Detection of Two Races of *Phialophora gregata* f. sp. *adzukicola*, the Causal Agent of Adzuki Bean Brown Stem Rot

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ABSTRACT

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Adzuki bean brown stem rot (BSR) is endemic on Hokkaido Island and has been controlled since 1985 by using resistant cultivars. BSR was reported on the resistant cultivar Kita-no-otome in a field near Memuro-cho, and this study was undertaken to determine if pathogenic races were present. The existence of avirulent and virulent isolates of the pathogen to cultivar Kita-no-otome was shown by comparing the virulence among six isolates (T96-1, T96-2, T96-3, T96-4, T96-5, and S95-1) obtained from diseased plants or naturally infested field soils. Three out of six isolates caused no disease on Kita-no-otome (DSI = 0), whereas the other three isolates were virulent on this cultivar (DSI = 1.2 to 2.6). In additional experiments, another three lines, Toiku No. 125, Toiku No. 132, and Toiku No. 140, derived from various gene sources, also revealed the same response to two representative isolates (T96-1 and T96-5) tested as with Kita-no-otome. Consequently, two races of *Phialophora gregata* f. sp. *adzukicola*, race 1 and race 2, can be distinguished by avirulence or virulence to Kita-no-otome, respectively.

Adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), which is a member of the subgenus *Ceratotropis*, is produced in four countries of northeast Asia: Japan, China, South Korea, and Taiwan (10). In Japan, adzuki bean is the second most important legume after soybean, and about 100,000 metric tons are produced annually. Hokkaido Island, the northernmost region of Japan, is where about two-thirds of the commercial production in Japan occurs.

One of the most common diseases of adzuki bean in Hokkaido is brown stem rot (BSR), caused by *Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams f. sp. *adzukicola* Kobayashi et al. (9). BSR outbreaks were reported in 1970, and the pathogen was first identified as *Cephalosporium gregatum* Allington & D.W. Chamberlain (12). Yield losses attributable to BSR have been estimated at up to a 69% decrease in the number of mature pods or a 77% reduction in seed weight per plant, based on the yield of susceptible versus resistant cultivars in soil infested with the pathogen (3). *P. gregata* f. sp. *adzukicola* invades young plants through the roots and extends systemically up the plant as mycelia and conidia (12). The disease is characterized by a reddish brown discoloration

of vascular and pith tissues of the stem and the petiole that often occurs together with leaf chlorosis, necrosis, or defoliation (12). Air temperature, especially a low temperature in June, favors disease development (4). Inoculum levels also may contribute to variation in the disease incidence and the severity of BSR (8).

The most effective means of controlling this disease is through resistant cultivars. Two adzuki bean cultivars resistant to BSR are commercially available (1,4-6). Hatsune-shozu (1) has been planted in Hokkaido since 1985. BSR severity in this cultivar was less than that of Erimo-shozu, a susceptible cultivar, when plants were planted in naturally infested fields. The production area of Hatsune-shozu in Hokkaido, however, decreased by about 45 ha (0.2%) in 1995 (*unpublished data*) because of its unfavorable qualities, such as susceptibility to low temperature or the dark color of the seed coat. Therefore, new BSR-resistant cultivars with good agronomic qualities were required. Another resistant cultivar, Kita-no-otome, developed from the cross Erimo-shozu × 2025(F5), was released in 1994 (6). Because this cultivar had a high yield and tolerance for cool weather, its cultivation was expected to make adzuki bean production in Hokkaido stable. The occurrence of BSR on this cultivar, however, was discovered in 1995. This could be due to variation in the pathogen in different locations. Therefore, the objective of this study was to determine if races of *P. gregata* f. sp. *adzukicola* existed.

MATERIALS AND METHODS

Field experiment. A recently released resistant cultivar, Kita-no-otome (6), a susceptible cultivar, Erimo-shozu (11), and a previously registered resistant cultivar, Hatsune-shozu (1), were cultivated to evaluate resistance in two fields infested with *P. gregata* in the Tokachi district of Hokkaido: a field in Tokachi Agricultural Experiment Station (A8), where adzuki bean and maize have been cultivated alternately for 20 years, and another field in Memuro (Ti), where a rotation of adzuki bean-soybean-sugar beet-maize-wheat had been carried out before this experiment. In the A8 field, 80 seeds of each cultivar were planted in 2-m rows in four-row plots on 27 May 1995. In the Ti field, 40 seeds of each cultivar were planted in 2-m rows in two-row plots on 29 May 1995. Rows were spaced 60 cm apart in both fields. The experimental design was a randomized complete block with three replications. Before planting adzuki beans, the levels of *P. gregata* population in these two fields were estimated by dilution plating on a selective medium (8).

