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FUNGAL DISEASES

Induction of phytoalexins in adzuki bean after inoculation with *Phytophthora vignae* f. sp. *adzukicola*

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Abstract To identify phytoalexins of adzuki bean elicited in response to attempted infection of *Phytophthora vignae* f. sp. *adzukicola*, we isolated compounds from adzuki bean and evaluated their antifungal activity. Seven flavonoids (daidzein, genistein, 2′-hydroxygenistein, coumestrol, dalbergioidin, kievitone, and phaseol) were identified from epicotyls wound-inoculated with a mycelial suspension of an avirulent race of *P. vignae* f. sp. *adzukicola*. Of those compounds, kievitone and dalbergioidin accumulated to higher levels in incompatible interactions compared to compatible interactions 48 h after inoculation. Kievitone strongly inhibited the germination of encysted zoospores, and dalbergioidin were slightly suppressive. From these results, we concluded that kievitone and dalbergioidin are phytoalexins in adzuki bean.

Keywords Phytophthora stem rot · *Phytophthora vignae* f. sp. *adzukicola* · Adzuki bean · *Vigna angularis* · Phytoalexin · Kievitone

Phytophthora vignae Purss f. sp. *adzukicola* Tsuchiya, Yanagawa et Ogoshi causes Phytophthora stem rot of adzuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] (Kitazawa et al. 1978, 1979; Tsuchiya et al. 1986), one of the major yield-limiting factors in the northern island of Hokkaido, Japan (Fujita 2007). It infects the roots, epicotyls, and stems of young seedlings and mature adzuki plants, eventually causing wilt or leaf blight (Kitazawa et al. 1978; Tsuchiya 1982). Leaf infection in vitro has also been observed (Harada and Kondo 2009). For controlling the disease, the use of resistant cultivars is considered to be one of the effective methods. However, the emergence of new races within a relatively short time has been a hindrance. Previous studies in Hokkaido revealed the emergence and wide distribution of a new race, designated race 4 (Kondo et al. 2004; Notsu et al. 2003), which is capable of infecting newly developed resistant cv. Syumari (Fujita et al. 2002). Recently, another new race, 5, was identified (Ogura 2008). For development of strategies to control the disease, understanding resistance mechanisms in host–pathogen interactions is essential. In legumes, studies have long focused on phytoalexins because of their significant roles in defense mechanisms. Varieties of compounds have been isolated from many plants and their antimicrobial actions on fungal pathogens have also been well documented (Deverall 1972; Grisebach and Ebel 1978; Ingham 1972; Iwashina 2003). Meanwhile, little is known about resistance mechanisms in adzuki bean against Phytophthora stem rot, and there have been relatively few studies on phytoalexins produced by adzuki bean. Flavonoid compounds (dalbergioidin, kievitone, phaseollidin, genistein, 2′-hydroxygenistein, dihydrowighteone, phaseol, lupiwighteone, lupinalbin A, (3R)-(-)-vestitol, demethylvestitol and naringenin) were identified from adzuki bean plant inoculated with an abiotic elicitor or a few microorganisms that are nonpathogenic to adzuki beans (Abe et al. 1987; Ingham 1990; Seneviratne and Harborne 1992). Yet, it is not conclusive as to whether these or other unidentified compounds are induced and function as phytoalexins against pathogenic microorganisms in adzuki beans. Therefore, to determine whether phytoalexins are produced by adzuki bean after inoculation with *P. vignae* f. sp. *adzukicola*, we isolated compounds that adzuki bean produced in response to attempted infection of the pathogen,

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evaluated their antifungal effect on germination of encysted zoospores and compared the amount of those compounds accumulated in adzuki bean epicotyls after inoculation with virulent or avirulent races.

