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ORIGINAL PAPER

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Accumulation of 2,5-dimethoxy-1,4-benzoquinone in suspension cultures of *Panax ginseng* by a fungal elicitor preparation and a yeast elicitor preparation

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Abstract Suspension cultures of *Panax ginseng* C.A. Meyer (Araliaceae) were treated with either an elicitor preparation from the culture broth of the phytopathogenic hyphomycete *Botrytis cinerea* or a yeast elicitor preparation, and the accumulation of a new compound, which was not detected in non-elicited cultures, was observed. The accumulated compound was isolated and shown to be 2,5-dimethoxy-1,4-benzoquinone by ^{1}H-NMR, ^{13}C-NMR and electron ionization (EI) mass spectra. While it is well known that this compound shows antibacterial activity against *Staphylococcus aureus*, its presence in ginseng root has not been reported to date. Levels of the compound in the media increased rapidly, reaching a maximum level of 65.10±4.96 μg/g fresh weight at approximately 12 h after treatment with the yeast elicitor preparation. The maximal level of the compound in medium from the culture treated with an elicitor preparation from the culture broth of *B. cinerea* was 46.13± 10.42 μg/g fresh weight after 24 h of incubation.

Introduction

Ginseng (the root of *Panax ginseng* C.A. Meyer) is one of the most valuable and widely acclaimed oriental medicines. It has been used by people in East Asia to cure a wide spectrum of diseases and conditions for more than 1,000 years (Tang and Eisenbrand 1992), and is presently is used world-wide as a tonic and adaptogenic. Ginseng generally requires 6 years of growth before yielding high-quality roots, during which, it is susceptible to pathogens. Among the ginseng diseases reported in ginseng-cultivating countries, a number of microorganisms, including *Fusarium solani*, *Cylindrocarpon destructans* and *Botrytis* spp., have been reported to be a serious threat to ginseng farming in Korea (Yu and Ohh 1993). Since ginseng is largely consumed as a food in oriental countries, uses of pesticides and other agrochemicals for eradicating these phytopathogenic microorganisms during cultivation are of limited value. As a result, considerable crop loss ensues by the time of harvest (Parke and Shotwell 1995). To resolve this problem, the development of new biopesticides as alternatives to the use of chemical pesticides and which do not cause damage to humans or the environment would be desirable (Khambay and O'Connor 1993; Addor 1999).

When plants are exposed to a pathogenic microorganism, they are often able to defend themselves against microbial attack. In many cases, the protective mechanism involves an inducible defense mechanism, such as the accumulation of phytoalexins (Yoshikawa et al. 1993). Phytoalexins are defined as antimicrobial secondary metabolites that are accumulated by plants in response to microbial attack (Müller 1956). In this study, in order to search for phytoalexins that accumulate by elicitation in suspension cultures of *P. ginseng*, a yeast elicitor preparation and an elicitor preparation from the culture broth of *Botrytis cinerea*, a known phytopathogen of ginseng, were added to cell suspensions. After elicitation, a new compound, which was not observed in the non-elicited medium, had accumulated in the suspension cultures. The chemical identification of the compound and its characterization by spectral methods are described.

Materials and methods

Chemicals and instruments

2,4-Dichlorophenoxyacetic acid (2,4-D), *p*-chlorophenoxyacetic acid (CPA), kinetin, and yeast extract were purchased from Sigma (St. Louis, Mo., USA). ^{1}H-NMR and ^{13}C-NMR spectra were obtained in $CDCl_3$ on a Varian VXR 300 spectrometer operating at 300 and 75 MHz, respectively. The chemical shift values are reported as ppm relative to tetramethylsilane (TMS) as internal standard. Electron ionization (EI) mass spectra were obtained on a VG Trio-2 spectrometer.

