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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2017.08.012>Biomass accumulation of *Panax vietnamensis* in cell suspension cultures varies with addition of plant growth regulators and organic additivesTuan Tran Trong^{1,✉}, Dieu-Hien Truong^{2,✉}, Hoang Chinh Nguyen², Dieu-Thai Tran¹, Huyen-Trang Nguyen Thi¹, Giap Do Dang², Ho Nguyen Huu³¹Plant Cell Technology Department, Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Ha Noi Highway, Linh Trung, Thu Duc, Ho Chi Minh City, Viet Nam²Faculty of Applied Sciences, Ton Duc Thang University, 19 Nguyen Huu Tho, Tan Phong, District 7, Ho Chi Minh City, Viet Nam³Genetic Engineering Department, Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Ha Noi Highway, Linh Trung, Thu Duc, Ho Chi Minh City, Viet Nam

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ABSTRACT

Objective: To evaluate the impact of plant growth regulators including kinetin (KN), benzyl adenine and naphthalene acetic acid, yeast extract and casein hydrolyzate on biomass accumulation of Vietnamese ginseng *Panax vietnamensis* (*P. vietnamensis*) in cell suspension culture.**Methods:** Cell suspension cultures were established from friable calluses derived from leaves and petioles of 3-year-old *in-vitro* *P. vietnamensis* plants. The cell suspension cultures were grown in Murashige and Skoog basal media supplemented with various concentrations of KN, benzyl adenine, naphthalene acetic acid, and yeast extract and casein hydrolyzate.**Results:** All tested factors generated an increase in the cell biomass of *P. vietnamensis* in suspension culture, but the impact of each varies depended on the factor type, concentration, and incubation period. Addition of 2.0 mg/L KN resulted in the largest biomass increase after 24 d, (57.0 ± 0.9) and (3.1 ± 0.1) mg/mL fresh and dry weight, respectively, whereas addition of benzyl adenine or naphthalene acetic acid produced optimum levels of *Panax* cell biomass at 1.0 and 1.5 mg/L, respectively. Addition of the elicitor yeast extract led to a 1.4–2.4 fold increase in biomass of *P. vietnamensis*, while addition of casein hydrolyzate enhanced biomass accumulation 1.8–2.6 fold.**Conclusions:** The addition of each factor causes significant changes in biomass accumulation of *P. vietnamensis*. The largest biomass accumulation is from cultures grown in MS media containing 2.0 mg/L KN for 24 d. The outcome of the present study provides new insights into the optimal suspension culture conditions for studies on the *in vitro* cell biomass production of *P. vietnamensis*.

1. Introduction

Since ancient times medical herbs have played a prominent role in human health. Recently the demand for complementary

and alternative medicine, particularly based on traditional medicine, has increased dramatically among the population worldwide [1–3]. According to an estimate from the United Nations World Health Organization, about 80% of the world's population have utilized herbal medicine for primary health care [4]. The King of all herbs, ginseng, has been used not only to treat physical conditions (*i.e.*, cardiovascular, immune, and neuronal) but also to treat sexual dysfunction and to enhance sexual behavior and gonadal functions [5]. However, ginseng is very expensive due to environmental and economic factors such as length of time to maturity, rarity, wild fires, drought, and high demand [6].

The 20th variety of ginseng discovered is a new *Panax* species, Vietnamese ginseng *Panax vietnamensis* (*P. vietnamensis*)

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Ha et Grushv., called “Sâm Ngọc Linh” in Vietnamese. This species contains not only the protopanaxatriol and protopanaxadiol saponins found in *Panax ginseng* (*P. ginseng*) but also dammarane saponins [2,7,8]. The natural extent of this species is limited to the Ngọc Linh mountain area where it was discovered in 1973 [2,9]. In recent years, the ability of *in vitro* cultivated *P. vietnamensis* plant leaf explants or thin layers of main roots to survive in their natural habitat has increased about 85% [10,11]. However, as with other ginseng species, the cultivation period for *P. vietnamensis* plants is long: it takes 5–7 years before the rhizomes and roots can be harvested. In light of the difficulties surrounding natural cultivation of ginseng, biotechnological alternatives like differentiated tissue culture (e.g., whole plant and organ cultures, calluses, cell suspensions, as well as protoplasts) are attractive alternatives for mass production of ginseng [6,12]. Plant cell and tissue culture methods have focused on large-scale production of *P. ginseng* or isolation of its chemical constituents. For example, ginsenosides are produced in the callus and in cell suspension cultures of *P. ginseng* and *Panax quinquefolius* [2,13,14]. Ma et al. observed the same results for *P. vietnamensis* [15]. Interestingly, changes in cell culture conditions can increase production of *P. vietnamensis* biomass [16,17]. This has been demonstrated in cell suspension culture of these plants in flasks [18] and in bioreactors [6,19]. It has been reported that some biotic and abiotic elicitors [e.g., yeast extract (YE), casein hydrolyzate (CH), chitosan, jasmonic acid, and salicylic acid] as well as valuable secondary metabolites can be added to plant cell suspension culture media to enhance the biomass yield [20,21]. A few studies have been conducted on *P. vietnamensis* to optimize growth conditions for *in-vitro* tissue culture and cell suspension culture. However, production of *P. vietnamensis* biomass and ginsenoside remains low due to slow growth [2]. Nguyen et al. obtained maximum adventitious root growth by adding 5% sucrose to the media of a cell suspension culture of *P. vietnamensis* [19]. In fact, optimization of cell suspension culture, and, more specifically, callus material, is a particularly powerful approach to maximize biomass and the production of compounds [12].

