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Furuya, Naruto
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Iiyama, Kazuhiro
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Shiozaki, Naomi
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Matsuyama, Nobuaki
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

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Phytotoxin Produced by *Burkholderia gladioli*

Naruto Furuya, Kazuhiro Iiyama, Naomi Shiozaki* and Nobuaki Matsuyama

Laboratory of Plant Pathology, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan
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The phytotoxin produced by *Burkholderia gladioli* was purified and characterized. The ultraviolet and visible absorption spectrum of the phytotoxin exhibited two maxima at 260 nm and 399 nm in 80% aqueous methanol. The molecular weight of this substance was determined as 193 and the molecular formula was decided as $C_7H_7N_5O_2$ by a high resolution FAB mass spectrometry. From these results, this substance was identified as toxoflavin.

INTRODUCTION

Bacterial diseases of plants are induced by various disease-principles such as toxin, enzyme, plant hormone and extracellular polysaccharide (EPS) which are produced by phytopathogenic bacteria. However, disease-principles in large part of plant pathogenic bacteria are still unclear. It was found that *Burkholderia glumae*, which is closely resembled to *B. gladioli* in many bacteriological properties, produced toxoflavin and fervenulin causing inhibition of seedling elongation and chlorosis on rice leaves (Sato *et al.*, 1989). The authors demonstrated that toxoflavin produced by *B. glumae* is closely associated with its virulence (Iiyama *et al.*, 1994; 1995). These facts led to the speculation that *B. gladioli* will also excrete toxoflavin. The present study was designed to clarify the disease-principle of *B. gladioli*.

MATERIALS AND METHODS

Purification and identification of phytotoxin extracted from culture filtrate

B. gladioli MAFF302424, donated by the Genetic Resources Center, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, was used in this study. The bacterium was pre-cultured in a test tube containing 10 ml of potato semi-synthetic broth (PS; $Na_2HPO_4 \cdot 12H_2O$ 2.0 g, $Ca(NO_3)_2 \cdot 4H_2O$ 0.5 g, polypeptone 5.0 g, sucrose 20.0 g in 1,000 ml decoction of potato 300 g, and the pH value was adjusted to 7.0 before autoclaving) at 30 °C for 1 day. Then 5 ml of bacterial suspension was inoculated into 200 ml of PS broth in a 500 ml Sakaguchi-flask and bacteria were grown at 30 °C for 4 days under shaking conditions. After incubation, the culture filtrate was collected by centrifugation (4,000*g*, 10 min). The culture filtrate was concentrated to one tenth of the original volume by evaporation *in vacuo* at less than 40 °C, and then extracted with chloroform. The extract was developed on a silica-gel

* Present address: Kunisaki Agriculture Extension Center, Oita 873-05, Japan

(Wako-gelB-10) TLC plate with dichloromethane-methanol (9:1, v/v). The substances were detected under UV (365 nm) immediately after the developing solvent had reached 10 cm-line from the origin. Every spot detected was scraped and eluted with methanol, and assayed for its phytotoxic activity. Silica-gel powder of the area (Rf 0.4–0.5) exhibiting dark orange-coloration was collected, and eluted with methanol to subject to rechromatography with chloroform-methanol (9:1, v/v) solvent system. The bioactive zone was scraped from the silica-gel plate and eluted with methanol. The eluate was concentrated *in vacuo* and subjected to TLC with ethylacetate-chloroform-acetic acid (20:8:1, v/v/v). Then, the active zone was scraped from the plate and eluted with distilled water. The eluate with distilled water was shaken with an equal volume of chloroform to remove ash. The chloroform layer was dried with an evaporator followed by nitrogen gas blowing. Purity of the phytotoxin was estimated using a high performance liquid chromatography equipped with Shodex DM614, MS C-18, YMC AQ-312 and Shim-pack CLC-NH_2 columns and a UV absorbance detector. Samples were injected as 80% aqueous methanol solution. The phytotoxin was eluted at a flow rate of 1 ml/min with 80% methanol Solution and detected at 260 nm. UV spectrum of the phytotoxin was recorded by a Shimadzu UV-2200 spectrophotometer and FAB mass spectrometry spectrum was obtained by a JEOL JMS-D300 spectrometer using a direct inlet system.

Bioassay for toxicity

The phytotoxic activity was bioassayed by the following method. Rice seeds (cv. "Asominori") were husked, surface disinfected with 70% ethanol for 1 min and 3% hypochlorite solution (Antiformin) for 90 min, and washed in sterile distilled water. Seeds were germinated at 28 °C for 2 days in the dark. Three pre-germinated seeds were incubated with 2 ml of the test solution passed through membrane filter (0.22μm) at 30 °C for 14 days under continuous light conditions (34,000–40,000 lux) in test tube. The seeds were similarly treated with sterile distilled water as the check. Toxicity was estimated by measuring the lengths of the sprout and root of the seedling.

