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FUNGAL DISEASES

Adzuki bean leaf infection by *Phytophthora vignae* f. sp. *adzukicola* and resistance evaluation using detached leaves inoculated with zoospores

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Abstract We examined the response of adzuki bean leaves to infection by *Phytophthora vignae* f. sp. *adzukicola* and determined whether inoculated leaves can be used to evaluate cultivar resistance. Detached adzuki bean leaves were inoculated with zoospores, and the resulting symptoms were diagnosed. Resistant reactions were characterized by dark brown, speckled lesions or a lack of symptoms, while susceptible reactions were characterized by water-soaked spreading lesions. In an inoculation experiment using a combination of three differential cultivars and three races, the response of 10-day-old primary leaves accurately differentiate between race-specific resistance and susceptibility of adzuki cultivars.

Keywords Phytophthora stem rot · *Phytophthora vignae* f. sp. *adzukicola* · Adzuki bean · *Vigna angularis* · Resistance evaluation · Zoospore inoculation

Phytophthora stem rot, caused by *Phytophthora vignae* Purss f. sp. *adzukicola* (Kitazawa et al. 1978, 1979; Tsuchiya et al. 1986), stands alongside brown stem rot (Kobayashi et al. 1991) and Fusarium wilt (Kitazawa and Yanagita 1989; Kondo and Kodama 1989) as one of the most economically important diseases that affects adzuki bean plants in Hokkaido, Japan (Fujita 2007). Because the pathogen is soilborne, cultural and chemical methods are insufficient for controlling the disease completely; instead, the use of resistant cultivars is considered one of the most important methods for disease control (Fujita 2007). A recent survey of race distribution in Hokkaido revealed the existence of a new race, designated race 4, which is capable of infecting currently resistant cultivars (Kondo et al. 2004; Notsu et al. 2003). Accordingly, screening for new resistant resources has been conducted (Fujita 2007). Root-dip inoculation (Notsu et al. 2003) and field experiments are the most frequently used procedures to assess resistance and evaluate races; however, these procedures require significant time and space. The root-dip method takes at least 4 weeks to complete, and field experiments require even more time and space. Thus, new methods that can evaluate resistance or races more easily and quickly are necessary for breeding and identifying novel resistant cultivars.

In studies using soybean and *P. sojae*, detached soybean leaves were inoculated and evaluated for resistance with satisfactory results (Morgan 1963). However, the infection of leaves of adzuki bean has received little attention, and consequently, few attempts to assess the resistance of adzuki bean with leaves have been undertaken. The objectives of our study were to examine whether infection of the leaves is accompanied by the formation of lesions and whether leaf inoculations are useful for testing cultivar resistance to races of *P. vignae* f. sp. *adzukicola*.

Experiments were conducted using three races of *P. vignae* (races 1, 3, and 4 corresponding to isolates Pv-nsk, Pv-kaes3, and Pv-o2, respectively), which were isolated in Hokkaido (Kondo et al. 2004), and the following set of differential cultivars: Erimo-shozu (universally susceptible), Kotobuki-shozu (resistant to race 1, susceptible to 3 and 4), and Syumari (resistant to races 1 and 3, susceptible to 4). The seeds were planted in vermiculite at 5-day intervals to produce leaves of different ages, and plastic

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containers (15 cm × 20 cm × 5 cm) were placed in a growth chamber at 25°C under fluorescent light for 15 h and 18°C in the dark for 9 h. The seedlings were watered every day, and the dates of germination and primary leaf expansion were recorded.

Primary leaves of different ages (5 or 10 days after expansion) were cut from the plants at the petioles and immediately placed onto moistened paper towels in plastic containers (15 cm × 20 cm × 5 cm). A 40-µl drop of zoospore suspension (10^3 zoospores/ml) was placed on the center of each leaf. Twenty leaves were inoculated for each combination of cultivar, leaf age, and race tested.

The zoospore suspension was prepared as follows: mycelia cultured in V8 juice media for 3 days were rinsed with 50 ml of sterile water five times, placed in Petri dishes filled with 15 ml of sterile water, and incubated overnight at 25°C until a sufficient number of zoospores were released. The suspension was then filtered through four layers of gauze to remove the mycelia; the zoospore concentration was adjusted with sterile water.