Ginseng propagation in cell culture has been reported previously [22–26]. However, to be economically competitive with field cultivated ginseng, especially *P. vietnamensis*, there is still a need to increase the productivity of the tissue culture process. In the present study, we aimed to evaluate the influence of varying concentrations of different elicitors [*i.e.*, plant growth regulators (PGRs), cytokinins [kinetin (KN), benzyl adenine (BA)], and auxin [naphthalene acetic acid (NAA)], YE and CH] on the growth of *P. vietnamensis* in cell suspension culture. The optimized media composition identified in this study is a significant step toward finding the best conditions for biomass production of the valuable medicinal plant *P. vietnamensis*.

2. Materials and methods

2.1. Plants, materials and establishment of cell suspension culture

Cell suspension cultures were established from friable calluses derived from leaves and petioles of 3-year-old *in-vitro* *P. vietnamensis* plants. *In-vitro* *P. vietnamensis* plants were

obtained by sterilizing the surface of leaves, then cutting them into pieces, and placing the fragments into Murashige and Skoog (MS) [27] containing 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L thidiazuron at pH 5.6 to induce callus formation. For surface sterilization, *Panax* healthy leaves were cut into 1.5 cm × 1.5 cm sections, then submerged in 96% ethyl alcohol for 1 min, 5 min in 1% NaOCl, 30 s in 96% ethanol, and rinsed three times with sterile distilled water. For maintenance, *P. vietnamensis* calluses were sub-cultured in the same media. All of the media was autoclaved for 25 min. Cultures were incubated in the dark at $(25 \pm 2) ^\circ\text{C}$.

The cell suspension cultures were grown in MS basal media supplemented with various concentrations of the PGRs (KN, BA, NAA), and organic elicitors (YE and CH). For cell culture experiments, 250 mL flasks containing 50 mL of media were inoculated with 2 mg of the leaf-derived calluses from *P. vietnamensis*. Cell suspensions were incubated on a rotary shaker at (120 ± 10) r/m at $(25 \pm 2) ^\circ\text{C}$ under a 16/8 h light/dark regime using fluorescent lamps with a light intensity of 35 $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$. To establish growth and production kinetics, the cultures were harvested at different times (3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 d) and analyzed for biomass accumulation. The fresh weight (FW) was determined by centrifuging the harvested suspension cells at 4000 r/m for 20 min. Subsequently, the dry weight (DW) of the cells was measuring following drying in an oven at 60 $^\circ\text{C}$ until a constant weight was achieved.

2.2. Plant growth regulator experiments

The various concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of two kinds of cytokinins (KN and BA) or one type of auxin (NAA) were incorporated into cell culture media [MS + vitamins (B1 and B6) + 30 g/L sucrose] at pH 5.6. The culture conditions were as described in Section 2.1. PGRs-free MS basal media was used as a control.

2.3. Yeast extract and casein hydrolyzate experiments

The callus-derived cells of *P. vietnamensis* were used to determine the influence of various concentrations of either YE or CH (Sigma, Germany) on plant biomass production. Appropriate concentrations of YE or CH were first dissolved in distilled water and added to the media before adjustment of the pH and sterilization (0.5, 1.0, 1.5, and 2.0 g/L). The effects of YE or CH on the fresh and dry weight were measured.

2.4. Statistical analyses

All experiments were repeated three times in triplicate. To reveal patterns of variation and clustering among treatments (either from different plant hormones or organic additives), a principle component analysis (PCA) followed by a hierarchical clustering analysis on principal components (HCPC) was performed using R 3.0.1 software (R-Development Core-Team 2013) and FactoMineR 1.25 package [28]. Multivariate analysis (PCA and HCPC) was performed on a dataset containing the average mean fresh/dry weight of cells. Principal components (PCs) were calculated using a correlation matrix. The optimal group number defined by hierarchical clustering of principal components was chosen automatically by the statistical software and was 3–10 clusters.

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