The severity of symptoms was assessed in terms of the mean disease severity index (DSI) of 56 or 28 arbitrarily collected plants per plot of the A8 or the Ti fields, respectively, with a scale where 0 = no symptoms, 1 = a slight vascular discoloration without foliar symptoms, 2 = foliar symptoms or severe vascular discoloration (above the first node or in petioles), and 3 = death. The severity was assessed on 9 September before plants reached maturity. Plants with DSI = 0.0 were rated resistant, plants with DSI > 0.0 but < 1.0 were regarded as intermediate (slightly susceptible), and plants with DSI ≥ 1.0 were considered susceptible. After drying seeds for about 1 month under natural conditions, seed weight per plot was measured. The data were analyzed by analysis of variance (ANOVA), and treatments were compared by Fisher's protected least significant difference (PLSD) ($P = 0.05$).

Greenhouse experiment. Isolates of *P. gregata* were obtained from diseased stem pieces or naturally infested soils using selective media (8). Isolates were as follows: T96-1 from soil in the Ti field, T96-2 from Erimo-shozu in a field at Obihiro, T96-3 from Kita-no-otome in the A8 field, T96-4 from Hatsune-shozu in the A8 field, T96-5 from soil in the A8 field, and S95-1

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from Erimo-shozu in a field at Sapporo. Single-spored isolates were stored in potato sucrose agar medium at 18 to 20°C. Inoculum was produced in liquid V8 juice medium at 25°C on a reciprocal shaker at 120 oscillations per min. After 3 weeks, mycelia and spores were collected by filtering through filter paper (Whatman No. 1) or by centrifugation at $1,400 \times g$ for 10 min, and washed by suspending in distilled water and centrifugation. The fungal pellets were homogenized with distilled water (10,000 rev/min $\times$ 3 min) and diluted to 10^7 spores and mycelial fragments per milliliter.

Adzuki bean cultivars used for determining races were Erimo-shozu, Hatsune-shozu, and Kita-no-otome. Seedlings were grown in sterilized vermiculite (about 10 to 14 days) and used for inoculations. Seedlings were removed and dipped into inoculum suspensions for 12 h and then transplanted into a soil (Pot-ace, Katakura Chikkarin K. K., Tokyo, Japan) : vermiculite mixture (1:1) in 12-cm-diameter plastic pots.

Disease severity was assessed 6 weeks after inoculation in greenhouse experiments as the mean DSI with a scale described above. The inoculation experiments were completely randomized, with three replications (pots) per isolate per cultivar and seven plants per pot. The test was repeated once with these cultivars, and nighttime low temperatures and daytime high temperatures during these tests were 20 to 27°C and 12 to 23°C in tests 1 and 2, respectively. An additional three genotypes of adzuki bean, Toiku No. 125, Toiku No. 132, and Toiku No. 140, which were derived from different resistant genotypes, Kariwano No. 68, Acc86, and Kuro-shozu, respectively, were also used for differential reactions to representative isolates. The experiments were completely randomized, with three replications (pots) per isolate per genotype and seven plants per pot. The test was repeated once with these genotypes, and nighttime low temperatures and daytime high temperatures during these tests were 18 to 22°C and 19 to 26°C in tests 1 and 2, respectively. The data were analyzed by ANOVA, and treatments (isolates and cultivars) were compared by Fisher's PLSD ($P = 0.05$).

RESULTS

Field experiments. Inoculum densities of *P. gregata* at sowing were 7.0×10^3 CFU/g of dry soil and 1.0×10^3 CFU/g of dry soil in the A8 and the Ti fields, respectively. A significant difference of BSR severity in cultivar Kita-no-otome was found between the two fields (Table 1). Kita-no-otome grown in the Ti field showed a resistant reaction (DSI = 0.0), whereas that cultivar grown in the A8 field suffered from BSR (DSI = 1.6). Accordingly, the yield (1,430 kg/ha) of Kita-no-otome grown in the A8 field was less than

half of that (2,920 kg/ha) in the Ti field. BSR symptoms were observed in both fields for cultivars Erimo-shozu and Hatsune-shozu. The susceptible cultivar Erimo-shozu developed extensive stem and leaf symptoms of BSR in both fields.

