Benzoic Acid Accumulation in the *Pinus thunbergii* Callus Inoculated with the Pine Wood Nematode, *Bursaphelenchus xylophilus*

Hong Zhang*, Hiroshi Kanzaki and Kazuyoshi Kawazu

Laboratory of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan

* The Graduate School of Natural Science and Technology, Okayama University, Okayama 700, Japan

Z. Naturforsch. **52 c,** 329–332 (1997); received December 18, 1996/January 31, 1997

Nematode Inoculation, Benzoic Acid, Phenylacetic Acid, *Bacillus* spp., Pine Wilt Disease

Phenylacetic acid (PA), a phytotoxic product of the bacteria accompanying the virulent nematode isolate OKD-3 was detected in the callus of *Pinus thunbergii* after inoculation with the nematode. The amount of PA detected was large enough to induce the formation and accumulation of benzoic acid (BA) and its conjugates in the callus. These results further support the hypotheses that PA is the pathogenic toxin and that the PA-producing bacterial strains accompanying the pathogenic nematode are the genuine pathogens of the pine wilt disease.

Introduction

Pine wilt disease has been spreading throughout Japan except Hokkaido for several decades (Kishi Y., 1995). Undoubtedly, this disease results from an invasion by the pine wood nematode, *Bursaphelenchus xylophilus* (Kiyohara *et al.*, 1971). Although a lot of research has been conducted on this disease since the discovery that the pine wood nematode is the responsible pathogen of this disease, no satisfactory results have been obtained to account for the wilting mechanism. Kawazu *et al.* (Kawazu *et al.*, 1987; Kawazu, 1990) reported that inoculation of a three-year-old tree of *Pinus thunbergii* with *B. xylophilus* resulted in a rapid increase in benzoic acid (BA) and its conjugate, glucosyl benzoate (GB), and in the prolonged accumulation of the former. It has been suggested that this phenomenon could be used as a chemical diagnosis of this disease (Kawazu *et al.*, 1987, 1996a; Kawazu, 1990). Based on some observations previously reported (Mamiya, 1980, 1982). Kawazu (1990) had assumed that microorganisms accompanying the nematode might be responsible for the pathogenesis of the pine wilting. From *B. xylophilus* he isolated three species of bacteria, *Bacillus cereus* (HY-3), *B. subtilis* (HY-16), and *B. megaterium* (HY-17), that were toxic to seedlings and cultured cells of *Pinus thunbergii*. The phytotoxin produced by these strains was isolated and identified as phenylacetic acid (PA)(Kawazu, 1990, 1992). The present authors also observed the typical chemical diagnosis in suspension-cultured cells of *P. thunbergii* treated with PA or with a freeze-dried supernatant of *B. cereus* (HY-3) culture (Kawazu *et al.*, 1996a). Consequently, they suggested that PA played a direct role in the formation of BA and GB in pine tissues (Kawazu, 1992; Kawazu *et al.*, 1996a), and proposed that PA might be a pathogenic toxin of the pine wilt disease and that the PA-producing bacterial strains might be the pathogens of the disease (Kawazu *et al.*, 1996a).

However, it is still unclear whether these strains of bacteria can produce enough PA to trigger the formation of BA when introduced into pine tissues by the nematode. To clarify this, inoculation of callus of *P. thunbergii* with the living pathogenic nematodes was tried, because inoculating suspension-cultured cells led to death of the pine cells due to the rapidly propagating bacteria.

This paper reports the formation and accumulation of BA and its conjugates in *P. thunbergii* callus after inoculation with the pathogenic nematodes.

* This study is part of the work for H. Z.'s doctoral thesis at Graduate School of Natural Science and Technology, Okayama University.

Reprint requests to Prof. Dr. K. Kawazu.
Telefax: +81-86-254-0714.

0939–5075/97/0500–0329 $ 06.00 D

Materials and Methods

Pine callus

The callus of *P. thunbergii* was initiated from its seedlings as described in the preceding paper (Kawazu *et al.*, 1996a). They were subcultured at ca. 1-month intervals with 16 h/d illumination at 25 °C.

Nematodes

A virulent isolate of *B. xylophilus*, OKD-3 (Kawazu *et al.*, 1996b), was obtained by the Baermann funnel method from a wilted pine tree in Handayama experimental forest, Okayama University. Subculture of this nematode and preparation of the nematode suspension for inoculation were carried out as previously described (Kawazu *et al.*, 1996b).