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Cell cultures

Panax ginseng callus was obtained from the Korean Collection for Type Cultures (strain no. 10121; Taejon, Korea) and maintained on Schenk and Hildebrandt (SH) solid medium (Schenk and Hildebrandt 1972) supplemented with 0.5 mg 2,4-D/l, 2.0 mg CPA/l and 0.1 mg kinetin/l at 25 °C in the dark by subculturing at 3-week intervals. Suspension cultures were established by suspending 4 g of callus (fresh weight) in 100 ml fresh SH medium (pH 5.8±0.1) with 2,4-D (0.5 mg/l), CPA (2.0 mg/l), kinetin (0.1 mg/l) and sucrose (30 g/l) in a 250-ml Erlenmeyer flask. Suspension cultures were subcultured at 3-week intervals (4 g fresh weight inoculum) and grown at 25 °C on a gyratory shaker at 100 rpm in the dark. To obtain a large quantity of culture media, 12 g of cells were inoculated into 500 ml of the same liquid medium in Fernbach flasks.

Measurement of growth

To measure the growth of cells, 2.0 g of callus (fresh weight) was inoculated and maintained on 50 ml of fresh SH medium in a 125-ml Erlenmeyer flask. Cell growth was measured by weighing the fresh weight of suspension-cultured cells at 4-day intervals throughout one batch-culture over a culture period of 24 days.

Preparation of yeast elicitor

Yeast extract was prepared by the method of Yoshikawa (1993) and Yamamoto (1995) with slight modifications. Ten grams of yeast extract (Sigma) was dissolved in 80 ml distilled water and added to 320 ml absolute ethanol. The resulting solution was allowed to stand at 4 °C for 24 h, and the precipitate that formed was dissolved in 50 ml distilled water, followed by ultrafiltration (Amicon, Mass., USA) using 10-kDa-cut-off membrane. The fraction below 10 kDa in molecular mass was lyophilized and dissolved in distilled water. The resulting solution was sterilized by filtration through a 0.22-μm membrane and then added to the 8-day-old cell suspension to an appropriate final concentration.

Preparation of fungal elicitor

The strain of *B. cinerea* 9802 used in this study was obtained from the Korea Ginseng and Tobacco Research Institute (Suwon, Korea). The strain was cultured on potato dextrose agar (Sigma) in petri dishes and incubated at 25 °C in the dark. Conidia suspensions of *B. cinerea* were inoculated on 500 ml of potato dextrose broth in Erlenmeyer flasks and incubated for 10 days at 25 °C on a reciprocal shaker (150 rpm) in the dark. The cell suspension was filtered after a cultivation period of 10 days, and the cell free medium was then autoclaved at 121 °C for 20 min and lyophilized (Ayers et al. 1976) prior to its addition to the suspension cultures at an appropriate concentration.

Elicitation of suspension cultures

The yeast elicitor, which had a molecular mass below 10 kDa (50 μg/ml), and the fungal elicitor (100 μg/ml) were added to separate 8-day-old suspension cultures, and the cultures were subsequently incubated for an additional 24 h. The media was collected by vacuum filtration and extracted three times with an equal volume of ethyl acetate. The extract was dissolved in methanol and examined by thin layer chromatography (TLC) on precoated silica-gel-60 F_{254} plates (Merck, Germany). The plates were developed with $CHCl_3$:MeOH (10:1) and the spots were detected by spraying with anisaldehyde-H_2SO_4 reagent.

Isolation and identification of the accumulated compound

The media (35 l) of the suspension cultures elicited with the yeast elicitor was extracted three times with an equal volume of ethyl acetate. The evaporated residue (1.7 g) was eluted from a silica-gel column with a gradient solvent system ($CHCl_3$:MeOH, v/v) to produce six subfractions, each of which showed a different pattern by TLC as described above. The third fraction, which contained a compound with R*f* 0.73, was collected and chromatographed on a Sephadex LH-20 column with a mixture of *n*-hexane:CH_2Cl_2:MeOH (10:10:1, v/v). The chemical structure of the purified compound was elucidated by ^{1}H-NMR, ^{13}C-NMR and EIMS analysis.

