(-)-Jasmonic Acid, a Phytotoxic Substance from Botryodiplodia theobromae: Characterization by NMR Spectroscopic Methods

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(−)-JASMONIC ACID, A PHYTOTOXIC SUBSTANCE FROM *BOTRYODIPLODIA THEOBROMAE*: CHARACTERIZATION BY NMR SPECTROSCOPIC METHODS[1]

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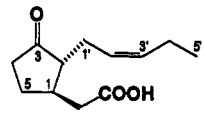
ABSTRACT.—*Botryodiplodia theobromae* causes die-back disease of ornamental roses. A phytotoxic substance isolated from the culture filtrate of this fungus was characterized as (1*S*,2*R*)-3-oxo-2-(2′-*cis*-pentenyl)cyclopentan-1-acetate [**1**] [(−)-jasmonic acid)] by spectral analysis (^{1}H and ^{13}C nmr, DEPT, ^{1}H- ^{1}H COSY, ^{1}H- ^{13}C COSY). The production of necrotic lesions was observed on the detached leaf surface of the rose plants with 5 μg concentrations. The toxic metabolite also inhibited germination of wheat seeds.

A die-back disease of ornamental roses, *Rosa* species, (Rosaceae) under waterlogged conditions was recently found to be caused by *Botryodiplodia theobromae* Pat. (order Sphaerosidales) (1). Decreased flower production, decreased quality of flowers, and reduced life span of rose bushes were the consequences. The disease first appears in the form of necrosis of twigs from the flowering ends; the necrosis progresses downward ultimately causing death of the entire cane. In later stages, severe defoliation also occurs in most of the diseased plants. The symptoms suggest the elaboration of toxic metabolites by this pathogen during pathogenesis (2). Therefore, we carried out a chemical investigation of the culture filtrate of *B. theobromae*. This study led to the isolation of a phytotoxin **1** possessing potent necrotic effects on leaves and twigs of rose.

RESULTS AND DISCUSSION

The phytotoxin **1** was assigned the molecular formula $C_{12}H_{18}O_3$ on the basis of ms $[M]^+$ at *m/z* 210 and by the analysis of the ^{13}C-nmr spectrum. The multiplicities of each carbon atom were determined by using DEPT experiments with polarization pulses at 45°, 90°, and 135°. These experiments (3) revealed the absence of aliphatic quaternary as well as hydroxylated carbon resonances. The ratio of carbons to hydrogens in the molecule indicated a monocyclic nature since it had a saturated keto group (^{13}C nmr δ 218:1; ir 1740 cm^{-1}), a carboxylic group (^{13}C nmr δ 177.69; ir 1700 cm^{-1}) and a disubstituted olefinic bond [^{13}C nmr δ 134.22 (CH), 124.82 (CH); ^{1}H nmr δ 5.26, 5.47, each 1H, dt, J=10.5, 5.5 Hz]. In addition to the above-mentioned functionalities, there was a terminal Me group (^{13}C nmr δ 14.05; ^{1}H nmr δ 0.97, 3H, t, J=6.0 Hz) and an unresolved multiplet integrating for 12 protons in the region δ 1.5–2.9 in the ^{1}H-nmr spectrum. The ^{13}C-nmr spectrum, in conjunction with DEPT edited spectral data, reflected the presence of five aliphatic methylenes (δ 38.65, 37.69, 27.15, 25.50, and 20.58) and two aliphatic methine resonances (δ 53.88 and 27.77). The parent carbon skeleton and the nature of the side chain moieties were deduced by decoupling experiments of the olefinic resonances followed by homonuclear



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[1]Part 4 in the series "Chemical Studies on Phytotoxic Metabolites." For part 3, see R.S. Shukla, P.K. Agrawal, R.S. Thakur, and A. Husain, *Plant Sci.*, **66**, 43 (1990). Part 32 in the series "NMR Spectral Investigations." For part 31, see P.K. Agrawal, A.W. Frahm, and M. Schneider, *Magn. Reson. Chem.* **30**, 1079 (1992). CIMAP Publication No. 93-12 J.

COSY spectral analysis in the region 0.5 to 3.5 ppm as discussed below.

