academicJournals

Vol. 7(24), pp. 3049-3055, 11 June, 2013
DOI: 10.5897/AJMR12.599
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of polysaccharides and oligosaccharides from endophytic fungus *Berkleasmium* sp. Dzf12 on diosgenin accumulation in *Dioscorea zingiberensis* cell and seedling cultures

Peiqin Li, Chao Luo, Ruiya Luo, Yan Mou, Weibo Sun and Ligang Zhou*

College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.

Accepted 3 June, 2013

The effects of three polysaccharides; exopolysaccharide, water-extracted mycelial polysacharide, and sodium hydroxide-extracted mycelial polysaccharide (EPS, WPS and SPS) and their corresponding oligosaccharides; oligosaccharide obtained by hydrolysis of EPS, oligosaccharide obtained by hydrolysis of WPS and oligosaccharide obtained by hydrolysis of SPS (EOS, WOS and SOS) as the elicitors prepared from endophytic fungus *Berkleasmium* sp. Dzf12 on growth and diosgenin accumulation in cell and seedling cultures of *Dioscorea zingiberensis* were investigated. Among all the elicitation treatments, EOS generated the most satisfactory effect on enhancing diosgenin production in both cell and seedling cultures. The highest diosgenin yield (2.89 mg/L) in the cell cultures treated with EOS at 20 mg/L was achieved, which was 6.88-fold of control (0.42 mg/L). Meanwhile, when the seedlings were treated with EOS at 40 mg/L, the maximum diosgenin yield (14.68 mg/L) was achieved, which was 4.60-fold as the control (3.19 mg/L). Oligosaccharides WOS and SOS also showed obvious effects on enhancing diosgenin accumulation in the cell and seedling cultures, which were stronger than their corresponding polysaccharides WPS and SPS. The diosgenin yield in the cell cultures separately treated with WOS at 40 mg/L or SOS at 20 mg/L reached the maximum, which were 4.27- and 2.86-fold of control, respectively. For the seedlings treated with WOS or SOS, the maximum diosgenin yield was 8.43 mg/L, which was 2.64-fold of control. The results indicate that the polysaccharides and oligosaccharides as the elicitors from *Berkleasmium* sp. Dzf12 could be applied in *D. zingibereneis* cell and seedling cultures to produce diosgenin.

Key words: Polysaccharide, oligosaccharide, endophytic fungus, *Berkleasmium* sp. Dzf12, *Dioscorea zingiberensis*, diosgenin accumulation, cell cultures, seedling cultures.

INTRODUCTION

Plant endophytic fungi are microorganisms that reside in the internal tissues of living plants without causing any immediate overt negative effects or external symptoms (Strobel, 2006). They have been considered as important and novel sources of natural bioactive compounds with potential application in agriculture, medicine and food

*Corresponding author. E-mail: lgzhou@cau.edu.cn.

Abbreviations: EPS, Exopolysaccharide; **WPS**, water-extracted mycelial polysaccharide; **SPS**, sodium hydroxide-extracted mycelial polysaccharide; **EOS**, oligosaccharide obtained by hydrolysis of EPS; **WOS**, oligosaccharide obtained by hydrolysis of WPS; **SOS**, oligosaccharide obtained by hydrolysis of **SPS**; **dw**, dry weight; **Dzf12**, endophytic fungus *Berkleasmium* sp. Dzf12.

industry (Strobel et al., 2004; Kharwar et al., 2011). During the long period of co-evolution, a friendly relationship was gradually set up between each endophytic fungus and its host plant (Zhao et al., 2011a). 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 the host plants to resist external biotic and abiotic stresses, and benefiting for the host growth in return (Rodriguez et al., 2009). Some endophytic fungi can stimulate host or non-host plants to produce secondary metabolites. Typical examples included paclitaxel (or namely taxol) production in *Taxus chinensis* cell cultures induced by the endophytic fungus *Aspergillus niger* (Wang et al., 2001a), artemisinin production in Artemisia annua hairy root cultures elicited by the endophytic fungus *Colletotrichum* sp. (Wang et al., 2001b), paclitaxel formation in Taxus cuspidata cell cultures treated with the endophytic fungus *Fusarium* mairei (Li and Tao, 2009), alkaloid production in *Catharanthus roseus* cell cultures induced by its endophytic *Fusarium oxysporum* F9 (Tang et al., 2011).

