Conidia production of *Exserohilum rostratum*, a biocontrol agent against red sprangletop, by a two-phase system using sponge matrix

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Received October 8, 2008; Accepted January 28, 2009

Bioherbicides have been developed as biological agents to offer non-chemical alternatives to reduce chemical discharge into the environment and are applied in a similar manner to chemical herbicides to control noxious weeds. The most common microorganism used as the active ingredient is a plant pathogenic fungus and its propagules are conidia or mycelial fragments. The fungal isolate MKY3010, *Exserohilum rostratum* (a synonym of *Setospharia rostrata* in telemorph), showing potential efficacy as a bioherbicide against red sprangletop (*Leptochloa chinensis*) were obtained in Kyushu, Japan. However, this fungus did not produce any conidia in liquid culture. Mycelia of *E. rostratum* grew well in sponge matrix submerged in liquid culture (the first phase). Consequently conidia were abundantly produced from the mycelia when the sponge matrix was exposed to the air (the second phase). This two-phase system consistently produced over 5 x 10^8 conidia L^{-1} within 5 days. In addition, the conidia obtained were more uniform in size and achieved higher percentages of germination and appressoria formation compared with those produced on agar media. These results showed that a two-phase system using sponge matrix could be a promising tool for providing the conidia of *E. rostratum* to develop a bioherbicide against red sprangletop, an emerging weed in agro-ecosystem.

Key words: *Exserohilum rostratum, Leptochloa chinensis*, biological control, conidia, sponge matrix.

INTRODUCTION

Biocontrol of weeds using plant pathogens has gained acceptance as a practical, safe, environmentally beneficial weed management method (Charudattan 2001). Biocontrol agents have been isolated from diseased weeds in the nature and kept in laboratories. Generally, this biological weed management has been practiced through either a classical or an augmentative approach. The classical strategy introduces highly host-specific agents from the weed's native geographic range into the weed-invaded regions where biocontrol has been required. On the other hand, the augmentative strategy employs bioherbicidal annual application of endemic or foreign agents similar to chemical herbicides. The active ingredient of a bioherbicide is a living microorganism and its propagules are applied in inundative doses. The most commonly used microorganism as a bioherbicide is a fungus and its propagules are conidia or mycelial fragments. Mass production and formulation have been key technologies to develop bioherbicides as well as to discover sufficient microbial agents (Auld and Morin 1995).

Red sprangletop [*Leptochloa chinensis* (L.) Nees] is a grassy weed originated in tropical Asia and distributed throughout South-East Asia, from East Africa to South Africa, Burma, Sri Lanka, India, Australia, and Japan. This weed has adapted to moist, swampy places in open habitats and is a prolific seed producer yielding more than 40,000 seeds per plant in the field. Therefore, it is a serious weed of rice, and troublesome weed in corn and sorghum fields. Around the Mekong delta, it has been an emerging major weed in direct-seeded rice fields, following barnyard grass (Chin, 2001). In Japan, the weed has appeared not only in direct-seeded rice fields but also in soybean areas converted from paddy (Matsuo et al. 1987). Moreover, the weed has spread even in transplanted rice fields in Kyushu, the southern part of Japan (Sumiyoshi et al. 2007).

In Vietnam, *Setospharia rostrata* Leonard was obtained as a promising agent for biologically controlling red sprangletop (Thi et al. 1999). This fungus is capable of causing more than 90% of mortality at the dose of 10^{13} conidia ha^{-1} in the field (Chin et al. 2003). Moreover, *Exserohilum rostratum* (Drechsler) Leonard & Suggs, a synonym of *S. rostrata* in telemorph, was isolated in Kyushu and determined to be pathogenic to red sprangletop in Japan (Yamaguchi et al. 2005). *E. rostratum* is capable of producing conidia on solid media such as potato dextrose agar but not in liquid media at all. In this study, we have examined the effect of a two-phase system using sponge matrix (Nakashima et al. 1988) on the

production of conidia of *E. rostratum* which show bio-control activity against red sprangeletop.