Inoculation of the callus with the nematode

A callus sample (1 g fresh weight) was transferred to each of 14 test tubes (25 x 200 mm) containing 10 ml of semi-solid fresh medium of the same composition as the subculture medium, and cultured at 25 °C with 16 h/d illumination. After 10 days, when the fresh weight of the callus reached approximately 2 g per tube, each callus was inoculated with 150 µl of a suspension of 3000 nematodes. An equivalent volume of sterile distilled water served as a control. The callus was harvested at 0, 1, 2, 3, 5, 7 and 14 days after inoculation, and subjected to quatification of PA and BA after extraction. Every inoculation was conducted in duplicate and the data in Figs. 1 and 2 were the averages of these two test tubes.

Procedures for extraction of PA and BA

The harvested callus was lyophilized and homogenized with MeOH. The homogenate was filtered through filter paper (Toyo No. 2) under suction. The filtrate was concentrated *in vacuo* and suspended in 1 ml of distilled water. This aqueous suspension, after being acidified to pH 4 by adding 1 N HCl solution, was extracted three times with 1 ml portions of EtOAc. The combined EtOAc extract was evaporated *in vacuo* to a residue which was dissolved in 1 ml of EtOAc for the quantification of PA and BA.

The EtOAc-insoluble aqueous phases contained PA and BA conjugates, which were hydrolyzed with acid and base to the free acids. The hydrolysate was made acidic with 1 N HCl to pH 4 and extracted three times with EtOAc. After removal of EtOAc, the residue was dissolved in 1 ml of EtOAc for the quantification of PA and BA. The quantitative analysis of PA and BA was carried out by GC-MS (Kawazu *et al.*, 1996a).

Results

Changes in superficial appearance of pine callus after nematode inoculation

The pine callus which had been green at the start began to turn brown 2 days after inoculation with nematode isolate OKD-3 and became almost brown on the 5th day due to hypersensitive response to the nematode.

Detection of PA in pine callus inoculated with the nematode

Phenylacetic acid (PA), a product of the bacteria accompanying the nematode, was detected in the inoculated pine callus. As shown in Fig. 1, free PA increased linearly after the inoculation, and reached a maximum level of 2.0 µg per tube on the 2nd day. Part of the PA in the callus was converted to its conjugates, which reached the highest amount of 0.67 µg per tube (about one third of the total amount of PA) on the 3rd day after the inoculation.

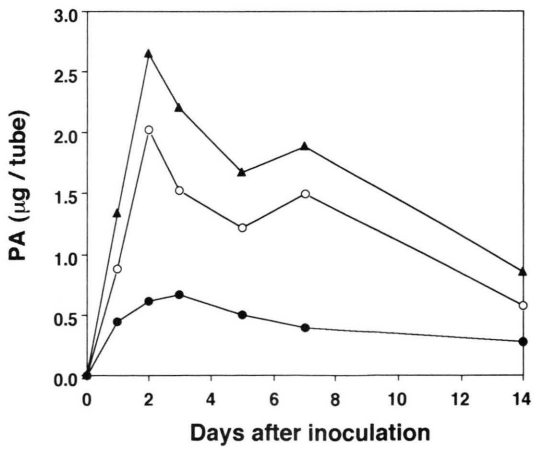

Fig. 1. Time course of phenylacetic acid (PA) produced by the bacteria accompanying a pathogenic nematode isolate, OKD-3 in *Pinus thunbergii* callus inoculated with the nematode. ○, free PA; ●, conjugate PA; ▲, total PA.

Formation and accumulation of BA and its conjugates in pine callus inoculated with the nematode

Inoculation of the pine callus with the nematode increased both free and conjugate BA dramatically (Fig. 2). The conjugate BA content was greater than the control level on the 1st day, reached a maximum of 174 nmol/g dry wt. (ca. 9 times as high as the control) on the 2nd day, and finally dropped to 36 nmol /g dry wt. on the 14th day after the inoculation. Free BA increased progressively in the callus after inoculation. The highest amount of free BA, 342 nmol/g dry wt., which was approximately 7-fold higher than the control, was detected on the 3rd day.