Dioscorea zingiberensis C. H. Wright (Dioscoreaceae) is a well-known traditional Chinese medicinal herb, indigenous to the south of China (Wang et al., 2008; Zhu et al., 2010). The rhizomes have a high content of diosgenin, which is an important precursor of semi-synthetic steroids such as corticosteroids, sex hormones (example progesterone) and other steroidal drugs in pharmaceutical industry (Saunders et al., 1986; Fernandes et al., 2003). Our previous investigations showed that a broad diversity of endophytic fungi existed in *D. zingiberensis* (Xu et al., 2008). From these fungi, some metabolites (that is, beauvericin from *Fusarium* redolens Dzf2, polysaccharides and oligosaccharides from *F. oxysporum* Dzf17) have been isolated to show their enhancing effects on diosgenin production in cell and seedling cultures of *D. zingiberensis* (Zhang et al., 2009; Yin et al., 2011; Li et al., 2011a, 2011b), some metabolites (that is, polysaccharides from *Berkleasmium* sp. Dzf12 and F. oxysporum Dzf17) to show antioxidant activities (Li et al., 2011c, 2012), some metabolites (that is, beauvericin from F. redolens Dzf2, spirobisnaphthalenes from *Berkleasmium* sp. Dzf12) to show antimicrobial activities (Cai et al., 2009; Xu et al., 2010; Zhao et al., 2011b).

Berkleasmium sp. Dzf12 was an endophytic fungus isolated from the healthy rhizomes of *D. zingiberensis* (Cai et al., 2009). In this work, the effects of three polysaccharides (EPS, WPS and SPS) and their corresponding oligosaccharides (EOS, WOS and SOS) prepared from endophytic fungus *Berkleasmium* sp. Dzf12 on growth and diosgenin accumulation in cell and seedling cultures of *D. zingiberensis* were investigated in order to search for the most effective polysaccharide or oligosaccharide to increase diosgenin yield as well as to further understand the interactions between *Berkleasmium* sp. Dzf12 and its host *D. zingiberensis*.

MATERIALS AND METHODS

Seedling and cell culture of *D. zingiberensis*

The calli were induced from the root explants of *D. zingiberensis* as described previously (Zhang et al., 2009), and were subcultured on Murashige and Skoog (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 an interval of 30 days in darkness (Li et al., 2011a). The medium pH was adjusted to 5.8 before autoclaving for 15 min at 121°C. All experiments were carried out in 125-mL Erlenmeyer flasks. The cell cultures were maintained on the above liquid medium on a rotary shaker at 120 rpm in darkness at 25°C.

The seedlings of *D. zingiberensis* were initially obtained by callus redifferentiation on MS medium supplemented with 6-benzyladenine (5.0 mg/L), kinetin (2.0 mg/L), sucrose (30 g/L) and agar (8 g/L) at 25°C under 12 h daily illumination of approximately 2000 lux provided by cool fluorescent tubes. The medium pH was adjusted to 5.8 before autoclaving. Subculture of the seedlings was conducted on MS medium supplemented with sucrose (30 g/L) and agar (8 g/L) at 25°C and an interval of 30 days under 12 h daily illumination of approximately 2000 lux (Yin et al., 2011). The seedlings subcultured for 5 generations were taken as the plant materials. Each 125-mL Erlenmeyer flask was filled with 50 mL of MS solid medium, and three seedlings (about 1.0 g fresh weight) were inoculated in each flask.

Cultivation of endophytic fungus *Berkleasmium* sp. Dzf12

The endophytic fungus *Berkleasmium* sp. Dzf12 (GenBank accession number EU543255) was isolated from the healthy rhizomes of D. zingiberensis in our previous study (Cai et al., 2009; Zhang et al., 2010). It was preserved on potato dextrose agar (PDA) slants at 4°C and subcultured every six months. The mycelia were firstly cultivated in a 150-mL flask containing 30 mL modified Sabouraud broth medium consisting of glucose (40 g/L), peptone (10 g/L), KH2PO4 (1.0 g/L), MgSO4•7H2O (0.5 g/L), FeSO4•7H2O (0.05 g/L), which was incubated at 25°C on a rotary shaker at 150 rpm for 4 days as the inoculated seed culture (Zhao et al., 2011b). And then 5 mL of homogeneous 4-day-old *Berkleasmium* sp. Dzf12 seed culture broth was inoculated to a 1000-mL Erlenmeryer flask containing 300 mL modified Sabouraud broth medium. All flasks were incubated at 25°C on a rotary shaker at 150 rpm for 12 days. A total of 55 L fermentation broth was obtained, and then centrifuged at 7,741 x g for 20 min. The supernatant and mycelia were collected separately. Mycelia were washed twice with deionized water, then lyophilized. About 250 g of mycelia in dry weight (dw) was obtained.

