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Production of desmethyl-gregatin A, a possible causative toxin of brown stem rot in adzuki bean, by *Phialophora gregata* f. sp. *adzukicola*

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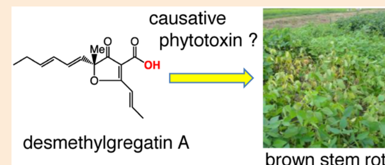
Supplementary material

To elucidate the cause of brown stem rot in the adzuki bean, we re-evaluated the phytotoxins produced in cultures of the causative agent, *Phialophora gregata* f. sp. *adzukicola*. The ethyl acetate–soluble acidic fraction of the culture, as well as the neutral fraction, inhibited the growth of alfalfa seedlings. In the neutral fraction, known phytotoxins gregatin A, B, and C or D and penicilliol A were present. Although the phytotoxins in the acidic fraction were unstable, liquid chromatography–mass spectrometry analysis of the partially purified material suggested that one phytotoxin present was the non-methylated gregatin desmethyl-gregatin A (gregatinic acid A).



Keywords: *Phialophora gregata*, brown stem rot, adzuki (*Vigna angularis*), gregatin, phytotoxin, desmethyl-gregatin A.

Introduction

Brown stem rot of adzuki beans [*Vigna angularis* (Willd.) Ohwi and Ohashi] is a typical vascular disease, characterized by browning of the stem's vascular bundles, wilting of the entire body, defoliation, and death of the plant. The causative agent of the disease is *Phialophora gregata* (Allington and Chamberlain) W. Gams f. sp. *adzukicola*,[1)] which produces phytotoxins, called gregatins[2–4)] (Fig. 1). Gregatin-deficient mutants of the causative agent showed reduced pathogenicity as compared with the parental isolates.[5)] However, gregatins were not detected in the diseased tissues of inoculated adzuki bean plants, and gregatins were not recovered from healthy plants injected with pure gregatin A.[6)] Furthermore, gregatin-deficient mutants generated by *Agrobacterium tumefaciens*–mediated transformation were pathogenic to adzuki beans with similar virulence to the wild-type strain, whereas the non-pathogenic mutant produced gregatin concentrations comparable to those of the wild-type strain.[7)] Accordingly, it is still uncertain whether gregatins are the cause of brown stem rot of adzuki beans.

As part of our research to elucidate the cause of crop diseases by bioassay-guided searching for phytotoxins,[8–10)] in this study we searched for phytotoxins other than gregatins produced by the causative agent of brown stem rot of adzuki bean, *Phialophora gregata* f. sp. *adzukicola*.

Materials and methods

1. Fungal strain and media

Phialophora gregata f. sp. *adzukicola* MAFF 241057 (originally described as strain T649[11)]) deposited at the Genetic Resources Center, National Agriculture and Food Research Organization, was used in this study.

The phytotoxin productivity of the pathogen was evaluated on

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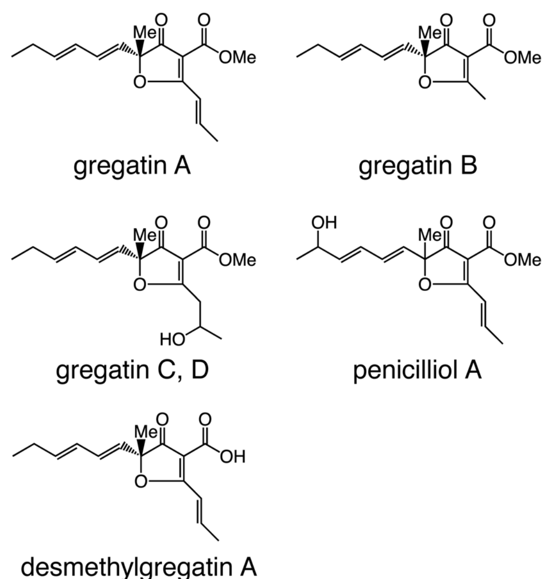

Fig. 1. Structures of gregatins and related compounds. Gregatins C and D are stereoisomers.

five agar media. Potato dextrose agar (PDA) medium was prepared in accordance with the standard method (broth of 200 g potato tubers, 20 g dextrose, and 15 g agar per liter of reverse osmosis water). Potato paste dextrose agar (PPDA) and potato paste sucrose agar (PPSA) media were prepared with boiled and mashed potato instead of potato broth in PDA medium. Oatmeal agar (OMA)[12] and V8 agar (V8A)[13] media were prepared in accordance with the literature.