Quantification of the accumulated compound

The media extract was evaporated, dissolved in $CHCl_3$, and subjected to GC analysis. An analytical GC (GC-353; GL Sciences, Japan), equipped with a flame ionization detector and a DB-1 capillary column (0.25 mm×30 m), was used for the separation. The GC conditions were as follows: split ratio 30:1, injection temperature 280 °C, detection temperature 280 °C. The column temperature was increased 2 °C/min from 140 °C to 180 °C and then held for 10 min. Helium was used as a carrier gas at a flow rate of 30 ml/min.

Results

Suspension cultures

Panax ginseng callus was maintained in the dark on SH medium containing 2,4-D (0.5 mg/l), CPA (2.0 mg/l), and kinetin (0.1 mg/l). Suspension cultures were established from the callus tissue in the same medium. Actively growing cells, selected from several passages in the liquid medium, were transferred to 125-ml Erlenmeyer flasks. In the suspension cultures, ginseng cells grew as a homogeneous, bright-yellow suspension. The growth pattern of the cells followed a typical sigmoid curve (Fig. 1). The lag phase in the cell-suspension cultures of *P. ginseng* was up to 8 days. Following the exponential phase, the cells reached a stationary phase in about 20 days.

Elicitation by yeast and fungal elicitors

The yeast elicitor (50 μg/ml) and fungal elicitor (100 μg/ml) preparations were added to separate suspen-

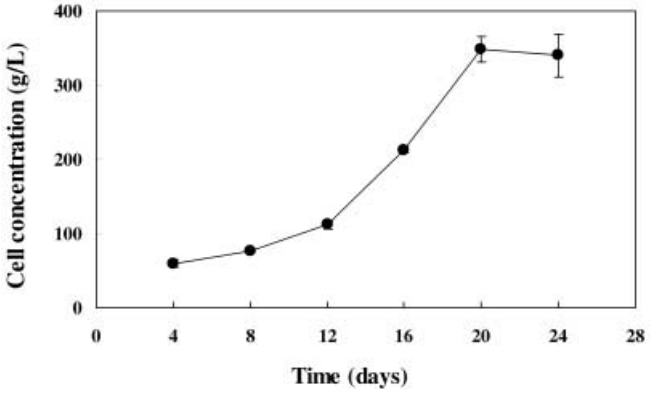

Fig. 1 Time course for growth in a cell suspension culture of *Panax ginseng* grown in a SH medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 mg/l *p*-chlorophenoxyacetic acid (CPA) and 0.1 mg/l kinetin. The results represent the mean of three independent assays

sion cultures of *P. ginseng* at day 8 of the cultivation period, when the cultures were in the early exponential phase of growth. As discussed in a review article by Eilert (1987), most cell cultures respond to elicitation only during the growth phase. Since *P. ginseng* cells at day 8 were the most responsive to elicitor treatment (compared to days 12 and 16), this time point was chosen for elicitation. The cultures were homogeneous in appearance, and on elicitation with the yeast elicitor the color changed from light yellow to light brown. The addition of the fungal elicitor resulted in a change to a lime-green color. Differences were also found in cell viability for the yeast-elicitor-treated cells compared with the fungal-elicitor-treated cells. The yeast elicitor resulted in a low level of cell death beginning at 24 h after treatment, while the fungal elicitor did not have any effect on cell viability and morphology for a period of up to 48 h after treatment. After incubating for an additional 24 h after elicitor treatment, each culture medium was extracted with ethyl acetate and analyzed by TLC. At least two compounds, with R*f* values of 0.82 and 0.73, respectively, had accumulated in the culture media of cells treated with yeast elicitors. In addition, in the fungal-elicitor-treated medium, two compounds, with R*f* values of 0.73 and 0.56, respectively, had accumulated. Since the compound with an R*f* of 0.73 had accumulated in the medium treated with yeast elicitor and in the medium treated with fungal elicitor, it was isolated and identified. When the R*f* 0.73 compound was analyzed by GC, a large peak, with a retention time of 9.6 min, was observed (Fig. 2). To identify optimum concentrations for the elicitation effect, different concentrations of yeast elicitor and fungal elicitor preparations were added to 8-day-old suspension cultures of *P. ginseng*. Yeast elicitor concentrations varied between 1.0 µg and 500 µg, the concentrations of the fungal elicitor between 1.0 µg and 1,000 µg per ml of suspension. The highest elicitation effect with respect to accumulation of the compound was observed at a concentration of 50 µg/ml yeast elicitor and 100 µg/ml fungal elicitor. These concentrations of elicitors were applied for all the elicitation experiments.