MATERIALS AND METHODS

Fungal isolate. The fungal isolate MKY3010 was used throughout the experiments. MKY3010 was isolated from severely diseased leaves of red sprangletop in Miyazaki prefecture, Kyushu. Based on the facts that conidia of this isolate had apparent hilum and septa, formed rostrate shape, and were germinated from the sub-hyaline region of the end cell, MKY3010 shown in Fig.1 was identified as *Exserohilum rostratum* (*Setospharia rostrata* in telemorph). In addition to the morphological characteristics of conidia formed, we confirmed that the sequences of its 18S rDNA were highly homologous to those of a standard isolate of *E. rostratum* (data not shown).

Conidia production on an agar medium. *E. rostratum* MKY3010 was cultured on autoclaved PDA (potato dextrose agar, Nissui Pharmaceutical Co. Ltd., Tokyo) in a 90-mm petri dish and incubated at 25°C in the dark for 12 days. Conidia formed on the agar plate were harvested using a plastic scrapper with sterile distilled water containing 0.1% Triton X-100 [polyoxethylene (10) octylphenyl, Wako Pure Chemical Industries Ltd., Osaka]. The suspension was filtrated through gauze to remove agar and mycelial debris. Conidia concentration was adjusted to $5x10^4$ mL^{-1} by diluting with 0.1% Triton X-100.

Conidia production by a two-phase system. Procedures of a two-phase system using sponge matrix for synchronous conidia production are schematically presented in Fig.2. In the first phase, quantitatively sufficient mycelia were cultured in submerged liquid culture. The basal medium contained 20 g of glucose, 2 g of $NaNO_3$, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.2 g of $CaCl_2$, 3.4 g of polypeptone, 3.4 g of yeast extract, 10 g of rice oil, and 20 g of polyurethane foam (5 mm-cubes, Bridgestone Co. Ltd. Tokyo), which provide approximately 3,500 cubes per 1 L of distilled water. The initial pH was adjusted to 5.5 by 0.1 N HCl. Each 500 mL-flask containing 150 mL of the medium containing polyurethane foam was inoculated with 0.1% (v/v) conidia suspension obtained by plate culture. Following autoclaving, these flasks were incubated on a rotary shaker at 100 rpm in the dark.

In the second phase, conidia were produced in the open air. After submerged in liquid culture, the sponge matrix covered with mycelia was separated from the culture broth through filtration using a sieve. To cause the conidiation of the fungus, mycelia on the surface of polyurethane foam were exposed to the air in a beaker at 25°C in a moist chamber. Conidia were harvested with

Fig. 1. ***Exserohilum rostratum*** **MKY3010 isolated from red sprangletop in Miyazaki.** 1) colony on PDA at 25°C for 5 days; 2) rostrate-shaped conidia harvested from PDA; Scale bar, meaning 50 μm; long arrow, indicating hilum; one-sided arrows, indicating septa.

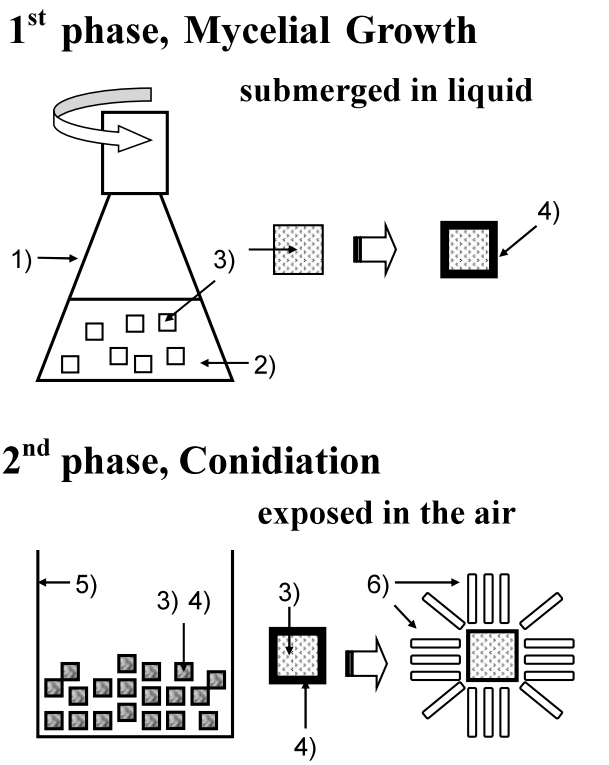

Fig. 2. Schematic illustration of a two-phase system using sponge matrix. 1) flask; 2) liquid medium; 3) polyurethane foam; 4) mycelia on the surface of polyurethane foam; 5) beaker; 6) conidia produced from mycelia.

