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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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 additives

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ARTICLE INFO

Article history:
Received 27 May 2017
Received in revised form 25 Jul 2017
Accepted 30 Jul 2017
Available online 15 Sep 2017

Keywords:
Panax vietnamensis
Plant cell suspension culture
Plant regulators
Yeast extract
Casein hydrolyzate
Biomass

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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Peer review under responsibility of Hainan Medical University.

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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 Ngoc 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 ± 2) °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 ± 2) °C under a 16/8 h light/dark regime using fluorescent lamps with a light intensity of 35 μmol/(m^2·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 4 000 r/m for 20 min. Subsequently, the dry weight (DW) of the cells was measuring following drying in an oven at 60 °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.

The experimental data were subjected to an analysis of variance (ANOVA) and a subsequent post hoc Tukey's test was applied to determine the variation in relative abundance of fresh/dry weight of ginseng cells. These tests were conducted with Minitab 16.1.1. software.

3. Results

3.1. Biomass accumulation of P. vietnamensis in cell suspension culture varies with addition of PGRs

In order to determine the rates of the cellular growth for *P. vietnamensis* among different types of culture media, the fresh and dry weight of cells accumulated in basal MS and MS supplemented with various concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of PGRs (KN, BA and NAA) was measured and a PCA was conducted using the mean data. The results showed that the first two components accounted for 90.3% of the observed variation (PC1 78.2% and PC2 12.1%, Figure 1a). In addition, HCPC using the mean data defined four clusters (Figure 1b). The first cluster (Group 1 in the PCA) contained biomass yield of *P. vietnamensis* callus-derived cells in MS media supplemented with the highest concentration of PGRs (2.0 mg/L) at 21, 24 and 27 d. *P. vietnamensis* cells in MS media supplemented with lower concentrations of PGRs (1.5 mg/L) at 18, 21, 24 and 27 d corresponded to the second cluster (Group 2 in PCA). Growth of *P. vietnamensis* after 24 d in MS media supplemented with 0.5 and 1.0 mg/L PGRs was also included in this group. The third cluster (Group 3 in PCA) contained *P. vietnamensis* samples at 6, 9, 12, 15, and 30 d on MS media supplemented with or without all PGRs. *P. vietnamensis* cells collected after 18 and 21 d in MS media with or without 0.5 and 1.0 mg/L PGRs were also located in this group, as well as the control cells collected after 24 d. Finally, the fourth cluster (Group 4 in PCA) included *P. vietnamensis* cells grown in control media and in MS media supplemented with 0.5, 1.0, 1.5, and 2.0 mg/L PGRs for 3 d.

A two-way ANOVA model was carried out for both fresh and dry biomass of *P. vietnamensis* cells to identify differences between cellular growth of plants cultured into MS media free of PGRs and media supplemented with various concentrations of PGRs and cultured for different lengths of time.

Considering the cellular weight of *P. vietnamensis* cell suspension cultures after each 3-day interval in MS media supplemented with different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of KN, we observed that the cells grew well for 24 d and their

