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Matsumoto, Masaru
Institute of Tropical Agriculture, Kyushu University

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A Qualitative Baiting Technique for Selective Isolation and DNA Diagnosis of *Rhizoctonia* spp., Causal Agents of Rice Sheath Diseases, from Soil

Masaru MATSUMOTO*

Institute of Tropical Agriculture, Kyushu University, Fukuoka 812-8581, Japan
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A baiting technique was evolved for selective isolation and DNA diagnosis of causal agents of rice sheath diseases by *Rhizoctonia* spp. from artificially inoculated and naturally infested soils using eleven plant stem segments and selective medium containing benomyl and metalaxyl. Rice, mat rush and wheat stem segments soaked in metalaxyl at 50 µg/ml were successfully used to isolate *R. solani* AG 1, *R. solani* AG 2, *R. oryzae*, *R. oryzae-sativae* and *R. fumigate* from artificially infested soils. Moreover, rice and wheat stem segments soused in metalaxyl at 100 µg/ml were successfully used to extract genomic DNA derived from all *Rhizoctonia* spp. Metalaxyl untreated stems were colonized in significantly higher numbers and contained in significantly higher concentration of DNA of these *Rhizoctonia* isolates than untreated stems. Untreated stems were also colonized isolates and extracted DNA derived from other common soil-inhabiting fungi.

INTRODUCTION

Sheath blight disease of rice plant is distributed widely in rice growing countries from tropics to the temperature regions and is now ranging next to blast in economic importance (Yamada, 1986). The incident of disease is belonging to the genus *Rhizoctonia* (deCandolle, 1815) and *R. solani* Kuhn anastomosis group (AG) 1 IA is recognized as the dominant pathogen of this disease. Several other fungi belonging to *Rhizoctonia* spp. have also been isolated from sheath blight-like lesions on rice plants. These are *R. solani* AG 2 IIB, *R. oryzae*, *R. oryzae-sativae* and *R. fumigate* (Matsumoto et al., 1997). Since these *Rhizoctonia* spp. are soil-inhabiting fungi and cause similar symptoms on rice sheaths, differentiation and identification based on visual observation is often difficult. Therefore, a simple method for qualitative determination of soil-inhabiting *Rhizoctonia* would assist in understanding their symptomatology.

Qualitative determination of *Rhizoctonia* spp. in infected plants is made by isolations from infected host plant tissues. Susceptible 'bait' plant is used for quantitative estimation of inoculum density and/or inoculum potential of the pathogen in soil. Baiting techniques is also used for studying the occurrence and distribution of *Rhizoctonia* spp. by using seeds, paper disk, or excised plant parts as baits (Herr, 1973; Papavizas et al., 1975; Papavizas and Davey, 1962; Sneh et al., 1966) and usually semiselective, and several *Rhizoctonia* spp. may be isolated. In this case, characterization of these fungi may be time-consuming because they are not readily distinguishable during initial growth in culture.

* Institute of Tropical Agriculture, Kyushu University (E-mail: mmatsu@agr.kyushu-u.ac.jp)

The aim of this study is to develop a baiting technique for the selective isolation of *Rhizoctonia* spp. and selective extraction of *Rhizoctonia* spp. DNA from soil samples.

MATERIALS AND METHODS

Soils

A Kasuya clay loam was collected from paddy field of Kasuya-cho of Fukuoka Prefecture, Japan. These soils were put through a sieve with an opening of 3 mm, moistened with an atomizer at moderate humidity and stored in a refrigerator at 5 °C until use.

Effect of fungicides on radial growth

Two fungicides were evaluated for their effect on radial growth of five isolates each of *R. solani* AG 1 IA, AG 2-2 III B, *R. oryzae*, *R. oryzae sativae* and *R. fumigate*. Stock culture was maintained on potato-dextrose agar (PDA) slants amended with streptomycin (20 mg/L) at 25 °C until use.

Benomyl (50% WP; Sumitomo Chem. Co., Japan 20889) and metalaxyl (0.5% WP; Sankyo Co., Japan 15702) were suspended in sterile distilled water and added at 10 and 50 µg/ml to sterile cool PDA before the medium was dispensed into plastic petri dish. A 3 days old PDA culture was transferred on PDA containing fungicides at 0, 10 and 50 µg/ml and incubated at 28 °C. The radius of growth on each plate was measured at 24 and 48 hours.