Preparation of the polysaccharides

Exopolysaccharide (EPS) was prepared from fermentation broth of *Berkleasmium* sp. Dzf12 according to the method of Li et al. (2011a). The fermentation supernatant without mycelia was concentrated to a proper volume under vacuum at 60°C by a rotary evaporator and mixed with three volumes of 95% ethanol. The mixture was stirred vigorously and then maintained at 4°C for 48 h. The precipitate was collected by centrifugation at 17,418 x g for 15 min from the ethanol dispersion and then washed twice with absolute ethanol and acetone respectively. The final precipitate was designated as the crude EPS, which was then subjected to succes-

sive deproteination with Sevag reagent (chloroform-n-butanol at 4:1, v/v), decolorization with H_2O_2, and removal of small molecular impurities by dialysis. Polysaccharide mixture with molecular weight greater than 8,000-14,000 Da was kept in the dialysis tube. The retentate was concentrated to a certain volume and then mixed with three volumes of 95% ethanol to precipitate EPS. The precipitate thus obtained was lyophilized and weighed. The purified EPS (34.58 g) was stored in a desiccator at room temperature.

Both the water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS) were prepared from the mycelia by successive extraction with hot water and then with sodium hydroxide solution (Tseng et al., 2008; Li et al., 2011a). The dry mycelia (250 g) of *Berkleasmium* sp. Dzf12 were powdered in a disintegrator, and then subjected to heat circumfluence extraction at 50°C by 95% ethanol-petroleum ether at 1:1 (v/v) as the refluxing solvent to remove monosaccharide, disaccharide and lipid. The ratio of mycelia powder (g) to refluxing solvent (mL) was 1:5 (w/v). Defatted mycelial powder was obtained by centrifugation (7,741 x g) for 20 min, dried to the constant weight in an oven at 40°C, and then immersed in hot water at 90°C for 2 h with the ratio of water (mL) to the material (g) as 30:1 (v/w). The water extraction was repeated twice. After that, centrifugation was carried out at 7,741 x g for 20 min to separate the residue and the supernatant. The supernatant was condensed to a certain volume under vacuum at 60°C, and then mixed with three volumes of 95% ethanol, then kept at 4°C for 48 h. The following procedures for polysaccharide preparation and purification were the same as the treatments of EPS. The gained polysaccharide (15.88 g) by water extraction was considered as WPS. The mycelial residue not containing WPS was further extracted with 10% sodium hydroxide (NaOH) solution at room temperature for 24 h. The remaining steps were the same as the treatments of WPS. The finally obtained polysaccharide (25.90 g) was designated as SPS.

Preparation of the oligosaccharides

Each crude oligosaccharide was obtained by hydrolysis of the corresponding polysaccharide with 2.17 mol/L of trifluoroacetic acid (TFA) at 85°C for 4 h according to the method of Li et al. (2011b). And then the acid hydrolytes were filtrated. TFA in the filtrate was evaporated in the form of an azeotrope with methanol under vacuum. The concentrate was designated as the crude oligosaccharide which was consisted of a series of oligosaccharide monomers. The crude oligosaccharides EOS, WOS and SOS were from their corresponding polysaccharides EPS, WPS and SPS, separately. The carbohydrate content of the polysaccharides and oligosaccharides was measured by the method of anthrone-sulfuric acid spectrophotometer (Li et al., 2011c), which involved sulfuric acid hydrolysis of the sample in the presence of anthrone agent at 100°C. The absorbance at 620 nm was measured and calibrated to carbohydrate content using glucose as a reference.

Elicitation treatment

The polysaccharides (that is, EPS, WPS and SPS) and oligosaccharides (that is, EOS, WOS and SOS) were respectively dissolved in sterile distilled water as the stock solutions, and then filtered through a sterile filter membrane (pore size, 0.45 μm). For the seedling cultures, each 125-mL flask was filled with 50 mL MS solid medium. After the MS medium was autoclaved, gradient concentrations of the stock sterile polysaccharide or oligosaccharide solutions were immediately added to MS medium with the final concentrations at 10, 20, 40, 80 and 160 mg/L of carbohydrate equivalent. The inoculated quantity was three seedlings (about 1.0 g fresh weight) in each flask. The seedlings were harvested after cultivation of 32 days. For the cell cultures, the polysaccharides and oligosaccharides elicitation solutions were prepared the same as the seedling cultures. The addition concentrations were also at 10, 20, 40, 80 and 160 mg/L of carbohydrate equivalent. Each 125-mL flask was filled with 30 mL liquid medium with 0.3 g of fresh cell cultures as the inoculum. When the suspension cell cultures were cultured for 25 days, the elicitors were added. The cell cultures were harvested on day 30 of cultivation.

