

Evaluation of indigenous fungi in Kyushu as biocontrol agents against red sprangletop (*Leptochloa chinensis*)

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Diseased red sprangletop (*Leptochloa chinensis*) was collected in nature in Kyushu and causal fungi were isolated from the lesion on necrotic parts of the plants. These fungi were estimated for their pathogenicity to red sprangletop, the paddy weed, and rice using *in vitro* assay. The isolates of *Bipolaris* and *Exserohilum* showed apparent pathogenicity specific to red sprangletop seedlings. In comparison of virulence using the conidia obtained by a two-phase system with sponge matrix, the isolates of *B. micropus* caused more severe lesions and necrosis on the leaves of red sprangletop than those of *Ex. rostratum* that has been reported as a potent biocontrol agent in Vietnam. *B. micropus* MKY3033 isolated in Miyazaki Prefecture was most pathogenic and showed potent herbicidal activity against the weed but not against other plants such as rice, corn, wheat and barnyard grass, which belong to the Gramineae family, suggesting that the fungus should be useful biocontrol agent for Integrated Weed Management.

Key words: biological weed control, *Bipolaris micropus*, *Exserohilum rostratum*, *Leptochloa chinensis*.

INTRODUCTION

Weed infestation is one of major constraints affecting rice production that is the most important crop in Japan being cultivated in 1.8 million ha of paddy fields. Although weeds have been eradicated using various cultural practices in current farming methods of paddies in Japan, chemical herbicides have been heavily used for weed control as the most effective and immediate method. In Japan, ¥3,500/ha of paddy fields were spent as herbicide costs (Shibayama 2001). Some problems, however, have been emerged in association with heavy use of herbicides such as the appearance of herbicide resistant weeds that leads to further increase of the cost for chemical weed control. Other problems are chemical residues in ground water and rainfall that cause environmental pollution. Furthermore the use of genetically modified plants for herbicide resistance has become common in the world. It is afraid that the escaping modified plants should be difficult to eradicate since the plants are known to thrive like weeds in nature. Increasing awareness of the general public about the safety of herbicides and its influence on food crops and environment has encouraged researchers to develop alternative weed control approaches such as biological control (Charudattan 2001).

Biological control (biocontrol), by definition, is the use of a biotic agent to suppress or reduce pest population. Weed control using this approach can complement and be integrated with traditional cultural and chemical methods

for weed control. Generally, biocontrol of weeds using plant pathogens consists of two strategies: 1) the classical strategy employing an ecological approach that involves an initial inoculation of self-sustaining agent to weed population; and 2) the augmentative or inundated approach utilizing annual application of endemic or foreign bioherbicultural agents similar to herbicide applications. In paddy fields, several fungi were reported to be effective in controlling some kinds of weeds (Yamaguchi 2006), for example *Exserohilum monoceras* (Drechsler) Leonard & Suggs to barnyard grass (*Echinochloa* spp.) (Tsukamoto et al. 1997, Hirase et al. 2003), *Epicoccossorus nematosporus* nom. inval. to Kuroguwai (*Eleokaris kuroguwai* Ohwi), and *Exserohilum rostratum* (Drechsler) Leonard & Suggs to red sprangletop (*Leptochloa chinensis* (L.) Nees) (Chin et al. 2003).

Red sprangletop, an annual grass originated in tropical Asia (Chin 2001), has been a major weed not only in soybean fields converted from paddies but also in paddy fields both for direct seeded and transplanted rice in Kyushu (Sumiyoshi et al. 2007). 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 fields (Matsuo et al. 1987). The seeds show no dormancy and germinate even in anaerobic conditions. Germination was influenced by temperature (minimum 15°C; optimal 25-35°C), and light (phytochrome dependent) (Benvenuti et al. 2004). Red sprangletop, therefore, is noxious weed both in rice paddies and some upland crop fields.

We showed the results of our study on the isolation of fungi from diseased red sprangletop collected in Kyushu district of Japan and their pathogenicity to determine their

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potential as biocontrol agents against red sprangletop.

MATERIALS AND METHODS

Fungal isolate. Diseased red sprangletop showing apparent necrosis and lesions on leaves in nature was collected in Takanabe town in 2003 and Miyazaki city in 2004 in Miyazaki Prefecture and Ogohri city in 2004 in Fukuoka Prefecture, those are located in Kyushu. Diseased tissue pieces (approx. 5x5 mm) were taken from the edges of necrotic areas and the lesions. These pieces were put into 70% ethanol solution for a few seconds, and then transferred to 1% hypochlorite solution for surface-sterilization. The pieces were washed in sterile distilled water for 3 times. Then they were placed on Petri dishes containing 1.5% water agar, and kept in the dark at 25°C. When fungal hyphae appeared from the pieces on agar plates, the edges of hyphae were picked up by a thin needle under a microscope, and transferred on 1/2 concentrated potato dextrose agar (PDA, Nissui, Tokyo) containing chloramphenicol at 100 ppm to remove bacterial contaminations. PDA plates were cultured in an incubator at 25°C in the dark for 1 week. Several fungal characteristics observed, such as growth speed, color, and pigmentation on the plates, were recorded for their primary identification.