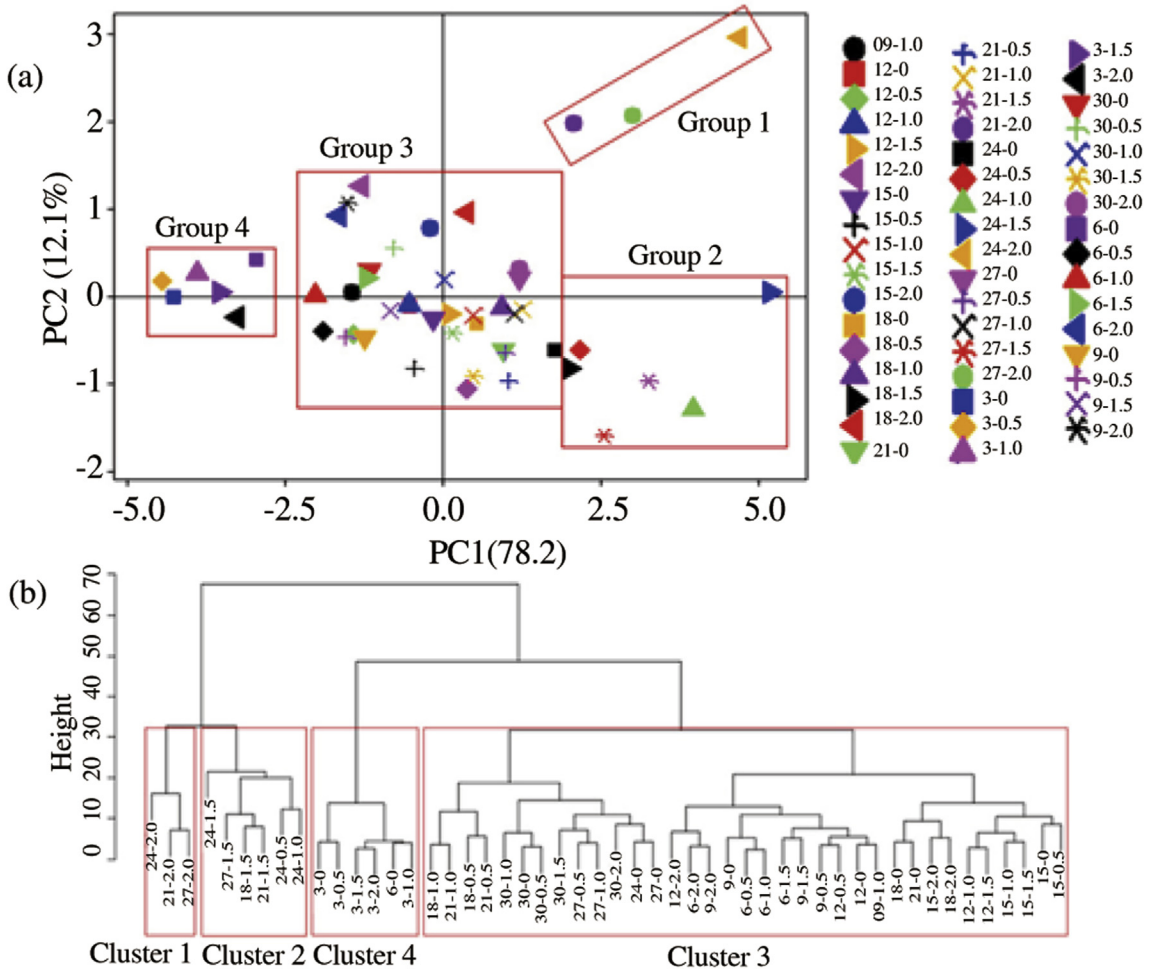

Figure 1. Principal component analysis (a) and Hierarchical cluster analysis on principal components (b) of the cell biomass of *P. vietnamensis* suspension culture on MS supplemented with or not different concentrations (*i.e.*, 0.5; 1.0; 1.5 and 2.0 mg/L) of plant growth regulators (*i.e.*, KN, BA, and NAA) from 3 to 30 d.

PCAs show the first (PC1) and second (PC2) principal components. Treatments were coded as follows: incubation period (days: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30) – concentration (0 (control), 0.5, 1.0, 1.5 and 2.0 mg/L) of PGRs.

fresh and dry weights increased significantly with incubation time ($P < 0.001$, Figure 2a and b). After 24 d, the biomass of *P. vietnamensis* decreased significantly in this media ($P < 0.001$). In general, the addition of KN to MS media led to a significant increase in the fresh and dry biomass of *P. vietnamensis* cells ($P < 0.001$). On the other hand, the dry weight of *P. vietnamensis* from cell suspension cultures grown in this media (except for MS media with 2.0 mg/L KN) significantly reduced output compared to control plants ($P = 0.002$, Figure 1b). Media with the highest KN concentration (2.0 mg/L) gave the highest fresh and dry weights of *P. vietnamensis* cells over the duration of the experiment ((57.00 ± 0.90) mg/mL FW and (3.10 ± 0.09) mg/mL DW (Figure 2a and b).

Among all tested BA concentrations, 1.0 mg/L BA was the optimal concentration for *P. vietnamensis* biomass accumulation at 24 d (Figure 2c and d). At this BA concentration, (46.20 ± 0.80) and (2.50 ± 0.05) mg/mL of fresh and dry cell weight respectively were obtained which was significantly more biomass than other tested BA concentrations ($P < 0.001$). In contrast to KN, media with the highest BA concentration (2.0 mg/L) led to a significant decrease in the weight of *P. vietnamensis* produced over time relative to MS media supplemented at other BA concentrations ($P < 0.001$). The MS media containing 0.5 and 1.5 mg/L BA proved to be more effective in *P. vietnamensis* biomass accumulation than those of other BA concentrations tested from 3 to 30 d in culture (with the exception of the 24th day). The cells continued to survive for 30 d but decreased in total weight (Figure 2c and d).

