



Characterization of nonpathogenic *Cadophora gregata*, a potential biological control agent, concomitantly isolated from soil infested with *Cadophora gregata* f. sp. *adzukicola*, the cause of adzuki bean brown stem rot

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ABSTRACT

We collected 555 isolates of *Cadophora gregata* from adzuki bean field soils in Hokkaido, Japan, from 1997 to 2000. To identify the brown stem rot (BSR) pathogen *C. gregata* f. sp. *adzukicola*, we screened these isolates for pathogenicity to adzuki beans. Of the isolates, all of which originated in Tokachi District, Hokkaido, Japan, 23 were avirulent to adzuki bean, soybean, or mung bean. However, polymerase chain reaction (PCR) with specific primers for *C. gregata* f. sp. *adzukicola* (BSRA1 and BSRA2) detected the specific identifying DNA fragment in these isolates, and cluster analysis with inter-simple sequence repeat markers showed that the isolates were phylogenetically closer to strains that are virulent to adzuki bean. Thus, we concluded that the isolates were nonpathogenic *C. gregata*. A few selected isolates of the nonpathogenic *C. gregata* were effective at reducing BSR *in vivo* and show potential for development as biological control agents.

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1. Introduction

Brown stem rot (BSR), an economically important disease of adzuki bean [*Vigna angularis* (Willd.) Ohwi et Ohashi], is caused by *Cadophora gregata* (Harrington and McNew, 2003). Isolates of *C. gregata* from adzuki bean and soybean are host-specific and are recognized as two different formae speciales, *C. gregata* f. sp. *adzukicola* (CGA) and *C. gregata* f. sp. *sojae* (CGS), respectively (Kobayashi et al., 1983, 1991). The pathogens can be differentiated from each other and other fungi based on isozyme banding patterns (Yamamoto et al., 1990), DNA sequences of internal transcribed spacer (ITS) regions of rDNA (Chen et al., 1996, 1999; Harrington et al., 2000), DNA sequences of intergenic spacer (IGS) regions of rDNA (Chen et al., 2000), microsatellite markers (Chen et al., 2002), and amplified fragment length polymorphisms (AFLPs) or inter-simple sequence repeats (ISSRs; Meng and Chen, 2001). Once soilborne CGA is inside adzuki bean plants by invading root tissues of young plants, it spreads into the vascular and pith tissue via mycelia and conidia production (Narita et al., 1971). This fungus infects vascular tissue and causes pith and vascular tissue discoloration of the stem and petiole, in conjunction with foliar chlorosis or necrosis. Consequently, the pathogen can cause susceptible plants to wilt and reduce adzuki bean yields. Although phytotoxic gregatins produced by CGA previously were thought to be associated with

these symptoms (Kobayashi and Ui, 1977), Tanaka et al. (2007) found that the toxins were unlikely to be essential for pathogenicity.

Extensive studies have been performed on phenotypical variations (races) of CGA isolates (Kondo et al., 1998, 2002), adzuki bean resistance to BSR (Adachi et al., 1988; Chiba, 1982, 1985; Chiba et al., 1987; Fujita et al., 1995, 2002, 2007), and the close ecological association of *C. gregata* with nematodes (Djiwanti et al., 1999; Sugawara et al., 1997a; Yamada et al., 2005a,b). To date, disease-resistant cultivars and crop rotation are the most practical means of controlling BSR. Indeed, Fujita et al. (2007) and Kondo et al. (2009) found one cultivated adzuki bean variety and one wild adzuki bean accession that tolerated all of the CGA races, making them useful for breeding BSR-resistant adzuki bean cultivars. Yamada et al. (2005b) showed that using wild oats [*Avena strigosa* Schreb.] as green manure decreased the degree of BSR damage by suppressing nematodes. However, as shown by the quick appearance of a strain virulent to recently developed resistant breeding lines (Kondo et al., 2005), BSR outbreaks in new races may be inevitable, due to the co-evolutionary relationship between crop varieties and their pathogens.

The distribution of CGA races has been examined using isolates collected from adzuki bean field soils in Hokkaido (Kondo et al., 2002). Pathogenicity tests revealed that most isolates were virulent to the susceptible cultivar Erimo-shozu, although several avirulent isolates were discovered. Therefore, we examined the pathogenicity of additional isolates to investigate the distribution of nonpathogenic *C. gregata* (NPC). Some nonpathogenic strains

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of plant pathogenic species may have the potential to protect the plant against infections caused by the virulent strains (Sneh, 1998). Moreover, understanding NPC may enable us to optimize ecological conditions to enhance its suppressive ability in crop rotations. Our objectives were to identify NPC strains, their characteristics, and their potential as biological control agents of adzuki bean BSR.

2. Materials and methods

2.1. Sources and cultivation of isolates

As described in a previous study (Kondo et al., 2002), *C. gregata* was isolated from 44 adzuki bean field soil samples collected from five districts in Hokkaido from 1997 to 2000 using a modified selective medium soil dilution method (Table 1). Single-spored isolates were stored in green-pea agar (GPA; 200 g frozen green peas boiled 15 min, filtered through four layers of cheesecloth, and solidified using 20 g agar/l) at 4 °C. Sporulation ability and cultural morphology were determined using V8-juice agar (200 ml V8 juice and 2 g CaCO₃ centrifuged at 5000 rpm for 15 min, with the supernatant diluted to 1 l with distilled water and solidified using 20 g agar/l) and potato dextrose agar (PDA; Difco, Lawrence, Kansas, USA), respectively. For long-term storage, agar disks containing spores of each isolate grown on GPA were placed in individual cryovials containing 20% sterile glycerol and maintained at –80 °C. In Table 2 four nonpathogenic isolates that were originally obtained as CGA and CGS from diseased adzuki bean and soybean in Japan, respectively, were included and also used in this study: a virulence-deficient mutant A'31-2 (Kobayashi et al., 1981); isolates A60K68 and A60To, which were originally identified as virulent (Yamamoto, 1994); and isolate A57T22, which was uncertain for virulence.