2. Culture conditions and extraction method

Phialophora gregata f. sp. *adzukicola* MAFF 241057 was inoculated to a 500 mL shaking flask containing 100 mL of PPD liquid medium, after which the flask was incubated at 22°C for 2 days on a reciprocal shaker (160 rpm). Portions of this culture (0.5 mL) were inoculated on petri dishes (9 cm diameter) containing 10 mL of agar medium, and the dishes were incubated at 22°C for 14 days. The cultured material was macerated with acetone, and the acetone extract was treated with ethyl acetate in accordance with the established method[14] to obtain an ethyl acetate–soluble neutral fraction and an acidic fraction.

3. Bioassays

The phytotoxic activities of fungal extracts and fractions during purification were assessed in alfalfa (*Medicago sativa* L.) seedlings following a previously reported method.[9]

4. HPLC conditions and LC–MS analysis

Crude extracts and purified fractions were analyzed using a high-performance liquid chromatography (HPLC) system (LC-10, Shimadzu, Kyoto, Japan) equipped with a Develosil ODS-UG-5 column (5 μm, 2.0×150 mm; Nomura Chemical, Aichi, Japan). The chromatographic conditions were as follows: flow rate 0.2 mL/min; detection UV 254 nm; solvent aqueous acetonitrile (30% for 5 min, 30–90% for 20 min, and 90% for 5 min) with or without 0.1% trifluoroacetic acid (TFA); and column oven temperature 40°C.

The liquid chromatography–mass spectrometry (LC–MS) analysis was confirmed with LC–TOF MS (JMS-T100LC, JEOL, Tokyo, Japan) using the same column and conditions with a TFA-containing solvent system as described for the HPLC analysis.

5. Methylation of ethyl acetate–soluble acidic fraction

To a solution of the ethyl acetate–soluble acidic fraction (*ca.* 23 mg) in methanol–toluene (1 : 4, 2 mL) was added trimethylsilyldiazomethane in *n*-hexane (*ca.* 0.6 M, 0.5 mL; Tokyo Chemical Industry, Tokyo, Japan) at room temperature. The mixture was stirred for 30 min at room temperature and concentrated to give the reaction products.

Results

1. Selection of culture medium

To re-evaluate phytotoxins produced by the causative agent of brown stem rot of adzuki bean, *Phialophora gregata* f. sp. *adzuki-*

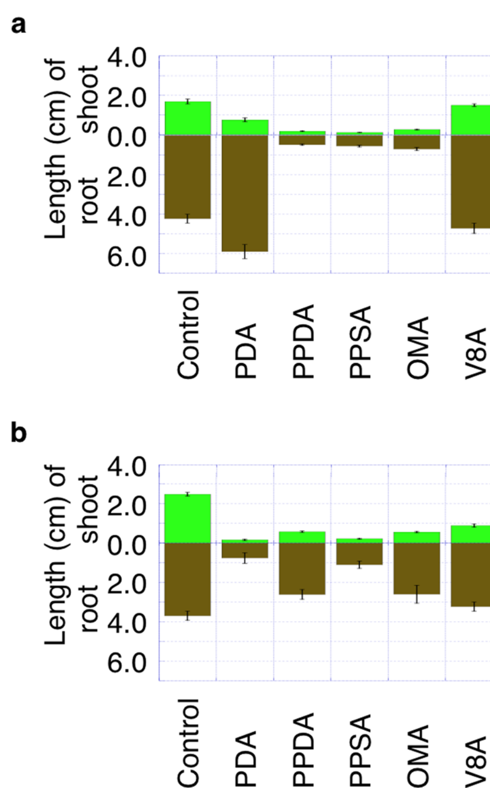

Fig. 2. Phytotoxic activity of ethyl acetate extracts of *Phialophora gregata* f. sp. *adzukicola* from five culture media. *PDA*, potato dextrose agar; *PPDA*, potato paste dextrose agar; *PPSA*, potato paste sucrose agar; *OMA*, oatmeal agar; *V8A*, V8 agar. Growth inhibitory activity of the crude ethyl acetate extracts was examined at dose equivalents to 1.0 mL (neutral fraction, **a**) and 5.0 mL (acidic fraction, **b**) of cultured material using alfalfa seedlings. Each bar represents the average of the mean values of eight seedlings. Error bars represent the standard deviation from the mean.

