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Original Research Article

Enhancement of Diosgenin Production in Plantlet and Cell Cultures of *Dioscorea zingiberensis* by Palmarumycin C_{13} from the Endophytic fungus, *Berkleasmium* sp. Dzf12

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Abstract

Purpose: *To study the effect of palmarumycin C_{13}, an elicitor from the endophytic fungus Berkleasmium sp. Dzf12, on growth and diosgenin production in plantlet or cell cultures of its host plant, Dioscorea zingiberensis.*
Methods: *Palmarumycin C_{13} was isolated from the ethyl acetate extract of the endophytic fungus Berkleasmium sp. Dzf12 using a combination of high-speed counter-current chromatography (HSCCC), Sephadex LH-20 chromatography and preparative high performance liquid chromatography (HPLC). The biomass of the plantlet and cell cultures of D. zingiberensis as well as their diosgenin content and yield were analyzed after treatment with palmarumycin C_{13}.*
Results: *Optimal elicitation of diosgenin production by palmarumycin C_{13} in D. zingiberensis plantlet and cell cultures was achieved when palmarumycin C_{13} was added to the medium at a concentration of 60 mg/L (for plantlet culture) at the beginning of culturing or 10 mg/L (for cell culture) on day 25 after inoculation. By using these optimal concentrations, the diosgenin yield of the cultured plantlets reached its maximum of 6.44 mg/L, that is, > 1.4-fold increase, while diosgenin yield of the cultured cells reached a maximum of 10.73 mg/L, which is an > 8.0-fold increase.*
Conclusion: *Addition of palmarumycin C_{13} from the endophytic fungus, Berkleasmium sp. Dzf12, is a potentially effective strategy for enhancing diosgenin production in D. zingiberensis plantlet and cell cultures.*

Keywords: *Endophytic fungus, Berkleasmium sp. Dzf12, Spirobisnaphthalene, Palmarumycin C_{13}, Dioscorea zingiberensis, Diosgenin, Elicitation, Cell culture, Plantlet culture*

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INTRODUCTION

Dioscorea zingiberensis C. H. Wright (Dioscoreaceae) is a well known traditional Chinese medicinal herb, and is indigenous to the south of China [1]. Its rhizomes have a high content (about 1.1 %) of diosgenin, which is an important precursor of various synthetic steroidal drugs [2]. In order to speed up the application, the enhancement of diosgenin yield in *D. zingiberensis* is required. Many strategies (i.e., medium optimization, elicitation, as well as two phase culture) have been well developed to enhance the metabolite production of the either micro-organism or plant cultures. Among them, the use of elicitors was regarded as a convenient and effective approach [3,4]. Plant endophytic fungi, the latent phytopathogens or mutualistic

symbionts presenting inter- and/or intra-cellularly in the normal tissue of host plants, have been proved to be a promising source of novel structures and/or strong bioactivities [5]. 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. *Berkleasmium* sp. Dzf12 was an endophytic and spirobisnapthalenes-producing fungus isolated from the rhizomes of *D. zingiberensis* in our previous studies [6-8]. This fungus was found to be a high producer of palmarumycin C_{13} [9-11], which has been shown to have obvious antibacterial and antifungal activity [6,12], anti-tumor activity and inhibitory activity on phospholipase D (PLD) [13].

The aim of this investigation was to study the effects of palmarumycin C_{13}, as an elicitor from endophytic *Berkleasmium* sp. Dzf12, on the growth of the plantlet and cell cultures of the host plant, *D. zingiberensis*, and diosgenin production. It will provide more information for further understanding the interactions between the endophytic fungus and its host plant as well as for enhancing diosgenin production by employing *D. zingiberensis* plantlet and cell cultures.