2.2. Cultural conditions and screening of nonpathogenic strains

The inoculum was grown in V8-juice broth at 25 °C on a reciprocal shaker at 120-oscillations/min. After 3 weeks of incubation, mycelia and spores were collected by filtration through Whatman No. 1 filter paper and washed by suspending them in distilled water, followed by centrifugation. The fungal pellets were homogenized in distilled water with a homogenizer (10,000 rpm for 3 min; AN-5, Shin-nihonseiki, Tokyo, Japan) and the concentration of mycelial fragments and spores was determined using a hemocytometer; mycelial fragments of all sizes were included in counts. Blended cultures were then diluted with distilled water to a concentration of 10⁷ propagules per milliliter. The adzuki bean varieties used to determine fungal races were cvs. Erimo-shozu (susceptible to all races), Kita-no-otome (resistant to races

1 and 3, but susceptible to race 2) and Acc259 (resistant to races 1 and 2, but susceptible to race 3). Seedlings were grown in plastic containers (15 × 20 × 5 cm) with sterilized vermiculite for about 10–14 days in greenhouse, then the roots were washed gently with running tap water. The roots of 10 seedlings of each cultivar were dipped into each inoculum suspension (50 ml) for 12 h, then transplanted into a soil (Pot-ace, Katakura Chikkarin K.K. Tokyo, Japan)/vermiculite mixture (1:1, v/v) in 12-cm diameter plastic pots. The response to the pathogen was evaluated after 8 weeks growth in greenhouse. Nighttime low and daytime high temperatures during these tests were 15/32 °C. Plants received supplemental lighting from metal halide sodium lamps (400 W) to maintain a 14-h photoperiod. The inoculation experiments were completely randomized, with two replications (pots) per isolate per cultivar and five plants per pot. Pathogenicity tests were repeated twice with these cultivars, and nonpathogenic isolates were determined. Soybean [*Glycine max* (L.) Merrill. cv. Sapporomidori] and mung bean [*Vigna radiata* (L.) R. Wilczek, susceptible to both CGS and CGA] were also used to determine formae speciales of *C. gregata*. The roots of 10 seedlings of each crop grown as described above were dipped into a suspension (50 ml) of each inoculum (Table 3) for 12 h. The seedlings were then transplanted into the sterilized mixed soil in 18-cm-diameter plastic pots. Isolates T96-1 (race 1 CGA), T96-5 (race 2 CGA), and S58KS (CGS) also were used as controls. For BSR assessment, the number of diseased plants in the greenhouse with foliar symptoms (stunted and necrotic) or vascular discoloration was counted 8 weeks after inoculation.

2.3. Production of gregatins

Isolates were grown in adzuki bean stem medium (5 g dry stem pieces boiled 15 min, filtered through four layers of cheesecloth, with the filtrate diluted to 1 l using distilled water) containing 5% glucose (Kobayashi and Ui, 1975) on a reciprocal shaker at 120-oscillations/min for 4 weeks at 25 °C. The culture filtrate (200 ml) was adjusted to pH 7.0, extracted once with an equal volume of ethyl acetate, and washed with distilled water. The ethyl acetate solution was evaporated, yielding an oily residue. This extract was dissolved in acetone (200 µl), and 3 µl was spotted onto a thin-layer chromatography (TLC) silica gel plate (Silica gel 60 F₂₅₄; Merck, Darmstadt, Germany). The solvent system was chloroform:methanol (98:2, v/v). Gregatins were visualized by UV irradiation (254 nm). For positive and negative controls, extracts from a culture of wild-type strain T96-5 and non-inoculated medium were used, respectively.

2.4. DNA extraction from mycelia

For DNA extraction, agar plugs were removed from the growing margin of 1-week-old cultures and transferred to V8-juice broth. After a 14-day incubation period at 25 °C, mycelial mats were harvested by filtering the broth through Whatman No. 1 filter paper in a Buchner funnel then cleaned by rinsing each mat several times with distilled water. After removing the excess water, all mycelial mats were frozen at –80 °C until they were ground to a fine powder in liquid nitrogen using a sterilized mortar and pestle. The DNA extraction was conducted using the DNeasy Plant Mini Kit (QIA-GEN, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was determined by UV/Vis spectrophotometer (DU640; Beckman Coulter Inc., Fullerton, CA, USA) and the purity of the DNA samples was examined by electrophoresis in 0.8% agarose (Wako Pure Chemical Industries, Osaka, Japan) gel (TBE buffer).

Table 1

Distribution of virulent and avirulent isolates of *Cadophora gregata* on adzuki bean collected from brown stem rot (BSR) infested fields during 1997 to 2000 in Hokkaido.

District	No. of fields surveyed	No. of isolates	
		Virulent	Avirulent
Shiribeshi	5	45 (45) ^b	0
Iburi	4	52 (52)	0
Kamikawa	8	43 (40)	0
Ishikari	1 ^a	36	0
Tokachi	26	379 (346)	23
Total	44	555 (483)	23

^a The experiment field of Hokkaido University, Sapporo.

^b Number in parenthesis shows the number of isolates described in the result (Table 1) of Kondo et al. (2002).

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