Determination of biomass and diosgenin analysis

D. zingiberensis seedlings were harvested from the Erlenmeyer flasks and washed with distilled water to remove residual medium. The suspension cell cultures were harvested by filtration under vacuum. Both the seedlings and cell cultures 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 cell or seedling cultures 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 for three times with petroleum ether. The combined petroleum ether solution was washed twice with 1 mol/L of NaOH solution, and then twice with distilled water. After dehydration with anhydrous sodium sulfate, the petroleum ether solution was then concentrated to dryness under vacuum. The extract was dissolved in acetonitrile, and then filtered through a filter (pore size, 0.22 μm) before analysis.

A high performance liquid chromatography (HPLC) 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-C18 column (250 mm × 4.6 mm i.d., particle size 5 μm) was used for chromatography by using acetonitrile-water (90:10, v/v) as the mobile phase 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. The 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 medium was negligible and not determined.

Statistical analysis

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

RESULTS AND DISCUSSION

Effects of polysaccharides and oligosaccharides on growth and diosgenin production in cell cultures of *D. zingiberensis*

The effects of three polysaccharides (that is, EPS, WPS and SPS) and their corresponding oligosaccharides (that is, EOS, WOS and SOS) as the elicitors from endophytic fungus *Berkleasmium* sp. Dzf12 on growth and diosgenin production of *D. zingiberensis* cell cultures are summarized

Table 1. Effects of polysaccharides and their corresponding oligosaccharides on growth and diosgenin accumulation in cell suspension cultures of *D. zingiberensis*.

Concentration (mg/L)		Dry weight (g dw/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
CK	0	3.29 ± 0.24 ^{ijkl}	0.13 ± 0.02 ^{j}	0.42 ± 0.09 ^{qrst}
EPS	10	3.77 ± 0.23 ^{fgh}	0.12 ± 0.02 ^{j}	0.47 ± 0.04 ^{pqrs}
	20	4.20 ± 0.19 ^{bc}	0.20 ± 0.02 ^{ghi}	0.83 ± 0.06 ^{jkl}
	40	4.78 ± 0.21 ^{a}	0.24 ± 0.02 ^{ef}	1.16 ± 0.09 ^{efg}
	80	3.56 ± 0.23 ^{hij}	0.11 ± 0.02 ^{jk}	0.40 ± 0.09 ^{rstu}
	160	2.84 ± 0.19 ^{m}	0.08 ± 0.01 ^{jk}	0.24 ± 0.06 ^{tu}
EOS	10	3.27 ± 0.15 ^{jkl}	0.32 ± 0.03 ^{d}	1.03 ± 0.14 ^{ghi}
	20	4.08 ± 0.13 ^{bcde}	0.71 ± 0.06 ^{a}	2.89 ± 0.31 ^{a}
	40	3.81 ± 0.10 ^{efgh}	0.55 ± 0.06 ^{b}	2.11 ± 0.25 ^{b}
	80	3.35 ± 0.12 ^{ijkl}	0.36 ± 0.04 ^{d}	1.20 ± 0.11 ^{efg}
	160	2.44 ± 0.10 ^{n}	0.09 ± 0.03 ^{jk}	0.23 ± 0.06 ^{tu}
WPS	10	3.39 ± 0.26 ^{ijk}	0.17 ± 0.02 ^{i}	0.59 ± 0.11 ^{nopq}
	20	3.89 ± 0.12 ^{def}	0.22 ± 0.02 ^{efgh}	0.86 ± 0.12 ^{ijkl}
	40	4.12 ± 0.13 ^{bcd}	0.25 ± 0.02 ^{ef}	1.01 ± 0.12 ^{ghij}
	80	4.34 ± 0.03 ^{b}	0.13 ± 0.01 ^{j}	0.55 ± 0.04 ^{opqr}
	160	2.91 ± 0.13 ^{m}	0.17 ± 0.00 ^{k}	0.21 ± 0.00 ^{u}
WOS	10	3.57 ± 0.23 ^{ghi}	0.21 ± 0.01 ^{fghi}	0.77 ± 0.07 ^{klmn}
	20	3.93 ± 0.11 ^{cdef}	0.34 ± 0.02 ^{d}	1.33 ± 0.10 ^{de}
	40	4.33 ± 0.10 ^{b}	0.42 ± 0.01 ^{c}	1.81 ± 0.09 ^{c}
	80	4.05 ± 0.03 ^{bcdef}	0.36 ± 0.01 ^{d}	1.45 ± 0.02 ^{d}
	160	2.84 ± 0.18 ^{m}	0.08 ± 0.01 ^{jk}	0.24 ± 0.02 ^{tu}
SPS	10	3.31 ± 0.05 ^{ijkl}	0.19 ± 0.03 ^{hi}	0.61 ± 0.09 ^{mnop}
	20	3.80 ± 0.12 ^{efgh}	0.24 ± 0.02 ^{efg}	0.92 ± 0.10 ^{hijk}
	40	4.14 ± 0.10 ^{bcd}	0.19 ± 0.02 ^{hi}	0.79 ± 0.06 ^{klm}
	80	3.07 ± 0.13 ^{lm}	0.11 ± 0.01 ^{jk}	0.34 ± 0.03 ^{stu}
	160	2.91 ± 0.14 ^{m}	0.09 ± 0.01 ^{jk}	0.26 ± 0.02 ^{tu}
SOS	10	3.36 ± 0.12 ^{ijk}	0.22 ± 0.02 ^{efghi}	0.73 ± 0.07 ^{klmno}
	20	3.86 ± 0.18 ^{defg}	0.32 ± 0.02 ^{d}	1.23 ± 0.03 ^{ef}
	40	4.12 ± 0.09 ^{bcd}	0.26 ± 0.02 ^{e}	1.08 ± 0.11 ^{fgh}
	80	3.20 ± 0.17 ^{kl}	0.22 ± 0.01 ^{efgh}	0.71 ± 0.08 ^{lmno}
	160	2.82 ± 0.23 ^{m}	0.11 ± 0.01 ^{jk}	0.30 ± 0.06 ^{stu}

