Note

Mycelial Preparation of *Nimbya scirpicola* for Biological Control of *Eleocharis kuroguwai* OHWI

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INTRODUCTION

In our previous report we described the conidial preparation of the phytopathogenic fungus, *Nimbya scirpicola* (Dothideales, Diademaceae), for the control of spikesedge, *Eleocharis kuroguwai* OHWI, a troublesome weed in paddy field. The prepared conidia showed sufficient stability and efficacy. However, the productivity by the exploited culture method was still unsatisfactory for commercial use; 318 m² medium was required for the surface culture to harvest enough conidia to apply to a 1 ha paddy field at an effective dose.¹⁾

The use of mycelia, mass-produced in liquid culture, should resolve the problem. In the case of the entomopathogenic fungus, *Verticillium lecanii*, brastospores, small mycelium-like fragments that propagate in liquid culture, have been practically used in the biological control for the aphids and white flies.^{2,3)} On the other hand, mycelia of phytopathogenic fungi have never been applied as biological control agents in practice, perhaps owing to their inferior stability during storage⁴⁾. In commercial products at present, *Colletotrichum gloeosporioides* for the control of *Cuscuta* spp., *Phytophthora palmivora* for the control of *Morrenia odorata*, *C. gloeosporioides* f.sp. *aeschynomene* for the control of *Aeschynomene virginica*, and *C. gloeosporioides* f.sp. *malvae* for the control of *Malva pusilla* are all used their spores, which have special forms such as chlamydospores, sporangia etc.⁵⁻⁹⁾

Therefore, we first determined the virulence of the mycelia under greenhouse condition and then investigated the method of mycelial preparation to achieve practical stability and efficacy, in order to develop a commercially feasible mycelial preparation of *N. scirpicola*.

MATERIALS AND METHODS

1. Culture of Mycelia

Conidia (10⁵) of *N. scirpicola* K-004 isolate, grown on V-8 agar at 25°C for 7 days under light condition, were inoculated into 100 ml of C-100 medium (100 g glucose, 15 g NaNO₃, 5 g K₂HPO₄, 2.5 g MgSO₄·7H₂O, 2.5 g KCl, 0.05 g FeSO₄·7H₂O, 5 g yeast extract, 1 l distilled water; pH 6.0) in a 500 ml conical flask, and cultured by shaking at 200 rpm, 25°C for 7 days under light condition. The propagated mycelia in culture liquid were comminuted in a Waring blender at 5000 rpm for 30 sec to obtain mycelial fragments, and rinsed twice by centrifugation (3500 rpm, 10 min).

To quantify the mycelia, the unit gram equivalent (gE) that was ascertained from the fresh weight (g) of the mycelial fragment obtained here, was used for the following tests.

2. Mycelial Preparation

The mycelial fragments were resuspended with sterilized water at the proportion of 1:4 by weight and sprayed onto a Teflon or glass plate with an air brush at ca. 1 mm thickness, and then dried for 16 hr at 25°C. The adherent mycelia were scraped off and sieved through a 22-mesh screen to result in dry mycelial powder. When the effect of additives on viability in the drying process was evaluated, the additives were dissolved into the sterilized water used for resuspension of the mycelial fragments.

The procedure was carried out twice for every preparation.

3. Evaluation of Viability

Viability of mycelia was evaluated by colony forming ability of the mycelia on agar plates. Prepared mycelia (70 mgE) were suspended in 10 ml of sterilized water and serially diluted to concentrations of 10², 10³ and 10⁴ mycelia/ml. The suspension (1 ml) was poured into a 9 cm ϕ plastic Petri dish, in which 9 ml APDA (acid-potato dextrose agar) had been previously placed and heated at 50°C to prevent setting, then mixed and cooled. After incubation at 25°C for 5-7 days, the formed colonies were counted and the numbers of the colonies from 1 gE mycelial fragments was calculated to estimate the colony forming unit (CFU)/gE.

Ten replications were performed for each sample.

4. Evaluation of Virulence

Virulence of mycelia was evaluated by the same method as described in our previous paper,¹⁾ except for the sample preparation and the test condition: the sample was prepared in 0.1% solution of Tween 80 at a concentration of 0.5 gE mycelia/ml, and the inoculated plants were kept in a greenhouse at 28°C/12 hr:23°C/12 hr for the first 24 hr, put in a dew chamber at 22°C for the next 24 hr, and then put back in the greenhouse.

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Five plants were used for each sample.

RESULTS AND DISCUSSION

1. Virulence of Mycelial Fragment

Mycelial fragments prepared with C-100 culture medium exhibited the same level of virulence as conidia to the *E. kuroguwai*; the percentage of infected plants and the severity of disease in individual infected plant treated with mycelia were not significantly different from those treated with conidia (Table 1). 5.2×10^5 mycelial fragments are equivalent to 0.472 gE mycelia, therefore it was shown that 0.5 gE mycelia were equal to 5×10^5 conidia in virulence.

