FUNGAL DISEASES

Alternaria alternata causing black spot of peach produces a host-specific toxin

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Abstract

Here we report a new host-specific toxin that is produced by *Alternaria alternata* and is the eight reported for the fungus. The causal pathogen of black spot of peach produced a toxin in culture filtrates and in spore germination fluids. Toxin productivity during spore germination was closely related to pathogenicity of the pathogen. The degree of sensitivity of host plants to the toxin was consistent with that of susceptibility to the pathogen. Further, the toxin enabled infection by conidia of saprophytic *A. alternata* in peach leaves. Several cultivars showed resistance to the toxin and the pathogen, although no cultivars had an immune response. The toxin fit the criteria of a host-specific toxin and was designated AP-toxin.

Keywords *Alternaria alternata* · AP-toxin · Black spot of peach

Introduction

A new disease of peach (*Prunus persica*) causing black spot has been found in a limited area in Okayama Prefecture, Japan since 1989 (Inoue and Nasu 2000) and severely damages cv. Shimizu Hakuto, one of the major peach cultivars in Okayama. The disease affects fruits, twigs and leaves, and an *Alternaria* sp. was consistently isolated from them. The isolates were pathogenic to peach fruits and leaves. Based on morphological characteristics, the causal fungus was identified as *Alternaria alternata* (Fr.) Keissler (Inoue and Nasu 2000). Black spot of peach was also reported recently in Niigata Prefecture (Anonymous 2017).

While *A. alternata* is generally saprophytic (Rotem 1994), seven plant pathogens that cause highly specific diseases (Alternaria black spot of strawberry, Alternaria blotch of apple, Alternaria stem canker of tomato, black spot of Japanese pear, brown spot of tangerine, brown spot of tobacco, and leaf spot of rough lemon) have been reported so far (Akimitsu et al. 2014; Kohmoto 1992; Nishimura and Kohmoto 1983; Tsuge et al. 2013). These host-specific isolates of *A. alternata* have been proposed as pathotypes that produce host-specific toxins (HSTs) (Nishimura and Kohmoto 1983). In a recent review, Akimitsu et al. (2014) described HSTs as compounds that (1) have host-selective toxicity (2) which matches the specific host range of the HST-producing pathogen (3) are produced only by the fungus that is pathogenic to plants that are sensitive to that HST (4) released at the site of infection when conidia of the producing fungus germinate (5) reproduce the initial physiological changes in host cells during infection by the HST-producing pathogen, which then lead to (6) penetration or initial colonization by the HST-producing pathogen.

Until the report of this disease in Niigata Prefecture, we have not found any report on peach diseases involving both *A. alternata* pathogen and a fungal toxin, although three reports of interest were found. One examined the cause of a leaf spot in India without any description of host specificity (Madan et al. 1979), the second was on fruit rot during and after maturation of fruits in China (Zhang et al. 1995), and the last was on brown spot disease of peach and apricot trees (Yousefi and Shahri 2009). We report here that the causal pathogen of black spot of peach produces a host-specific toxin (AP-toxin) and propose the pathogen as a new pathotype of *A. alternata*.

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Materials and methods

Plant materials

Peach plants, cultivars (cvs.) Shimizu Hakuto (susceptible to black spot of peach) and Kawanakajima Hakuto (resistant) were grown in a greenhouse of Okayama University. Young leaves were detached and immediately used in experiments. Twenty-three other peach cultivars were also used to determine the host range of the pathogen. Leaves of peach cultivars in a cultivar-collection orchard were kindly provided by the Agricultural Experiment Station, Okayama Prefectural General Agriculture Center. We also tested three nonhost species: apple cv. Orin from the cultivar collection, susceptible to the apple pathotype of *A. alternata* and sensitive to AM-toxin; Japanese pear cv. Nijisseiki, susceptible to the Japanese pear pathotype of *A. alternata* and sensitive to AK-toxin; strawberry cv. Morioka 16 from the grown the greenhouse of Okayama University, susceptible to the strawberry pathotype of *A. alternata* and sensitive to AF-toxin.