Each value was expressed as mean ± standard deviation (n = 3). Different letters in each column indicated significant differences at p = 0.05.

in Table 1. Among all the treatments, the highest dry weight of the cell cultures was caused by polysaccharide EPS at 40 mg/L with the value of 4.78 g dw/L, which was 1.45-fold of control (3.29 g dw/L). The maximum values of dry weight of the cells treated with EOS, WPS, WOS, SPS and SOS were 4.08, 4.34, 4.33, 4.14 and 4.12 g dw/L, respectively, which showed no significant differences each other.

As shown in Table 1, the oligosaccharides showed more desirable effects on enhancing diosgenin content in cell cultures than their corresponding polysaccharides. The highest diosgenin content (0.71 mg/g dw) was observed in the cell cultures treated with oligosaccharide EOS at 20 mg/L, which was 5.46-fold of control (0.13 mg/g dw). The maximum diosgenin content (0.24 mg/g dw) was obtained in cell cultures treated with polysaccharide EPS at 40 mg/L, which was just 1.85-fold of control.

Diosgenin yield (mg/L) was the synthesized result of dry weight (g dw/L) and diosgenin content (mg/g dw) of the cultures. Of all the treatments, the maximum enhancement of diosgenin production was achieved in the cell cultures treated with EOS at 20 mg/L. Diosgenin yield was 2.89 mg/L and 6.88-fold of control (0.42 mg/L). Accordingly, polysaccharide EPS showed a lower

Table 2. Effects of polysaccharides and their corresponding oligosaccharides on growth and diosgenin accumulation in seedling cultures of *D. zingiberensis*.