Greenhouse experiments. Within each test in greenhouse experiments, isolate, cultivar, and isolate $\times$ adzuki bean cultivar (genotype) effects were significant. All six *P. gregata* isolates were virulent on Erimo-shozu (Table 2). Three isolates (T96-3, T96-4, and T96-5) collected from the A8 field were virulent to Kita-no-otome (DSI = 1.2 to 2.6), whereas apparent immunity was observed in Kita-no-otome inoculated with three other isolates (DSI = 0.0). The DSI ratings for Hatsune-shozu varied among the isolates (DSI = 0.1 to 1.7), but disease severity was generally less than that of Erimo-shozu for each isolate.

An additional set of adzuki bean genotypes also revealed differences between

two representative isolates for virulence (Table 3). Lines Toiku No. 125 and Toiku No. 140 were susceptible to isolate T96-5, but both lines were resistant to isolate T96-1, similarly to Kita-no-otome. Line Toiku No. 132 showed resistant and intermediate (slightly susceptible) reactions on isolates T96-1 and T96-5, respectively.

DISCUSSION

The results support the conclusion that there are two races of *P. gregata* f. sp. *adzukicola* on Hokkaido Island. Isolates avirulent to Kita-no-otome and isolates virulent to this cultivar should be designated race 1 and race 2, respectively. In a previous report (15), variation in virulence among isolates of *P. gregata* f. sp. *adzukicola* was described, but this report concluded that further investigations were required to designate the existence of races. No contradiction between these results and the results in the previous report

Table 1. Comparison of disease severity for adzuki bean cultivars in two fields naturally infested with *Phialophora gregata* f. sp. *adzukicola*

Adzuki bean cultivar	Field			
	A8		Ti	
	DSI ^y	Yield (kg/ha)	DSI	Yield (kg/ha)
Ermo-shozu	2.3 a ^z (S)	1,390 b	2.0 a (S)	1,360 c
Hatsune-shozu	1.1 a (S)	1,840 a	1.3 b (S)	1,750 b
Kita-no-otome	1.6 a (S)	1,430 b	0.0 c (R)	2,920 a

^y Disease severity index was assessed on a scale of 0 to 3 as follows: 0 = no symptoms, 1 = slight vascular discoloration without foliar symptoms, 2 = foliar symptoms or severe vascular discoloration (above the first node or in petioles), and 3 = death. Reaction class in parentheses was based on the DSI scale: 0.0 = resistant (R), >0.0 but <1.0 = intermediate (I), and ≥ 1.0 = susceptible (S).

^z Means with the same letter in a column are not significantly different ($P = 0.05$) as determined by Fisher's protected least significant difference.

Table 2. Disease reactions of adzuki bean cultivars to different isolates of *Phialophora gregata* f. sp. *adzukicola*

Isolate ^y	DSI ^z		
	Ermo-shozu	Kita-no-otome	Hatsune-shozu
Test 1			
T96-1	3.0 (S)	0.0 (R)	1.2 (S)
T96-2	3.0 (S)	0.0 (R)	1.1 (S)
S95-1	1.0 (S)	0.0 (R)	0.7 (I)
T96-3	3.0 (S)	2.0 (S)	0.8 (I)
T96-4	1.6 (S)	1.6 (S)	0.9 (I)
T96-5	1.2 (S)	2.6 (S)	0.7 (I)
		LSD(0.05) = 0.9	
Test 2			
T96-1	2.7 (S)	0.0 (R)	1.7 (S)
T96-2	2.2 (S)	0.0 (R)	1.4 (S)
S95-1	1.0 (S)	0.0 (R)	0.2 (I)
T96-3	1.6 (S)	1.7 (S)	0.6 (I)
T96-4	1.5 (S)	1.2 (S)	0.6 (I)
T96-5	2.1 (S)	1.7 (S)	1.0 (S)
		LSD(0.05) = 1.0	

^y Isolates originated from soils or diseased plants as follows: T96-1 from soil in the Ti field, T96-2 from Ermo-shozu in a field at Obihiro, S95-1 from Ermo-shozu in a field at Sapporo, T96-3 from Kita-no-otome in the A8 field, T96-4 from Hatsune-shozu in the A8 field, and T96-5 from soil in the A8 field. Nighttime low temperatures and daytime high temperatures during these tests were 20 to 27°C and 12 to 23°C in tests 1 and 2, respectively.

^z Disease severity index was assessed on a scale of 0 to 3 as follows: 0 = no symptoms, 1 = slight vascular discoloration without foliar symptoms, 2 = foliar symptoms or severe vascular discoloration (above the first node or in petioles), and 3 = death. Reaction class in parentheses was based on the DSI scale: 0.0 = resistant (R), >0.0 but <1.0 = intermediate (slightly susceptible) (I), and ≥ 1.0 = susceptible (S).