Because some investigators have considered flavonoids to be the most common phytoalexins of legumes (Grisebach and Ebel 1978; Ingham 1972), we focused on and attempted to isolate flavonoids from adzuki bean epicotyls inoculated with an avirulent race. The following isolates of *P. vignae* f. sp. *adzukicola* collected in Hokkaido (Kondo et al. 2004) were used and deposited in NIAS Genebank (National Institute of Agribiological Science, Tsukuba, Japan): Pv-nsk (race 1, MAFF 241391), Pv-kaes3 (race 3, MAFF 241390), and Pv-o2 (race 4, MAFF 241389). Fourteen-day-old seedlings of cv. Syumari (planted in vermiculite and grown in growth chamber at 25°C under fluorescent light for 15 h and 18°C in the dark for 9 h) were removed from the vermiculite and placed horizontally in plastic containers ($15 \times 20 \times 5$ cm, length × depth × height) with their roots covered with moistened paper towels. An approximately 1.5 cm long slit was cut in the upper part of the epicotyl (about 1 cm below the primary node) with a sterilized razor blade. Then, 200 μl of a suspension of minced mycelia (10 mg/ml distilled water) from 3-day-old cultures of Pv-kaes3 (avirulent to cv. Syumari) grown in V8 juice medium (200 ml V8 juice and 2.6 g $CaCO_3$ centrifuged at 5000 rpm for 15 min; the supernatant was diluted to 1 L with distilled water) was placed in the wound. After inoculation, the containers were kept closed with plastic (poly vinylidene chloride) cling film (Asahi kasei chemicals, Tokyo, Japan) to maintain high humidity and incubated at 25°C in the dark. For the control, noninoculated healthy epicotyls were treated in a similar way. At 48 h after inoculation (hai), the inoculated sites of 50 epicotyls and comparable tissue from control epicotyls were excised and immersed in 50 ml of 90% ethanol for 3 days. The ethanol extracts were then filtered, dried in vacuo at 38°C and extracted with ethyl acetate three times. The combined ethyl acetate extracts were concentrated in vacuo at 38°C. The concentrated organic fractions were loaded on silica gel preparative thin layer chromatography (TLC) plates (PLC Silica gel 60 F_{254}, 0.5 or 1.0 mm Glass plate 20×20 cm; Merck KGaA, Darmstadt, Germany) and developed with toluene:ethyl acetate:methanol (25:8:1, v/v). The developed plate was inspected under UV light at 254 and 365 nm. Each separated band was scraped from the plate, extracted with ethanol, and further purified with preparative TLC in following solvent systems: chloroform:methanol (100:3, v/v) or hexane:ethyl acetate:methanol (60:40:1, v/v). Purified compounds were identified on the basis of their UV spectra [DU640 spectrophotometer (Beckman Coulter, Tokyo, Japan)] and electron impact-mass spectrometry (EI-MS) [JEOL JMS-AX500 (GC-MS & MNR Laboratory, Graduate School of Agriculture, Hokkaido University)] by comparison with authentic standards (dalbergioidin, 2′-hydroxygenistein and phaseol were purchased from Apin Chemicals, Oxonia, UK; daidzein, genistein and coumestrol were from Sigma-Aldrich Japan, Tokyo, Japan) or previously reported values (Abe et al. 1987). Kievitone as a standard was extracted and purified from mung bean seedlings as previously described (O'Neill et al. 1983), with some modifications. Briefly, mung bean sprouts were sprayed with $CuCl_2$ (3×10^{-3} M), incubated at 25°C in the dark for 24 h, and extracted with 90% ethanol. The extracts were concentrated in vacuo and purified through preparative TLC. The identities of the isolated compounds were confirmed using high-performance liquid chromatography (HPLC) and EI-MS. HPLC was performed on LC-10Avp systems (Shimadzu, Kyoto, Japan) with a UV-VIS detector SPD-10Avp and a Shim-Pack CLC-C8 15 M column (4.6 mm i.d. × 150 mm; Shimadzu) using a linear gradient elution of 30–100% methanol in 0.1% acetic acid with a flow rate of 1.25 ml/min for 35 min. The column was maintained at 30°C. Effluent was monitored at 254 and 280 nm.

Consequently, six isoflavonoids, daidzein, genistein, 2′-hydroxygenistein, coumestrol, kievitone and phaseol, were identified spectroscopically and confirmed by comparison of the HPLC retention times with standards. Their spectral characteristics are as follows: *daidzein*: UV λ_{max} (MeOH, nm): 234sh, 249, 261sh, 305sh. EI-MS *m/z* (rel. int.): 254 (100), 137 (49), 118 (24); *genistein*: UV λ_{max} (MeOH, nm): 261, 336sh. EI-MS *m/z* (rel. int.): 270 (100), 269 (24), 153 (49), 135 (13); *2′-hydroxygenistein*: UV λ_{max} (MeOH, nm): 261. EI-MS *m/z* (rel. int.): 286 (100), 269 (23), 153 (69), 134 (30); *coumestrol*: UV λ_{max} (MeOH, nm): 242, 344. EI-MS *m/z* (rel. int.): 268 (100), 240 (8); *kievitone*: UV λ_{max} (MeOH, nm): 291. EI-MS *m/z* (rel. int.): 356 (100), 338 (46), 221 (57), 205 (30), 192 (23), 177 (24), 165 (85), 136 (16); *phaseol*: UV λ_{max} (MeOH, nm): 253, 264sh, 303, 345. EI-MS *m/z* (rel. int.): 336 (59), 281 (44), 280 (100). In addition to these compounds, dalbergioidin, which we could not isolate at high enough purity for spectral analysis, was identified using co-chromatography (TLC and HPLC) with the standard. None of these flavonoids were detected from noninoculated healthy epicotyls on HPLC.