Fungal materials

Thirty field isolates were obtained from diseased leaves of cv. Shimizu Hakuto (Inoue and Nasu 2000) and maintained on potato sucrose agar (PSA; 200 ml of extract from boiled potatoes, 20 g of sucrose, 800 ml of water). Each isolate was grown in potato sucrose broth (PSB; 200 ml of extract from boiled potatoes, 20 g of sucrose, 800 ml of water) at 25 ℃. After 14 days of culture, the medium was recovered by filtration using four layers of gauze and was used as culture filtrates. Spores formed on mycelial mats on the PSB were used for inoculation. Among the field isolates, isolate 9506, which produces culture filtrates with the highest toxicity was also kindly provided by the Agricultural Experiment Station, Okayama Prefectural General Agriculture Center, and was used in a large-scale culture for toxin preparation. A saprophytic isolate (O-94) was used as a nonpathogen. Conidia that were obtained from the culture of isolates on PSA plates at 25 °C for more than 14 days under dark, were suspended by paint brush in distilled water to use as inoculum.

Bioassays

Virulence was evaluated by spraying detached susceptible peach leaves with a suspension of 5×10^5 spores/ml distilled water. Necrotic lesions were observed after 24 h of incubation at 25 °C in a moist chamber. Virulence was rated based on the necrotic area on the leaf as follows: 0, no necrotic spots on the inoculated leaf; 1, necrotic spots with area less than 30% of leaf; 2, necrotic spots on 30–60% of total area;

3, necrotic spots on more than 60% of total area. Susceptibility of the leaves to the pathogen was assayed using the same procedure and index. Leaves were incubated for 72 h to observe any delayed response in resistant plants.

A leaf necrosis assay was used to detect toxin activity. The center of the abaxial side of leaves was injured by gently pressing with tweezers, and a drop of toxin solution (20 μl) was placed on each leaf. The leaves were incubated in a moist chamber for 24 h at 25 ℃. Toxicity was then evaluated as the maximum dilution for causing necrosis on susceptible (cv. Shimizu Hakuto) leaves and characterized in units (U), with 1 U defined as the minimum concentration to induce necrosis. For example, a 16-fold dilution of the sample solution (produced by successive twofold dilutions with distilled water) and induced necrosis, so the toxicity of that sample was determined to be 16 U. Leaves were incubated for 72 h to observe any delayed response in resistant plants.

Toxin preparation

For testing toxin productivity during germination, 20-μl droplets of a suspension of 1×10^6 conidia/ml were placed in a moist chamber, incubated for 24 h at 25 °C, then collected with a pipette and centrifuged at $1000 \times g$ for 10 min to pellet the spores. The cell-free supernatants were evaporated under reduced pressure (< 40 °C). The residues were dissolved with a small percentage of methanol (less than 5%) and distilled water. The volume of solution was adjusted to 10% of the volume of the incubated spore suspensions, and the resulting solutions were used as spore germination fluids (SGFs). Toxin activity of SGFs was determined using the leaf necrosis assay.

Culture filtrates were adjusted to pH 3.0 with 0.2 M HCl and were extracted with ethyl acetate. The ethyl acetate extracts were evaporated under reduced pressure at 40 °C. Extracts were purified by open silica gel column chromatography (Silica Gel 60, Nacalai Tesque, Kyoto, Japan) with the following solvents: hexane-ethyl acetate (80–100% ethyl acetate) and ethyl acetate-methanol (0–5% methanol, twice). Toxin activity was determined using the leaf necrosis assay. In the case of lipophilic samples, a small percentage of methanol (less than 5%) was used to re-dissolve the residue. Distilled water containing the same percentage of methanol as the toxin sample was used as a control.

Bioassay for toxin induction of susceptibility

Conidia of nonpathogenic *A. alternata* (O-94) were suspended in an aqueous toxin solution, prepared as described above, and the resulting suspension was sprayed onto one half of detached susceptible peach leaves for 24 h. The suspension was adjusted to provide a toxicity of 10 U. The other half of the leaves acted as controls and were

inoculated with spores of the nonpathogenic isolate in distilled water. Each bioassay was carried out with a set of two replications and repeated twice. Leaves were cleared in a solution of lactic acid-phenol-distilled water (4:1:1, v/v/v), then stained with a standard cotton blue solution, and 1×1 cm pieces were cut to examine 50 spores per replication (200 total) for germination and appressoria and infection hyphae formation with a microscope (BH2, Olympus, Tokyo, Japan). The percentage germination was calculated as (Number of germinated spores/total spores observed) $\times$ 100, percentage appressoria formation as (number of appressoria/total germ tubes observed) $\times$ 100, and percentage infection-hyphae formation as (number of infection hyphae/total appressoria observed) $\times$ 100.