2. Mycelial Preparation Assuring Survival during the Drying Process

Drying procedure damaged the viability and virulence of mycelia. However, when mycelia were suspended in C-100 culture medium before drying, the damage did not occur (Experiment I of Table 2). In order to determine the factors that affect viability during the drying procedure, mycelial fragments were dried, after suspended in whole C-100 medium

Table 1 Virulence of mycelia and conidia of *Nimbya scirpicola* to *Eleocharis kuroguwai*.

($n=6$, mean $\pm$ SD)

Inoculum	Diseased stalks (%)	Disease index ^{a)}
Mycelia (5.2×10^5) ^{b)}	88.99 $\pm$ 6.27	2.88 $\pm$ 0.83
Conidia (4.7×10^5)	88.39 $\pm$ 3.73	2.80 $\pm$ 0.54

^{a)} Disease index: 0, no symptoms on any stalks; 1, 0–25% diseased area; 2, 26–50% diseased area; 3, 51–75% diseased area; 4, 76–100% diseased area; 5, completely withered. ^{b)} 5.2×10^5 mycelial fragments are equivalent to 0.472 gE mycelia.

or its individual components, 10% glucose, 0.5% yeast extract and inorganic salts. As shown in Experiment II of Table 2, out of the tested components only when suspended in 10% glucose, mycelia kept viability after the drying procedure at almost the same level as when suspended in C-100 medium. The effect of other sugars, fructose (monosaccharide), sucrose (disaccharide), trehalose (disaccharide), maltose (disaccharide) were further investigated. Dried mycelia, suspended in each 10% sugar solution before drying, were stored at 4°C and their viability were checked periodically. As shown in Fig. 1, the viability of mycelia dried with lactose, maltose, trehalose, or sucrose was almost constant during the first 8 weeks, whereas the same but dried with

Fig. 1 Effect of sugars added in drying procedure on viability of stored mycelia of *N. scirpicola*.

Symbols indicate as follows: ■, fresh mycelia (before drying); □, glucose; ◆, fructose; ◇, sucrose; ●, trehalose; △, lactose; ▼, maltose.

Table 2 Effects of culture-medium components on viability and virulence of mycelia of *N. scirpicola* during drying procedure.

(mean $\pm$ SD)

Additive	Viability (CFU/gE ^{a)})	Diseased stalks (%)	Disease index ^{b)}
Experiment I ($n=6$)			
No additives	2.1×10^2	0.40 $\pm$ 0.97	0.03 $\pm$ 0.07
C-100 medium (whole)	8.7×10^5	99.90 $\pm$ 0.32	2.81 $\pm$ 0.64
Fresh (before drying)	3.3×10^6	100.00 $\pm$ 0.00	2.92 $\pm$ 0.38
Experiment II ($n=10$)			
C-100 medium (whole)	5.5×10^5	88.08 $\pm$ 8.76	2.58 $\pm$ 0.67
C-100 medium component			
10% glucose	5.0×10^5	84.81 $\pm$ 9.03	2.49 $\pm$ 0.58
0.5% yeast extract	1.6×10^2	1.00 $\pm$ 3.16	0.01 $\pm$ 0.03
Inorganic salts	5.4×10^2	3.11 $\pm$ 5.02	0.03 $\pm$ 0.05
Fresh (before drying)	9.1×10^5	92.83 $\pm$ 7.89	2.89 $\pm$ 0.72

^{a)} CFU/gE=Colony forming unit per gram equivalent. ^{b)} Disease index: 0, no symptoms on any stalks; 1, 0–25% diseased area; 2, 26–50% diseased area; 3, 51–75% diseased area; 4, 76–100% diseased area; 5, completely withered.

Table 3 Virulence of prepared mycelia *N. scirpicola* stored at 4°C.
($n = 10$, mean $\pm$ SD)

Storage period (weeks)	Inoculation date ('92-'93)	Prepared mycelia		Fresh mycelia	
		Diseased stalks (%)	Disease index ^{b)}	Diseased stalks (%)	Disease index ^{b)}
0	4/ 1, 4/ 8	54.08 $\pm$ 13.40	0.87 $\pm$ 0.26	25.30 $\pm$ 8.10	0.31 $\pm$ 0.09
4	4/30, 5/ 7	83.81 $\pm$ 11.36	1.31 $\pm$ 0.49	42.46 $\pm$ 10.87	0.40 $\pm$ 0.21
8	5/27, 6/ 3	87.50 $\pm$ 6.29	1.40 $\pm$ 0.54	60.91 $\pm$ 46.15	0.67 $\pm$ 0.54
12	6/24, 6/30	94.76 $\pm$ 8.99	1.40 $\pm$ 0.54	79.63 $\pm$ 26.25	1.17 $\pm$ 0.60
16	7/22, 7/28	98.43 $\pm$ 3.73	2.63 $\pm$ 0.23	92.13 $\pm$ 1.04	2.13 $\pm$ 0.48
20	8/19, 8/25	100.00 $\pm$ 0.00	3.04 $\pm$ 0.18	98.58 $\pm$ 0.98	2.78 $\pm$ 0.83
24	9/16, 9/24	92.04 $\pm$ 7.27	1.55 $\pm$ 0.34	77.94 $\pm$ 18.69	0.88 $\pm$ 0.26
28	10/14, 10/21	88.89 $\pm$ 7.87	1.29 $\pm$ 0.21	30.00 $\pm$ 8.66	0.39 $\pm$ 0.22
52	3/24, 3/30	42.46 $\pm$ 10.87	0.61 $\pm$ 0.28	23.33 $\pm$ 2.89	0.28 $\pm$ 0.09

^{a)} Fresh mycelia was prepared anew for each inoculation test. ^{b)} Disease index: 0, no symptoms on any stalks; 1, 0–25% diseased area; 2, 26–50% diseased area; 3, 51–75% diseased area; 4, 76–100% diseased area; 5, completely withered.