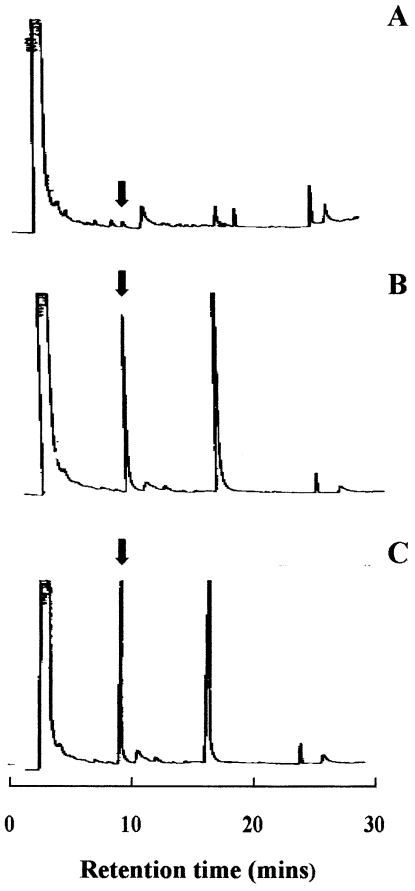

Fig. 2A–C GC-chromatogram of a media extract of *P. ginseng* which had been treated with yeast and fungal elicitors. **A** Non-elicited media extract; **B** media extract treated with yeast elicitor; **C** media extract treated with fungal elicitor. *Arrows* denote the 2,5-dimethoxy-1,4-benzoquinone peak, which accumulated in the medium of suspension cultures

Isolation and identification of 2,5-dimethoxy-1,4-benzoquinone by elicitation

In order to identify the compound that had accumulated in the culture media, the suspension culture was treated with yeast elicitor (50 µg/ml) and incubated for an additional 24 h in 500 ml-Fernbach flasks. The evaporated residue of the extracted media was subjected to silica-gel column chromatography. The fractions that showed spots with R*f* 0.73 were combined and further purified by chromatography on Sephadex LH-20. The purified compound was a yellow-colored powder and was determined to be 2,5-dimethoxy-1,4-benzoquinone by ^{1}H-NMR, ^{13}C-NMR, and EIMS analysis. The spectral data were as follows; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.83 (6H, s, $OCH_3\times2$), 5.83 (2H, s, H-3,6); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 56.5 ($OCH_3\times2$), 107.4(C-3,6), 157.3(C-2,5), 186.3 (C-1,4); EIMS *m*/*z* (rel int.): 168$[M]^+$ (81.05), 153$[M-15]^+$ (2.98), 138(23.07), 125(14.18), 112(5.75), 97(11.26), 80(32.39), 69(100). The spectral data of the compound were also compared with previously reported (Hofle 1976; Thomson 1971) data and reconfirmed by comparison with that of a synthetic standard (obtained from Dr. S.H. Kim, Natural Products Research Institute, Seoul National University, Seoul, Korea).