The irradiation of the low-field olefinic methine resonance of **1** at δ 5.47 resulted in a collapse of the olefinic methine resonance at δ 5.26 to a triplet (J=5.5 Hz) and led to the identification of allylic methylene resonances at δ 2.03 and 1.64. In the COSY spectrum, the resonance at δ 2.03 exhibited a cross peak to an Me resonance at δ 0.97. In an analogous manner, irradiation of the olefinic methine at δ 5.26 simplified the multiplicity pattern of the olefinic resonance at δ 5.47 to a triplet (J=5.5 Hz) and located an allylic methylene resonance at δ 2.38 which exhibited, in turn, a cross peak to a methine resonance at δ 2.13 in the COSY spectrum. Thus, the existence of a *cis*-pentenyl ($CH_2CH{=}CHCH_2CH_3$) group was inferred. The resonance at δ 1.95 displayed cross peaks at δ 2.13 and to a carboxylic group substituted methylene resonance at δ 2.31, revealing the presence of a CH_2COOH group at the adjacent position with respect to the position substituted with the cis-pentyl group. The one-bond ^1H- ^{13}C correlation of the assigned ^1H resonances in the ^1H- ^{13}C COSY spectrum led to the assignment of the carbon resonances belonging to the cis-pentenyl and CH_2COOH groups. The ^{13}C resonances left to be assigned were a CO at δ 218.1 and two methylenes at δ 38.65 and 27.15, which suggested the existence of a cyclopentanone nucleus as the parent skeleton. The deshielded methine resonance at δ 53.88 was found to be correlated with the cispentenyl substituted ^1H resonance at δ 1.95; therefore, the cis-pentenyl substituent was located at the α position and the CH_2COOH at the β position with respect to the keto group of the cyclopentanone nucleus. The chemical shift (δ 20.58) of the allylic methylene was consistent with the cis configuration of the olefinic bond (4,5), whereas the trans relationship between the cispentenyl and CH_2COOH substituents was evident by ^{13}C nmr chemical shifts (6,7). Furthermore, the negative optical rotation was in agreement with the assigned stereochemistry (6–9). Thus, the phytotoxin **1** could be identified as (1*S*,2*R*)-3-oxo-2-(2′-*cis*-pentenyl)-cyclopentan-1-acetate[(−)-jasmonic acid].

Application of **1** on rose leaves induced the development of brown necrotic lesions within 1 h of the treatment. The visible symptoms could be observed 24 h after treatment with application of as little as 5 μg (Figure 1). At 100 μg, severe leaf necrosis resulted.

In spite of the fact that cyclopentane-derived fatty acids of the jasmonic acid

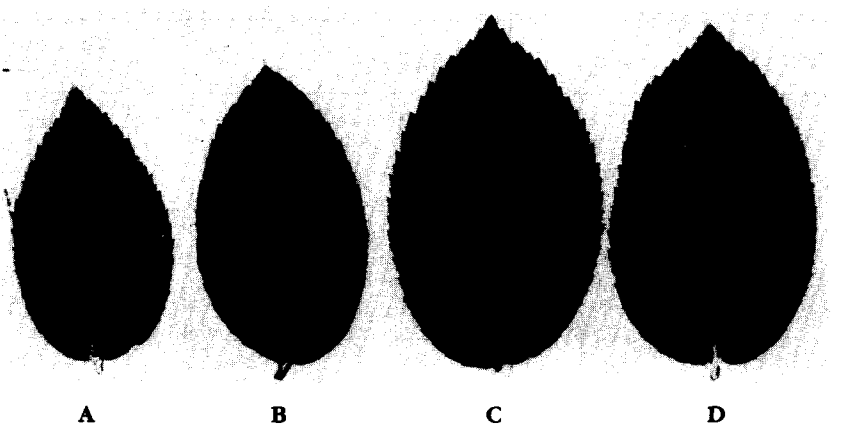

FIGURE 1. Phytotoxic effect (necrotic lesion) at 5 μg concentration of the synthetic **1** and natural **1** on the host (*Rosa* species, variety Golden Gate) after 24 h of treatment. (A) control, (B) synthetic **1**, (C) natural **1**, and (D) control.

type were the target of many synthetic and spectroscopic studies (8–14), unambiguous ^{1}H- and ^{13}C-nmr assignments were not available. Jasmonic acid has already been reported by various workers (8,11), to be produced by *B. theobromae*, and it has been found to be a growth hormone (9–15) and a plant defense signal (16). However, this is the first time that *trans*-jasmonic acid has been found to be a potent toxin responsible for production of toxic symptoms in dieback in infected rose plants. A synthetic sample of jasmoni acid (racemic mixture) was also found to produce similar toxic symptoms at 10 μg but exhibited negligible phyotoxic activity at 5 μg. The (−)-jasmonic acid isolated from the present pathogenic strain of *B. theobromae* was found to exhibit significant phytotoxic activity at 5 μg.