Preparation of artificially inoculated soils and bait plants for baiting technique

The Kasuya clay loam soils from paddy files were used in this experiment. Tested culture grown on fusuma medium amended with barley juice at 28 °C for 5 days was mixed to equal volume of heat-sterilized soil in beakers covered with aluminum foil and incubated at 28 °C for 10 days.

Total 11 of plant materials of soybean, tobacco, eggplant, tomato, cucumber, rice, radish, potato, wheat, mat rush and mung bean were used as the bait plant segments for isolation and DNA extraction of *Rhizoctonia* rice sheath pathogens. Plant materials were cut into 10 cm stems, dried with oven at 150 °C for 1 hr and autoclaved at 121 °C for 30 min. Autoclaved plant segments were stocked at room temperature until use.

Baiting technique for isolation and DNA extraction of *Rhizoctonia* spp. from soils

A technique previously used for isolating *R. zoeae* (Windham and Lucas, 1987) was modified in this experiment. Plant stems were cut into 10 mm segments and autoclaved at 120 °C for 30 min. Stem segments were soaked for 2 hr in a suspension of benomyl at 500 µg/ml plus metalaxyl at 100 µg/ml or in a benomyl suspension at 1000 µg/ml and blotted dry. Nontreated stem segments were soaked in sterile distilled water for 2 hr used as a control. Treated and nontreated stem segments were used to the following bait experiment.

The Kasuya clay loam of naturally infected and artificially infested soils were used in this experiment. Twenty stem segments treated with two fungicides of each plant materials were added to an equivalent of 150 g of oven-sterilized soil in 600 ml beaker. The

beakers were covered with cellophane film and incubated at 25 °C for 72 hr. The stems were removed, washed in tap water for 20 min, blotted dry with sterilized paper towels and placed on 2% water agar. Plates were observed at 24 and 48 hrs for *Rhizoctonia* spp. mycelium, transferred on PDA with hyphal tip from mycelial colonies and grown at 28 °C for 2 weeks. *Rhizoctonia* spp. were identified using biological characteristics previously published (Boerma *et al.*, 1977; Voorhees, 1934).

DNA extraction and PCR

Genomic DNA used in this experiment was derived from *Rhizoctonia*-like fungal DNA adsorbed by the baiting plant materials. Plant materials were cut into 5 cm stems and sterilized with oven at 150 °C for 2 hr. The segments were soaked for 2 hr in a suspension of benomyl at 250 µg/ml plus metalaxyl at 50 µg/ml or in a benomyl suspension at 500 µg/ml and blotted dry. Five stems were added to an equivalent of 150 g of oven-sterilized soil in 600 ml beaker. The beakers were covered with cellophane film and incubated at 25 °C for 72 hr. The stems were removed, washed in tap water for 20 min, blotted dry with sterilized paper towels and used for DNA extraction.

Extraction of genomic DNA from bait plant materials was performed based on the alkaline DNA extraction method (Matsumoto *et al.*, 1997). Before DNA extraction, each plant stem segment was soaked for 5 min in 1 ml of sterile 0.05M phosphate buffer, pH 7.0, containing 0.05% NaCl and 0.01% Tween 20 [phosphate buffered saline (PBS)]. These samples were dissolved in 300 µl of 0.5N NaOH with 0.5% (wt/vol) polyvinylpyrrolidone (PVP; Sigma Chem. Co.). Five microliters of the lysate sample was transferred to a new tube containing 495 µl of 20mM Tris-HCl, pH 8.0. Aliquots of 5 µl were used for individual PCR amplifications. Water was used as a negative control.

PCR amplification was performed in a reaction mixture containing 5 µl of DNA extract in 20 mM Tris-HCl, pH 8.0., 2.5 units of *Taq* DNA polymerase (Promega), 10 µl of 10× *Taq* PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 100 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 50 pmol each of forward and reverse primers, 2.5 mM MgCl₂, and sterile water in a total volume of 100 µl in a 0.5-ml of microfuge tube. Fifty microliters of mineral oil (Katayama Chem. Co.) were added to each tube to prevent evaporation. For identification of *Rhizoctonia* species, species-specific primers were used (Matsumoto, 2002). The program for amplification consisted of 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 2 min and DNA extension at 72 °C for 3 min. Aliquots (10 µl of the amplification products) were subjected to electrophoresis using a 2.0% agarose gel with Tris-borate-EDTA buffer at 100V for 1 h and the gel was stained with ethidium bromide.

