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Destruxin B produced by *Alternaria brassicae* does not induce accessibility of host plants to fungal invasion

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Abstract

It has been reported that *Alternaria brassicae*, the causal agent of gray leaf spot in *Brassica* plants, produces a host-specific or host-selective toxin (HSTs) identified as destruxin B. In this study, the role of destruxin B in infection of the pathogen was investigated. Destruxin B purified from culture filtrates (CFs) of *A. brassicae* induced chlorosis on host leaves at 50–100 μg ml^{-1}, and chlorosis or necrosis on non-host leaves at 250–500 μg ml^{-1}. Destruxin B was detected in spore germination fluids (SGFs) on host and non-host leaves, but not in a sufficient amount to exert toxicity to host plants. When spores of non-pathogenic *A. alternata* were combined with destruxin B at 100 μg ml^{-1} and inoculated on the leaves, destruxin B did not affect the infection behavior of the spores. Interestingly, SGF on host leaves allowed non-pathogenic spores to colonize host leaves. Moreover, a high molecular weight fraction (>5 kDa) without destruxin B obtained by ultrafiltration of SGF had host-specific toxin activity and infection-inducing activity. From these results, we conclude that destruxin B is not a HST and does not induce the accessibility of the host plant which is essential for colonization of the pathogen. In addition, the results with SGF imply that a high molecular weight HST(s) is involved in the host–pathogen interaction.

Keywords: *Alternaria brassicae*; *Brassica* plants; Destruxin B; Host-specific or host-selective toxins; Spore germination fluid; Infection-inducing activity; Accessibility; Colonization

1. Introduction

The fungus *Alternaria brassicae* (Berk.) Sacc. is the causal agent of gray leaf spot on *Brassica* plants (canola, oilseed, cabbage, mustards, etc.) and other cruciferous plants [1,2]. These diseases are known by many names, including black spot, dark leaf spot, and Alternaria blight. The visible symptoms on leaves are lesions with grayish, brownish or blackish centers, and chlorotic margins [3]. The pathogen affects all aerial plant parts, reducing the photosynthetic area and accelerating senescence and defoliation [1]. In several countries, the pathogen causes serious yield losses, especially in oilseed crops. In 1987, for example, the yield loss was up to 30% in heavily infected oilseed fields in western Canada [2].

It has been reported that *A. brassicae* produces four cyclic depsipeptide phytotoxins belonging to the family of compounds named destruxins [1,3–6]. Destruxin B ($C_{30}H_{51}$, N_5O_7, MW = 593) [3,7] is the major phytotoxin produced by this fungus *in vitro* [1,5] and *in planta* [4,6]. Three other phytotoxins, homodestruxin B, destruxin B_2, and desmethyldestruxin B, are produced in much smaller amounts [3]. Bains and Tewari [1] reported that destruxin B is phytotoxic to different *Brassica* plants, and the degree of sensitivity to the toxin correlates with the degree of susceptibility to the pathogen. Therefore, they classified destruxin B as a host-specific toxin according to the terminology established by Pringle and Scheffer [8]. However, Buchwaldt and Green [6] suggested that both host and non-host plants of *A. brassicae* are sensitive to destruxin B, although *Brassica* plants are the most sensitive and the sensitivity decreases as relatedness to the *Brassica* group becomes more distant. On this basis, they proposed

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that destruxin B is a concentration-dependent host-selective toxin and a virulent factor contributing to the aggressiveness of the pathogen.

Certain fungal pathogens, especially the genus *Alternaria*, are known to produce host-specific or host-selective toxins (HSTs) as agents of virulence or pathogenicity [9]. From studies on HSTs produced by fungal pathogens [10], a mechanism that determines specificity in the diseases involving HST has been proposed as follows: (i) germinating spores of fungal pathogen release HST as a host recognition factor at the infection site, (ii) the released signal factor affects selectively host cells, and (iii) the accessible state of host cells to possible hyphal invasion is disposed by the signal transduction. Although Buchwaldt and Green [6] have reported that destruxin B is released from germinating spores of *A. brassicae*, no information is yet available on whether the released destruxin B is involved in the initial colonization of the plant by the pathogen. We here show evidence that the destruxin B produced by *A. brassicae* is not responsible for inducing accessibility to host plants. We also propose the possibility that other toxin(s) different from destruxin B may contribute to the induction of the disease.