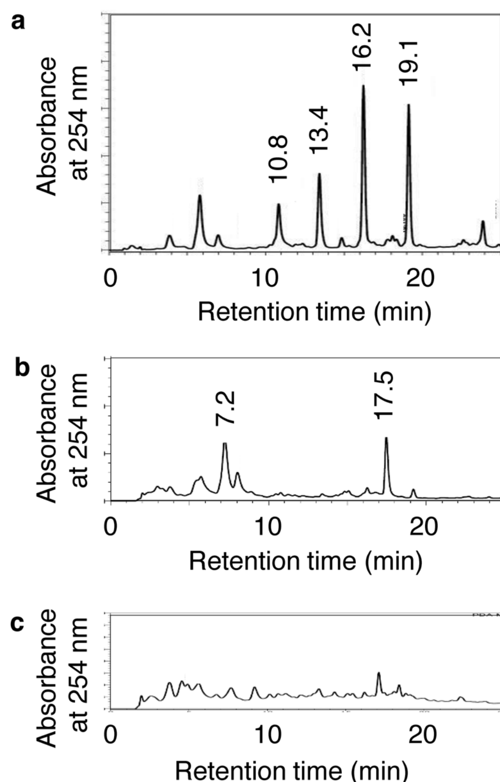

Fig. 3. HPLC profiles of the ethyl acetate–soluble neutral fraction (**a**), acidic fraction (**b**), and the methylated acidic fraction (**c**). HPLC analysis was performed using aqueous acetonitrile (**a**) or aqueous acetonitrile containing 0.1% trifluoroacetic acid (**b**, **c**).

cola MAFF 241057 was cultured on five types of culture media. The growth inhibitory activities of the ethyl acetate–soluble neutral and acidic fractions of the cultured material were examined using alfalfa seedlings, which are the same legume as adzuki beans and need fewer samples than adzuki beans, as an indicator.

Among the neutral fractions, those of PPDA, PPSA, and OMA showed strong activity (Fig. 2a). In contrast, for acidic fractions, that of PDA medium showed the strongest activity, followed by that of PPSA medium (Fig. 2b). The other three fractions showed moderate activity, *i.e.*, they inhibited hypocotyl growth but had little effect on root growth.

The neutral fraction is recognized to contain known phytotoxins gregatins. However, the acidic fraction may contain a new phytotoxin. Therefore, the PDA medium was used to search for a new phytotoxin.

2. *HPLC analysis of neutral and acidic extracts of cultured material in PDA medium*

The HPLC analysis of the neutral fraction with aqueous acetonitrile as the eluent revealed four major peaks (Fig. 3a). Based on a comparison of the UV spectra (Fig. S1b–e) and retention times (t_R) of the separately isolated specimens identified by NMR and MS (Figs. S3–S5), the peaks were identified as gregatins A (t_R=19.1 min), B (t_R=16.2 min), and C or D (t_R=13.4 min).[2,4) The peak at t_R=10.8 min was deduced to be penicilliol A by UV spectrum and MS data (*m/z* 293.1381, calcd. for $C_{16}H_{21}O_5$: 293.1384).[4,15) Although gregatins C and D are stereoisomers that differ in configuration of the hydroxyl group, we did not confirm the stereochemistry of the detected compound.

Analysis of the acidic fraction under chromatographic conditions the same as those of the neutral fraction yielded only broad peaks (Fig. S2a). When 0.1% trifluoroacetic acid (TFA) was added to the solvent, two peaks (t_R=7.2 and 17.5 min) were resolved (Fig. 3b). The UV spectra of the two peaks showed absorption maxima at 230 and 300 nm (Fig. S2c, d), which closely resembled those of gregatin A (Fig. S1b).

These results revealed that the acidic fraction was not contaminated with gregatins, and that acidic substances with the same chromophore as gregatin A were present. Given that the t_R of the posterior peak (t_R=17.5 min) preceded that of gregatin A (t_R=19.1 min), it was considered to be more polar than gregatin A and was presumed to be desmethyl-gregatin A,[16) a non-methylated (carboxylic acid) form of gregatin A. By a similar presumption, the peak at t_R=7.2 min was assumed to be the unmethylated form of penicilliol A (t_R=10.8 min).