In vitro screening for pathogenicity. The isolated fungi were examined in test tubes for their ability to cause diseases on red sprangletop and rice. Each of surface-sterilized and germinated seeds was put on a water agar (5 mL) in a test-tube (ϕ 25x120 mm). Then sterile distilled water (1 mL) was added into test tubes. They were kept in a plant growth chamber (12h/12h, day/night) at 25°C until seedlings grew at 1-2 leaf stage. The mycelial agar disks were put into test tubes where red sprangletop (obtained from Miyazaki Prefecture in 2003 and propagated in the greenhouse) or rice (*Oryza sativa* L. cv. Koshihikari) had been grown. Two weeks after the inoculation, the inoculated seedlings of red sprangletop and rice were observed to confirm pathogenicity of the isolated fungi.

Conidia production. The fungal isolates of *Bipolaris* sp. and *Exserohilum* sp. were used in our study. Conidia of the isolates as inocula were produced by the two-phase system using sponge matrix (Yamaguchi et al. 2009). The basal medium contained 20g of glucose, 2g of NaNO₃, 1g of K₂HPO₄, 1g of KH₂PO₄, 0.5g of MgSO₄, 0.2 g of CaCl₂, 3.4g of polypeptone, 3.4g of yeast extract, and 20g of sponge matrix (5mm-cubes of polyurethane foam, Bridgestone Co. Ltd. Tokyo) per 1 L of distilled water. The initial pH was adjusted to 5.5 by adding 0.1 N HCl. Mycelial suspension obtained from plate culture was added (0.1% v/v) to each flask (500 mL), which contained 150 mL of the medium. These flasks were incubated on a rotary shaker at 100 rpm at 25°C in the dark for 5 days. Then, the sponge matrix covered with mycelia was separated from the culture broth by sieve filtration. To induce the conidiation of the isolates, sponge matrix covered with mycelia was exposed to the air in sterilized beakers placed in a moist chamber at 25°C in the dark for 3 days. Conidia harvested from the surface of sponge matrix were put on an agar plate or kept in suspension for the following experiments.

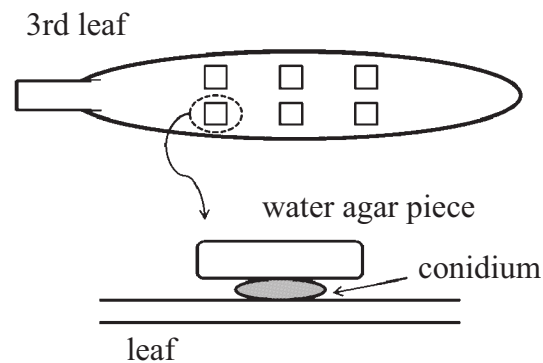


Fig. 1. Single spore inoculation method for assessment of virulence of the isolates.

Comparison of virulence. To assess the virulence of each isolate to red sprangletop, a single spore inoculation method (Tsukiboshi and Sato 1986) was used. Six single conidia of each isolate obtained from sponge matrix were placed on the surface of the third leaf of red sprangletop which had been grown for 1.5-2 months in a pot containing nursery soil in a plant growth chamber (12h/12h, day/night) at 25°C. Schematic illustration of the procedure was shown in Fig. 1. The inoculated plants were kept in a moist chamber for 24 hours at 25°C, and then returned to the plant growth chamber. Seven days after the inoculation, the number of lesions on the leaves and the lesion areas were estimated.

Host range test. Three to five leaf stage seedlings of rice cv. Hinohikari and cv. Minamiyutaka (feed for livestock), wheat (*Triticum aestivum* L.) cv. Nishihonami, corn (*Zea mays* L.) cv. Ohisama-corn and cv. Snow-dent (feed for livestock), two species of barnyard grass (*Echinochloa oryzicola* Vasing. and *E. crus-galli* Beauv.) and red sprangletop, all of which belong to the Gramineae family and had been grown in 1/5000 a pots containing nursery soil for 1-2 months in the greenhouse, were used in our study. They were sprayed with approx. 10mL/plant of conidial suspension (approx. $10^{4.5}$ conidia/mL) containing 0.1 % Triton X-100 using a sprayer, and then kept in a moist chamber at 25°C for 24 hours and transferred to the plant growth chamber (12h/12rh, day/night) at 25°C. Symptoms of diseases caused by the fungi in the inoculated seedlings were assessed 2 weeks after inoculation.

