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Full Length Research Paper

Enhancement of diosgenin production in *Dioscorea zingiberensis* seedling and cell cultures by beauvericin from the endophytic fungus *Fusarium redolens* Dzf2

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Diosgenin production in seedling and cell suspension cultures of *Dioscorea zingiberensis* C. H. Wright was enhanced by treatment with beauvericin obtained from its endophytic fungus *Fusarium redolens* Dzf2. Optimal elicitation of diosgenin production by beauvericin in *D. zingiberensis* seedling and cell cultures was achieved when beauvericin was added to the medium at a concentration of 100 mg/L (in seedling culture) or 40 mg/L (in cell culture). By using these optimal concentrations, the diosgenin yield of the cultured seedlings reached its maximum of 5.72 mg/L that was over a 1-fold increase and the diosgenin yield of the cultured cells reached its maximum of 5.15 mg/L that was over a 3.6-fold increase.

Key words: Dioscoreaceae, *Dioscorea zingiberensis*, endophytic fungus, *Fusarium redolens* Dzf2, beauvericin, seedlings, suspension cells, diosgenin production.

INTRODUCTION

Dioscorea zingiberensis C. H. Wright (Dioscoreaceae) is a traditional Chinese medicinal herb which is indigenous to the south of China (Chen et al., 2003). The rhizomes have a high content of diosgenin which is an important precursor of semi-synthetic steroids such as corticosteroids, sex hormones (for example progesterone) and other steroidal drugs produced by the pharmaceutical industry (Qin et al., 2006; Liu et al., 2010; Li and Ni, 2011). However, overexploitation of natural *D. zingiberensis* leads to rapid decrease of this plant resource and sharp shortage of diosgenin in pharmaceutical synthesis. Furthermore, agricultural production of *D. zingiberensis* requires 3 to 4 years from seedling to mature rhizome during which time plant growth is highly susceptible to a number of environmental factors as well as occupying large areas of cultivation land. The tissue and cell culture of *D. zingiberensis* has been regarded as an alternative means for efficient and controllable production of diosgenin (Zhou and Wu, 2006). Plant endophytic fungi, the latent phytopathogens or mutualistic symbionts presenting inter- and/or intra-cellularly in the normal tissue of host plants have proved to be a promising source of novel structures and/or strong bioactivities (Schulz et al., 2002; Zhang et al., 2006). They have been found in each plant species. During the long period of co-evolution, a mutually beneficial relationship was formed between each endophyte and its host plant. The host plant can supply plenteous nutriment and easeful habitation for the survival of its endophytes.

On the other hand, the endophytes would produce a number of bioactive constituents for helping their host plants to resist external biotic and abiotic stresses (Silvia et al., 2007; Rodriguez et al., 2009; Zhao et al., 2011). To the best of our knowledge, there were few reports about the effects of endophytic fungi on the secondary metabolism of their host plants (Wang et al., 2001; Zhang et al., 2009; Tang et al., 2011). *Fusarium redolens* Dzf2 was an endophytic and beauvericin-producing fungus isolated from the rhizomes of *D. zingiberensis* in our previous study (Xu et al., 2008, 2009, 2010a, 2010b). Beauvericin, a cyclic hexadepsipeptide mycotoxin most widely produced by many *Fusarium* fungal species (Logrieco et al., 1998; Fotso et al., 2002) had strong antibacterial, antifungal and insecticidal activities (Hamill et al., 1969; Grove and Pople, 1980; Castlebury et al.,

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1999; Xu et al., 2010b), and has also shown cytotoxic activity to various human cancer cell lines (Jow et al., 2007). The aim of this investigation was to study the effects of beauvericin as an elicitor from endophytic *F. redolens* Dzf2 on the growth and diosgenin production in seedling or cell cultures of its host plant *D. zingiberensis*.

MATERIALS AND METHODS

Seedling and cell suspension culture of *D. zingiberensis*

The seedlings of *D. zingiberensis* C. H. Wright were subsequently subcultured on Murashige and Skoog (MS) medium supplemented with agar (8 g/L) and sucrose (30 g/L) at 25°C and an interval of 32 days under 12 h daily illumination of approximately 2000 lux provided by cool fluorescent tubes. The calli were induced from the root explants of *D. zingiberensis* as described previously (Zhang et al., 2009), and were subsequently subcultured on MS medium supplemented with 6-benzyladenine (1.5 mg/L), naphthalene acetic acid (1.0 mg/L), agar (8 g/L), and sucrose (30 g/L) at 25°C and an interval of 30 days in darkness. The medium pH was adjusted to 5.8 before autoclaving. Each 125 ml Erlenmeyer flask was filled with 30 ml of MS medium without agar and 0.3 g of fresh cells was inoculated. The flask was then maintained on a rotary shaker at 120 rpm and 25°C in darkness.