Time course for the accumulation of 2,5-dimethoxy-1,4-benzoquinone after the addition of yeast and fungal elicitors

At day 8 of the culture period, 50 µg/ml of the yeast elicitor or 100 µg/ml of the fungal elicitor was added to suspension cultures of *P. ginseng*, and the level of 2,5-dimethoxy-1,4-benzoquinone was monitored for a 24-h period. The compound accumulated in the media treated with yeast as well as fungal elicitors; however, the time courses of the accumulation of the compound differed considerably from each other (Fig. 3). The amount of 2,5-

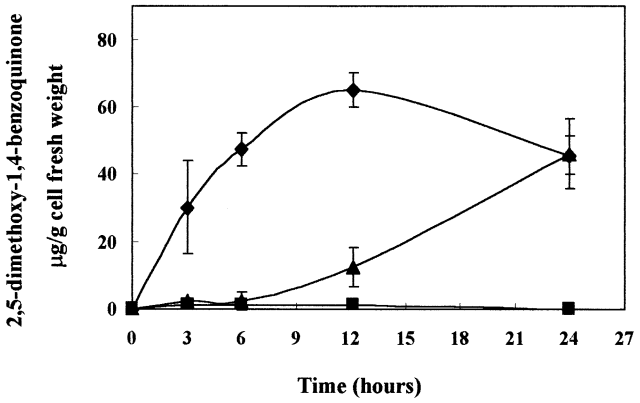

Fig. 3 Time course of 2,5-dimethoxy-1,4-benzoquinone accumulation after the addition of yeast and fungal elicitors. ■ Non-elicited media extract, ◆ media extract treated with yeast elicitor, ▲ media extract treated with *Botrytis cinerea* elicitor. The results represent the mean of three independent assays

dimethoxy-1,4-benzoquinone increased rapidly, reaching a maximum level of 65.10±4.96 μg/g fresh weight at around 12 h after addition of the yeast elicitor, whereas maximal production in the media treated with the fungal elicitor was 46.13±10.42 μg/g fresh weight after 24 h.

Discussion

Since the work reported on by Luo (1964) using cell and tissue cultures of *P. ginseng*, plant cell culture methods have been used as potentially more efficient alternatives for the mass production of ginseng and its bioactive components (Wu and Zhong 1999). However, these early studies focused largely on the production of ginsenosides and not on the production of phytoalexins accumulated by elicitors. The present results show that, in response to pathogenic microbes, cultured ginseng cells activate a self-defense mechanism that involves production and accumulation of a secondary metabolite. Since the accumulated compound is known to have antimicrobial activity (Kitanaka and Takido 1986) against *S. aureus*, and since it has never been isolated from *P. ginseng* or detected in non-elicited culture media, it is proposed that the compound is a phytoalexin that is produced in *P. ginseng* in response to pathogens.

As stated above, TLC analysis showed that the yeast-elicitor-treated media contained a compound with an R*f* value of 0.82, while the fungal-elicitor-treated media contained a compound with an R*f* value of 0.56, in addition to the isolated 2,5-dimethoxy-1,4-benzoquinone. In addition, the time course of 2,5-dimethoxy-1,4-benzoquinone accumulation in response to the two elicitors differed greatly. At this point, the issue of what specifically causes these discrepancies in the accumulated chemicals and the time course of accumulation can not yet be explained. For elucidating the cause of these discrepancies, studies of signal transduction mechanisms should be pursued, and the active component of each elicitor preparation should be characterized. As shown in the study of Song et al. (1995), the active component of yeast-derived elicitor is a sugar and/or peptide moiety. If the active agent in the *B. cinerea* fungal elicitor is characterized, the molecular basis of the elicitation mechanism and the cause of the discrepancies in the elicitation time may be elucidated. Such studies are now in progress, as is isolation of the compounds with R*f* values of 0.82 and 0.56. After isolating the compounds, studies to determine their defensive role in protecting *P. ginseng* against phytopathogenic microbes should be pursued.

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