These results are supported by previous studies on adzuki bean inoculated with an abiotic elicitor or non-pathogenic microorganisms. Dalbergioidin, 2′-hydroxygenistein and phaseol were isolated from roots of adzuki bean treated with *Cephalosporium gregatum* type B, which was considered to be an avirulent strain of brown stem rot pathogen (Abe et al. 1987). However, later the type B strain was demonstrated to belong to *Acremonium* spp., a non-pathogen of adzuki beans, by the analysis of protein

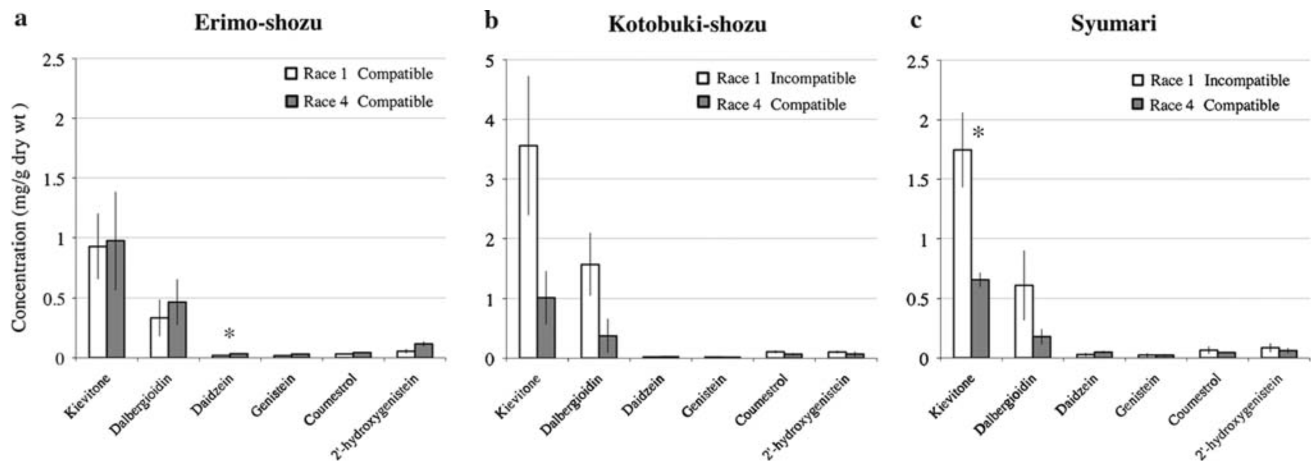

Fig. 1 Levels of six isoflavonoids in adzuki bean epicotyls wound-inoculated with *Phytophthora vignae* f. sp. *adzukicola.* **a** cv. Erimo-shozu inoculated with races 1 and 4 (both virulent). **b** cv. Kotobuki-shozu inoculated with races 1 (avirulent) and 4 (virulent). **c** cv. Syumari inoculated with races 1 (avirulent) and 4 (virulent) Data are expressed as means of three independent experiments. *Error bars* indicate SEM. Data were compared using two-sided Student's *t* test (*$P = 0.05$). Adzuki bean epicotyls were wound-inoculated with a mycelial suspension and incubated in dark at 25°C. The levels of isoflavonoids in epicotyls at 48 h after inoculation were analyzed with HPLC

and isozyme banding patterns and the base composition and homology of DNA (Yamamoto 1994). Genistein was found in the leaves of adzuki bean inoculated with *Helminthosporium carbonum*, a non-pathogen of adzuki bean (Ingham 1990). Kievitone was detected in adzuki bean epicotyls and leaves treated with $CuSO_4$ or with *Cladosporium herbarum* and *Helminthosporium carbonum*, nonpathogens of adzuki bean (Ingham 1990; Seneviratne and Harborne 1992). Kievitone is also a known phytoalexin of *Phaseolus vulgaris* and some other legumes (Burden et al. 1972; Ingham 1990; O'Neill et al. 1983; Partridge and Keen 1976; Seneviratne and Harborne 1992), and daidzein and coumestrol are also commonly detected in legumes (Brooks and Watson 1985; Ingham 1972).