After inoculation, the containers were kept closed to maintain humidity and incubated at 25°C in the dark for 4 days. The leaves were observed every day, and if the drop of zoospores dripped off a leaf, the leaf was removed.

Macroscopically visible responses appeared on the leaves 2 days after inoculation and became more distinct 4 days after inoculation. Dark reddish-brown, speckled lesions (Fig. 1a) or a lack of symptoms (Fig. 1b) were representative of the resistant reactions. The susceptible reactions were characterized by the formation of pale brown, water-soaked lesions (Fig. 1c). These lesions expanded rapidly with the rotting of the tissues, accompanied by the production of oospores (Fig. 1e), while most lesions on the resistant cultivars were restricted to areas of contact with the inoculum.

Microscopic observation of the inoculated sites 12 h after inoculation revealed the germination and appressorium formation by encysted zoospores (Fig. 1f). Most appressoria became attached between leaf cells. The leaf cells to which appressoria were attached also browned. This location is inconsistent with previous observations (Tsuchiya 1982) showing that germinated zoospores invaded through the stomata on inoculated epicoytls, suggesting that the course of infection on leaves might differ from that on epicotyls.

The result of the experiment with various combinations of three cultivars, two leaf ages, and three races is shown in Table 1. In the compatible combinations, more than 80% of the 5-day-old leaves showed susceptible reactions. Even in incompatible combinations, 15–50% of the 5-day-old leaves had susceptible reactions, but the incidence was still lower than that for compatible combinations. Clear and distinct responses were observed on the 10-day-old primary leaves. The incidence of susceptible reactions in the

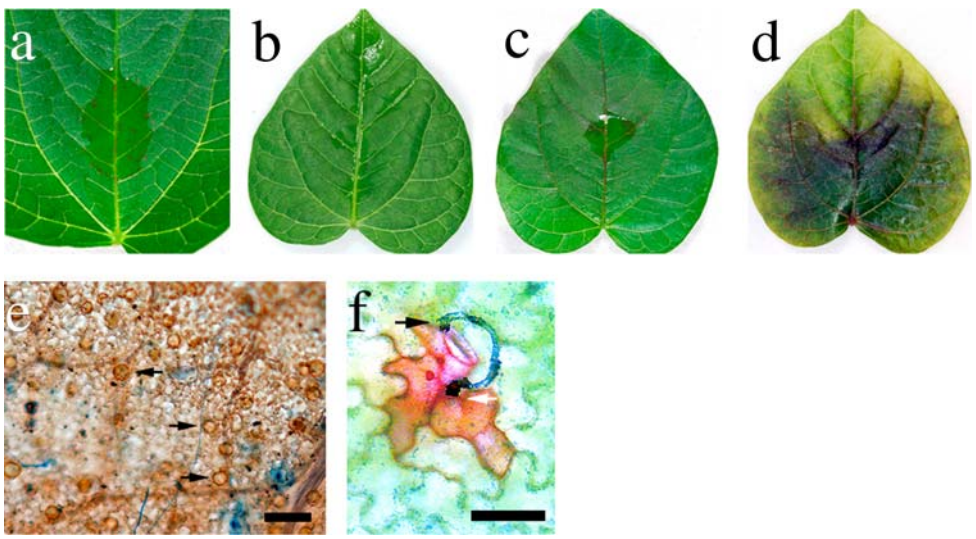

Fig. 1 Responses of detached adzuki bean primary leaves to inoculation with zoospores of *Phytophthora vignae* f. sp. *adzukicola*. **a** Restricted speckled lesions on 10-day-old Syumari leaves 96 h after inoculation with race 1 (avirulent). **b** Symptomless reactions on 10-day-old Syumari leaves 96 h after inoculation with race 1 (avirulent). **c** Spreading, water-soaked lesions on 10-day-old Erimo-shozu leaves 96 h after inoculation with race 1 (virulent). **d** Discoloration and spreading, water-soaked lesions cover almost the entire 5-day-old leaf of Erimo-shozu 96 h after inoculation with race 1 (virulent). **e** Oospores produced within the tissues of the water-soaked lesions. Leaves of Syumari inoculated with race 4 (virulent) were fixed with lacto-phenol and stained with aniline blue 96 h after inoculation. *Arrows* indicate representative oospores. *Scale bar* 100 µm. **f** Germ tube and appressorium formation inform germinated encysted zoospores on Syumari leaves 12 h after inoculation (stained with aniline blue). *Black arrow* indicates the encysted zoospore. *White arrow* indicates the dark appressorium. *Scale bar* 50 µm

