



## 3,5-DI-*O*-CAFFELOYLQUINIC ACID, AN INFECTION-INHIBITING FACTOR FROM *PYRUS PYRIFOLIA* INDUCED BY INFECTION WITH *ALTERNARIA ALTERNATA*

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(Received: July 29, 1996; in revised form: 16 June 1997)

**Key Word Index**—*Pyrus pyrifolia*; Rosaceae; Japanese pear; *Alternaria alternata* Japanese pear pathotype; black spot of Japanese pear; disease resistance; infection-inhibiting factor; 3,5-di-*O*-caffeoylquinic acid

**Abstract**—The isolation and structure determination of an infection-inhibiting factor, which belongs to a new type of plant metabolite for disease resistance, is described. The compound, 3,5-di-*O*-caffeoylquinic acid was isolated from Japanese pear leaves inoculated with spores of nonpathogenic *Alternaria alternata*. This compound inhibited lesion formation on pear leaves by *A. alternata* Japanese pear pathotype and also suppressed infection hyphae formation of *A. alternata*. However, spore germination, appressorium formation and mycelial growth of *A. alternata* were not affected by this compound. © 1997 Elsevier Science Ltd. All rights reserved

### INTRODUCTION

*Alternaria alternata* (Fr.) Keissler includes many plant pathogens which are associated with severe economical crop losses. Among these pathogens, there are at least seven known host–parasite combinations, in which host-specific toxins are responsible for determining host-specific pathogenicity [1–3]. Several lines of evidence from studies with AK-toxin produced by *A. alternata* Japanese pathotype, the cause of black spot of Japanese pear, suggest that the key role of host-specific toxins in pathogenesis is to suppress the general resistance mechanisms in the host plant and consequently facilitate fungal invasion in the host cells [4–6]. Pre-inoculation with spores of various fungi nonpathogenic to Japanese pear or pre-treatment with spore-germination fluids of these nonpathogens reduced the subsequent infection by *A. alternata* Japanese pear pathotype [4–7]. The appearance of induced resistance as a result of pre-inoculation was documented by a decrease in the number of lesions detected and in the formation of infection hyphae by the pathogen. These results suggest that pear leaves possess a latent resistance mechanism to fungal invasion. However, there has been no evidence for supporting the involvement of phytoalexins in defense reactions in pear leaf tissues against the invasion of *Alternaria* pathogen [4, 5, 8]. Instead of phytoalexins, production of infection-inhibiting factor (IIF), which

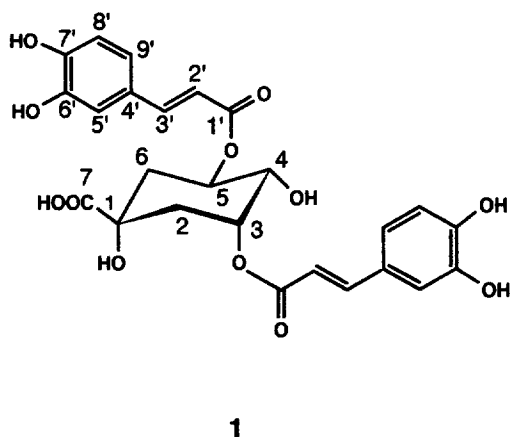
belongs to a new type of plant metabolite for disease resistance, has been reported in pear leaves [5, 8, 9]. IIF does not affect spore germination and appressorium formation but specifically inhibits the formation of the infection hypha of fungi [5, 8, 9]. In this paper, we describe the isolation and structure elucidation of the IIF-produced in Japanese pear leaves in response to infection with nonpathogenic *A. alternata*.

### RESULTS AND DISCUSSION

HPLC separation of ethanol extracts from Japanese pear leaves inoculated with spores of nonpathogenic *A. alternata* yielded a compound showing infection-inhibiting activity. The UV spectrum showed the presence of a caffeoyl residue. The <sup>1</sup>H and <sup>13</sup>C NMR spectra indicated the presence of quinic acid and two caffeoyl groups. The IR spectrum supported these results. By comparing the <sup>1</sup>H and <sup>13</sup>C NMR spectral data with those in the literature [10, 11], the purified compound was identified as 3,5-di-*O*-caffeoylquinic acid (**1**). The position of the two caffeoyl groups was determined to be at the 3 and 5 positions of quinic acid by comparison with the published spectral characteristics.

Compound **1** specifically inhibits formation of infection hyphae by the pathogen, but it does not affect spore germination and appressorium formation. The ED<sub>50</sub> value of **1** for inhibition of infection hypha formation was  $3 \times 10^{-4}$  M, while mycelial growth of

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*A. alternata* on PDA medium was not inhibited even at a concentration of  $2 \times 10^{-3}$  M. These results suggest that **1** which inhibits infection hypha formation, without exhibiting anti-fungal activities typical of phytoalexins, fulfills the conditions of IIF and plays an important role in general resistance mechanisms in pear leaves against infection with *A. alternata*. Production of **1** may be a part of resistant mechanisms of Japanese pear against fungal infection, because it is known that plant resistance in general is the result of the altered multiple chemical and physical profile in the infected tissues. Further biological and pathological testing of **1** will be described elsewhere.