Preparation and application of beauvericin

Endophytic fungus *F. redolens* Dzf2 (accession number DQ44621 in GenBank) was isolated from the rhizomes of *D. zingiberensis* and characterized as described in our previous report (Xu et al., 2008). Beauvericin was prepared by the method described by Xu et al. (2010b). It was dissolved in acetone as the concentrate stock solution, filter-sterilized through a 0.45 µm membrane and stored at 4°C. The sterilized beauvericin solution was added to the medium with its different concentrations at the beginning of the culture. The harvested time was determined based on the time courses of the cell or seedling growth and diosgenin production.

Determination of biomass and diosgenin yield

The seedlings were harvested from the Erlenmeyer flasks and washed with distilled water to remove residual medium. The suspension cells were harvested by filtration under vacuum. Both the seedlings and cells were lyophilized to a constant weight. Diosgenin extraction and determination were carried out as previously described with some modifications (Zhang et al., 2009; Zhu et al., 2010; Yin et al., 2011). Briefly, 100 mg of powdered dry cultured seedlings or cells was added into a tube with 20 ml of 95% ethanol and then subjected to ultrasonic treatment for 1 h. After that, 20 ml of 1 mol/L sulfuric acid was added to each tube and hydrolyzed at 121°C for 2 h. The hydrolyte was extracted three times with petroleum ether. The combined petroleum ether solution was washed twice with 1 mol/L NaOH and then twice with distilled water. After dehydration with anhydrous sodium sulfate, the petroleum ether solution was then concentrated to dryness under vacuum on a rotary evaporator. The extract was dissolved in acetonitrile and then filtered through a 0.22 µm filter before analysis. A high performance liquid chromatography system (Shimadzu, Japan) which consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector and CBM-20Alite system controller was employed. A reversed-phase Agilent TC-C_{18} column (250 mm × 4.6 mm i.d., particle size 5 µm) was used for separation by using a mobile phase of acetonitrile-water (90:10, v/v) at a flow rate of 1 ml/min at 30°C, and an LCsolution multi-PDA workstation was employed to acquire and process chromatographic data. The injection volume was 20 µL. Changes in absorbance at 203 nm were recorded. The peak area was calibrated to diosgenin content with a chemical standard (Sigma). Diosgenin content in the culture medium was negligible and not determined.

Statistical analysis

All the experiments were carried out three times. Each treatment was performed in triplicate, and the results were represented by their mean values and standard errors (S.E.). The data were submitted to analysis of variance to detect significant differences by PROC ANOVA of SAS version 8.2.

RESULTS AND DISCUSSION

Time courses of the seedling and cell suspension cultures of *D. zingiberensis*

The time courses of seedling growth and diosgenin production of *D. zingiberensis* are shown in Figure 1. The seedling biomass increased slowly in the first 20 days, and more rapidly between day 20 and day 28 reaching a maximum of 7.90 g/L around day 32. Diosgenin yield of the seedlings exhibited a similar time course to that of seedling weight, achieving the maximum yield around day 32. The results suggested that day 32 was a suitable time for harvesting diosgenin from *D. zingiberensis* seedling culture. The time courses of cell growth and diosgenin production in suspension culture of *D. zingiberensis* are shown in Figure 2. Cell dry weight increased slowly during the first 6 days and then exhibited a rapid increase as time up to day 30 with the highest value of 5.16 g/L. When the cultivation time was longer than 30 days, cell dry weight did not increase any more but a slight decrease. The yield of diosgenin exhibited the same trend as the cell dry weight.

Diosgenin yield reached the maximum with the value of 1.18 mg/L on the 30th day after inoculation. Thus, day 30 was the suitable time for harvesting *D. zingiberensis* cultured cells to produce diosgenin.

Effects of beauvericin on diosgenin production in seedling culture of *D. zingiberensis*

Table 1 shows the biomass, diosgenin content and diosgenin yield of *D. zingiberensis* seedling cultures fed with beauvericin at different concentrations. The seedling dry weight among the treatments had no significant differences. However, beauvericin at concentration of 0 to 100 mg/L stimulated diosgenin production in a concentration-dependent manner, giving the maximum response when added at 100 mg/ml. Compared with those of the control, diosgenin content increased by more than 1-fold (0.72 vs. 0.33 mg/g dw). Correspondingly,