3. *Methylation of the acidic fraction*

If the active substances in the acidic fraction were the unmethylated form of gregatin, it may be possible to identify it by methylation leading to gregatin. Therefore, the acidic fraction was treated with trimethylsilyldiazomethane.

The HPLC analysis of the methylated acidic fraction showed that the peak intensities of t_R=7.2 and 17.5 min were greatly reduced, and several smaller peaks appeared, whereas the peaks of predicted gregatin A (t_R=19.1 min) and penicilliol A (t_R=10.8 min) were absent (Fig. 3c). These results suggested that the active substance in the acidic fraction did not become gregatin by methylation but was transformed or degraded into multiple other compounds.

In addition, the phytotoxic activity was weakened by methylation (Fig. S6). Given that the decrease in intensity of the two main peaks detected by HPLC analysis of the acidic fraction correlated with the decrease in activity, these peaks were presumed to be new phytotoxins.

4. *Purification of phytotoxins in the acidic fraction*

Next, we aimed to purify the phytotoxic substances in the acidic fraction as is (without any treatment) using chemically modified silica gel with a diol group (Chromatorex DIOL, Fuji Silysia Chemical, Aichi, Japan) (Fig. 4), because the substances were considered to be unstable. As a result, the activity was recovered in 20% acetone in *n*-hexane containing 0.1% TFA fraction in the first chromatography (Fig. 5a). However, comparisons of the HPLC chromatograms before and after purification showed that the peak at t_R=17.5 min remained, but the peak at t_R=7.2 min disappeared, and a new peak appeared at t_R=14.2 min (Fig. 6a, b). These results suggested that the peak compound at t_R=7.2 min was unstable and was disrupted during the purifica-

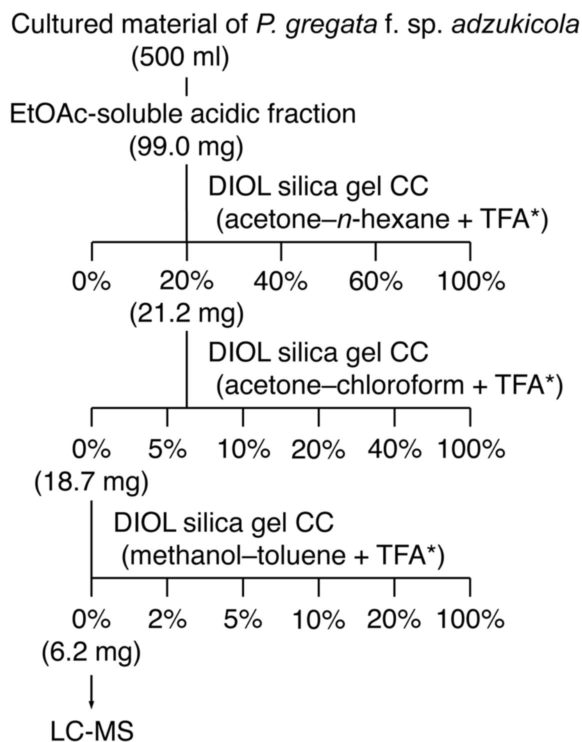

Fig. 4. Purification procedure for the acidic fraction. *All solvents for chromatography contained 0.1% trifluoroacetic acid (TFA). *CC*, Column chromatography.

tion process, whereas the peak compound at t_R=17.5 min was less unstable.

Next, purification was attempted using the acetone–chloroform+TFA system, but purification of the active substance did not proceed in this solvent system because most of the sample was recovered in the chloroform fraction (0% fraction, Fig. 4) and activity was also detected in that fraction (Fig. 5b). The HPLC profile was also unchanged before and after purification (Fig. 6c).

Purification by the methanol–toluene+TFA system resulted in the disappearance of the fraction showing distinct activity (Fig. 5c). HPLC analysis showed that the toluene+TFA fraction (0% fraction) gave a chromatogram similar to that of the 20% acetone–hexane+TFA fraction, but with about half the peak intensity (Fig. 6d). Therefore, the assay was performed at twice the dose, and weak activity was detected in the toluene+TFA fraction (Fig. 5d). However, subsequent HPLC analysis showed a further decrease in peak intensity (Fig. 6e).