EXPERIMENTAL

Plantlet and cell suspension culture of *D. zingiberensis*

The roots of *D. zingiberensis* were cut into 0.5 cm-long explants which were used for callus induction as described previously [14]. The calli were subcultured every 30 days 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) in darkness [15]. 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 suspension cell cultures were maintained on the above liquid medium on a rotary shaker at 120 rpm in darkness at 25 °C. The plantlet, callus and cell suspention cultures of *D. zingiberensis* are shown in Figure 1.

The plantlets 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 2,000 lux provided by cool fluorescent tubes. The medium pH was adjusted with 1 mol/L of HCl solution to 5.8 before autoclaving. Subculture of the plantlets 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 2,000 lux [14]. The plantlets subcultured for 5 generations were used as the plant materials. Each 125 mL Erlenmeyer flask was filled with 50 mL of MS solid medium, and three plantlets (about 1.0 g fresh weight) were inoculated in each flask.

Isolation and application of palmarumycin C_{13}

Endophytic fungus *Berkleasmium* sp. Dzf12 (accession number EU543255 in GenBank) was isolated from the healthy rhizomes of *D. zingiberensis* as reported previously [6]. The mycelia were grown in a 1000 mL Erlenmeryer flask containing liquid medium (300 mL) consisting of glucose (50 g/L), peptone (13 g/L), NaCl (0.6 g/L), K_2HPO_4 (0.6 g/L), and $MgSO_4 \cdot 7H_2O$ (0.2 g/L). 500 flasks were maintained on a rotary shaker at 150 rpm and 25 °C for 14 days. A total of 150 L of fermentation broth was obtained and centrifuged at 7,741 × g for 20 min. The supernatant and mycelia were collected separately. Mycelia were washed twicewith deionized water, then lyophilized. 600 g of mycelia in dry weight (dw) was obtained.

Palmarumycin C_{13} was isolated from the ethyl acetate extract of the mycelia of the endophytic fungus *Berkleasmium* sp. Dzf12 using a

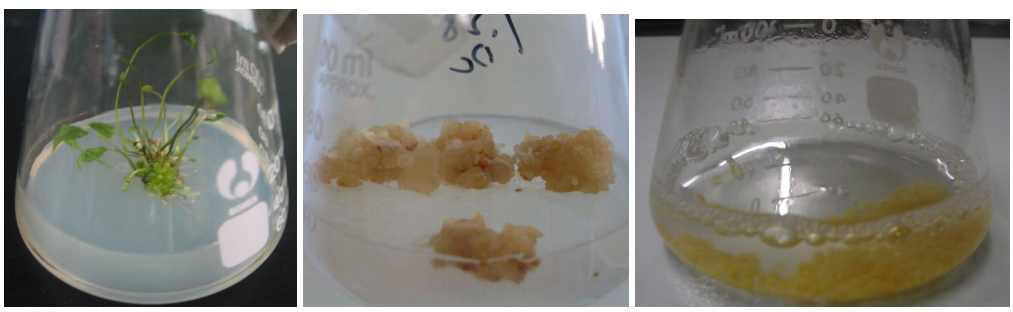

Figure 1: Plantlet (A), callus (B) and cell suspension (C) cultures of *D. zingiberensis*

combination of high-speed counter-current chromatography (HSCCC), Sephadex LH-20 chromatography and preparative HPLC [16]. The purified palmarumycin C_{13} was structurally identified by NMR and MS as well as the authentic sample [6]. 50 mg of purified palmarumycin C_{13} was dissolved in 5 mL acetone as the concentrate stock solution, filter-sterilized through a 0.45 µm membrane and stored at 4 °C. The sterilized palmarumycin C_{13} solution was added to the medium at different concentrations (10 to 100 mg/L) either at the beginning of culture for the plantlet cultures or on days 15, 20 and 25 of culture for the cell cultures. The same volume of acetone was used as the control (CK). The plantlet and cell cultures were harvested on day 32 and day 30 of culture, respectively except for the time course study.