With respect to the effect of NAA (0.5, 1.0, 1.5, and 2.0 mg/L) on biomass accumulation of *P. vietnamensis* over the culture period, the largest fresh and dry biomass of cells was obtained from callus-derived cells grown in MS media containing 1.5 mg/L NAA after 24 d (Figure 2e and f). For most of the NAA concentrations tested, *P. vietnamensis* fresh cell weights were higher than those from cultures grown in control media for the same number of days ($P < 0.001$; Figure 2e). The fresh and dry cell weight of *P. vietnamensis* dropped significantly between 27 and 30 d in all media tested ($P < 0.001$; Figure 2e and f).

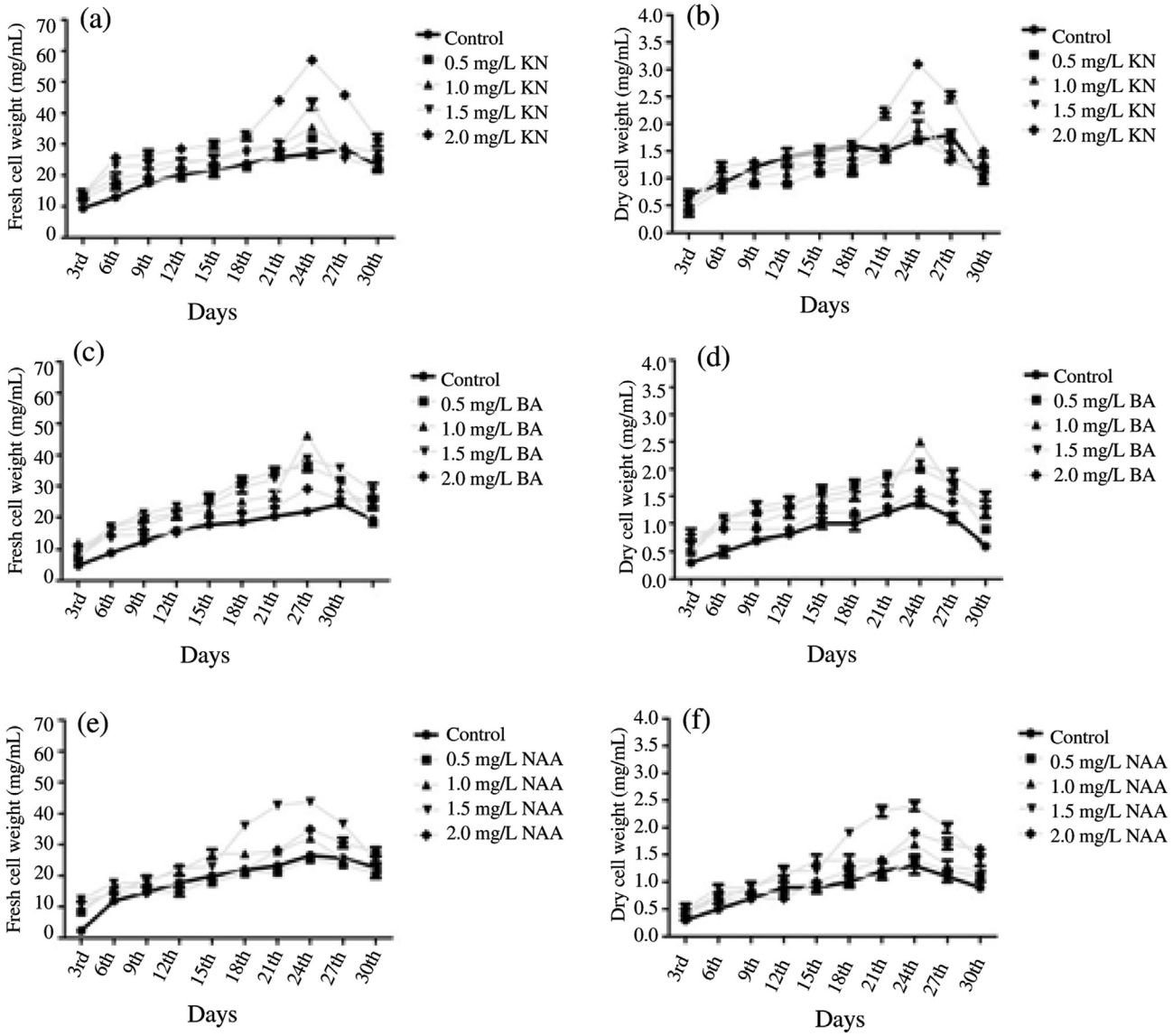

Figure 2. Effect of different concentrations (0.5, 1.0, 1.5 and 2.0 mg/L) of PGRs on fresh (a, c & e) and dry (b, d & f) weight of *P. vietnamensis* cells from 3 to 30 d.

The PGR-free MS basal medium was considered as the control treatment. Results are the mean of three replicates ± SD ($P < 0.05$). *Panax* cell derived callus on MS supplemented or not with various concentrations of KN (a, b), BA (c, d) and NAA (e, f).

3.2. Changes in biomass of *P. vietnamensis* in cell suspension culture due to YE and CH

The influences of additive elicitors (YE or CH) on biomass accumulation of *P. vietnamensis* over the culture period are depicted in Figures 3 and 4. A significant increase in the biomass of *P. vietnamensis* plants was achieved in MS media supplemented with various concentrations of elicitors (0.5, 1.0, 1.5, and 2.0 g/L) ($P = 0.01$; Figure 4). PCAs confirmed variations in the cell weight of *P. vietnamensis* after the same number of days cultured in MS media lacking or supplemented with elicitors (Figure 3a). First, the mean data used by PCA captured 95.8% of the total variance on a score plot constructed with the two first PCs (PC1 80.0% and PC2 18.5%, Figure 3a). In addition, the hierarchical clustering analysis performed on PCs (HCPC) separated the biomass accumulation of *P. vietnamensis* in different media over the culture time period into four clusters (Figure 4b). The first cluster consisted of *P. vietnamensis* cells grown in basal MS and MS media