RESULTS AND DISCUSSION

As shown in Fig. 1 and Table 1, remarkable toxic activity was located at the area of Rf 0.4–0.5, where a dark orange-coloration spot appeared, on the silica-gel TLC plate. The eluate from the corresponding area of a blank chromatogram showed no toxic activity. When the eluate was subjected to the preparative TLC with chloroform-methanol (9:1, v/v), two bioactive spots were detected at Rf 0.14 and 0.34. The substance at Rf 0.34 was confirmed as an artifact which appeared on the silica-gel plate during TLC. The fraction at Rf 0.14 was subjected to TLC with ethylacetate-chloroform-acetic acid (20:8:1, v/v/v) and the bioactive substance appeared at Rf 0.12. This substance was eluted with deionized water, and was transferred to chloroform by shaking with an equal volume of chloroform. The chloroform layer was evaporated and the purity of this sample was estimated by HPLC (Fig. 2). As shown in Fig. 3, the ultraviolet and visible absorption spectrum of the phytotoxin exhibited two maxima at 260 nm and 399 nm in 80% aqueous methanol. The molecular weight of this substance was determined as 193 and the molecular formula was decided as $C_7H_7N_5O_2$ by a high resolution FAB mass spectrometry

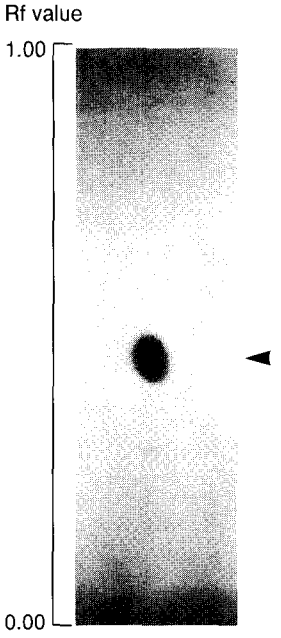

Fig. 1. TLC of the phytotoxin produced by *Burkholderia gladioli* MAFF302424.

Solvent: dichloromethane-methanol (9:1, v/v).
Plate: Silica-gel (Wako-gel B-10).
Arrowhead indicates the spot of the phytotoxin.

Table 1. Growth inhibition of rice seedlings in each fraction of TLC[a] of the chloroform extract from culture filtrate of *Burkholderia gladioli* MAFF302424

Fraction (Rf value)	Degree of growth inhibition	
	Sprout	Root
0.6 - 0.8	–[b]	–
0.5 - 0.6	–	–
0.4 - 0.5	++++	++++
0.3 - 0.4	+	+
0.2 - 0.3	+	+
0.1 - 0.2	–	–

a) TLC was conducted with dichloromethane-methanol (9:1, v/v).
b) The degree of inhibition of rice growth is presented as –: 0%, +: 0–25%, ++: 25–50%, +++: 50–75%, ++++: 75–100%. The percent inhibition of rice growth was calculated as follows; % inhibition = (A –B)/A×100.
A = the length of sprout or root in sterilized distilled water. B = the length of sprout or root in the solution of each fraction at various Rf values.

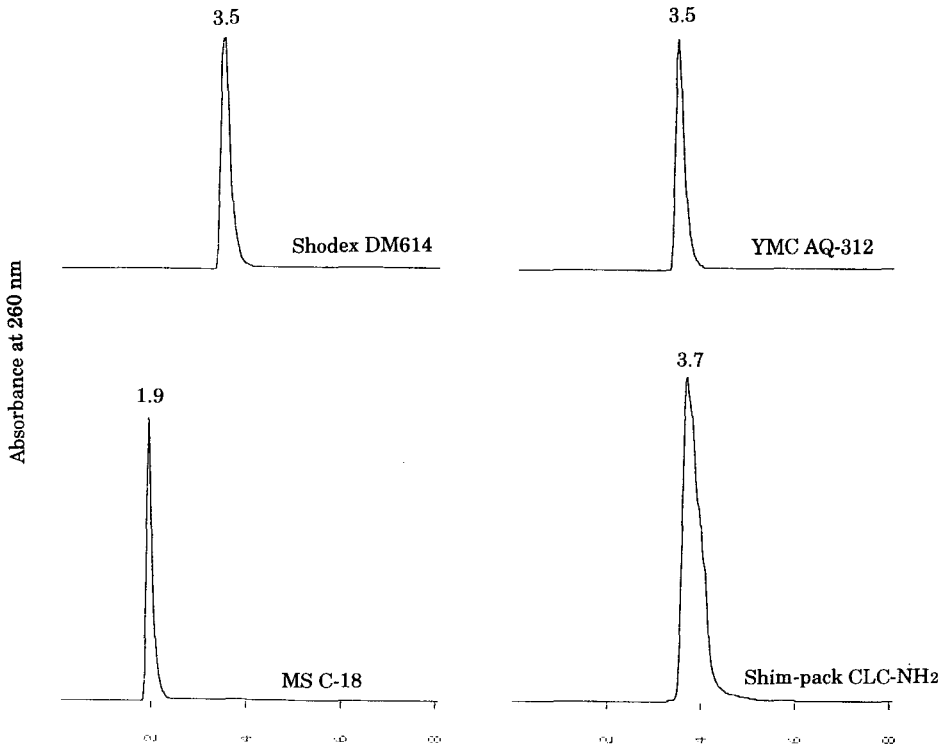

Fig. 2. HPLC chromatograms of the phytotoxin produced by *Burkholderia gladioli* MAFF302424.

