoropad (1967) reported that appressoria can remain dormant in unfavorable environments, and that the shoot epidermis is penetrated

Table 4. Effect of temperature and dew period on the formation of germ tube of *Epicoccosorus nematosporus* on *Eleocharis kuroguwai* seedlings

Dew period (h)	Percentage of germ tube[a]				
	15°C	20°C	25°C	30°C	LSD (0.05)
4	2.1	8.9	9.8	10.1	1.3[b]
8	11.8	44.3	55.7	52.4	3.7
12	36.0	71.3	82.9	80.9	3.8
16	41.7	79.2	84.2	81.9	3.8
20	52.8	79.6	85.6	85.9	3.2
LSD (0.05)	3.2	3.0	3.5	3.3	

[a] The number of germ tube was counted 24 h after inoculation. Conidial suspension at concentration of 105 conidia/ml containing 0.5% (w/w) dextrose was sprayed on 20-30-day old stem of *E. kuroguwai* until run off. Ten shoots were counted for each pot.

[b] Values are not significantly different at $p \leq 0.05$ according to least squares difference test.

Table 5. Effect of temperature and dew period on appressoria formation of *Epicoccosorus nematosporus* on *Eleocharis kuroguwai* seedlings

Dew period (h)	Appressoria (%)[a]				
	15°C	20°C	25°C	30°C	LSD (0.05)
4	1.6	4.9	6.0	4.6	0.9[b]
8	6.2	29.2	31.0	29.0	1.9
12	21.7	51.0	68.3	66.5	3.3
16	27.9	61.6	71.2	71.0	3.1
20	33.0	61.7	73.7	71.3	5.2
LSD (0.05)	3.6	2.7	3.4	3.0	

[a] The number of appressoria were counted 24 h in each dew after inoculation. Conidial suspension at concentration of 105 conidia/ml containing 0.5% (w/w) dextrose was sprayed on 20-30-day old stem of *E. kuroguwai* until run off. Ten shoots were counted for each pot. The suspension containing appressoria were counted five times per shoot.

[b] Values are not significantly different at $p \leq 0.05$ according to least square difference test.

when the environments again become favorable. Many pathogenic fungi require various periods of free moisture and favorable temperatures for spore germination, appressoria formation, and infection (Tebeest et al., 1978). Among climatic factors studied, period of wetness was the most important one (Arny and Rowe, 1991; Walker, 1981; Wastie, 1972).

In a study on the effect of dew period on biological weed control of field bindweed (*Convolvulus arvensis*) caused by *Phoma proboscis*, infection structures were evident after 2 h of dew period, and significant increases in plant mortality were evident after 4 h of dew period (Heiny and Templeton, 1991). However, in biological agents of *Colletotrichum* spp., 95-100% of reduction in weeding efficiency was

obtained with 8-12 h of dew period (Walker and Riley, 1982). In another study, an isolate of *A. macrospora* pathogenic to cotton (*G. barbadense* L.) produced maximum weed control after 9 h of dew at 25°C, whereas, another isolate of the fungus pathogenic to spurred anoda (*Anoda cristata*) required dew periods of over 24 h at 25°C (Walker, 1981). In biological weed control of spurred anoda (*A. cristata*) with *Alternaria macrospora* reported by Walker, 18 h of dew period with a temperature range of 20-30°C appeared most effective in killing weeds, but there were sharp decreases for temperatures below 10°C and over 35°C (Walker, 1981). Mean daily maximum temperature in rice canopies was 30°C from early July to mid August in the southern area of Korea. This temperature is sufficient for the disease development by the fungus to kill water chestnut plants.

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