Results

Toxin detection

The causal pathogen of black spot of peach produced a toxin in culture filtrates and in SGFs. Droplets of culture filtrate and SGFs of the pathogenic isolate (9506) induced more necrosis on susceptible leaves than on resistant leaves (Fig. 1). The toxin separated into the ethyl acetate during extraction and dissolved easily in methyl alcohol. Purification of the toxin is now in progress.
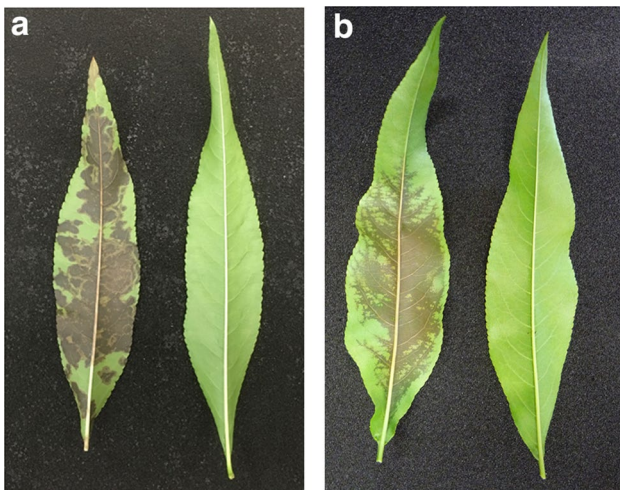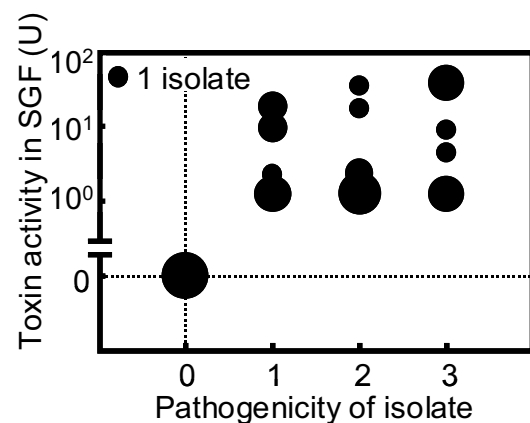
Correlation between pathogenicity and toxin productivity of the pathogen

When leaves of cv. Shimizu Hakuto were inoculated with spores of pathogenic isolates, spots that appeared often fused with each other and soon formed a large necrotic lesion (Fig. 1a). SGFs of these pathogenic isolates also induced necrosis on leaves. Among the 30 field isolates, 24 were pathogenic on leaves of cv. Shimizu Hakuto. The remaining six isolates may have lost their pathogenicity. In the test for a correlation between pathogenicity and toxin productivity, all the pathogenic isolates produced the toxin, and the six nonpathogenic isolates did not produce (Fig. 2). Toxin activity in SGFs, however, varied among the pathogenic isolates, but no activity was detected in SGFs of the six nonpathogenic isolates.

Correlation between susceptibility of plants to the pathogen and sensitivity to the toxin

All 25 peach cultivars, which are stocks from the Experiment Station, were susceptible to the pathogen, but the necrosis index varied from 1 to 3 (Table 1). Shimizu Hakuto and Shanhai Suimitsuto were the most susceptible of the cultivars tested, Kawanakajima Hakuto and Tenshin Suimitsuto appeared to be the most resistant, and the others had intermediate responses. The plant species that are susceptible to the apple, Japanese pear, or strawberry pathotype of *A. alternata* were immune (index 0) (Table 1).

Sensitivity of plants to the toxin was positively correlated with susceptibility of plants to the pathogen (Table 1).

Fig. 1 Necrosis on susceptible (cv. Shimizu Hakuto, left) and lack of symptoms on resistant (cv. Kawanakajima Hakuto, right) peach leaves 24 h after **a** inoculation with 1×10^6 conidia/ml of pathogenic isolate or **b** aqueous solution of 10 U toxin (tenfold dilution that induced necrosis) was placed on wound site

Fig. 2 Comparison between virulence and toxin productivity of the black spot pathogen of peach. Virulence of each field isolate was based on percentage of necrotic area on inoculated susceptible leaf. Toxin productivity was determined by leaf-necrosis assay with two-fold dilution of spore germination fluids (SGFs). The smallest circle represents one isolate; the largest circle represents six isolates in this graph

Table 1 Necrosis index on leaves 72 h after inoculation with the black leaf spot pathogen (5×10^5 conidia/ml) or its toxin (10 U)