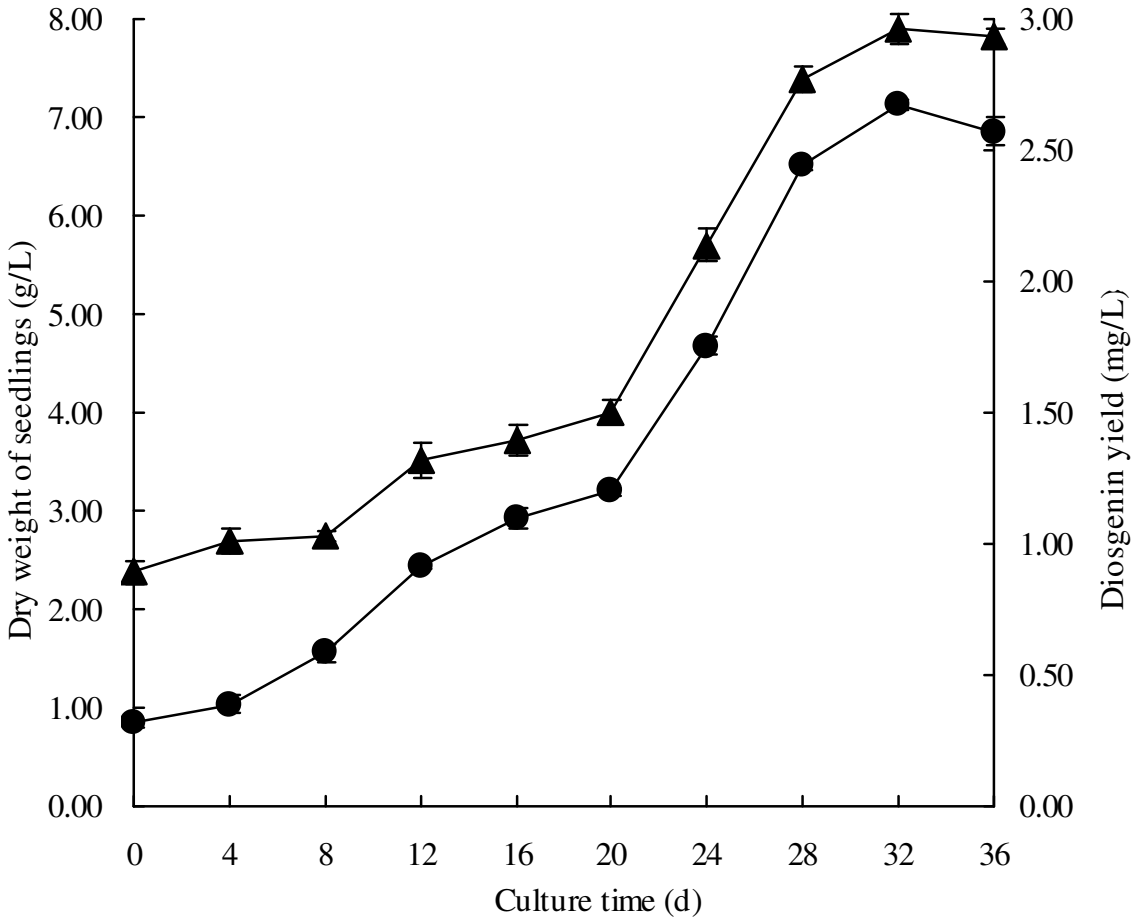

Figure 1. Time courses of biomass (▲) and diosgenin yield (●) of *D. zingiberensis* seedlings in solid culture (error bars represent standard errors, n = 3).

diosgenin yield also increased by more than 1-fold (5.72 vs. 2.67 mg/L).

Effects of beauvericin on diosgenin production in cell suspension culture of *D. zingiberensis*

Table 2 shows the biomass, diosgenin content and diosgenin yield of *D. zingiberensis* cell cultures fed with beauvericin at different concentrations. When the concentration of beauvercin was at 60 to 100 mg/L, the cell growth was obviously inhibited. When the concentration of beauvericin was at 0 to 40 mg/ml, the cell growth was not obviously affected and the diosgenin production was obviously stimulated in a concentration-dependent manner giving the maximum response when added at 40 mg/ml. Compared to the control, diosgenin content increased by more than 3.6-fold (1.07 vs. 0.23 mg/g dw) and diosgenin yield increased by more than 3.3-fold (5.15 vs. 1.19 mg/L). In summary, we first reported the enhancement of diosgenin production by beauvericin from the endophytic *F. redolens* Dzf2 in seedling or cell suspension cultures of *D. zingiberensis*. The results are further suggestive of the regulatory activities of beauvericin in plant growth and defense response (secondary metabolism) as well as understanding the co-evolution relationship between endophytic *F. redolens* Dzf2 and its host plant *D. zingiberensis*. On the other hand, the stimulating effects of beauvericin observed in our *D. zingiberensis* seedling or cell cultures may be useful for the production of diosgenin in future.