From these results, the degradation of the active substance, either during the purification process or over time thereafter, was considered to be the cause of the decrease in phytotoxic activity. In addition, the decrease in activity correlated with the decrease in the intensity of the peak at t_R=17.5 min in the HPLC analysis, thus increasing the certainty that this peak was the active substance. A peak at t_R=*ca.* 15 min, incidentally, was observed along with the peak at t_R=17.5 min in the purification process shown in Fig. 6. However, that peak was not observed in another batch of extracts (Fig. 3b or Fig. 7a); therefore, it could not be considered an active substance. The possibility of the presence of active substances that do not show UV absorption maxima was also considered low based on the analysis of the chromatogram at UV 210 nm (Fig. S7).

5. LC–MS analysis of the partially purified acidic fraction

Given that the peak intensity at t_R=17.5 min correlated with the intensity of activity, the mass spectrum of the peak was measured by LC–MS. A newly grown culture was purified using the scheme shown in Fig. 4, and the toluene+TFA fraction (0% fraction) was subjected to LC–MS analysis.

The MS spectrum of the peak of interest (t_R=17.5 min by HPLC analysis (Fig. 7a), t_R=21.9 min by LC–MS analysis (Fig. 7b)) gave a peak at *m*/*z* 263.1295 (Fig. 7c), which was presumed to be a protonated molecule ($[M+H]^+$). From this accurate mass, the molecular formula of the compound was estimated to be $C_{15}H_{18}O_4$ (calcd. for $C_{15}H_{19}O_4$ 263.1283). The peak at *m*/*z* 245.1190 could be attributed to the dehydration peak of the protonated molecule ($C_{15}H_{17}O_3$, calcd. 245.1178) that at *m*/*z* 285.1109 was the sodium-cationized molecule ($C_{15}H_{18}O_4Na$, calcd. 285.1103) and that at *m*/*z* 301.0845 was the potassium cationized molecule ($C_{15}H_{18}O_4K$, calcd. 301.0842). Based on these results, the substance producing the peak was presumed to be desmethyl-gregatin A, the unmethylated form of gregatin A.[16)]

Discussion

To elucidate the cause of brown stem rot of adzuki bean, we reevaluated phytotoxins in cultures of the pathogen, *Phialophora gregata* f. sp. *adzukicola*.

The phytotoxin productivity of the pathogen was examined on five different media. Both the neutral and acidic fractions of the ethyl acetate–soluble fraction showed growth-inhibitory activity on alfalfa. The neutral fraction contained the known phytotoxin gregatins. However, this is the first report to demonstrate the production of an acidic phytotoxin by this pathogen.

The phytotoxic activity of the extract and relative activity between ethyl acetate–soluble neutral and acidic fractions were different among media. Interestingly, the acidic fraction of PDA cultures showed the strongest activity, while the neutral fraction showed very weak activity. On the other hand, the neutral fraction of PPDA cultures showed strong activity, while the acidic fraction was moderately active. The difference between the two media is the exclusion or inclusion of the water-insoluble components of potato. Although the microbial findings were taxonomically different, the production of the phytotoxin, thaxtomin, by *Streptomyces scabiei* in the common potato scab disease is known to be triggered by plant components such as suberin[17)] and cello-oligosaccharides.[18,19)] A similar phenomenon may be involved in the toxin production of this fungus.

The acidic phytotoxin found in this study was unstable and was degraded by methylation with trimethylsilyldiazomethane or purification by DIOL silica gel considered a milder purifica-

