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A simple selective medium for the primary isolation of *Bipolaris*, *Drechslera* and *Exserohilum* species

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***Bipolaris* Shoemaker, *Drechslera* Ito and *Exserohilum* Leonard and Suggs are known to be pathogenic to food crops, foliage, and turf grasses. Moreover, some of these fungi have been reported as potential bioherbicides against grassy weeds, for example barnyard grass, goose grass, and red sprangletop. A selective culture medium for fungal isolation will be useful for studies of diseases caused by the fungi. The sensitivity of *Bipolaris, Drechslera* and *Exserohilum* to 11 different fungicides and 9 kinds of carbon sources were tested *in vitro*. Thiophanate-methyl, a chemical fungicide, had little impact on the growth of *B. oryzae* (Breda de Haan) Shoemaker, *D. avenacea* (Curtis ex Cooke) Shoemaker, and *E. rostratum* (Drechsler) Leonard and Suggs. While D-mannose, a carbon source, enhanced the growth of those fungi, especially it was effective in growing *Bipolaris* and *Drechslera* that grew a little bit slowly on potato sucrose agar. We have developed a selective medium based on potato extract broth containing D-mannose (1%), agar (1.5%), thiophanate-methyl (100mg・L^{-1}), and chloramphenicol (100mg・L^{-1}) as an antibiotic, and the pH was adjusted to 4.8. Plant pathogenic fungi, *Bipolaris, Drechslera* and *Exserohilum* were consistently isolated from diseased rice, oat, and red sprangletop respectively by using the selective medium.**

Key words: *Bipolaris, Drechslera, Exserohilum,* selective medium, thiophanate-methyl, mannose.

INTRODUCTION

The fungal genera, *Bipolaris*, *Drechslera* and *Exserohilum*, all of which belonged to former *Helminthosporium* Link, are known as graminicolous species which are pathogenic to Oryzoideae, Arundinoideae, Festucoideae, Eragrostoideae, and Panicoideae in the family of Gramineae. These fungi cause leaf spots, blights, and a variety of symptoms depending on the kinds of diseases and hosts. Some of these fungal species caused severe economic losses to crops all over the world (Ueyama et al. 1975). For example, *B. maydis* (Nishikado and Miyake) Shoemaker (teleomorph: *Cochliobolus heterostrophus* (Drechsler) Drechsler) caused southern corn leaf blight in the USA. *B. oryzae* damaged more than 90% of rice yields in South Asia. Furthermore, several isolates of the fungi showed pathogenicity to grassy weeds, for example, *E. monoceras* (Drechsler) Leonard and Suggs (= *D. monoceras* Ellis, Tsukamoto et al. 1997, Zhang and Watson 1997, Hirase et al. 2003) to barnyard grass (*Echinochloa* spp.), *B. setariae* (Sawada) Shoemaker (Figliola et al. 1988) to goose grass (*Eleusine indica* (L.) Gaertn.), *E. rostratum* (Drechslera) Leonard and Suggs (teleomorph: *Setosphaeria rostrata* Leonard, Chin et al. 2003) and *Exserohilum* sp. (Yamaguchi et al. 2005) to red sprangletop (*Leptochloa chinensis* (L.) Nees).

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Although *Bipolaris*, *Drechslera* and *Exserohilum* grow well on a standard medium such as potato sucrose agar, this medium is not suitable for the primary isolation of the fungi from diseased plants because its growth is disturbed due to faster growth of other fungi and bacteria. Therefore, it is important to develop a selective medium for isolating these fungi, which enables easier detection of the plant pathogens by suppressing the growth of saprophytic microorganisms to facilitate the enumeration of the pathogens. The present study aims to develop a simple selective medium for the primary isolation of *Bipolaris, Drechslera* and *Exserohilum* from diseased Gramineae plants.

MATERIALS AND METHODS

Fungal isolates. In this study, fungal isolates used were obtained from diseased plants. *B. oryzae* MKY4059, *D. avenacea* MKY4118 and *E. rostratum* MKY3010 were isolated from severely diseased rice (*Oryza sativa* L.), oat (*Avena sativa* L.) and red sprangletop, respectively, all of which were grown in Miyazaki prefecture, Kyushu. The isolates of these fungi, which had been shown to be pathogenic based on Koch's postulates, were stored potato sucrose agar slants under room temperatures in the laboratory.