Determination of biomass and diosgenin yield

The plantlets 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 plantlets and cells were lyophilized to a constant dry weight (dw).

Diosgenin extraction and determination were carried out as previously described with some modifications [14]. Briefly, 100 mg of powdered dry cultured plantlets 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 to cause hydrolysis at 121 °C for 2 h. The hydrolyte was extracted three times with petroleum ether. The combined petroleum ether solution was washed twice with 20 mL of 1 mol/L NaOH, and then twice with 20 mL of distilled water. After dehydration of the petroleum ether layer with anhydrous sodium sulfate, the petroleum ether solution was then concentrated under vacuum on a rotary evaporator. The petroleum ether extract was dissolved in acetonitrile, and then filtered through a 0.22 µm filter before analysis [14].

Diosgnenin quantification

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-C18 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 ºC, and an LC solution 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 [14].

Statistical analysis

All the experiments were carried out three times. Each treatment with palmarumycin C_{13} was performed in triplicate, and the results represented as mean ± standard deviations. The data were submitted to analysis of variance to detect significant differences by PROC ANOVA of SAS version 8.2.

RESULTS

Effect of palmarumycin C_{13} on diosgenin production in plantlet culture of *D. zingiberensis*

Table 1 shows the biomass (plantlet dry weight), diosgenin content and yield of *D. zingiberensis* plantlet cultures fed palmarumycin C_{13} with different concentrations at the beginning of culture. The plantlet cultures were harvested on day 32 of culture. The growth of the plantlets was slightly inhibited by palmarumycin C_{13} at 10 to 40 mg/L, and greatly inhibited at 60 to 100 mg/L. However, palmarumycin C_{13} at 10 to 80 mg/L obviously stimulated diosgenin production. When palmarumycin C_{13} was at 60 mg/L, both diosgenin content (0.95 mg/g dw) and yield (6.44 mg/L) reached the maximum values which were 2.88-fold and 2.41-fold of those (0.33 mg/g dw and 2.67 mg/L) of the control, respectively.

Figure 2 shows the time courses of growth, diosgenin content and yield in plantlet culture of *D. zingiberensis* after addition of palmarumycin C_{13} at 60 mg/L. The plantlet biomass increased slowly in the first 20 days, and more rapidly between day 20 and day 28, reaching a maximum around day 32 (Figure 1A). Diosgenin yield of the plantlets exhibited a similar time course to that of plantlet weight, achieving the maximum yield around day 32 (Figure 1C). The results verified that day 32 was a suitable time for harvesting plantlet cultures for diosgenin production by treatment with palmarumycin C_{13} at 60 mg/L.

Table 1: Effects of palmarumycin C_{13} on biomass, diosgenin content and yield in plantlet culture of *D. zingiberensis*

Palmarumycin C_{13} concentration (mg/L)	Dry weight of plantlets (g/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
0	8.08 ± 0.44 [a]	0.33 ± 0.01 [d]	2.67 ± 0.26 [d]
10	7.94 ± 0.16 [a]	0.58 ± 0.03 [c]	4.61 ± 0.17 [b]
20	7.91 ± 0.27 [a]	0.77 ± 0.02 [b]	6.09 ± 0.34 [a]
40	7.83 ± 0.18 [a]	0.79 ± 0.01 [b]	6.19 ± 0.53 [a]
60	6.78 ± 0.55 [b]	0.95 ± 0.03 [a]	6.44 ± 0.68 [a]
80	5.05 ± 0.46 [c]	0.76 ± 0.02 [b]	3.84 ± 0.34 [c]
100	4.18 ± 0.36 [c]	0.65 ± 0.02 [c]	2.72 ± 0.33 [d]

Note: *All values represent mean ± standard deviation; values marked with different letters in each column indicate significant differences between treatments with palmarumycin C_{13} at p = 0.05; Plantlet cultures were harvested on day 32 of culture*

Effect of palmarumycin C_{13} on diosgenin production in cell suspension culture of *D. zingiberensis*

The effects of palmarumycin C_{13} on cell growth and diosgenin production in cell suspension culture of *D. zingiberensis* are shown in Table 2. To investigate the impacts of additional time of palmarumycin C_{13} in combination with its concentration in medium, the treatment was carried out at different growth stages (days 15, 20 and 25) in cell suspension culture with various concentrations (5 to 100 mg/L) in the medium, and cultures were harvested on day 30 of culture.