Table 1. Mycelial growth and conidia production of *E. rostratum* MKY3010 by a two-phase system using sponge matrix

1st phase	Days after inoculation			
Submerged in liquid	2	3	4	5
Mycelia grown (dried g L^{-1})	<10	15.7±0.3	15.5±0.6	15.3±0.7
2nd phase[1)]	Hours after incubation			
Exposed in the air	24	48	72	96
Conidia produced (x10^8 L^{-1})	<1	5.3±0.2	5.3±0.3	5.2±0.3

Data present means±SD derived from 5 replicates.
1) mycelia at 3 days after inoculation, which were used for conidia production.

Table 2. Morphological characteristics of conidia produced by a two-phase system using sponge matrix compared to those produced by a plate culture

Culture method	Conidial length (μm)						No. of septa
	Average	Distribution (%)					
		<70	70-89	90-109	110-129	>129	
Two phase system[1)]	101.6	0	6	86	8	0	8-10
Plate culture[2)]	99.7	8	22	40	26	4	5-11

Approximately 300 conidia produced by each method, respectively, were examined.
1) 5-day culture used; 2) 12-day culture used.

distilled water containing 0.1% Triton X-100 using a magnetic stirrer, then filtrated, and kept in suspension.

Measurements of conidia. The net weight of mycelia in the sponge matrix in the first phase was calculated based on differences in the weight of polyurethane with or without mycelia. The number of conidia in the second phase was counted under a microscope. Moreover, the length of conidia and the number of septa were measured microscopically.

To assess the germination and appressoria formation of conidia produced, suspension at 5x10^4 conidia mL^{-1} containing 0.1% Triton X-100 was dropped on both water agar and the third expanded-leaves of red sprangletop seedlings, and the specimens were incubated at 25°C in the dark. After 6 hours, the germination of approximately 100 conidia on agar plate was checked under a stereoscopic microscope. After 12 hours, the appressoria formation of approximately 50 conidia on detached leaves was observed under a light microscope following lactophenol cotton blue staining.

Pathogenicity test. Four to five leaf-stage seedlings of red sprangletop grown in a pot were sprayed with sufficient amount of suspension of 5x10^4 conidia mL^{-1} containing 0.1% Triton X-100 using a sprayer (approximately 10 mL per seedling), and then kept in a moist chamber at 25°C with 12 hours of daily illumination. The inoculated 3 leaves were collected and the number of lesions appeared was counted at 4 days after inoculation.

RESULTS AND DISCUSSION

Effect of a two-phase system on conidia production. The results of conidia production by a two-phase system using sponge matrix were summarized and shown in Table 1. In the first phase, the conidia of *E. rostratum* MKY3010 applied as seeds were germinated in liquid, and then hyphae grew well and formed thin filmform mycelia on the surface of the polyurethane foam in liquid medium. There were few free mycelia observed in liquid medium when the polyurethane foam was inserted. The mycelia produced in the sponge matrix reached a maximum yield of over 15 dried g L^{-1} within 3 days. In the second phase, these mycelia were exposed to the air and consequently conidia were formed. The number of the conidia produced within 48 hours in a moist chamber was 5 x 10^8 L^{-1}, which was almost equivalent to 3 dried g L^{-1}. The estimated ratio of conidia/mycelia in dried weight was around 20%. The results revealed that a two-phase system could massively produce the conidia of *E. rostratum* within 5 days, which is much shorter than conidia production by a plate culture using PDA (12 days).