More detailed studies such as addition time of beauvericin in medium, beauverin in combination with other factors to stimulate diosgenin production, the stimulating mechanism of beauvericin on diosgenin biosynthesis in *Dioscorea* cultures and how to isolate diosgenin from *Dioscorea* cultures containing beauvericin are needed to be further studied.

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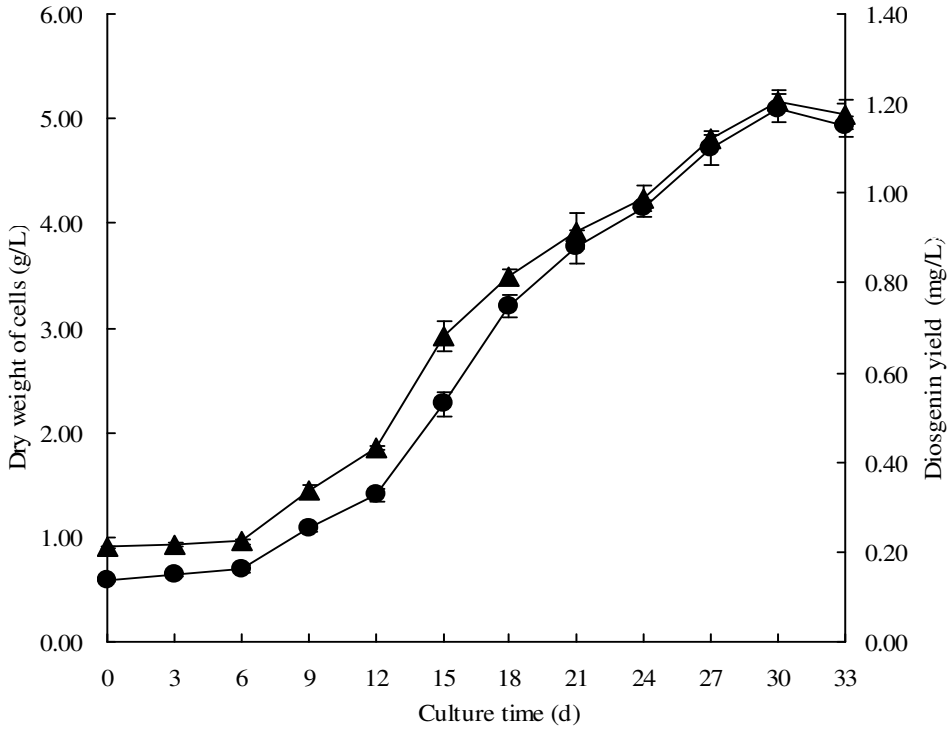

Figure 2. Time courses of biomass (▲) and diosgenin yield (●) of *D. zingiberensis* cells in suspension culture (error bars represent standard errors, n = 3).

Table 1. Effects of beauvericin on biomass, diosgenin content and diosgenin yield in seedling culture of *D. zingiberensis*.

Beauvericin concentration (mg/L)	Dry weight of seedlings (g/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
0	8.08 ± 0.44a	0.33 ± 0.01d	2.67 ± 0.26e
40	6.79 ± 0.55a	0.59 ± 0.02abc	4.01 ± 0.07bc
60	6.84 ± 0.48a	0.61 ± 0.02abc	4.17 ± 0.08b
80	7.58 ± 0.61a	0.66 ± 0.01ab	5.00 ± 0.33ab
100	7.95 ± 0.25a	0.72 ± 0.04a	5.72 ± 0.12a
120	8.07 ± 0.54a	0.47 ± 0.03bcd	3.79 ± 0.17cd

Note: Values represent mean ± S.E., n = 3. Culture time was in 32 days. Different letters in each column indicated significant differences among the treatments at p = 0.05.

Table 2. Effects of beauvericin on biomass, diosgenin content and diosgenin yield in cell suspension culture of *D. zingiberensis*.

Beauvericin concentration (mg/L)	Dry weight of cells (g/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
0	5.17 ± 0.13a	0.23 ± 0.01g	1.19 ± 0.04g
10	5.17 ± 0.18a	0.40 ± 0.01f	2.07 ± 0.03e
20	4.95 ± 0.09a	0.71 ± 0.03d	3.52 ± 0.07c
40	4.81 ± 0.05a	1.07 ± 0.03a	5.15 ± 0.08a
60	4.13 ± 0.08b	0.93 ± 0.03b	3.84 ± 0.09b
80	4.01 ± 0.09b	0.81 ± 0.03c	3.25 ± 0.03d
100	3.80 ± 0.13b	0.46 ± 0.01e	1.75 ± 0.03f

Note: Values represent mean ± S.E., n = 3. Culture time was in 30 days. Different letters in each column indicated significant differences among the treatments at p = 0.05.

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