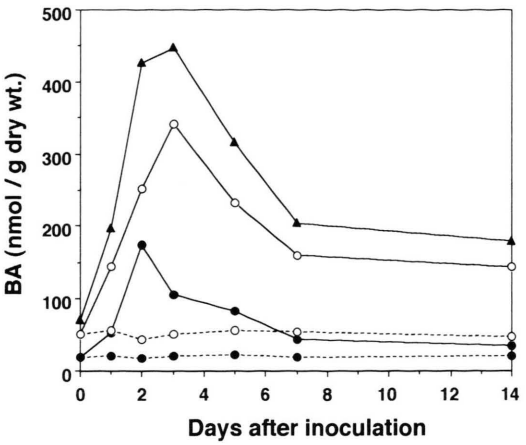

Fig. 2. Time course of benzoic acid (BA) accumulated in *Pinus thunbergii* callus (——) inoculated with a pathogenic nematode isolate OKD-3, and in a control (-----). ○, free BA; ●, conjugate BA; ▲, total BA.

Discussion

After inoculation of *P. thunbergii* callus with the virulent isolate, OKD-3, the accompanying bacteria propagated quickly in the callus and produced PA. About one third of the PA produced was detoxified by conversion into its conjugates in the callus on the 3rd day. BA conjugates increased remarkably in the callus one day after the inoculation, and peaked on the 2nd day, indicating that some of the BA formed was quickly converted into its conjugates. Previously, it was shown that administration of PA to *P. thunbergii* suspension-cultured cells induced rapid formation of BA and its conjugates (Kawazu *et al.*, 1996a). The present study showed that also in the pine callus inoculated with the nematode, a sufficient amount of PA for induction of BA formation was produced by the accompanying bacteria.

Total BA (both free and conjugate forms) in the callus after inoculation with the nematode reached a maximum one day later than in suspension-cultured cells provided with PA (Kawazu *et al.*, 1996a). It is conceivable that the PA concentration in the pine callus just after the nematode inoculation was very low because of small bacterial population accompanying the nematode at that time, but PA was produced by the bacteria in a sufficient amount for induction of BA formation later.

The data presented in this paper further support the hypotheses that PA is the pathogenic toxin and that the PA-producing bacterial strains accompanying the pathogenic nematode are the genuine pathogens of the pine wilt disease.

Kawazu K. (1990), Changes in constituents of pine wood by infection of pine wood nematode. Nippon Nogeikagaku Kaishi (in Japanese), **64**, 1262–1264.

Kawazu K. (1992), A few strains of bacteria accompanying the pathogenic nematode isolated from the wilting pine wood. In: Studies on the Natural Character of Suburban Forests and their Function in Protecting the Environment (VI) (in Japanese). Report of Specified Research at Okayama University in 1991, pp. 67–72.

Kawazu K., Ozaki Y., Fujiwara M. and Kobayashi A. (1987), Changes in constituents of pine wood at the early stage of pine wilt disease caused by invasion of the pine wood nematode. In: Studies on the Natural Character of Suburban Forests and their Function in Protecting the Environment (I) (in Japanese). Reports of Specified Research at Okayama University in 1986, pp. 81–86.

Kawazu K., Zhang H. and Kanzaki H. (1996a) Accumulation of benzoic acid in suspension cultured cells of *Pinus thunbergii* Parl. in response to phenylacetic acid administration. Biosci. Biotech. Biochem. **60**, 1410-1412.

Kawazu K. Zhang H., Yamashita H. and Kanzaki H. (1996b), Relationship between the pathogenicity of the pine wood nematode, *Bursaphelenchus xylophilus*, and phenylacetic acid production. Biosci. Biotech. Biochem., **60**, 1413- 1415.

Kishi Y. (1995), The pine wood nematode and the Japanese pine sawyer, Thomas Company Ltd., Tokyo, Japan.

Kiyohara T. and Tokushige Y. (1971), Inoculation experiments of a nematode, *Bursaphelenchus* sp. onto pine trees. J. Jpn. For. Soc. (in Japanese with English summary) **53**, 210–218.

Mamiya Y. (1980), Inoculation of the first year pine (*Pinus densiflora*) seedlings with *Bursaphelenchus lignicolus* and the histopathology of diseased seedlings. J. Jpn. For. Soc. (in Japanese with English summary), **62**, 176–183.

Mamiya Y. (1982), Pine wilt and pine wood nematodes: histological aspects of disease development. In: Resistance to Disease and Pests in Forest Trees (H. M. Heyerock, B. R. Stephan, and K. von Weisenberg ed.). Wageningen, Hol. PUDOC., pp. 153–160.