Fig. 2 Changes in viability of prepared mycelia during 4°C storage.

Symbols indicate as follows: ■, fresh mycelia (before drying); ●, prepared and stored mycelia, adding 10% lactose and 1% silica gel before drying and at 4°C.

glucose or fructose clearly declined. These results agree with those of Yanagita (1981)¹⁰⁾. The former sugars are all categorized as disaccharide and the latter as monosaccharide. Disaccharide (sucrose), trisaccharide (raffinose) and inositol have already been described as the best protectants for microorganisms being used for wet dissemination through aerosol.¹⁰⁾

3. Stability in Efficacy and Viability of Prepared Mycelia

Mycelial fragments were dried after suspended in 10% lactose, as a drying protectant, and 1% silica gel, as a carrier, for use in the stability evaluation. The prepared mycelia were stored at 4°C and their viability and virulence were evaluated periodically. Changes in viability during 76-week's storage at 4°C are shown in Fig. 2. Viability was sustained almost at the initial level, *ca.* 2×10^6 CFU/gE, for 36 weeks and then gradually decreased but even after 76-week's storage the prepared mycelia exhibited viability at *ca.*

3×10^5 CFU/gE. High durability of the prepared mycelia was also proved by the virulence evaluation (Table 3). The condition of disease prevalence fluctuated depending upon the season, perhaps owing to the variations of humidity conditions in the greenhouse and of susceptibility of the host plants. However, the prepared and stored mycelia exhibited the same, or even a higher level of virulence compared with the fresh mycelia prepared anew for each inoculation test in all cases. The virulence was maintained even after 52-week's storage when the viability was shown to reduce by 1/4 (*ca.* 5×10^5 CFU/gE; Fig. 2).

Durability and virulence of the present preparation of mycelia are considered to be sufficient for the practical use. In general, submerged culture is technically established and easy to be scaled up: it has an advantage over surface culture in mass production. However, productivity in the present culture method (submerged culture), 18.1 gE mycelia from 1 l medium, is still not satisfactory: 5525 l medium is required to produce the mycelia for application to a 1 ha paddy field in calculation. Further research on culture should be necessary for the practical use.

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REFERENCES

- 1) T. Matsuki, H. Negishi, M. Honda & T. Fujimori: *J. Pesticide Sci.* **22**, 39 (1997)
- 2) T. Saito: *Jpn. J. Appl. Entomol. Zool.* **32**, 224 (1988)
- 3) "Biopesticides in Crop Protection," PJB Publications Ltd., Richmond, pp. 32 & 35, 1994
- 4) Nihon Seikagaku Kai: "Biseibutsu Jikkenhou," Tokyo Kagaku Doujin, Tokyo, p. 48, 1992
- 5) D. S. Kenny: *Weed Sci.* **34** (Suppl. 1), 15 (1986)
- 6) E. Feichtenberger, G. A. Zentmyer & J. A. Menge: *Phytopathology* **74**, 50 (1984)

- 7) D. O. TeBeest, G. E. Templeton & R. J. Smith, Jr.: *Phytopathology* **68**, 389 (1978)
- 8) G. E. Templeton: "Microbes and Microbial Products as Herbicides," ed. by R. E. Hoagland, Am. Chem. Soc., Washington, D. C., pp. 320-329, 1990
- 9) R. M. D. Makowski & K. Mortensen: *Proc. First Int. Weed Control Congr.* **2**, 298 (1992)
- 10) T. Yanagita: "Biseibutsu Kagaku 2," Japan Scientific Societies Press, Tokyo, p. 494, 1981

要 約

クログワイの病原菌 *Nimbya scirpicola* の微生物除草剤としての菌糸調製法

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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 菌の菌糸を活性成分とした微生物除草剤の開発を目的に, *N. scirpicola* 菌糸が, 長期間, 生存力と病原力を維持し得る調製法を検討した. 液体培養で生産した菌糸を破碎し, 10%ラクトースと 1%シリカゲルを添加してテフロン板に噴霧し, 一晩風乾した. 得られた乾燥菌糸粉末は, 胞子と同等の病原力を有し, 4°C の保存において, 乾燥前の生存力を 36 週間, 対象雑草であるクログワイに対する病原力を 52 週間維持していた. 胞子同様, 糸状菌の菌糸を用いた微生物除草剤の開発が可能であることがわかった.