To compare flavonoid accumulation levels between compatible and incompatible race–cultivar combinations, we inoculated wounded epicotyls of cvs. Erimo-shozu (universally susceptible), Kotobuki-shozu (resistant to race 1, susceptible to races 3 and 4) and Syumari (resistant to races 1 and 3, susceptible to race 4) with Pv-nsk (race 1) and Pv-o2 (race 4) as described before. For the control, noninoculated healthy epicotyls of each cultivar were used. The inoculated sites and comparable tissue from noninoculated healthy epicotyls were excised and immersed in 10 ml of 90% ethanol 48 hai, and subsequent extraction was as described. After the extraction, epicotyls were dried at 105°C overnight and weighed. Each ethyl acetate extract was evaporated to dryness, dissolved with 25% methanol (10 μl/mg dry mass of extracted epicotyls), and subjected to HPLC analysis. Compounds were identified and quantified by calibration with standards. The experiment was repeated three times, and in each experiment not less than three epicotyls were used in each race–cultivar combination. Two-sided Student's *t* tests were used to compare the differences in the accumulation levels of each compound between the inoculated races.

In the preliminary test, the amount of kievitone was less than 0.8 mg/g dry mass in the epicotyls of cv. Syumari at 12 and 24 hai with virulent or avirulent races (data not shown). Accordingly, no significant difference was observed in the amount of kievitone between those race–cultivar combinations (two sided Student's *t* test, $P = 0.05$). Higher levels of kievitone and dalbergioidin were measured at 48 hai (Fig. 1). In incompatible race–cultivar combinations, kievitone (1.7–3.6 mg/g dry mass) and dalbergioidin (0.6–1.6 mg/g dry mass) levels were higher than in compatible combinations (kievitone: 0.6–1.0 mg/g dry mass; dalbergioidin: 0.2–0.5 mg/g dry mass). Of all race–cultivar combinations examined, however, we identified significant differences in the amount of kievitone only when cv. Syumari was inoculated with virulent and avirulent races. No significant difference was found between virulent and avirulent races in other cases for the levels of kievitone and dalbergioidin in cv. Kotobuki-shozu and for dalbergioidin in cv. Syumari. Under these conditions, levels of these flavonoids failed to fully reveal any difference in the resistance of the cultivars to races perhaps from the lack of measurements at the critical incubation time. Much less daidzein, genistein, 2′-hydroxygenistein and coumestrol accumulated (<0.1 mg/g dry mass) compared with the levels of kievitone and dalbergioidin, and no consistent relation was observed between their levels and race–cultivar combinations. There also seemed to be no decisive differences in phaseol between the race–cultivar

Table 1 Effect of six isoflavonoids, produced by adzuki bean in response to *Phytophthora vignae* f. sp. *adzukicola* on germination of encysted zoospores of the pathogen

Treatment	Germination (%)[a]
Control	90.7 ± 1.4
Daidzein 100 ppm	90.4 ± 1.4
Genistein 100 ppm	92.9 ± 1.1
Coumestrol 100 ppm	91.2 ± 2.7
Dalbergioidin 100 ppm	69.9 ± 4.8*
Kievitone 100 ppm	3.8 ± 1.6*
Kievitone 10 ppm	4.8 ± 1.1*

[a] Germination of isolate Pv-o2 after 18 h incubation in microwell-plates in dark at 25°C. Data are mean ± SEM of four replications

*Statistically significant ($P < 0.05$) difference from control was observed with Dunnett's multiple comparison using arcsine-transformed data

combinations, although we could not quantify the amounts of phaseol exactly because we lacked enough of the purified compound for the HPLC calibration. Instead, the amount of phaseol was estimated using area of the HPLC peaks. None of these flavonoids were detected from intact healthy epicotyls on HPLC.