Cell growth was inhibited when palmarumycin C_{13} was added on the either 15th day or 20th day of culture at all test concentrations. The dry weight of the treated cell cultures was decreased to 2.51 g/L when palmarumycin C_{13} was added on day 15 at 100 mg/L, and to 3.50 g/L when palmarumycin C_{13} was added on day 20 at 100 mg/L. An enhancing effect on cell growth was only observed when palmarumycin C_{13} was added on day 25 at 5 mg/L.

Diosgenin yield (mg/L) is the result of the synthesized cell dry weight (g dw/L) and diosgenin content (mg/g dw). When palmarumycin C_{13} was added either on the 20th day or on the 25th day at all test concentrations (5 to 100 mg/L), diosgenin yield was obviously improved. The highest diosgenin yield (10.73 mg/L), which was 9.02-fold of control (1.19 mg/L), was obtained when palmarumycin C_{13} was added on the 25th day at 10 mg/L.

Figure 3 shows the time courses of growth, diosgenin content and yield in cell suspension culture of *D. zingiberensis* after addition of palmarumycin C_{13} at 10 mg/L on day 25 of culture. The results (Figure 3A) showed that dry weight of the treated cell cultures was always higher than control during the days after treatment with palmarumycin C_{13}, and reached the maximum on day 30, and then remained almost constant.

Diosgenin content of the treated cell cultures began to increase significantly from the 2nd day after elicitation, and reached the maximum on day 30 and then decreased slightly. The varied trend of diosgenin yield was almost identical to that of diosgenin content, and the highest diosgenin yield was also observed on day 30.

It was concluded that an optimal cultivation time for *D. zingiberensis* cell cultures to produce diosgenin was set at 30 days by the addition of palmarumycin C_{13} to the medium at 10 mg/L on day 25 after inoculation.

DISCUSSION

There were some reports about the effects of endophytic fungi on the secondary metabolism of their host plants. Typical examples included paclitaxel (taxol) enhancement in *Taxus chinensis* cell cultures induced by the endophytic fungus *Aspergillus niger* [17], artemisinin production in *Artemisia annua* hairy root cultures elicited by the endophytic *Colletotrichum* sp. [18], paclitaxel formation in *Taxus cuspidata* cell cultures treated by the endophytic *Fusarium mairei* [19], and alkaloid production in *Catharanthus roseus* cell cultures induced by its