#### EXPERIMENTAL

**Induction of IIF.** A spore suspension ( $1 \times 10^6$  spores  $\text{ml}^{-1}$ ) of nonpathogenic *A. alternata* (O-94) was sprayed onto young leaves of Japanese pear (*Pyrus pyrifolia* Nakai) cv. Nijisseiki. The inoculated leaves were incubated in a moist chamber for 18 hr at  $26^\circ$ .

**Bioassay for infection-inhibiting activity.** Each test soln was evapd to dryness, dissolved in 0.2 ml of EtOH, and mixed with 3.8 ml of a spore suspension ( $2 \times 10^5$  spores  $\text{ml}^{-1}$ ) of *A. alternata* Japanese pear pathotype. The midribs of susceptible young pear leaves were removed and half of the lower surface of the leaves was sprayed with spore suspensions containing the test soln. The other half was sprayed with the same concn of spore suspension in 5% EtOH as a control. After incubation in a moist chamber for 18 hr at  $26^\circ$ , the number of necrotic lesions on the leaves was counted. The inhibition rate of lesion formation by the sample soln was calcd by comparing the number of lesions developed on sample-treated and control leaves. Inhibiting activity of the samples to fungal penetration was determined by the following method. Spores of O-94 were suspended in test solns and sprayed on a cellulose membrane (Wako, Visking tube). After incubation for 24 hr at  $26^\circ$  in a moist chamber, infection behaviour of the spores was observed under a light microscope. The rates of spore germination, appressorium formation per germ tube

and infection hypha formation per appressorium were calcd.

**Extraction and isolation.** Japanese pear (cv. Nijisseiki) leaves (50 g) inoculated with spores of *A. alternata* were dipped in EtOH for 24 hr at  $4^\circ$ . Leaf extracts were rotary-evapd until  $\text{H}_2\text{O}$  remained. The aq. soln was passed through a preconditioned ODS column (ODS-Q3, Wako,  $25 \times 150$  mm). The column was rinsed with 200 ml of  $\text{H}_2\text{O}$  and the IIF was eluted with 50% MeOH. The IIF was finally purified on prep. HPLC. The column was a  $25 \times 250$  mm Lichrosorb ODS-7 (Merck) and eluted with MeOH- $\text{H}_2\text{O}$ -AcOH (50:50:1) at a constant flow rate of  $5 \text{ ml min}^{-1}$ , and detection was by  $A_{330}$ . The active substance was eluted with a *R*<sub>f</sub> 26.5 min as a single peak. The pure IIF was used for the biological studies and the following structural determination data.

**3,5-di-O-Caffeoylquinic acid.** Amorphous powder. UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm:242, 300sh, 328. IR  $\nu_{\text{max}}^{\text{KBr}}$   $\text{cm}^{-1}$ :3418, 1692, 1605, 1524, 1448, 1284, 1183, 1120, 980, 812.  $^1\text{H}$  NMR (270 MHz,  $\text{CD}_3\text{COCD}_3$ ):  $\delta$  2.20 (4H, br. s, H-2, H-6), 3.99 (1H, br. s, H-4), 5.40 (2H, br. s, H-3, H-5) (quinic acid moiety);  $\delta$  6.19 (1H, d,  $J = 15.9$  Hz, H-2'), 6.20 (1H, d,  $J = 15.9$  Hz, H-2'), 6.81 (1H, d,  $J = 7.8$  Hz, H-8'), 6.83 (1H, d,  $J = 7.8$  Hz, H-8'), 6.97 (1H, dd,  $J = 7.8, 1.9$  Hz, H-9'), 7.00 (1H, dd,  $J = 7.8, 1.9$  Hz, H-9'), 7.11 (1H, d,  $J = 1.9$  Hz, H-5'), 7.11 (1H, d,  $J = 1.9$  Hz, H-5'), 7.50 (1H, d,  $J = 15.9$  Hz, H-3'), 7.53 (1H, d,  $J = 15.9$  Hz, H-3') (caffeoyl groups).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{COCD}_3$ ): 74.8 (C-1), 38.1 (C-2), 72.1 (C-3), 70.6 (C-4), 72.4 (C-5), 36.3 (C-6), 176.3 (C-7) (quinic acid moiety); 167.2, 167.5 (C-1'), 116.8, 116.8 (C-2'), 146.1, 146.4 (C-3'), 128.1, 128.2 (C-4'), 115.6, 115.7 (C-5'), 146.8, 146.8 (C-6'), 149.2, 149.3 (C-7'), 116.1, 116.7 (C-8'), 122.9, 123.1 (C-9') (caffeoyl groups).

**Acknowledgements**—We are grateful to H. Tanaka, Y. Kusakari, and S. Mitani for technical assistance.

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