Table 3. Comparison of disease reactions of adzuki bean genotypes to two representative isolates of *Phialophora gregata* f. sp. *adzukicola*

Race (isolate ^w)	DSI ^{x,y}					
	Erimo- shozu	Kita-no- otome (Kariwano No. 63)	Hatsune- shozu (Akamame)	Toiku No. 125 (Kariwano No. 68)	Toiku No. 132 (Acc 86)	Toiku No. 140 (Kuro- shozu)
Test 1 ^z						
1 (T96-1)	1.9 (S)	0.0 (R)	0.8 (I)	0.0 (R)	0.0 (R)	0.0 (R)
2 (T96-5)	1.7 (S)	1.2 (S)	0.8 (I)	1.9 (S)	0.9 (I)	1.5 (S)
		LSD(0.05) = 0.4				
Test 2 ^z						
1 (T96-1)	2.7 (S)	0.0 (R)	0.3 (I)	0.0 (R)	0.0 (R)	0.0 (R)
2 (T96-5)	2.1 (S)	2.0 (S)	0.3 (I)	1.4 (S)	0.8 (I)	1.7 (S)
		LSD(0.05) = 0.4				

^w Isolates T96-1 and T96-5 originated from soil in the Ti field and from soil in the A8 field, respectively.

^x Disease severity index was assessed on a scale of 0 to 3 as follows: 0 = no symptoms, 1 = slight vascular discoloration without foliar symptoms, 2 = foliar symptoms or severe vascular discoloration (above the first node or in petioles), and 3 = death. Reaction class in parentheses was based on the DSI scale: 0.0 = resistant (R), >0.0 but <1.0 = intermediate (slightly susceptible) (I), and ≥1.0 = susceptible (S).

^y Genotype in parentheses indicates the predicted gene source for brown stem rot resistance.

^z Nighttime low temperatures and daytime high temperatures during experiments were 18 to 22°C and 19 to 26°C in tests 1 and 2, respectively.

(15) was found, with the response of Kariwano No. 68 (mentioned as Maruba in that report) to two isolates being similar to that of Toiku No. 125 which was derived from Kariwano No. 68.

Kita-no-otome was registered in the Ministry of Agriculture, Forestry and Fisheries of Japan in 1994 (6). In the process of developing this cultivar, a number of field experiments were carried out in various regions where 3- to 5-year rotations were done. The incidence of BSR was not found in this cultivar in the A8 field until 1987 (6). During the absence of Kita-no-otome in the A8 field after 1987, the emergence of virulent isolates or changes in frequency of virulent isolates in the population in this field may have occurred due to selection pressure imposed by BSR resistance genes in the adzuki bean genotypes planted. Supposedly, the distribution of race 2 is restricted to a few regions, with race 1 being prevalent in adzuki bean fields in Hokkaido (6).

Hatsune-shozu was previously developed as a BSR-resistant cultivar (1) based on field experiments that determined total dry weight ratio; comparing the yields of seeds between highly infested fields and clean fields. The reaction of Hatsune-shozu to BSR, however, was not immune. Although foliar or stem symptoms were observed, the rating of BSR severity in this cultivar was less than that of Erimo-shozu when plants were inoculated with each of the races in these tests. This characteristic

may reflect other genetic mechanisms of resistance to BSR. Hatsune-shozu may have some minor or modifier genes with weak resistant effects that were different from a dominant gene of Kita-no-otome (*unpublished data*). BSR infection causes the severe yield losses in adzuki bean demonstrated in the field experiment. Undoubtedly, the best way to reduce damage is by breeding resistant cultivars. Since adzuki bean genotypes resistant to race 2 have not been found, the use of intermediate resistance might be useful for BSR control. Line Toiku No. 132 was suggested to be a gene source for breeding resistant cultivars, because of both complete resistance to race 1 and intermediate resistance to race 2 in these tests.

In the case of the related BSR pathogen on soybean, *P. gregata* f. sp. *sojae* (7,9), a high degree of variability in aggressiveness to some cultivars among isolates was described even in the same field, but no races have been identified (13,14). Further investigations of the regional distribution of races and variability in aggressiveness within adzuki bean BSR isolates are needed for the control of BSR. Molecular biological methods, one of which was used in a study of soybean BSR (2), might be useful for this purpose.

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