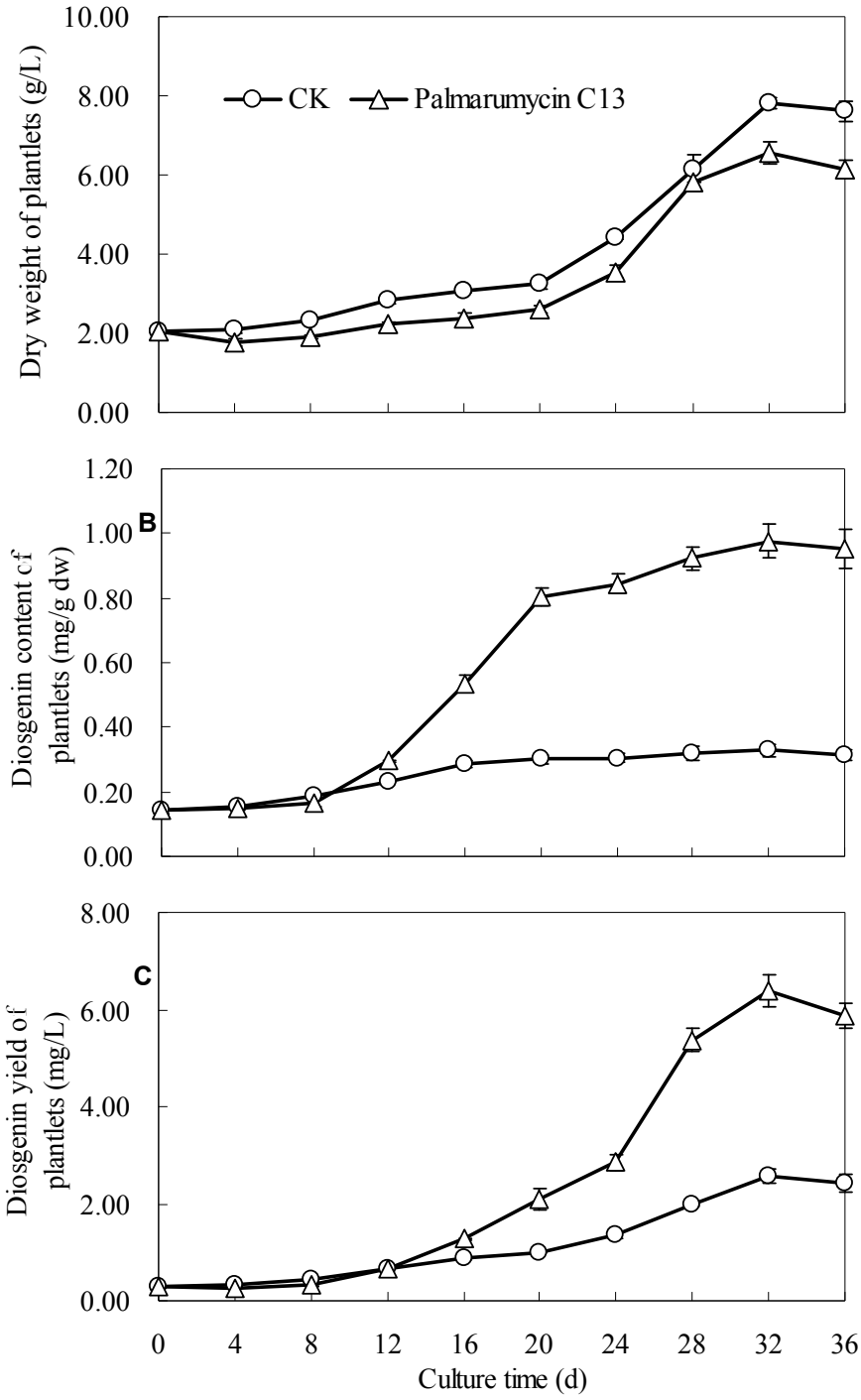

Figure 2: Time courses of (A) growth, (B) diosgenin content (C) and yield in plantlet culture of *D. zingiberensis* after addition of palmarumycin C_{13} at 60 mg/L. Each value is expressed as mean ± standard deviation (n = 3)

endophytic *Fusarium oxysporum* F9 [20]. In this work, we first reported the enhancement of diosgenin production by palmarumycin C_{13} from the endophytic *Berkleasmium* sp. Dzf12 in plantlet or cell suspension cultures of *D. zingiberensis*. Palmarumycin C_{13} could be used as an elicitor to increae diosgenin yield of *D. zingiberensis* cultures to show its potential application. More detailed studies such as palmarumycin C_{13} in combination with other factors such as metal ions and oligosaccharide elicitors to stimulate diosgenin production, and the stimulating mechanism of palmarumycin C_{13} on diosgenin biosynthesis in *D. zingiberensis* cultures are needed to be further studied.