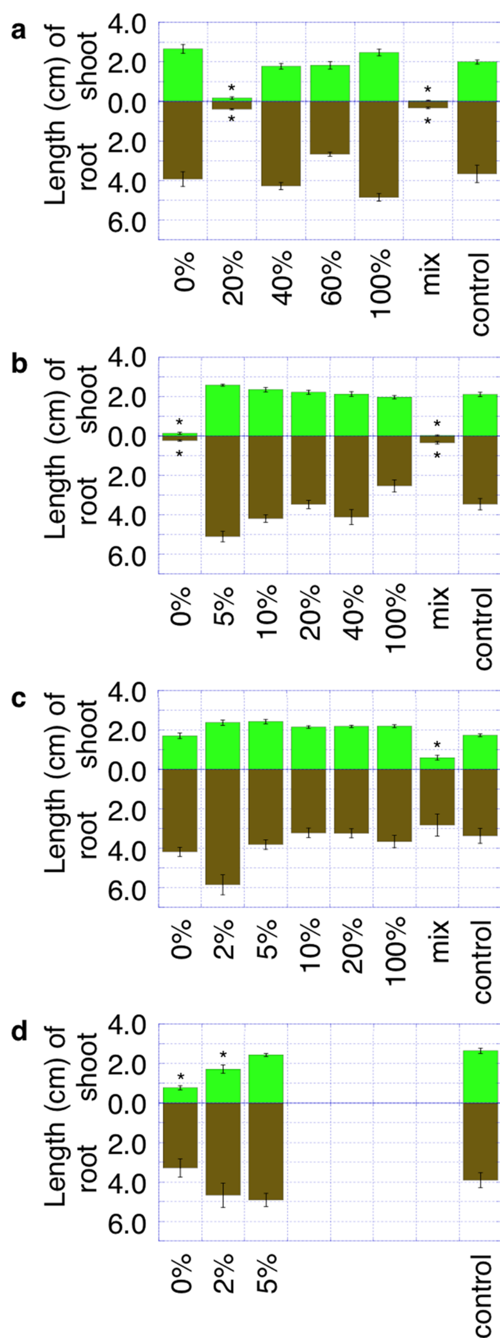

Fig. 5. Phytotoxic activity of fractions purified by column chromatography with acetone–*n*-hexane+TFA (**a**), acetone–chloroform+TFA (**b**), or methanol–toluene+TFA (**c**, **d**) as the solvent system. Each fraction was examined at a dose equivalent to 10 mL (**a**–**c**) or 20 mL (**d**) of the cultured material. Each bar represents the average of the mean values of eight seedlings. Error bars represent the standard deviation from the mean. * An asterisk indicates significant growth-inhibitory activity as compared with the control ($p<0.01$, Dunnett's test).

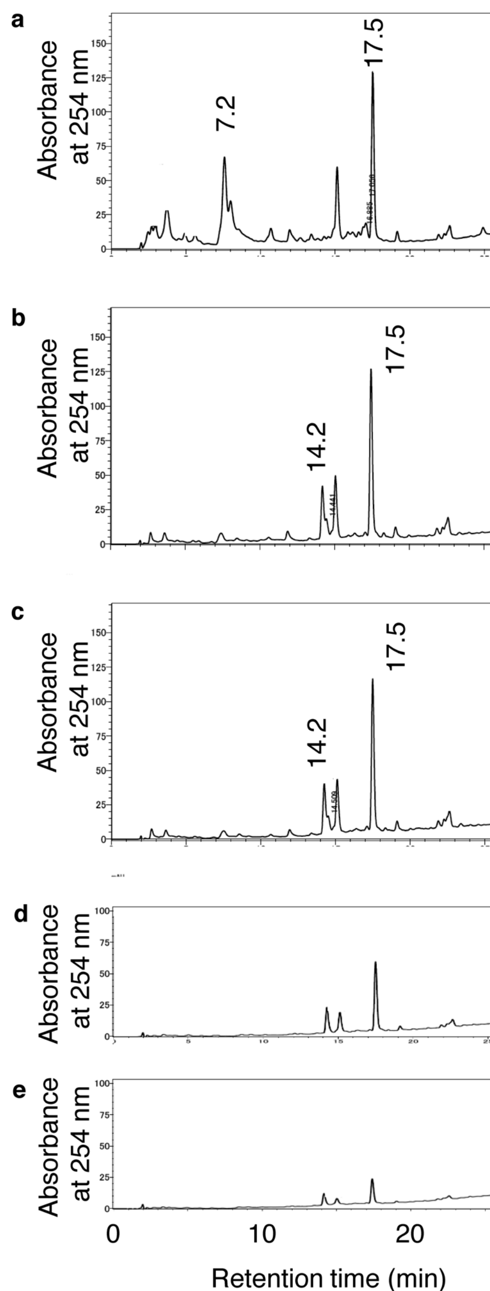

Fig. 6. HPLC profiles of the ethyl acetate–soluble acidic fraction (**a**) and partially purified acidic fractions (**b**–**e**); **b**: 20% acetone–*n*-hexane+TFA fraction, **c**: chloroform+TFA (0%) fraction, **d**: toluene+TFA (0%) fraction, **e**: second analysis of toluene+TFA (0%) fraction. HPLC analysis was performed using aqueous acetonitrile containing 0.1% TFA. The amount of sample injected was equivalent to 14 μL of the cultured material.