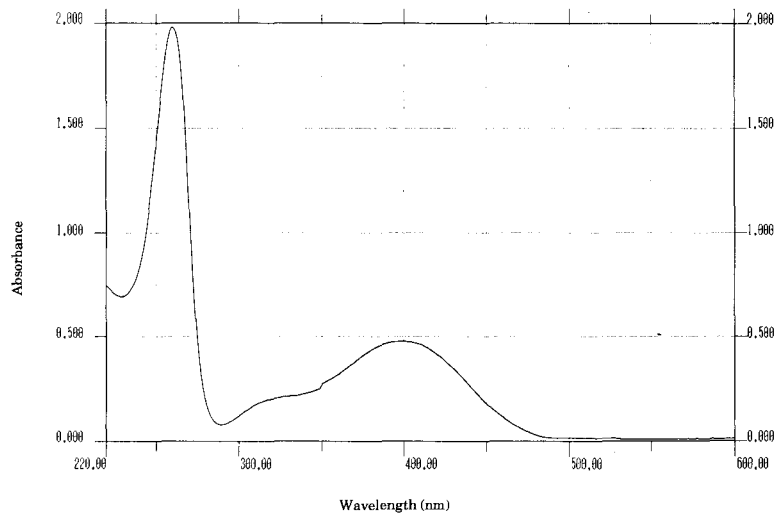

Fig. 3. Visible and ultraviolet absorption spectrum of the phytotoxin produced by *Burkholderia gladioli* MAFF302424.

(Fig. 4). From these results, it is clear that *B. gladioli* also produces toxoflavin as well as *B. glumae*. Further investigation to clarify the role of toxoflavin in pathogenicity of *B. gladioli* is in progress.

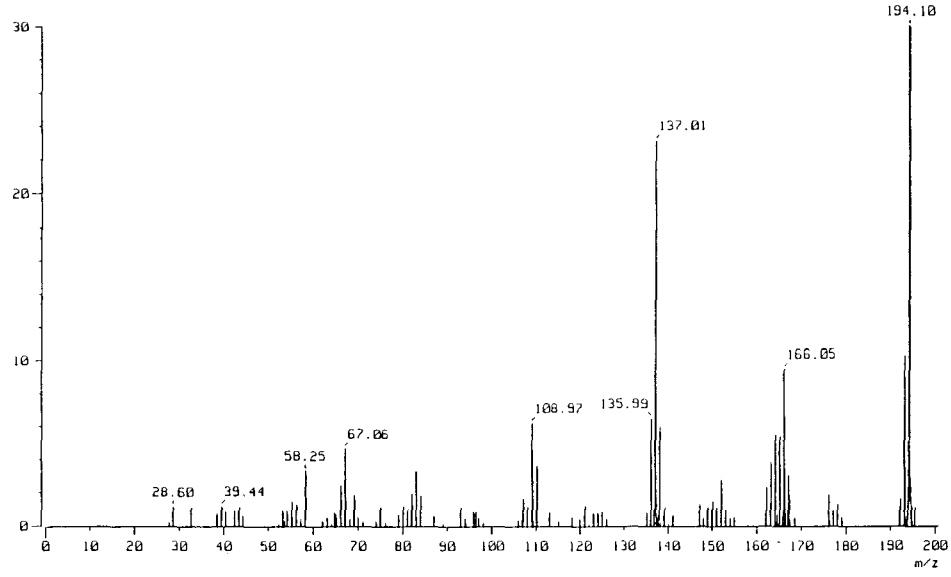

Fig. 4. FAB-MASS spectrum of the phytotoxin produced by *Burkholderia gladioli* MAFF302424.

REFERENCES

Sato, Z., Y. Koiso, S. Iwasaki, I. Matsuda and A. Shirata 1989 Toxins produced by *Pseudomonas glumae*. *Ann. Phytopathol. Soc. Jpn.*, **55**: 353-356

Iiyama, K., N. Furuya, K. Hara, N. Nakashima, Y. Takanami and N. Matsuyama 1994 Phytotoxin produced by *Pseudomonas glumae* Kurita *et* Tabei, a causal bacterium of the grain and seedling rot of rice. *J. Fac. Agr., Kyushu Univ.*, **38**: 175-181

Iiyama, K., N. Furuya, Y. Takanami and N. Matsuyama 1995 A role of phytotoxin in virulence of *Pseudomonas glumae* Kurita *et* Tabei. *Ann. Phytopathol. Soc. Jpn.*, **61**: 470-476