Cultivar	Necrosis index[a]	
	Pathogen	Toxin
Peach		
Shimizu Hakuto	3	3
Shanhai Suimitsuto	3	3
Akatsuki	2	2
Asama Hakuto	2	2
Benishimizu	2	2
Chikuma	2	2
Chiyohime	2	2
Chiyomaru	2	2
Golden peach	2	2
Hakuto	2	2
Hakuho	2	2
Hakurei	2	2
Kamogawa Hakuto	2	2
Kanougan Hakuto	2	2
Masahime	2	2
Matsuki Hakuto	2	2
Okubo	2	2
Shunrai	2	2
Takei Hakuho	2	2
Wase Ogonto	2	2
Yahata Hakuho	2	2
Yamato Hakuto	2	2
Yuzora	2	2
Kawanakajima Hakuto	1	1
Tenshin Suimitsuto	1	1
Apple		
Orin	0	0
Japanese pear		
Nijisseiki	0	0
Strawberry		
Morioka 16	0	0

[a]Necrosis index was rated by percentage of necrotic area on entire leaf: 0, no necrosis; 1, <30%; 2, 30–60%; 3, >60%.

Shimizu Hakuto and Shanhai Suimitsuto were the most sensitive to the toxin, cvs. Kawanakajima Hakuto and Tenshin Suimitsuto were the most resistant, and the rest were intermediate. Nonhost plants were immune.

Differences in responses between susceptible and resistant cultivars

All the peach cultivars examined were susceptible to the pathogen and sensitive to the toxin, although there were quantitative differences in susceptibility and sensitivity. Effects of inoculation density and toxin dose on leaf responses were compared between one of the most susceptible cvs., Shimizu Hakuto, and one of the most resistant cvs., Kawanakajima Hakuto. There was an apparently quantitative difference between susceptible and resistant cultivars. Resistant cv. Kawanakajima Hakuto was less susceptible to the pathogen and less sensitive to the toxin than the susceptible cv. Shimizu Hakuto was (Figs. 3, 4). Further, we reconfirmed that sensitivity of plants to the toxin was positively correlated with susceptibility of plants to the pathogen.
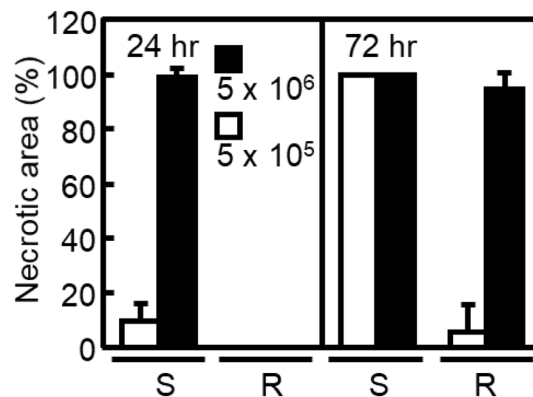
Fig. 3 Relative necrotic area on leaves of susceptible cv. Shimizu Hakuto (S) and resistant cv. Kawanakajima Hakuto (R) at 24 and 72 h after inoculation with conidia of the black spot pathogen. For the half-leaf bioassay using a set of three leaves, one leaf half was inoculated with 5 ml of 5×10^5 conidia/ml and the other half with 5×10^6 conidia/ml); the assay was done three times. Necrotic and green areas were measured using 1-mm-grid tracing paper, to calculate the percentage area that was necrotic area (100% = whole leaf was necrotic). Bars indicate standard error
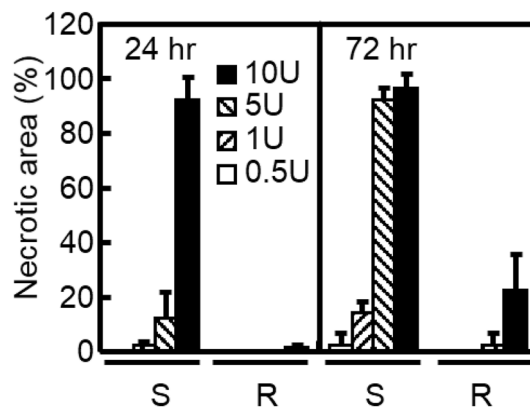
Fig. 4 Relative necrotic area on leaves of susceptible cv. Shimizu Hakuto (S) and resistant cv. Kawanakajima Hakuto (R) at 24 and 72 h after treatment with toxin (0.5, 1, 5 and 10 U). The percentage of necrotic area was used as a measure of toxin sensitivity. Each bioassay consisted of a set of three leaves; four leaf sections were cut from each leaf and treated with 0.5, 1, 5, or 10 U of toxin solution. The necrotic area was obtained as described for Fig. 3. The assay was done three times. Bars indicate standard error.