tion material than silica gel and the subsequent storage period. Based on the UV spectrum and LC–MS analysis, this phytotoxin was presumed to be desmethyl-gregatin A.[16] The unstable nature of the phytotoxin was also shown by the fact that the strong peak of the dehydrated ion (*m*/*z* 245) was observed in the electrospray ionization time-of-flight mass spectrometric analysis, which is a soft ionization method. The instability of gregatin is thought to be due to the susceptibility of the double bond conjugated with two carbonyl groups by the attack of nucleophilic reagents. The finding that gregatin A spontaneously converted to gregatins C and D in water[16] supports this idea. In the chlamydospore-like cell-inducing activity of *Cochliobolus lunatus*, the reactivity of azaphilone compounds, which contain a simi-

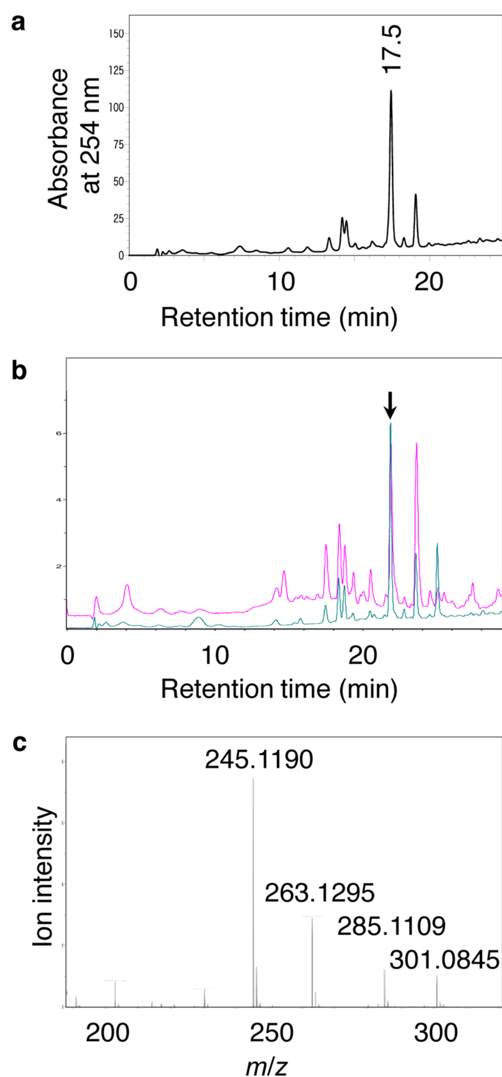

Fig. 7. HPLC (**a**) and LC–MS (**b**) profiles of the partially purified acidic fraction and MS spectrum (**c**) of the peak indicated with an arrow in the LC–MS profile. Profiles **a** and **b** are arranged to compensate for differences in the retention time due to the different pipelines. The blue plot in the LC–MS profile represents the UV_{254} chromatogram, and the red plot shows a total ion chromatogram. The numbers in the MS spectrum represent accurate masses.

lar cross-conjugated system, with nucleophilic reagents has been shown to correlate well with biological activity.[20]

Until now, the proof that a plant disease is caused by a phytotoxin has been based on the isolation and structural determination of the phytotoxin from a culture of the pathogen and whether the external administration of the phytotoxin to plants reproduces the disease symptoms caused by the pathogen. However, the application of this traditional methodology may lead to erroneous conclusions when examining whether desmethyl-gregatin A is involved in this disease, because desmethyl-gregatin A is unstable and will change into other compounds or be degraded during the period of administration.

Recently, the biosynthetic genes for gregatin A were identified, and desmethyl-gregatin A was shown to be produced as a precursor to gregatin A.[16] However, that study does not address the biological activity of desmethyl-gregatin A or its contribution to lesion formation.

The involvement of gregatin in this disease is questionable.[7] However, to rigorously verify the contribution of gregatin, desmethyl-gregatin, or other unknown compounds to lesion formation, we consider it is necessary to compare the disease symptoms caused by the wild-type strain with those caused by mutant strains in which the *O*-methyltransferase (methylation) gene (*grgD*) of the pathogen is knocked out and desmethyl-gregatin accumulates without the production of gregatin, or strains in which crucial genes involved in desmethyl-gregatin biosynthesis are mutated (*e.g.*, *grgF*, which controls the condensation of two polyketide chains, or *grgG*, which catalyzes the formation of a furanone ring).

Electronic supplementary materials

The online version of this article contains supplementary material (Figs. S1–S7) which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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