Each of the fungal isolates was grown in 9-cm Petri dishes containing potato sucrose agar (PSA) at 25˚C in the dark for 5-7 days. This standard PSA medium was

Table 1. Growth of *Bipolaris, Drechslera* and *Exserohilum* on the media containing chemical fungicides

Fungicides	Fungal growth		
	B. oryzae	*D. avenacea*	*E. rostratum*
Benomyl	6.1±0.7(39.6)	9.7±0.9(39.4)	22.1±0.6(40.0)
Captan	7.0±0.7(45.5)	10.7±0.9(43.5)	26.1±0.7(47.2)
Chinomethionat	6.2±0.8(40.3)	11.6±1.0(47.2)	27.8±0.6(50.3)
Copper oxychloride	6.9±0.7(44.8)	11.1±1.0(45.1)	25.9±0.6(46.8)
Maneb	0(0)	0(0)	0(0)
Manzeb	0(0)	0(0)	0(0)
Polycarbamate	0(0)	0(0)	0(0)
Polyoxins	<2(<13.0)	<2(<8.1)	7±0.5(12.7)
Potassium bicarbonate	8.0±0.7(51.9)	13.6±1.0(55.3)	33.0±0.5(59.7)
Thiophanate-methyl	15.1±1.1(98.1)	24.2±1.1(98.4)	55.9±0.6(101.1)
Triflumizole	0(0)	0(0)	0(0)

Data, diameters of each colony except for agar disc 5 days after incubation at 25℃, are shown as means±SD derived from 9 replicates (mm).
( ): growth % on the control (without fungicide)

Table 2. Effects of the carbon sources on the growth of *Bipolaris*, *Drechslera* and *Exserohilum* species

Carbon sources	Fungal growth		
	B. oryzae	*D. avenacea*	*E. rostratum*
D-arabinose	14.3±0.8	23.7±0.8	55.0±0.7
D-fructose	12.0±1.1	23.3±1.0	53.0±0.7
D-galactose	14.0±1.1	24.0±1.1	54.4±0.8
D-glucose	15.3±0.8	24.7±1.1	54.9±0.6
D-mannose	17.1±0.9	25.7±0.9	56.1±0.9
D-ribrose	14.3±0.9	23.9±0.7	54.6±0.7
L-sorbose	3.1±0.3	3.8±0.4	4.8±0.6
D-xylose	14.7±0.9	24.2±0.9	54.8±0.6
Sucrose	15.4±0.8	24.6±1.1	55.3±0.7

Data, diameters of each colony except for agar disc 5 days after incubation at 25℃, are shown as means±SD derived from 9 replicates (mm).

prepared using the following materials: 200 g potato, 10 g sucrose, 15 g agar, and 1000 ml distilled water. The potatoes were boiled in distilled water until cooked and strained through a double layer of gauze to collect the broth. The volume was adjusted to one liter.

Fungicides. Chemical fungicides examined were copper oxychloride (Sun-Bordeaux, 73.5% active ingredient, Sumitomo Chem., Tokyo), benomyl (Benlate, 50% active ingredient, Sumitomo Chem., Tokyo), captan (Osocide, 80% active ingredient, Sumitomo Chem., Tokyo), chinomethionat (Morestan, 25% active ingredient, Sumitomo Chem., Tokyo), maneb (M-Dipher, 75% active ingredient, Sumitomo Chem., Tokyo), manzeb (Ziman-Dithane, 75% active ingredient, Dow Chem. Japan, Tokyo), polycarbamate (Bis-Dithane, 85% active ingredient, Hokko Chem., Tokyo), polyoxins (Polyoxin AL, 10% active ingredient, Nihon Noyaku, Tokyo), thiophanate-methyl (Topsin M, 70% active ingredient, Nippon Soda, Tokyo), potassium bicarbonate (Kari-Geen, 37% active ingredient, Sumitomo Chem., Tokyo), and triflumizole (Trifmine, 30% active ingredient, Nippon Soda, Tokyo). All fungicide formulations used in this study were wettable powders.

When the PSA medium was autoclaved for 20 minutes at 1.01×10^5 N/m^2 and cooled to around 60℃, each fungicide was added to the medium and suspended. The PSA containing each fungicide at a concentration of 100 ppm (active ingredient) was dispensed immediately into Petri dishes. After solidified enough, agar discs containing the mycelia of each fungus were cut from the margin of mycelial colonies using a 6-mm cork borer; placed on the surface of the medium; and then incubated at 25℃ for 5 days in the dark. The diameter of colony was measured as a fungal growth.