Although over 100 microorganisms have been reported as candidates for bioherbicidal agents and researchers have succeeded in discovering potential bioherbicides, only a few fungal agents have been registered for a practical use (Charudattan and Dinoor 2000). There must be some technological constraints in mass production of fungal agents. A submerged fermentation has been preferred to solid-substrate fermentation systems due to its cost-effectiveness and availability (Jackson et al. 1996). Moreover, a two-phase system using sponge matrix could be an alternative to a large-scale production method for fungal agents, in which conidia production using liquid medium is not applicable.

Morphological characteristics of the conidia. The size of the conidia produced in the sponge matrix was determined in comparison with those produced on PDA. Although the averages of conidial length were almost the same (101.6 and 99.7 μm, respectively), the conidia pro-

Table 3. Germination, appressoria formation and infection of the conidia produced by a two phase system using sponge matrix compared to those produced by a plate culture

Culture method	Germinated Conidia (%)	Appressoria formed conidia (%)	No. of induced lesions (cm^{-2})
Two phase system[1)]	99.4	89.2	8.1
Plate culture[2)]	76.8	71.6	7.7

Data based on 5 replicates.
1) 5-day culture used; 2) 12-day culture used.

duced by a two-phase system were more uniform in size than those produced by a plate culture (Table 2). More than 85% of the conidia produced in the sponge matrix were in the range of 90-109 μm in length. While the conidia produced on PDA showed more variation in size. Conidia production by a two-phase system was determined to be more synchronous than a plate culture.

Adequate formulation represents another technical constraint on the development of reliable and efficacious bioherbicides. Recently, new formulations including liquid formulations such as multiple emulsions of water in oil in water have been demonstrated to enhance their bioherbicidal efficacies (Auld et al. 2003). Considering the characteristics, the conidia produced by a two-phase system may be available even for newly defined formulations.

Biological characteristics of the conidia. The results of the germination, appressoria formation, and infection of the conidia produced were shown in Table 3 in comparison with the conidia produced on PDA. Almost all the conidia produced by a two-phase system were germinated and showed higher percentage (89.2%) of appressoria formation than those produced by a plate culture (71.6 %). These data showed statistically significant differences ($P<0.05$).

The conidia of *E. rostratum* induced necrotic lesions in the leaves of red sprangletop seedlings at 4 days after inoculation (Table 3), which revealed that the conidia were apparently pathogenic to red sprangletop. It is expected that the conidia produced by a two-phase system show herbicidal efficacy equivalent to the established efficacy of the conidia produced by a plate culture.

Several publications have reported that *Helminthosporium* species including *Exserohilum* (*Setosphaeria* in telemorph), *Biporalis* (*Cochliobolus* in telomorph) and *Drechslera* (*Pyrenophora* in telemorph) were effective in controlling grassy weeds (Yamaguchi 2006). A two-phase system using sponge matrix may be useful for producing conidia of other *Helminthosporium* species and may serve as a promising tool for mass-production of bioherbicides.

ACKNOWLEDGMENTS

The authors thank Dr. T. Tsukiboshi of the National Institute of Livestock and Grass land Science, Nasu, Japan, for providing standard fungal isolates to identify *E. rostratum*.

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アゼガヤの防除に有効な *Exserohilum rostratum* のスポンジを基材とした二段階培養による分生子生産

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要　約

微生物除草剤は，雑草を対象に化学農薬と同様の方法で施用されるが，環境中への影響が小さいことが期待される．用

いられる微生物の多くは植物病原糸状菌で，有効成分として分生子や菌糸片が使われている．アゼガヤの防除に有効な *Exserohilum rostratum* MKY3010を九州において分離したが，本菌は液体培地中で分生子を形成しない．そこで，スポンジを基材とした二段階培養を検討した結果，*E. rostratum*は液体培地中のスポンジで菌糸体を十分に増殖し（第1段階），その後，空気中に曝露させることによって多数の分生子を形成した（第2段階）．この二段階培養法により，5日間で培地1リットル当り5億個の分生子が生産された．得られた分生子は，寒天平板培地で培養した場合に比べて大きさが極めて均一で，発芽率や付着器形成率が高く，アゼガヤに対する病原性が認められた．これらから，スポンジを基材とした二段階培養法は *E. rostratum* の分生子生産に有効で，本菌を微生物除草剤として展開するツールとなる可能性が示された．