Concentration (mg/L)		Dry weight (g dw/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
CK	0	11.94 ± 0.41 ^{fghij}	0.27 ± 0.02 ^{mn}	3.19 ± 0.27 ^{jkl}
EPS	10	14.57 ± 0.63 ^{b}	0.28 ± 0.01 ^{lm}	4.07 ± 0.32 ^{ghi}
	20	13.03 ± 0.47 ^{def}	0.37 ± 0.02 ^{ijk}	4.77 ± 0.38 ^{fg}
	40	12.15 ± 0.93 ^{efghi}	0.41 ± 0.04 ^{hi}	4.99 ± 0.74 ^{f}
	80	10.84 ± 0.83 ^{jklmn}	0.32 ± 0.02 ^{kl}	3.51 ± 0.45 ^{ijk}
	160	10.24 ± 0.28 ^{mn}	0.23 ± 0.03 ^{nop}	2.33 ± 0.31 ^{mn}
EOS	10	12.35 ± 0.32 ^{efgh}	0.37 ± 0.02 ^{ij}	4.62 ± 0.16 ^{fg}
	20	13.33 ± 0.56 ^{cde}	0.55 ± 0.05 ^{cd}	7.34 ± 0.72 ^{d}
	40	14.63 ± 0.44 ^{b}	1.00 ± 0.02 ^{a}	14.68 ± 0.16 ^{a}
	80	12.81 ± 0.51 ^{defg}	0.84 ± 0.05 ^{b}	10.70 ± 0.63 ^{b}
	160	11.06 ± 0.74 ^{ijklm}	0.42 ± 0.02 ^{gh}	4.70 ± 0.51 ^{fg}
WPS	10	12.37 ± 0.46 ^{efgh}	0.28 ± 0.01 ^{mn}	3.40 ± 0.36 ^{ijk}
	20	13.86 ± 1.05 ^{bcd}	0.32 ± 0.03 ^{kl}	4.49 ± 0.68 ^{fg}
	40	12.87 ± 0.69 ^{defg}	0.36 ± 0.03 ^{jk}	4.59 ± 0.55 ^{fg}
	80	10.83 ± 0.52 ^{jklmn}	0.33 ± 0.02 ^{jk}	3.62 ± 0.32 ^{ij}
	160	9.76 ± 0.27 ^{n}	0.25 ± 0.03 ^{mno}	2.46 ± 0.32 ^{lm}
WOS	10	11.60 ± 0.62 ^{hijk}	0.32 ± 0.02 ^{kl}	3.76 ± 0.32 ^{hij}
	20	12.97 ± 0.80 ^{defg}	0.48 ± 0.02 ^{ef}	6.19 ± 0.53 ^{e}
	40	14.23 ± 0.33 ^{bc}	0.59 ± 0.02 ^{c}	8.43 ± 0.51 ^{c}
	80	13.05 ± 0.67 ^{def}	0.46 ± 0.03 ^{fg}	6.02 ± 0.35 ^{e}
	160	10.70 ± 0.68 ^{klmn}	0.34 ± 0.03 ^{jk}	3.73 ± 0.50 ^{hij}
SPS	10	11.81 ± 0.71 ^{ghijk}	0.24 ± 0.02 ^{mnop}	2.85 ± 0.05 ^{klm}
	20	11.43 ± 0.79 ^{hijklm}	0.22 ± 0.01 ^{op}	2.50 ± 0.39 ^{lm}
	40	11.26 ± 0.75 ^{hijklm}	0.21 ± 0.01 ^{op}	2.38 ± 0.42 ^{m}
	80	10.98 ± 0.62 ^{ijklm}	0.20 ± 0.02 ^{p}	2.18 ± 0.29 ^{mn}
	160	10.47 ± 0.56 ^{lmn}	0.16 ± 0.02 ^{q}	1.63 ± 0.35 ^{n}
SOS	10	11.93 ± 0.60 ^{efghi}	0.37 ± 0.02 ^{ijk}	4.38 ± 0.14 ^{fg}
	20	14.71 ± 0.67 ^{b}	0.45 ± 0.01 ^{fgh}	6.61 ± 0.69 ^{e}
	40	16.33 ± 0.68 ^{a}	0.52 ± 0.03 ^{de}	8.43 ± 0.13 ^{c}
	80	12.41 ± 0.43 ^{efgh}	0.28 ± 0.03 ^{lm}	3.53 ± 0.33 ^{ijk}
	160	11.03 ± 0.63 ^{ijklm}	0.23 ± 0.01 ^{nop}	2.54 ± 0.38 ^{lm}

Each value was expressed as mean ± standard deviation (n = 3). Different letters in each column indicated significant differences at p = 0.05.

enhancement of diosgenin yield than EOS, with the maximum diosgenin yield as 1.16 mg/L at concentration of 40 mg/L. Both oligosaccharides WOS and SOS also showed more satisfactory stimulating effects on diosgenin yield than their corresponding polysaccharides. The maximum diosgenin yield caused by WOS and SOS were respectively 1.81 and 1.23 mg/L, both of which were lower than that caused by EOS. It is suggested that EOS may be the most efficient elicitor to enhance diosgenin accumulation in *D. zingiberensis* cell cultures.

Effects of polysaccharides and oligosaccharides on growth and diosgenin accumulation in seedling cultures of *D. zingiberensis*

The effects of three polysaccharides and their corresponding oligosaccharides as the elicitors on growth and diosgenin production of *D. zingiberensis* seedling cultures are summarized in Table 2. The most efficient elicitor to enhance seedling growth was SOS at 40 mg/L with dry weight as 16.33 g dw/L which was 1.37-fold of control

(11.92 g dw/L). Meanwhile, its corresponding polysaccharide SPS showed obvious effects on seedling growth at all concentrations. The maximum values of dry weight caused by EPS and EOS were respectively 14.57 g dw/L and 14.63 g dw/L, which showed no significant differences. For WOS, the highest dry weight was obtained at 40 mg/L, which was 14.23 g dw/L and higher than that of WPS.