RESULTS AND DISCUSSION

Isolation and screening of the fungi. A total of more than 100 fungal isolates were obtained from lesions and necrotic tissues of red sprangletop collected in nature. Most fungal isolates belonged to the fungal genera, *Bipolaris* Shoemaker, *Drechslera* Ito, *Exserohilum* Leonard & Suggs, and *Curvularia* Boedijn (data not shown). Among the isolates tested for pathogenicity, only MKY-3010, MKY3033, MKY4009 and MKY4027 produced large lesions on leaves and caused necrosis of the red sprangletop seedlings but not to rice seedlings. These

Table 1. Characteristics of the isolates from red sprangletop in Kyushu

Isolate No.	species	Origin	Hyphal growth ¹⁾ (mm/ day)	Conidium ²⁾	
				Length(μ m)	No. of septa
MKY3010	<i>Ex. rostratum</i>	Takanabe in 2003	11.4 \pm 0.9	104.5	9.6
MKY3033	<i>B. micropus</i>	Takanabe in 2003	7.2 \pm 0.4	48.2	4.3
MKY4009	<i>B. micropus</i>	Miyazaki in 2004	7.0 \pm 0.3	47.0	4.1
MKY4027	<i>Ex. rostratum</i>	Ogohri in 2004	11.2 \pm 0.5	102.3	9.3

1) Data were derived from 9 replicates grown on PDA at 25°C in the dark for 5 days.

2) Data were derived from 50 conidia obtained by the two-phase system with sponge matrix.

Table 2. Comparison of virulence among the isolates obtained from red sprangletop

Tested fungal isolate		Virulence on leaf of red sprangletop	
		No. of lesions	Lesion area ¹⁾ (mm ²)
MKY3010	<i>Ex. rostratum</i>	5.0	6.4a
MKY3033	<i>B. micropus</i>	5.8	13.0b
MKY4009	<i>B. micropus</i>	5.6	12.8b
MKY4027	<i>Ex. rostratum</i>	4.8	6.2a

Data were derived from 5 replicates (6 conidia on the third leaf).

1) Different letters indicate significant differences (Turkey's test, $P < 0.01$)

isolates were re-isolated from the lesion produced and fulfilled the Koch's postulates.

MKY3033 and 4009 produced conidia that were fuliginous, almost straight, slightly tapering from middle, elliptical and had obconical basal cell with a short protuberant hilum (Fig.2). Based on these characteristics, size of conidia and number of septa (Table 1), these isolates were identified as *B. micropus* (Drechsler) Shoem. (Ueyama et al. 1975, Sivanesan 1987). On the other hands, MKY3010 and 4027 produced conidia that were slightly curved, rostrate, thick-walled and had basal septum darker and thicker than the other septa with a distinct protuberant hilum. Based on these characteristics, size of conidia and number of septa (Table 1), these isolates were identified as *Ex. rostratum* (Ueyama et al. 1975, Sivanesan 1987). *B. micropus* is known as a pathogen of *Leptochloa* and *Paspalum* and *Ex. rostratum* as a common parasite of many kinds of grasses (Sivanesan 1987).

Comparison of virulence of the isolates. The virulence of *Ex. rostratum* MKY3010 and 4027, and that of *B. micropus* MKY3033 and 4009 to red sprangletop seedlings were compared using the single spore inoculation method. Although there were no differences on number of lesions, the isolates of *B. micropus* caused more severe symptom on the leaves than those of *Ex. rostratum* (Table 2). Lesion area caused by *B. micropus* showed twice compared to *Ex. rostratum* and there were statistically significant differences between them ($P < 0.01$). Our bioassay has indicated that *B. micropus* is more pathogenic to red sprangletop than *Ex. rostratum* based on the virulence of the conidia which could be the active agent of bioherbicides in formulation.