RESULTS

Effect of fungicides on radial growth

Radial growth of *Rhizoctonia* spp. has been completely inhibited by the treatment of 10 and 50 µg/ml of Benomyl compound expect for *R. oryzae* (Table 1.). Although benomyl showed the radical growth of *R. oryzae* isolates by about 30% percentage inhibition at a treatment of 50 µg/ml, another isolates of *Rhizoctonia* were completely inhibited or almost inhibited in radial growth at the treatment of 10 and 50 µg/ml. Metalaxyl showed

Table 1. Effect of selected fungicides on radial growth of *Rhizoctonia* spp.

Rhizoctonia specie	Isolates	Percentage inhibition of growth treated with 1)			
		Benomyl		Metalaxyl	
		10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
<i>R. solani</i> AG1IA	RS114	95	99	0	3
	RS117	98	100	0	0
	RS138	99	100	0	1
<i>R. solani</i> AG22III B	RS232	93	99	0	0
	RS237	94	100	0	0
	RS246	96	99	0	0
<i>R. oryzae</i>	RO11	15	37	2	16
	RO17	18	35	1	17
	RO20	20	33	4	12
<i>R. oryzae-sativae</i>	CO14	87	95	5	9
	CO36	90	98	6	11
	CO39	85	94	3	10
<i>R. fumigata</i>	CF55	99	100	0	2
	CF67	95	100	0	0
	CF71	100	100	0	0

1) Growth at 48hr compared with growth on potato-dextrose agar

the non inhibition or the slight inhibition of radial growth of isolates of *Rhizoctonia* spp. at a concentration of 10 µg/ml.

Isolation of *Rhizoctonia* isolates from artificially inoculated and soils using baiting technique

The use of benomyl and metalaxyl treated bait plants relatively increased the colonization of *Rhizoctonia* spp. from artificially inoculated soils with plant baits, compared with the use of benomyl-treated and fungicide-free segments (Table 2.). High percentage of colonization of *Rhizoctonia* spp. was observed by using stem segments of wheat, rice and mat rush stems as the bait plants. Tested bait plants of cucumber, potato and tomato were also slightly isolated from stem segments presoaked with benomyl plus metalaxyl. However the other plant stem segments, like as soybean, eggplant, radish, and mung bean were not observed the colony growth of *Rhizoctonia* spp. (data not shown). The inclusion of benomyl in the stem presoak effectively inhibited the isolation of rice sheath fungus *Rhizoctonia* spp. from artificially inoculated soils. Many common soil-borne fungi, such as species of *Alternaria*, *Fusarium*, *Phthium* and *Trichoderma* were isolated from untreated stem segments of all tested plant stem segments.

There were significant differences in isolation of *Rhizoctonia* spp. as determined by linear contrasts with a single degree of freedom among benomyl, benomyl plus metalaxyl, and fungicide-free treatments. There were slight differences in isolation of *Rhizoctonia* spp. from three plant segments, such as wheat, rice and mat rush from artificially inoculated soils. There were not also significant differences in isolation of *Rhizoctonia* spp. from plant stem segments such as cucumber, potato and tomato among presoaked fungicide treatments and non-fungicide treatment.

Table 2. Isolation of *Rhizoctonia* spp. from artificially inoculated soil with 6 plants stems presoaked in benomyl and metalaxyl on 2% water agar.

Fungicide	Rate ($\mu\text{g/ml}$)	Species	Stem segment colonization from plant baits of (%) ¹⁾					
			Wheat	Rice	Mat rush	Cucumber	Potato	Tomato
Benomyl + Metalaxyl	500,100	<i>R. solani</i> AG1IA	48.0	71.0	68.0	18.0	21.0	11.0
		<i>R. solani</i> AG22III B	45.0	74.0	65.0	31.0	20.0	19.0
		<i>R. oryzae</i>	44.0	68.0	61.0	15.0	5.0	11.0
		<i>R. oryzae-sativae</i>	43.0	54.0	69.0	18.0	8.0	5.0
		<i>R. fumigata</i>	41.0	63.0	63.0	19.0	12.0	7.0
Benomyl	1000	<i>R. solani</i> AG1IA	23.0	20.0	31.0	14.0	8.0	5.0
		<i>R. solani</i> AG22III B	31.0	24.0	24.0	15.0	10.0	8.0
		<i>R. oryzae</i>	24.0	31.0	30.0	20.0	4.0	6.0
		<i>R. oryzae-sativae</i>	27.0	24.0	20.0	9.0	10.0	10.0
		<i>R. fumigata</i>	11.0	15.0	24.0	11.0	11.0	6.0
Control		<i>R. solani</i> AG1IA	15.0	23.0	27.0	15.0	7.0	2.0
		<i>R. solani</i> AG22III B	12.0	14.0	15.0	12.0	9.0	1.0
		<i>R. oryzae</i>	19.0	11.0	12.0	9.0	6.0	5.0
		<i>R. oryzae-sativae</i>	13.0	10.0	13.0	12.0	9.0	0.0
		<i>R. fumigata</i>	12.0	11.0	11.0	10.0	8.0	0.0