Carbon source. D-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-ribrose, L-sorbose, D-xylose, and sucrose (All substances were obtained from Wako Pure Chem., Osaka) were evaluated as a carbon source of the fungal growth in the agar medium described above, which contained 10g of each carbon substance. The isolates of *B. oryzae*, *D. avenacea and E. rostratum* were inoculated as agar discs with mycelia on the agar containing each carbon source, and incubated at 25℃ for 5 days in the dark. The fungal growth was estimated as a

Table 3. Fungal isolation from diseased plants with lesions by using the selective medium

Diseased Plants	No. of leaf-sections examined	No. of isolated fungi			
		Bipolaris	*Drechslera*	*Exserohilum*	Others
Oat	30	0	25	0	5
Red sprangletop	45	0	0	45	0
Rice	30	26	0	0	4

same manner described above.

Selective medium. The cultural plate in 9-cm Petri dish was designated as a selective medium for isolating *Bipolaris, Drechslera and Exserohilum*, which contained the following components per liter: 200g potato (used for extracting broth), 10g mannose, 15g agar, 100mg thiophanate-methyl (added after the medium was autoclaved), and 100mg chloramphenicol. The initial pH was adjusted to 4.8 by use of 0.1 N HCl.

The efficacy of the selective medium was assessed on the basis of recovery of *Bipolaris, Drechslera and Exserohilum* from diseased plants. Leaves with apparent lesions in oat, red sprangletop, and rice plants grown in the field were washed in running tap water to remove surface contaminants. Leaf-sections were cut at 2-3mm from the margin of actively growing lesions. The cut leaf-sections were placed on the cultural plates in Petri dishes; incubated at 25°C; and observed daily until the fungi emerged.

RESULTS AND DISCUSSION

Tests of the fungicides. The sensitivity of each fungus to the chemical fungicides *in vitro* was shown in Table 1. *B. oryzae*, *D. avenacea* and *E. rostratum* did not grow on the media containing 100ppm of maneb, manzeb, polycarbamate, and triflumizole. The fungal growth was mostly suppressed by polyoxins. It was suggested that the *Bipolaris*, *Drechslera and Exserohilum* species were susceptible to those fungicides. On the media containing 100ppm of benomyl, captan, chinomethionat, copper oxychloride, and potassium bicarbonate, the fungi grew in some extents; however, the growth was slower compared to those on control media without fungicides. On the other hand, the fungal growth was vigorous on the medium containing thiophanate-methyl. The results revealed that *Bipolaris, Drechslera* and *Exserohilum* were resistant to thiophanate-methyl at a concentration of 100ppm.

Therefore, thiophanate-methyl was suggested to be useful for the selective medium. Moreover, as thiophanate-methyl has been reported to be effective against a wide range of fungi including *Monilia*, *Gloeosporium, Botrytis, Sclerotinia, Corticiium, Fusarium, Pyricularia,* this chemical fungicide is expected to suppress the growth of saprophytic fungi on Gramineae such as oat, red sprangletop, and rice plants.

Evaluation of the carbon sources. All carbon sources listed above except L-sorbose seemed to be suitable for the growth of *B. oryzae, D. avenacea* and *E. rostratum* (Table 2). Among the 9 kinds of carbon sources, D-mannose was indicated to be the best for the isolation of these fungi, especially for that of *Bipolaris* and *Drechslera* which grew slower than *Exserohilum* on the PSA medium. The data on *B. oryzae* and *D. avenacea* showed statistically significant differences between D-mannose and sucrose ($P<0.05$).