As shown in Table 2, the oligosaccharides also caused higher diosgenin content than the corresponding polysaccharides in seedling cultures. Of three oligosaccharides, EOS at 40 mg/L showed the most effective to increase diosgenin content in seedling cultures with the value as 1.00 mg/g dw which was 3.70-fold of control (0.27 mg/g dw) and far higher than that (0.41 mg/g dw) treated with polysaccharide EPS. Other oligosaccharides WOS and SOS also increased diosgenin content in seedling cultures. When the seedlings were treated with WOS or SOS at 40 mg/L, diosgenin content increased to the highest, which were 0.59 mg/g dw and 0.52 mg/g dw, respectively. When the seedlings were treated with EOS at 40 mg/L, the highest diosgenin yield (14.68 mg/L) was gained, which was 4.60-fold of control (3.19 mg/L) and far higher than that (4.99 mg/L) treated with EPS. When the seedlings were treated with WOS or SOS at 40 mg/L, the maximum diosgenin yield was the same with the value of 8.43 mg/L, which was 2.64-fold of control, and higher than their corresponding polysaccharides. This indicated that oligosaccharide EOS was also the most efficient elicitor to enhance diosgenin accumulation in *D. zingiberensis* seedling cultures.

The results in this study showed that the polysaccharides and oligosaccharides from endophytic fungus *Berkleasmium* sp. Dzf12 had enhancing effects on diosgenin production in cell and seedling cultures of *D. zingiberensis*. Furthermore, the oligosaccharides showed stronger effects on improving diosgenin accumulation in *D. zingiberensis* cell or seedling cultures than their corresponding polysaccharides. Enhancing effects of the oligosaccharides and polysaccharides as the elicitors from endophytic fungus *Berkleasmium* sp. Dzf12 on diosgenin accumulation in *D. zingiberensis* cultures could provide more information to understand the symbiotic relationships between the endophyte fungus and its host plant. However, we still do not know how these elicitors from endophytic fungi mediate the biosynthesis of diosgenin in *D. zingiberensis*. The enhancing effects of these oligosaccharide and polysaccharide elicitors on diosgenin biosynthesis may be related to the elicitor type, elicitation dose, the age of plant culture and the plant culture system (Dornenburg and Knorr, 1995; Zhou and Wu, 2006). Ebel and Mithofer (1998) reported that oligoglucoside elicitor might first bind to certain proteins in the cell membrane, which could function as a signal transduction receptor to initiate a series of subsequent defense-related responses. The oligosaccharide elicitors from endophytic *Berkleasmium* sp. Dzf12 were found to be more effective than the polysaccharide elicitors to increase diosgenin accumulation in *D. zingiberensis* cell and seedling cultures. It is possible that the added polysaccharides in medium were decomposed into the oligosaccharides which stimulate the biosynthesis of diosgenin in *D. zingiberensis* cultures, that are worthy of further study. The oligosaccharides used in this study were composed of different oligosaccharide monomers. It is necessary to further isolate and purify the main active oligosaccharide monomers with significantly enhancing diosgenin accumulation in *D. zingiberensis* cultures. As a whole, the application of the polysaccharide and oligosaccharide elicitors from endophytic fungus *Berkleasmium* sp. Dzf12 to stimulate diosgenin accumulation in *D. zingiberensis* cultures could be an effective strategy for future diosgenin production.

ACKNOWLEDGMENTS

This work was co-financed by the grants from the National Natural Science Foundation of China (31071710), and the Hi-Tech R and D Program of China (2011AA10A202).

REFERENCES

Cai X, Shan T, Li P, Huang Y, Xu L, Zhou L, Wang M, Jiang W (2009). Spirobisnaphthalenes from the endophytic fungus Dzf12 of *Dioscorea zingiberensis* and their antimicrobial activities. Nat. Prod. Commun. 4:1469-1472.

Dornenburg H, Knorr D (1995). Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microb. Technol. 17:674-684.

Ebel J, Mithofer A (1988). Early events in the elicitation of plant defense. Planta 206:335-348.

Fernandes P, Cruz A, Angelova B, Pinheiro HM, Cabral JMS (2003). Microbial conversion of steroid compounds: recent developments. Enzyme Microb. Technol. 32:688-705.

Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011). Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat. Prod. Rep. 28:1208-1228.

Li P, Luo C, Sun W, Lu S, Mou Y, Peng Y, Zhou L (2011c). In vitro antioxidant activities of polysaccharides from endophytic fungus *Fusarium oxysporum* Dzf17. Afr. J. Microbiol. Res. 5:5990-5993.