Table 2: Effects of palmarumycin C_{13} on biomass, diosgenin content and yield in cell suspension culture of *D. zingiberensis*

Addition time of palmarumycin C_{13}	Palmarumycin C_{13} conc. (mg/L)	Dry weight of cells (g/L)	Diosgenin content (mg/g dw)	Diosgenin yield (mg/L)
	0 (CK)	5.17±0.17 ^{b}	0.23±0.02 ^{J}	1.19± 0.11 ^{h}
Day 15	5	4.89±0.27 ^{bc}	0.22±0.02 ^{J}	1.08±0.04 ^{h}
	10	4.42±0.11 ^{d}	0.27±0.01 ^{IJ}	1.19±0.20 ^{h}
	20	3.93±0.01 ^{e}	0.43±0.01 ^{h}	1.69± 0.03 ^{g}
	40	2.80±0.13 ^{g}	0.53±0.04 ^{g}	1.48± 0.05 ^{gh}
	60	2.64±0.12 ^{g}	0.38±0.03 ^{hi}	1.00± 0.11 ^{hi}
	80	2.59±0.02 ^{g}	0.30±0.03 ^{I}	0.78±0.15 ^{I}
	100	2.51±0.05 ^{g}	0.23±0.01 ^{J}	0.58±0.14 ^{J}
Day 20	5	5.25±0.26 ^{ab}	0.49±0.02 ^{gh}	2.57±0.11 ^{f}
	10	5.17±0.21 ^{b}	0.65±0.02 ^{f}	3.36±0.07 ^{e}
	20	4.77±0.11 ^{cd}	0.79±0.03 ^{e}	3.77±0.08 ^{de}
	40	3.95±0.15 ^{e}	1.05±0.13 ^{d}	4.15±0.36 ^{cd}
	60	3.85±0.04 ^{et}	0.65±0.03 ^{f}	2.50±0.13 ^{f}
	80	3.65±0.04 ^{f}	0.52±0.01 ^{g}	1.90±0.07 ^{g}
	100	3.50±0.11 ^{f}	0.52±0.01 ^{g}	1.82±0.25 ^{g}
Day 25	5	5.71±0.35 ^{a}	1.39±0.04 ^{c}	7.94±0.47 ^{b}
	10	5.26±0.37 ^{ab}	2.04±0.07 ^{a}	10.73±0.40 ^{a}
	20	5.18±0.45 ^{b}	1.98±0.01 ^{a}	10.26±0.37 ^{a}
	40	4.89±0.03 ^{bc}	1.60±0.05 ^{b}	7.82±0.18 ^{b}
	60	4.46±0.36 ^{d}	1.03±0.03 ^{d}	4.59±0.11 ^{c}
	80	4.16±0.22 ^{d}	0.94±0.02 ^{de}	3.91±0.08 ^{d}
	100	4.10±0.14 ^{de}	0.81±0.02 ^{e}	3.32±0.07 ^{e}

Note: *All values are mean ± standard deviations; values marked with different letters in each column indicate significant differences among the treatments at p = 0.05; suspension cell cultures were harvested on day 30 of culture.*

CONCLUSION

The addition of palmarumycin C_{13} from the endophytic fungus *Berkleasmium* sp. Dzf12 is a potentially effective strategy for enhancing diosgenin production in *D. zingiberensis* plantlet and cell cultures. The results also provide an insight into the interaction between endophytic *Berkleasmium* sp. Dzf12 and its host plant *D. zingiberensis*. Furtthermore, the stimulating effect of palmarumycin C_{13} observed in *D. zingiberensis* plantlet and cell cultures may be useful in field cultivation for the production of diosgenin.