2. Materials and methods

2.1. Plant materials

Brassica spp. and other cruciferous and non-cruciferous plants were used in this study (Table 1). All plant seeds, except for those of Japanese pear, were grown in pots containing a mixture of soil and vermiculite (2:1) in a glasshouse for 3–4 weeks. Japanese pear seedlings were planted in pots and maintained in a glasshouse. Because plant leaves that overheat during the summer season become temporarily insensitive to some HSTs [11], the second and third leaves of all plants were detached and used for bioassays from October to April.

2.2. Fungal isolates

Two isolates, S-193-11 and O-265 of *A. brassicae* and a non-pathogenic isolate, O-94 of *A. alternata*, were used in this study. Stock cultures of these isolates were maintained on potato dextrose agar (PDA) slants at room temperature (25±2 °C) in the dark. Subcultures of the isolates S-193-11 and O-265 were grown on V-8 juice agar medium containing 0.004% Rose Bengal in Petri dishes (9 cm diameter), and incubated for 2–3 weeks at 20 °C under a 16 h dark and 8 h UV light (black fluorescent light, FL-20SBLB, Toshiba, Tokyo, Japan) period. A subculture of the isolate O-94 was grown on PDA in Petri dishes (9 cm diameter) and incubated for 2–3 weeks at 25 °C in the dark. After the incubation period, the spores of all isolates were collected separately, as described by Otani et al. [12]. The dishes were flooded with distilled water (DW), and the surfaces were brushed with a small paintbrush. The spore suspensions were filtered through three layers of cheesecloth to remove the mycelium debris, and the spores were washed twice in DW by centrifugation (Himac CF 7D2, Hitachi, Tokyo, Japan) at 800*g* for 5 min. The spores collected on Whatman filter paper no. 50 were air-dried and stored at −80°C until use.

Table 1
Toxicity of destruxin B purified from CF of *A. brassicae* (S-193-11) to host and non-host plant leaves

Plant species	Dilution-end point for toxicity of destruxin B[a] ($\mu g\,ml^{-1}$)
Host	
Brassica napus cv. Westar (oilseed)	50.0
B. campestris cv. Taibyo-Rokujunichi (Chinese cabbage)	50.0
B. campestris cv. Natsurakuten (Komatsuna)	50.0
B. oleracea cv. Shoshu (cabbage)	50.0
Raphanus sativus cv. Taibyo-Soubutori (Japanese radish)	50.0
R. sativus cv. Kometto (red radish)	100.0
Non-host	
Cucumis sativus cv. Tsubasa (cucumber)	250.0
Lycopersicon esculentum cv. Momotaro (tomato)	250.0
Pisum sativum cv. Horunsunakku (pea)	250.0
Pyrus pyrifolia cv. Nijisseiki (Japanese pear)	500.0

[a]The purified destruxin B was serially diluted with 10% MeCN to give 1000, 500, 250, 100, 50, 25, and 10 $\mu g\,ml^{-1}$. The toxicity of destruxin B was determined by leaf assay. Visible symptoms were recorded after 48 h of incubation at 25 °C in continuous light.

2.3. Purification of destruxin B from culture filtrates (CFs)

Due to the low concentration of destruxin B in the spore germination fluids (SGFs) of *A. brassicae*, purification of destruxin B was carried out *in vitro* using Frie's liquid medium supplemented with yeast extract ($1\,g\,l^{-1}$). Frie's liquid medium (200 ml) in a 500 ml glass bottle was inoculated with five pieces (1 cm^2) of mycelia of the isolate S-193-11, and incubated at 25 °C in the dark. The culture medium was harvested after incubation for 40 days and filtered through four layers of Miracloth and Whatman filter paper no. 2. The CF was extracted three times with ethyl acetate, and the extract was evaporated to dryness with a rotary vacuum evaporator (Model N-1, Eyela, Tokyo, Japan) at 40 °C and dissolved in 100% methanol (HPLC grade). After filtration through a 0.45-μm chromatodisc unit 13N (Kurabo, Tokyo, Japan), the sample (20 μl) was injected into a column (Develosil ODS-UG-5, 4.6/150 mm, NW) (Nomura Chemical, Tokyo, Japan). Eluent from the column was monitored at 220 nm with an L-7100 pump and 655A variable wavelength UV monitor (Hitachi, Tokyo, Japan). The running solution