It is possible that in low concentration jasmonic acid acts as a growth hormone, but under high concentration it proves to be toxic, causing chlorosis, necrosis of leaves and twigs, and abscission of leaves. The phytotoxic activity of various organic acids that are produced by fungi pathogenic to plants, such as oxalic acid (17), fumaric acid (18), 3-methythiopropionic acid (19), and 3-nitropropionic acid (20), has already been reported.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H and ^{13}C nmr were obtained with a Bruker WM-400 (400 MHz) nmr spectrometer in $CDCl_3$ with TMS as internal standard. Chemical shift values are reported in ppm downfield from TMS. The homo- and heteronuclear correlation experiments were carried out with the standard pulse sequence.

CULTURING AND TOXIN PRODUCTION.—*B. theobromae* was isolated from the diseased rose twigs as described earlier (1). The identification of *B. theobromae* was confirmed by C.A.B. International Mycological Institute, Kew, England, where a voucher is deposited. A single spore isolate of the fungus was used for the studies. The fungus was grown for 15 days at 26±1° in Roux bottles (1 liter) containing 100 ml of modified Czapek's solution supplemented with 0.1% yeast extract. The pH of the medium was adjusted to 7.0 before autoclaving.

TOXIN ISOLATION AND PURIFICATION.—The culture broth was filtered through four layers of cheesecloth and reduced to one fifth of its original volume by rotary evaporation in vacuo at 40–45°. The pH of the culture filtrate was then adjusted to 4 with 1 N HCL, and it was extracted thrice with an equal amount of EtOAc. The EtOAc extracts were pooled, dried (anhydrous Na_2SO_4), and concentrated at 40° in vacuo to yield a dark brown viscous mass (crude toxin).

The crude toxin was subjected to cc (Si gel) and eluted with hexane and increasing amounts of EtOAc. Since the toxicity was confined to the fractions eluted with hexane-EtOAc (3:1–1:1), these were combined, concentrated, and purified by preparative tlc on Si gel using $CHCl_3$-MeOH

TABLE 1. ^{1}H and ^{13}C Spectral Assignments of (−)-Jasmonic Acids [**1**].

Carbon	^{13}C	Multiplicity[a]	^{1}H[b]	Multiplicity and coupling constants[c]
C-1	37.77	CH	2.13	m
C-2	53.88	CH	1.95	m
C-3	218.10	C	—	—
C-4	38.65	CH_2	2.79, 2.37	
C-5	27.15	CH_2	2.33, 1.52	
C-1′	25.51	CH_2	2.38	
C-2′	124.82	CH	5.26	dt, J=10.5, 5.5
C-3′	134.22	CH	5.47	dt, J=10.5, 5.5
C-4′	20.58	CH_2	2.03, 1.64	m
C-5′	14.05	Me	0.97	t, J=6.0
CH_2	37.69	CH_2	2.31	
COOH	177.69	C	—	—

[a]Based on the DEPT analysis.
[b]Based on the ^{1}H- ^{1}H COSY and ^{1}H- ^{13}C COSY.
[c]In Hz.

(19:1). Tlc exhibited several spots, but the major phytotoxic activity was confined to a band at R_f 0.46, which was removed and eluted with EtOAc to afford **1** (0.3 g). The purity of the toxin was ascertained by ms and nmr spectroscopic data (Table 1).

PHYTOTOXICITY TEST.—The phytotoxic activity of the phytotoxin was tested on the detached leaves of the host plant (variety Golden Gate). Two needle punctures 5 mm apart were made on the upper leaf surface for two replicates of each concentration, and 10 μl of the phytotoxin samples, dissolved in a few drops of acetone and then diluted with distilled H_2O (5, 10, 20, 40, 60, 80, 100, 150, and 200 μg), were placed on the puncture wounds. In the control, an equal amount of distilled H_2O was used for monitoring the reaction. The toxicity was determined by monitoring the area of necrotic lesions on the leaves 24 h after treatment. Root-growth-inhibiting activity of the toxin was assayed with germinating wheat seeds (21) using various concentrations.

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