Efficacy of the developed selective medium. *B. oryzae, D. avenacea* and *E. rostratum* grew well on the developed

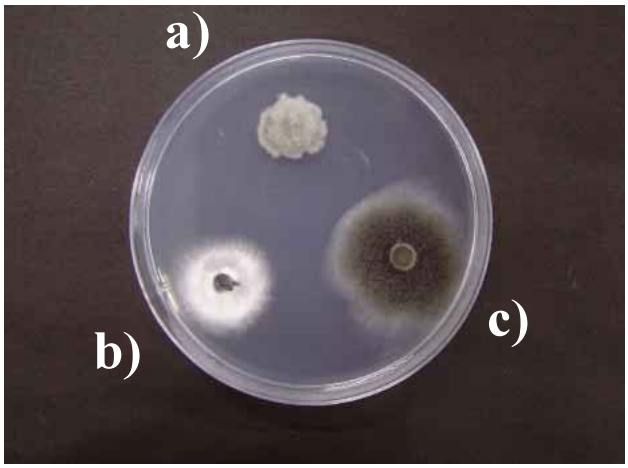

Fig. 1. Fungal growth on the selective medium 4 days after inoculation. a) *B. oryzae*; b) *D. avenacea*; c) *E. rostratum*

Fig. 2. Development of *Exserohilum* from diseased red sprangletop on the selective medium 6 days after incubation.

selective media containing thiophanate-methyl (Fig.1). By using this selective medium, the fungi were consistently isolated from the diseased tissues of rice, oat and red sprangletop (Table 3). The fungal isolates from rice, oat and red sprangletop were identified as *Bipolaris*, *Drechslera* and *Exserohilum*, respectively, under microscopic observations by checking conidium formation and germination (Nishihara 1991). In fact, *Exserohilum* was recovered from all lesions formed on leaves of red sprangletop collected in Kumamoto prefecture in 2007 (Fig.2, Table 3). *Bipolaris* and *Drechslera* were isolated from approximately 85% of diseased rice and oat, collected in Miyazaki prefecture in 2008. However, other fungi such as *Rhizopus* sp. and *Mucor* sp., which grew much faster than *Bipolaris* and *Drechslera* sometimes appeared on the media, and disturbed the growth of *Bipolaris* and *Drechslera*. Suppression of the emergence of *Rhizopus* sp. and *Mucor* sp. on the medium should be focused in future studies. In addition to thiophanate-methyl, the chloramphenicol at a concentration of 100ppm successfully suppressed the growth of bacteria in the selective medium (pH 4.8). Therefore, the selective medium was effective for isolating *Bipolaris*, *Drechslera* and *Exserohilum* from diseased plants even when many saprophytic microorganisms were present under natural conditions.

Bipolaris, Drechslera and Exserohilum cause epidemic and catastrophic diseases in several kinds of crops, and are still recognized as important plant pathogens. Moreover, it has been reported that several isolates of these fungi were pathogenic to grassy weeds and shown as potential bioherbicides (Yamaguchi 2006). The simple selective medium developed in this study may be useful not only for ecological and epidemiological studies of *Bipolaris, Drechslera* and *Exserohilum* but also for the development of biocontrol agents for grassy weeds. Further experiments will be needed to determine the efficacy of the selective medium for isolating another species of *Bipolaris, Drechslera* and *Exserohilum*.

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*Bipolaris, Drechslera, Exserohilum*属菌の
一次分離に有効な簡易選択培地

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要　約

*Bipolaris, Drechslera, Exserohilum*属の糸状菌は，何れもトウモロコシなどの作物や牧草類，芝などに病害を引き起こす重要な植物病原菌として知られている．一方，最近では，これらの糸状菌をヒエやアゼガヤなどイネ科雑草の生物的防除に利用する研究が精力的に進められている．本研究では，*Bipolaris, Drechslera, Exserohilum*の分離に有効な選択培地の確立を試みた．腐生菌を抑制することを目的として供試した11種類の殺菌剤の中で，広範囲の植物病害に有効とされるチオファネートメチルは，100ppmの濃度でこれら3属菌（*B. oryzae, D. avenacea, E. rostratum*）の菌糸成長に影響を及ぼさなかった．また，9種類の炭素源の中では，Dマンノースが，PSA培地上で*E. rostratum*に比べて生育の遅い*B. oryzae* 及び*D. avenacea* の菌糸成長を促進した．ジャガイモ煎汁液にDマンノース（10g/L）を加え，抗菌剤としてチオファネートメチル（有効成分で100mg/L）を，更に抗生物質としてクロラムフェニコール（100mg/L）を加用し，培地中のpHを酸性（pH4.8）としたものを，これら3属菌の一次分離を目的とする選択培地とした．この選択培地を利用することにより，自然条件下で発病したイネ，エンバク，アゼガヤの各罹病葉組織から簡便に*Bipolaris, Drechslera, Exserohilum*属菌が分離され，その有効性が確認された．