Li P, Mao Z, Lou J, Li Y, Mou Y, Lu S, Peng Y, Zhou L (2011b). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell cultures by oligosaccharides from its endophytic fungus *Fusarium oxysporum* Dzf17. Molecules 16:10631-10644.

Li P, Mou Y, Shan T, Xu J, Li Y, Lu S, Zhou L (2011a). Effects of polysaccharide elicitors from endophytic *Fusarium oxysporum* Dzf17 on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis*. Molecules 16:9003-9016.

Li P, Sun W, Luo C, Shan T, Mou Y, Lu S, Mao Z, Zhou L (2012). In vitro evaluation of antioxidant activities of polysaccharides from the endophytic fungus *Berkleasmium* sp. Dzf12. Afr. J. Microbiol. Res. 6:471-477.

Li Y-C, Tao W-Y (2009). Effects of paclitaxel-producing fungal endophytes on growth and paclitaxel formation of Taxus cuspidata cells. Plant Growth Regul. 58:97-105.

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009). Fungal endophytes: diversity and functional roles. New Phytol. 182:314-330.

Saunders R, Cheetham PSJ, Hardman R (1986). Microbial transformation of crude fenugreek steroids. Enzyme Microb. Technol. 8:549-555.

Strobel G, Daisy B, Castillo U, Harper J (2004). Natural products from endophytic microorganisms. J. Nat. Prod. 67:257-268.

Strobel GA (2006). Harnessing endophytes for industrial microbiology. Curr. Opin. Microbiol. 9:240-244.
Tang Z, Rao L, Peng G, Zhou M, Shi G, Liang Y (2011). Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*. J. Med. Plants Res. 5:2192-2200.
Tseng Y-H, Yang J-H, Mau J-L (2008). Antioxidant properties of polysaccharides from Ganoderma tsugae. Food Chem. 107:732-738.
Wang C, Wu J, Mei X (2001a). Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl. Microbiol. Biotechnol. 55:404-410.
Wang J, Zhang Z, Tan R (2001b). Stimulation of artemisinin production in Artemisia annua hairy roots by the elicitor from the endophytic *Colletotrichum* sp. Biotechnol. Lett. 23:857-860.
Wang Y, Liu H, Bao J, Hong Y, Yang A, Zhang C (2008). The saccharification-membrane retrieval-hydrolysis (SMRH) process: a novel approach for cleaner production of diosgenin derived from *Dioscorea zingiberensis*. J. Clean. Prod. 16:1133-1137.
Xu L, Wang J, Zhao J, Li P, Shan T, Wang J, Li X, Zhou L (2010). Beauvericin from the endophytic fungus, *Fusarium* redolens, isolated from *Dioscorea zingiberensis* and its antibacterial activity. Nat. Prod. Commun. 5:811-814.
Xu L, Zhou L, Zhao J, Li J, Li X, Wang J (2008). Fungal endophytes from *Dioscorea zingiberensis* rhizomes and their antibacterial activity. Lett. Appl. Microbiol. 46:68-72.
Yin C, Li P, Li H, Xu L, Zhao J, Shan T, Zhou L (2011). Enhancement of diosgenin production in *Dioscorea zingiberensis* seedling and cell cultures by beauvericin from the endophytic fungus *Fusarium* redolens Dzf2. J. Med. Plants Res. 5:6550-6554.
Zhang R, Li P, Xu L, Chen Y, Sui P, Zhou L, Li J (2009). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell culture by oligosaccharide elicitor from its endophytic fungus *Fusarium oxysporum* Dzf17. Nat. Prod. Commun. 4:1459-1462.
Zhao J, Shan T, Mou Y, Zhou L (2011a). Plant-derived bioactive compounds produced by endophytic fungi. Mini-Rev. Med. Chem. 11:159-168.
Zhao J, Zheng B, Li Y, Shan T, Mou Y, Lu S, Li P, Zhou L (2011b). Enhancement of diepoxin ζ production by yeast extract and its fractions in liquid culture of *Berkleasmium*-like endophytic fungus Dzf12 from *Dioscorea zingiberensis*. Molecules 16:847-856.
Zhou L, Wu J (2006). Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. Nat. Prod. Rep. 23:789-810.
Zhu Y-L, Huang W, Ni J-R, Liu W, Li H (2010). Production of diosgenin from *Dioscorea zingiberensis* tubers through enzymatic saccharification and microbial transformation. Appl. Microbiol. Biotechnol. 85:1409-1416.