1) Percentage of 50 stem segments of each plant bait colonized by *Rhizoctonia* spp.

Table 3. DNA diagnosis assay for rice sheath pathogens by *Rhizoctonia* spp. from artificially inoculated and naturally infested soils baited with 11 plants stems segments using PCR analysis

Bait plant	Soil sample ²⁾	DNA diagnosis using PCR analysis ³⁾				
		<i>R. solani</i> AG1IA	<i>R. solani</i> AG22III B	<i>R. oryzae</i>	<i>R. oryzae-sativae</i>	<i>R. fumigata</i>
Wheat	Artificially	+ ³⁾	+	+	+	+
	Naturally	+	+	+	+	+
Rice	Artificially	+	+	+	+	+
	Naturally	+	+	+	+	+
Mat rush	Artificially	-	+	-	-	+
	Artificially	-	+	-	-	-
Cucumber	Naturally	-	-	-	-	-
	Artificially	-	-	-	-	-
Potato	Naturally	-	-	-	-	-
	Artificially	-	-	-	-	-
Tomato	Artificially	-	-	-	-	-
	Naturally	-	-	-	-	-
Eggplant	Artificially	-	-	-	-	-
	Naturally	-	-	-	-	-
Radish	Artificially	-	-	-	-	-
	Artificially	-	-	-	-	-
Tobacco	Naturally	-	-	-	-	-
	Artificially	-	-	-	-	-
Soy bean	Naturally	-	-	-	-	-
	Artificially	-	-	-	-	-
Mung bean	Artificially	-	-	-	-	-
	Naturally	-	-	-	-	-

1) Fungicide-treated stem segments were presoaked in suspension containing benomyl at 500 $\mu\text{g/ml}$ and metalaxyl at 100 $\mu\text{g/ml}$ before adding to soil samples

2) Soils were used the Kasuya clay soil with artificially inoculated and naturally infested samples

3) +; *Rhizoctonia* spp. was detected -; *Rhizoctonia* spp. was not detected

DNA detection assay for *Rhizoctonia* spp. from soil

When the fungicide-treated plant stem segments of wheat and rice were used for DNA diagnosis, rice sheath pathogens caused by *Rhizoctonia* spp. were detected from artificially inoculated and naturally infested soils by PCR analysis (Table 3 and Figure 1.). In this experiment, PCR analysis also revealed that DNA diagnosis with bait plant of mat rush was detected 5 species of *Rhizoctonia* from artificially inoculated soil and also detected *R. solani* AG 2 2 III B and *R. fumigata* from naturally infested soil, respectively (Table 3.). When the other plant baits were used for DNA diagnosis, target species of *Rhizoctonia* were not detected from artificially inoculated and naturally infested soils.

Figure 1 shows electrophoretic pattern of PCR amplified rDNA from plant stem segments of rice (A) and wheat (B) in artificially inoculated soils. Stringent PCR conditions allowed amplification of a single fragment of rDNA with the same size of about 500 bp in *R. solani* AG 1 IA (a), AG 2 2 III B (b) and *R. oryzae* (e) and one with a size of about 450 bp of *R. oryzae-sativae* (c) and *R. fumigata* (d) (Fig. 1A and 1B). Homologous PCR amplification products related to the five *Rhizoctonia* spp. were also obtained from rice and wheat stem segments in naturally inoculated soil.