Table 1 Reactions of detached adzuki bean primary leaves of different ages to inoculation with zoospores of *Phytophthora vignae* f. sp. *adzukicola*

Race (isolate)	Leaf age (days)[a]	Cultivar	Race-cultivar interaction	No. of leaves with reaction[b]	
				S	*R*
1 (Pv-nsk)	5	Erimo-shozu	C	16	4
		Kotobuki-shozu	I	5	15
		Syumari	I	3	17
	10	Erimo-shozu	C	3	13
		Kotobuki-shozu	I	0	20
		Syumari	I	0	20
3 (Pv-kaes3)	5	Erimo-shozu	C	18	2
		Kotobuki-shozu	C	17	3
		Syumari	I	10	10
	10	Erimo-shozu	C	7	10
		Kotobuki-shozu	C	6	14
		Syumari	I	0	20
4 (Pv-o2)	5	Erimo-shozu	C	20	0
		Kotobuki-shozu	C	19	1
		Syumari	C	20	0
	10	Erimo-shozu	C	6	8
		Kotobuki-shozu	C	4	16
		Syumari	C	8	12

C compatible, *I* incompatible

[a] Days after expansion

[b] Number of leaves with a resistant (*R* = nonspreading, dark brown lesion or a lack of symptoms) or susceptible (*S* = spreading lesion) reaction 4 days after inoculation. Detached primary leaves of different ages were inoculated with zoospores from each race and incubated in the dark

compatible interactions was 19–43%, while all leaves in the incompatible combinations had resistant reactions. It should be noted that leaf age affected both the magnitude and the type of the reactions. On the 10-day-old leaves, the spread of the susceptible lesion in the compatible combinations was limited to some extent as shown in Fig. 1c, whereas expansion of the water-soaked lesions on the 5-day-old leaves was so great that sometimes entire leaves became water-soaked (Fig. 1d). Moreover, the number of the leaves with resistant reactions increased as leaf age increased for both the compatible and incompatible combinations. Interestingly, some 10-day-old leaves in the compatible combinations had resistant type of reactions similar to Fig. 1a or b. Therefore, this age-related resistance might be involved in the inhibition of not only the spread of the pathogen within the leaf tissue but also of the initial invasion of the fungus.

Adzuki bean stem rot is primarily a disease of the roots, epicotyls, and stems. The pathogen invades adzuki bean plants at the roots, epicotyls, and stems. It then causes damping-off of the seedling and reddish-brown lesions on the stems and petioles of mature plants (Tsuchiya 1982). Therefore, to date, the infection through the leaves had not been focused on. The increased resistance associated with leaf age observed in this study may partially account for the lack of the remarks about leaf infection in the field.

In this study, we analyzed the responses of adzuki bean leaves inoculated with zoospores; these responses were similar to those of soybean leaves inoculated with zoospores of *P. sojae* (Morgan 1963). Race specificity using three differential cultivars and three races was properly assessed on 10-day-old leaves, suggesting that zoospore inoculation of adzuki bean leaves may be used with other established procedures to evaluate resistance. This method can finished in only about 2 weeks, less time than is required for other procedures. It also requires comparatively less space. In addition, zoospore inoculation of leaves has another merit that multiple races could be assessed on a single leaf as was demonstrated in the soybean-*P. sojae* interactions (Abe et al. 2002). For practical applications, however, further experiments with more strains would be needed because only three strains representing each race were tested in this experiment.

In the soybean-*P. sojae* system, several other physiological studies have been conducted using zoospore inoculation (Bhattacharyya and Ward 1986; Mohr and

Cahill 2001). However, few studies have investigated the mechanism of resistance in adzuki bean plants against pathogenic microorganisms, even though the understanding of resistance mechanisms and interactions between host and pathogen is important for developing disease control strategies. Thus, inoculation of leaves with zoospores, in which the soil does not interfere with pathogen invasion, may also be useful for further physiological studies of adzuki bean resistance.

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