Note

An Improved Method for Preparation and Preservation of Conidia for *Nimbya scirpicola*, Pathogenic Fungus for *Eleocharis kuroguwai*

Tomoko MATSUKI,* Hideaki NEGISHI,**
Minoru HONDA and Takane FUJIMORI

Plant Protection Research Laboratory, Japan Tobacco Inc.,
Aoba-ku, Yokohama 227, Japan

(Received June 19, 1996; Accepted August 22, 1996)

INTRODUCTION

Public concerns over biological control agents, as a substitution of chemicals, have been growing for these years because of their environmental friendliness: Biological control agents adopting specific pathogens as active ingredients are in general not toxic to human and other non target lives and free from the problems of pesticide resistance. Biological control agents for insect pests, such as *Bacillus thuringiensis*, *Beauveria bassiana*, and *Verticillium lecanii*, have been widely accepted by growers and consumers. However, those for weeds are still unpopular: Only four agents, *Colletotrichum gloeosporioides* for the control of *Cuscuta* spp. in China, *Phytophthora palmivora* for the control of *Morrenia odorata* in the United States, *C. gloeosporioides* f.sp. *aeschynomene* for the control of *Aeschynomene virginica* in the United States, *C. gloeosporioides* f.sp. *malvae* for the control of *Malva pusilla* in Canada, have been developed and been commercially available.¹⁻⁷⁾

The spikesedge is one of the most troublesome paddy weeds in Japan. It overwinters in paddy soil as tubers. Germination occurs during spring but the timing is irregular and extended; some tubers remain dormant for years; so that the chemical control could hardly be achieved and persistent treatments are required for the complete control. We have observed the fungal diseases of this weed for these years and selected *Nimbya scirpicola*, which was isolated and identified in 1989 and was found out their capacity of the pathogen to cause disease under natural condition at paddy field in 1991, as a candidate for a biological control agent.^{8,9)} In this report, we describe the methods for larger-scale production and preservation of the conidia, which enable extensive shelf-life for the practical use, and the results of greenhouse trials, by which the virulence of the produced conidia was checked.

Present address: * Tobacco Science Research Laboratory, JT, Aoba-ku, Yokohama 227, Japan; ** Leaf Tobacco Research Laboratory, JT, Oyama, Tochigi 323, Japan.

MATERIALS AND METHODS

1. Preparation of Conidia by Mesh Screen Method

A *N. scirpicola* K-004 isolate, which was isolated from the diseased spikesedge⁸⁾ and had been maintained on V-8 agar, was used for test. Conidia (10^5) were inoculated into 100 ml 3-5-5-5 medium (60 g glucose, 15 g NaNO_3 , 5 g K_2HPO_4 , 2.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g KCl, 0.05 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g yeast extract, 1 l distilled water; pH 6.0) in a 500 ml flask and shake-cultured at 25°C for 7 days under light condition. The propagated mycelia were filtrated, and were comminuted in a Waring blender at 10,000 rpm for 10 sec after resuspended in water that contained 2.25% sodium alginate and 8% cellulose, to produce 4% mycelial suspension. The mycelial suspension was spread over a nylon-mesh screen (350×255 mm, 1.5 mm mesh) and then 0.2 M CaCl_2 solution was sprayed to form gel. The mounted mycelia on the screen were cultured in a moist chamber for 7 days at 25°C under a 16 L : 8 D photophase to form conidia, and then air-dried for 4 hr at room temperatures. Conidia formed on the screen were collected by suction with membrane filter.

2. Evaluation of Virulence

Virulence of the conidia formed on the screen was evaluated under greenhouse condition, by the same method as described by Tanaka *et al.*⁹⁾ Conidial suspension in 0.1% Tween 80 solution (1 ml) was sprayed over the spikesedge (*ca.* 2-3 weeks after emergence) that was planted in a plastic pot (10 cm square). The inoculated plants were kept for 20 hr under conditions of 22°C, 100% r.h. The plants were then grown under greenhouse conditions of 28°C/12 hr : 23°C/12 hr for 2 weeks. Diseased and intact area on the stalks was measured at 1 and 2 weeks after inoculation, and the percentage of diseased stalks was calculated. Also average rate of diseased area for each plant was calculated and converted into disease index according to the following basis:

Disease index 0: no symptoms on any stalks, 1: 1-25% diseased area, 2: 26-50% diseased area, 3: 51-75% diseased area, 4: 76-100% diseased area, 5: completely withered.

Three replications were carried out for a series of test, and the tests were conducted twice.

3. Preservation Conditions of Conidia

Collected conidia were kept under various temperature and humidity conditions and periodically checked their viability, in order to validate the fittest conditions to preserve: Some conidia in lidded bottles were stored in refrigerators or thermocontrolled incubators at temperatures of -20, 4, 20, 25, 30, 35 and 40°C, and others in lidless bottles were stored at 25°C in desiccators, relative humidities inside which were adjusted at 20, 42, 51, 78 and 90% with saturated solutions of $\text{CH}_3\text{-COOK}$, $\text{Zn}(\text{NO}_3)_2$, $\text{Ca}(\text{NO}_3)_2$, $\text{Na}_2\text{S}_2\text{O}_3$ and ZnSO_4 , respectively.

Table 1 Incidence and severity of disease in *Eleocharis kuroguwai* inoculated with *Nimbya scirpicola* conidia under greenhouse condition.