Antimicrobial activity of daidzein, genistein, coumestrol, dalbergioidin and kievitone to *P. vignae* f. sp. *adzukicola* was assayed with encysted zoospores. The zoospore suspension was prepared as follows: mycelia of race 4 (Pv-o2) 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 sterilized gauze to remove the mycelia; the zoospore concentration was adjusted to 5×10^3 zoospores/ml with sterile water using a hemacytometer. Authentic standards of daidzein, genistein, coumestrol and dalbergioidin obtained from commercial suppliers, and kievitone purified from mung bean as previously described (O'Neill et al. 1983) were used. They were dissolved in ethanol (1 mg/ml), and 10 or 100 µl of each solution was put into wells of 24-well micro-plates (four wells for each treatment as replicates); the ethanol was then removed by air-drying. Zoospore suspensions were vortexed for 1 min to promote zoospore encystment, and 1 ml of encysted zoospore suspension was added into each well and incubated at 25°C in the dark. Spore germination was observed with an inverted microscope 18 h after treatment, and the germination rate was evaluated. Data were arcsine-transformed and analyzed by Dunnett's multiple comparison tests. In controls, 90.7% of the encysted zoospores germinated (Table 1), and hyphae were also observed 18 h after treatment (Fig. 2a). Kievitone strongly inhibited the germination of encysted zoospores, even at 10 ppm (Fig. 2b), while treatment with daidzein, genistein and coumestrol at 100 ppm showed no significant differences from the control. Dalbergioidin was also slightly inhibitory and reduced spore germination by 21% at 100 ppm. Results with race 1 isolate (Pv-nsk) were similar: kievitone at 10 ppm suppressed spore germination to 3.6%, while 37.0% of spores germinated in controls.

Many studies on phytoalexins have demonstrated their important roles in plant defense responses. A previous study, which assessed the relative role of phytoalexin and other resistance components (i.e., hypersensitive reaction and deposition of lignin and phenolics) in soybean–*P. sojae* interactions, demonstrated that the phytoalexin glyceollin was the major factor in restricting pathogen spread (Mohr and Cahill 2001). During the interactions between adzuki beans and *P. vignae* f. sp. *adzukicola* in this study, kievitone accumulated to higher levels in incompatible combinations (Fig. 1) and strongly inhibited spore germination (Table 1; Fig. 2). Dalbergioidin, which is assumed to be a precursor of kievitone (Ingham 1977; Woodward 1979), also accumulated to higher in incompatible combinations (Fig. 1) than in compatible combinations and was slightly suppressive to spore germination (Table 1). Meanwhile, the levels of daidzein, genistein, coumestrol and 2′-hydroxygenistein were much lower than those of kievitone and dalbergioidin regardless of the race–cultivar combinations (Fig. 1). Daidzein, genistein and coumestrol had no significant antifungal activity (Table 1). These results demonstrated that kievitone and dalbergioidin meet the definition of a phytoalexin, that is, they are low molecular

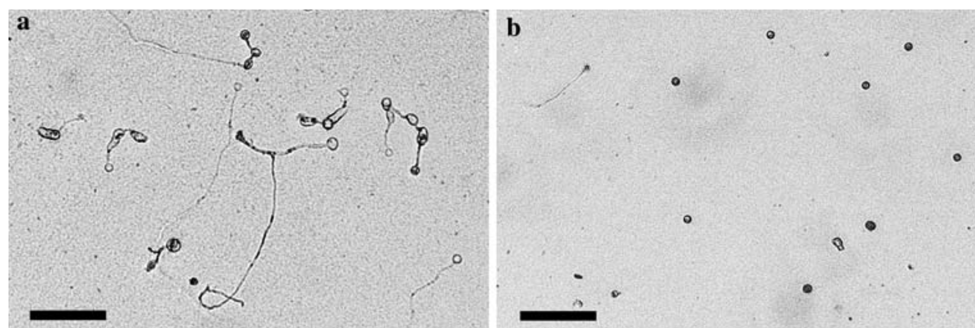

Fig. 2 Germination of encysted zoospores of *Phytophthora vignae* f. sp. *adzukicola* after 18 h incubation in microwell-plates in dark at 25°C. **a** Untreated control. **b** Kievitone at 10 ppm. *Scale bar* 100 µm

weight, antimicrobial compounds that are synthesized and accumulated by the plant in response to exposure to a microbe (Paxton 1980), and thus can be identified as phytoalexins of adzuki bean produced in response to attack by *P. vignae* f. sp. *adzukicola*.

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