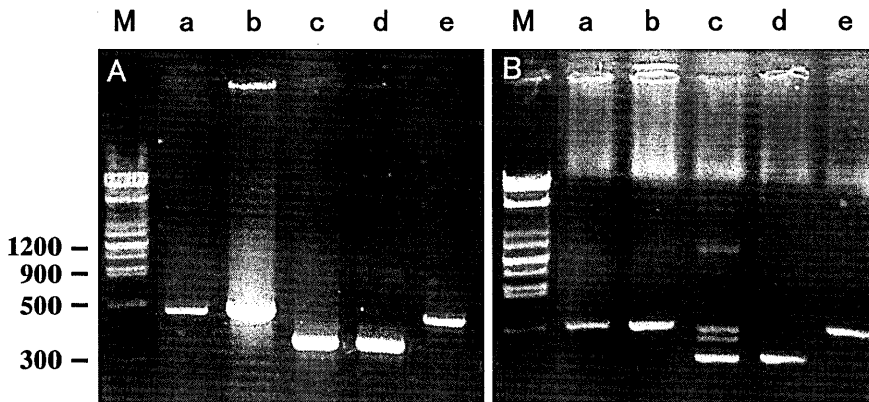


Fig. 1. Electrophoretic pattern of PCR-amplified rDNA of five *Rhizoctonia* spp. obtained from plant baits of rice (A) and wheat (B) using the *Rhizoctonia* species-specific primer sets in a 2% agarose gel. M, molecular marker (λ -DNA digested with *Bam*HI and *Hind*III); lane a, *R. solani* AG1IA; lane b, *R. solani* AG22III B; lane c, *R. oryzae-sativae*; lane d, *R. fumigata*; lane e, *R. oryzae*. Sizes of DNA fragments are indicated on the left.

DISCUSSION

Use of fungicide-treated baits not only enhanced isolation of *Rhizoctonia* spp. but also carried out DNA diagnosis of their fungal DNA from artificially inoculated and naturally infested soils. Selection of bait plants was assessed for investigating compatibility between bait plants and the species of *Rhizoctonia*. Common soil-inhabiting fungi that colonize bait plants were inhibited by the benomyl and metalaxyl-treated baits.

Effectively of benomyl treatment was tested for evaluating fungicide sensitivity of *R. solani* and *Rhizoctonia*-like fungi in vitro by Martin *et. al.* (1984). Moreover previous report suggested that the recovery of *R. zae* were effectively enhanced by using benomyl and metalaxyl treated cotton plant as the bait (Windham and Lucas, 1987). The use of cotton plant was not only brought the colonization of *R. zae* but also inhibited the colonization of *R. solani* and binucleate *Rhizoctonia*-like fungi. Although cotton plant as the bait was not used for isolation of rice sheath pathogens by *Rhizoctonia* spp. in this experiment, benomyl and metalaxyl treatment of bait plants effectively enhanced the isolation of species of *Rhizoctonia* (Table 1.). Rice, wheat and mat rush plants as the baits were effectively affected isolation of rice sheath pathogens caused by *Rhizoctonia* and inhibited isolation of *Rhizoctonia*-like fungi. The other tested bait plants used in this experiment were also effectively inhibited isolation of not only *Rhizoctonia* spp. but also *Rhizoctonia*-like fungi (Table 2.).

The differences in the bait plants for DNA diagnosis of rice sheath pathogens caused by *Rhizoctonia* were obaseved by PCR analysis from polluted Kasuya loam soils. DNA extraction method used for detection of soil-inhabiting microorganisms in the soil is usually accompanied a time and labor consuming. In addition to procedure of DNA extraction is disposed to complex operations and small mistakes. DNA diagnosis of soil-inhabiting fungi such as *Rhizoctonia* spp. from polluted soils with plant baits is used for the first time in this experiment and obtained interesting results. PCR analysis revealed that benomyl and metalaxyl-treated plant baits of rice, wheat and mat rush were successfully detected isolates of *R. solani* AG 1 IA, AG 2 2 III B, *R. oryzae*, *R. oryzae-sativae* and *R. fumigata* from artificially inoculated and naturally infested soils (Table 3., Fig. 1A and 1B). DNA extractions obtained from these bait plants were not detected isolates of *Rhizoctonia*-like fungi, common soil-inhabiting fungi and common soil bacteria.

The techniques described in this experiment would be satisfactory for use in direct isolation and DNA diagnosis of rice sheath pathogens caused by *Rhizoctonia* spp. using baiting techniques such as distribution studies. Before the technique can be used to estimate population of *Rhizoctonia* spp. in soils using molecular biological methods, studies will need to be conducted to extract directly total genome DNA from soils.

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