Inoculum	% of diseased stalks		Disease index ^{a)}	
	1 week	2 weeks	1 week	2 weeks
Conidia (10^4)	93.1±8.6	98.2±4.5	0.95±0.09	2.22±0.09
Conidia (10^5)	95.7±6.7	97.9±5.1	0.99±0.11	2.33±0.15

^{a)} Disease index 0: no symptoms on any stalks, 1: 1–25% diseased area, 2: 26–50% diseased area, 3: 51–75% diseased area, 4: 76–100% diseased area, 5: completely withered.

Data are expressed as the means±S. D. of 6 replications.

The tests were conducted twice.

4. Evaluation of Viability

Viability of conidia was checked by germination. Conidia were suspended in 1% glucose solution at a concentration of 5×10^3 conidia/ml. The prepared suspension (20 μ l) was dropped onto a glass slide coated with collodion and kept at 25°C for 3.5 hr. Conidia on slides were observed with a microscope, and the germinated and the intact ones were separately counted to calculate the germination rate.

Three replications were carried out for a series of test.

RESULTS AND DISCUSSION

The conidia were produced more effectively by the mesh screen method (3.14×10^4 conidia/cm²) than by the conventional method, based on simple culture on V-8 agar plate (3.77×10^3 conidia/cm²). The virulence of the conidia that were recovered from the screen was almost as high as that described in the previous report⁹⁾ (Table 1). In practical, 10^{11} conidia should be required to apply to a 1 ha paddy field,⁹⁾ which are estimated to be harvested from 318 m² mesh screen by this method. Therefore, further improvement on culture must be necessary for commercial production.

The periodical changes of germination rate of conidia that were stored at 25°C under various humidity conditions are shown in Fig. 1. The humidity greatly affects the viability of conidia: the higher humidity rises the faster the viability declines; the germination rate diminished by half within two weeks above 78% of humidity, eight weeks at 51%, 10 weeks at 42%, and 20 weeks at 20%. These results suggest that dry condition might be required to maintain the resting state of conidia. At any case, prepared conidia proved not possible to keep for a long term in room temperature (25°C) even if the condition was dry enough. The storage temperature also strongly affects the stability of conidia: the higher temperature rises the faster the viability declines (Fig. 2). Although the germination rate diminished by half within 20 weeks above 20°C, 80% of conidia that stored at 4°C and –20°C remained viability for 10 and 12 months, respectively. These results strongly indicate that the conidia of *N. scirpicola* should not be stored at room conditions in practical, but that they would maintain their viability for a year, without any additives, when stored under chilled or freezing condition.

Fig. 1 Viability of *Nimbya scirpicola* conidia stored at 25°C, under various humidity conditions.

Fig. 2. Viability of *Nimbya scirpicola* conidia stored at various temperatures.

ACKNOWLEDGMENTS

The authors wish to thank Mr. B. Shatter, Mycogen Corporation, for his technical advice in the fermentation of conidia using a nylon-mesh screen, and give many thanks to Mr. Imai, JT, for the critical reading of the manuscript.

REFERENCES

- 1) D. S. Kenney: *Weed Sci.* **34** (Suppl. 1), 15 (1986)
- 2) W. H. Ridings: *Weed Sci.* **34** (Suppl. 1), 31 (1986)
- 3) R. C. Bowers: *Weed Sci.* **34** (Suppl. 1), 24 (1986)
- 4) R. J. Smith, Jr.: *Weed Sci.* **34** (Suppl. 1), 17 (1986)

- 5) J. T. Daniel, G. E. Templeton, R. J. Smith, Jr. & W. T. Fox : *Weed Sci.* **21**, 303 (1973)
- 6) G. E. Templeton : "Microbes and Microbial Products as Herbicides," ed. by R. E. Hoagland, Am. Chem. Soc., Washington, D.C., pp. 320-329, 1990
- 7) R. M. D. Makowski & K. Mortensen : *Proc. First Int. Weed Control Congr.* **2**, 298 (1992)
- 8) Y. Harada, S. Imaizumi, H. Tanaka, H. Negishi, T. Fujimori, M. Yamada, R. Honkura & Y. Miura : *Ann. Phytopathol. Soc. Jpn.* **58**, 766 (1992)
- 9) H. Tanaka, S. Imaizumi, H. Negishi, N. Nitta, M. Honda, M. Sawaji, M. Yamada & T. Fujimori : "Integrated Management of Paddy and Aquatic Weeds in Asia," ed. by J. Bay-Petersen, Food and Fertilizer Technology Center for the Asian and Pacific Region, Taipei, pp. 202-209, 1994

要 約

クログワイの病原菌 *Nimbya scirpicola* の胞子の調製法と保存法

松木知子, 根岸秀明, 本田 実, 藤森 嶺

難防除雑草の一つであるクログワイ (*Eleocharis kuroguwai* OHwi) の罹病葉の病斑から単離した病原菌 *Nimbya scirpicola* K-004 菌を活性成分とした微生物除草剤の開発の一環として, *N. scirpicola* 胞子の調製法, 保存法を検討し, その病原力を温室において試験した. ナイロンメッシュスクリーンに塗布した菌糸をゲル化し, 培養して生産した胞子を乾燥して吸引回収したところ, 通常の固体培地上で培養した胞子より生産性に優れ, また, 保存性も優れていた(生産性: 8.3 倍, 保存性: 4°C で 10 か月, -20°C で 12 か月). また, 本法により調製した胞子を, 対象雑草であるクログワイへ接種したところ, 通常の固体培地上で培養した胞子と同等の病原力を示した.