Herbicidal activity. The fungal host range of *B. micropus* MKY3033 and *Ex. rostratum* MKY3010, both

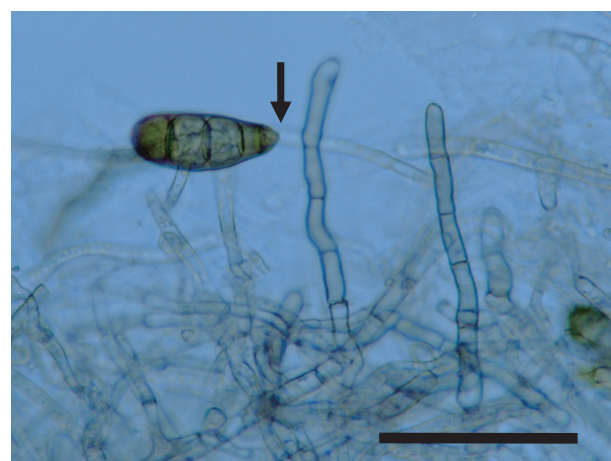


Fig. 2. *Bipolaris micropus* MKY3033 isolated from red sprangletop in Miyazaki prefecture. Conidium and conidiophores harvested from PDA; scale bar represents 50 μ m; arrow indicates hilum.

of which were determined to be pathogenic to red sprangletop, was investigated using the spray inoculation test (Table 3). When inoculum density was high (10^5 conidia/mL), both species killed red sprangletop seedlings. However, when inoculum density was low (10^4 conidia/mL), only *B. micropus* killed the plants and showed herbicidal activity. Even when conidia density was high, *B. micropus* caused no symptoms on rice, corn, wheat and barnyard grass, belonging to the Gramineae family. Also, we confirmed the isolates of *B. micropus* did not show any symptoms on the seedlings of several

Table 3. Pathogenicity of the isolates to various plants belonging to the Gramineae family

Tested plants	Sub-family	<i>B. micropus</i> MKY3033		<i>Ex. rostratum</i> MKY3010	
		10 ⁴	10 ⁵	10 ⁴	10 ⁵ (conidia/ mL)
red sprangletop	Chloridoideae	S	S	L	S
rice	Oryzoideae				
	cv. Hinohikari	N	N	N	HR
	cv. Minamiyutaka	N	N	N	HR
wheat	Pooideae				
	cv. Nishihonami	N	N	N	N
corn	Panicoideae				
	cv. Ohisama-corn	N	N	HR	HR
	cv. Snow-dent	N	N	N	HR
barnyard grass	Panicoideae				
	<i>E. oryzicola</i>	N	N	HR	HR
	<i>E. crus-galli</i>	N	N	HR	L

Visible assessment was based on 2 replicates (5-10 seedlings in a pot) at least.

N, no symptoms; HR, hypersensitive response (pinpoint lesions that did not enlarge); L, light infection (some lesions present and secondary spread limited); S, severe infection (many lesions present and some plants killed).

broad-leaf plants including soybean (*Glycine max* (L.) Merr.) (data not shown). These data have indicated that *B. micropus* has high host specificity on red sprangletop and no pathogenicity on other plants. Host specificity of a biocontrol agent is one of the most important factors to consider in a biological weed control.

In Vietnam, *Ex. rostratum*, the species we isolated in this study, has been expected to use as a biocontrol agent against red sprangletop (Chin et al. 2003). In Kyushu where the weed has started to increase in paddy fields, *B. micropus*, which shows more host specificity and potent herbicidal activity, was newly isolated in this study. The indigenous fungus that was most pathogenic to red sprangletop, *B. micropus* MKY3033, should be a biocontrol agent that can be used in Integrated Weed Management (IWM) system, combining with other cultural practices and mechanical, chemical, and biological intervention methods. In fact germination and growth of red sprangletop could be suppressed by deeper water condition in paddy fields (Benvenuti et al. 2004, Sumiyoshi et al. 2007). The management of water in irrigation may also be effective for the control of red sprangletop with the biocontrol using *B. micropus*. Follow-up experiments, especially field trials, will be needed for the development of a desired bioherbicide.

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アゼガヤの生物的防除に有効な九州に土着する
糸状菌の探索

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要 約

九州における水田雑草として問題となっているアゼガヤ (*Leptochloa chinensis*) の自然発病個体から病原糸状菌を分離するとともに, 分離菌の選択的な除草活性に関する評価を行った. 分離された糸状菌の中で, アゼガヤに特異的に病原性を示した糸状菌は, *Bipolaris micropus* 及び *Exserohilum rostratum* であった. 二段階培養法により得られた均質な分生子を用いて精密な接種実験を実施した結果, *B. micropus* が *E. rostratum* に比べてアゼガヤ葉に対する病原性が有意に高かった. また, この菌は, 噴霧接種による検定の結果, イネやトウモロコシ, コムギ, ヒエなど他の植物に全く病原性を示さなかった. 以上の結果から, 九州地方のアゼガヤから分離した *B. micropus*, 特に宮崎県で分離し病原性の強かった MKY3033 菌株は, IWM (Integrated Weed Management, 総合的雑草管理) における防除手段の一つとして有用である可能性が示された.