Table 2 Percentage of infection structures formed and subsequent penetration by nonpathogenic isolate of *Alternaria alternata* with and without toxin (10 U) on leaves

Treatment	Germination[b]	Appressorial formation[c]	Penetration[d]
Nonpathogen + toxin[a]	95.0 ± 4.7	43.7 ± 8.4	22.4 ± 7.1
Nonpathogen	94.0 ± 10.5	38.3 ± 10.3	0.0 ± 0.0

[a]Mean % ± SE (50 conidia, 4 replications)

[b]Mean % ± SE of germinated conidia in total conidia observed

[c]Mean % ± SE of appressoria per number of germ tubes observed

[d]Mean % ± SE of infection hyphae per number of appressoria observed

By 24 h after inoculation or treatment with toxin, resistant cv. Kawanakajima Hakuto showed no response although necrotic lesions were observed 72 h after treatment with the higher concentration of the toxin.

Induction of infection by the toxin

Microscopic observation revealed that adding the toxin (10 U) to the inoculum suspension did not influence either germination or appressorial formation by the nonpathogenic isolate on leaves. While the nonpathogenic isolated did not form infection hyphae without the toxin present, addition of the toxin resulted in 22.4% of appressoria formed infection hyphae (Table 2).

Discussion

The toxin found in the present study induced black spots (necrotic lesions) on leaves of the host (Fig. 1). In addition, productivity of the toxin was correlated with pathogenicity of the pathogen (Fig. 2), and susceptibility of the plants to the pathogen was correlated with their sensitivity to the toxin (Table 1). Induction of colonization by the toxin is suggested to be an important pathological criterion of HSTs (Akimitsu et al. 2014; Kohmoto et al. 1989), and HSTs are released during spore germination (Nishimura and Scheffer 1965; Nishimura et al. 1979; Otani et al. 1975). The toxin of peach pathogen was detected in SGFs and in culture filtrates. Adding the toxin to inoculum of the nonpathogenic *A. alternata* resulted in the induction of infection hyphae (Table 2). The results supports the evidence that the toxin produced during germination made the plants cells accessible to invasion and initial colonization by suppression of nonhost resistance (Yamamoto et al. 1984,2000). Thus, we concluded that the pathogenic *A. alternata* in this study produces a new HST to peach only and designated the HST as an AP-toxin (*Alternaria* and *Prunus*). This toxin is the eighth HST toxin reported for *A. alternata*. The term pathotype was proposed for HST-producing isolates of *A. alternata* by Nishimura and Kohmoto (1983) and applied to a new pathotype, *A. alternata* strawberry pathotype (Maekawa et al. 1984; Watanabe and Umekawa 1977; Watanabe et al. 1978). The validity of these pathotypes was further supported by molecular analyses of nuclear ribosomal DNA and mitochondrial DNA (Kusaba and Tsuge 1994, 1995, 1997). In addition, genes involved in toxin biosynthesis, which controls pathogenicity, have been identified in the Japanese pear pathotype, and homologous genes are conserved among isolates of the tangerine pathotype and the strawberry pathotype (Tanaka et al. 1999; Tsuge et al. 2013, 2016), but have not been detected among saprophytic isolates of *A. alternata*. The hypothesis that HST-producing *A. alternata* is an intraspecies variant for toxin production is supported by genetic aspects. Although a classification guideline for small-spored *Alternaria* species and the naming by forma specialis have been proposed (Nishikawa and Nakashima 2019; Woudenberg et al. 2015), in the first report on black spot disease, the causal pathogen was identified as *A. alternata* based on morphological characteristics (Inoue and Nasu 2000). Because it produces a new HST, AP-toxin, we thus propose that this pathogen is the peach pathotype of *A. alternata*.

In seven previous examples of HST-producing *A. alternata* and their host plants, the specificity was remarkably high: resistant cultivars were immune to the pathogen and tolerant to the HST at concentrations up to 10^4 higher than concentrations tolerated by susceptible cultivars (Nakashima et al. 1985; Namiki et al. 1986). In the present study, cultivar Kawanakajima Hakuto was one of the most resistant among peach cultivars examined but not completely immune to the pathogen and tolerant to AP-toxin in the laboratory conditions and so far has not been affected by the disease in orchards. Information on the chemical structure of AP-toxin and the primary target site of action in host cells will help us understand toxin specificity among peach cultivars.

Compliance with ethical standards

Human/animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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