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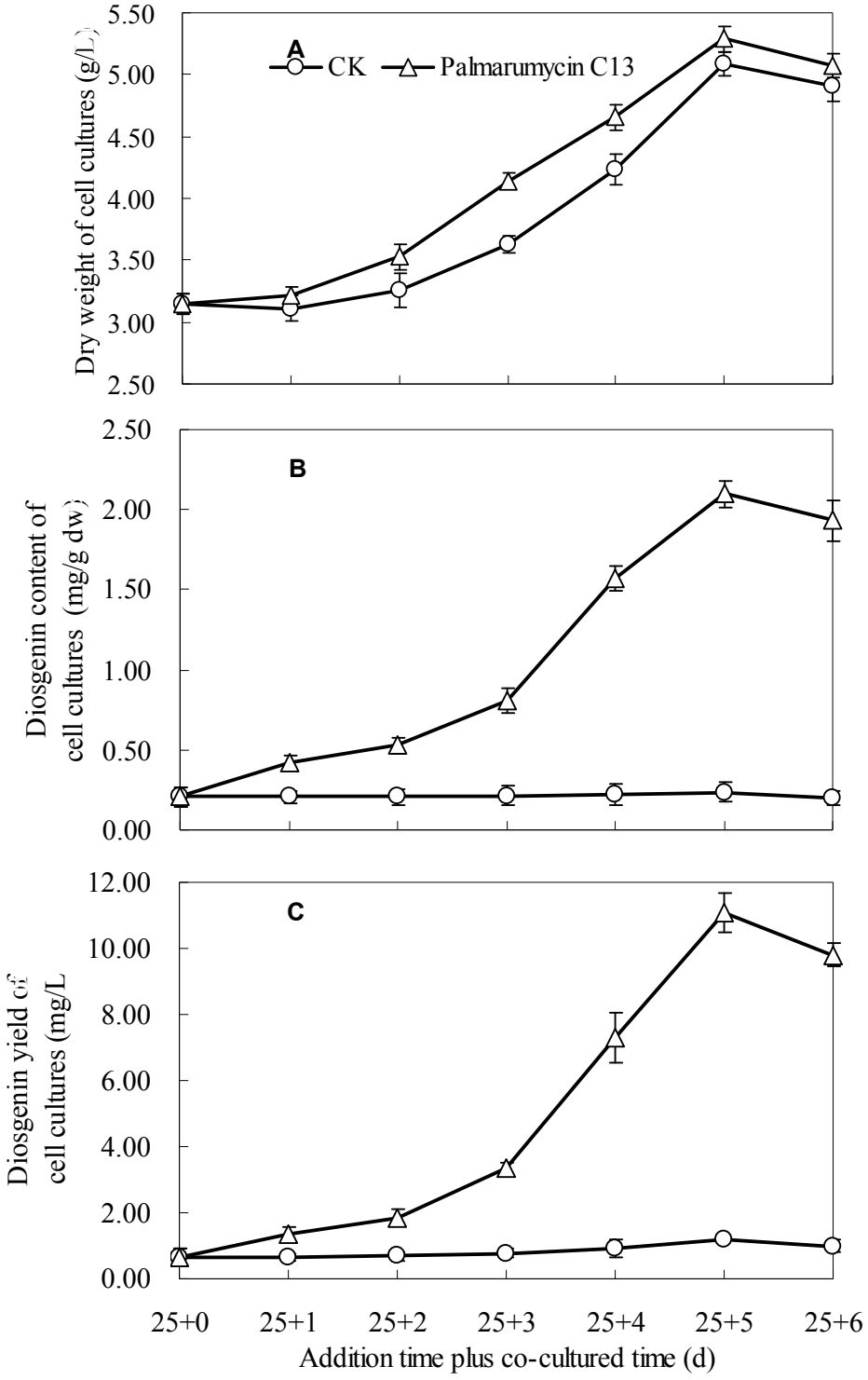

Figure 3: Time course of (A) growth, (B) diosgenin content and(C) yield in cell suspension culture of *D. zingiberensis* following addition of palmarumycin C_{13} at 10 mg/L on day 25 of culture. Each value is mean ± standard deviation (n = 3)

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