supplemented with all tested concentrations of elicitors after 3 d. This cluster also included cells cultured in control MS media for 6 and 9 d and *P. vietnamensis* cells cultured in MS media supplemented with the highest concentration of elicitors (2.0 g/L). Biomass obtained from growth in MS media containing 0.5 and 1.5 g/L elicitors after 6–15 d corresponded to the second cluster. Cluster 2 also included growth from cultures treated with 1.0 g/L elicitor and harvested between 6 and 12 d. It is noteworthy that this cluster also contained the biomass accumulation of *P. vietnamensis* exposed to MS media supplemented with the highest concentration of elicitor (2.0 g/L) for 9–15 d. Biomass from cultures grown in MS media without added elicitors for 12–18 d was also included in this group. The growth in biomass of *P. vietnamensis* callus treated with 0.5, 1.0, and 1.5 g/L elicitors and harvested between 18 and 30 d was contained in the third cluster, except for callus grown with 1.5 g/L elicitor for 21 d. This cluster also contained cell weights in MS media supplemented with 2.0 g/L elicitor for 27–30 d. Biomass of *P. vietnamensis* callus cultured on basal MS towards the end of the culture period (21–30 d) and plant callus grown with 1.0 g/L elicitor for 15 d were also members of this group. Finally, the fourth cluster represented *P. vietnamensis* cells grown in MS media supplemented with the highest concentration of elicitor (2.0 g/L) for 18–24 d and the biomass of plant suspension grown in 1.5 g/L elicitor for 21 d.

To identify the differences in growth of *P. vietnamensis* in cell suspension culture with basal MS and MS supplemented with various concentrations of YE over 30 d, a two-way ANOVA was conducted. The addition of YE to culture media led to a significant increase in biomass when compared to control media ($P < 0.001$, Figure 4a and b). The fresh and dry weights of *P. vietnamensis* cells increased slightly over the first 12 d in MS media treated with different concentrations of YE (0.5, 1.0, 1.5, and 2.0 g/L). The largest increase in biomass was achieved on day 21 d in MS supplemented with 1.0 g/L YE: dry

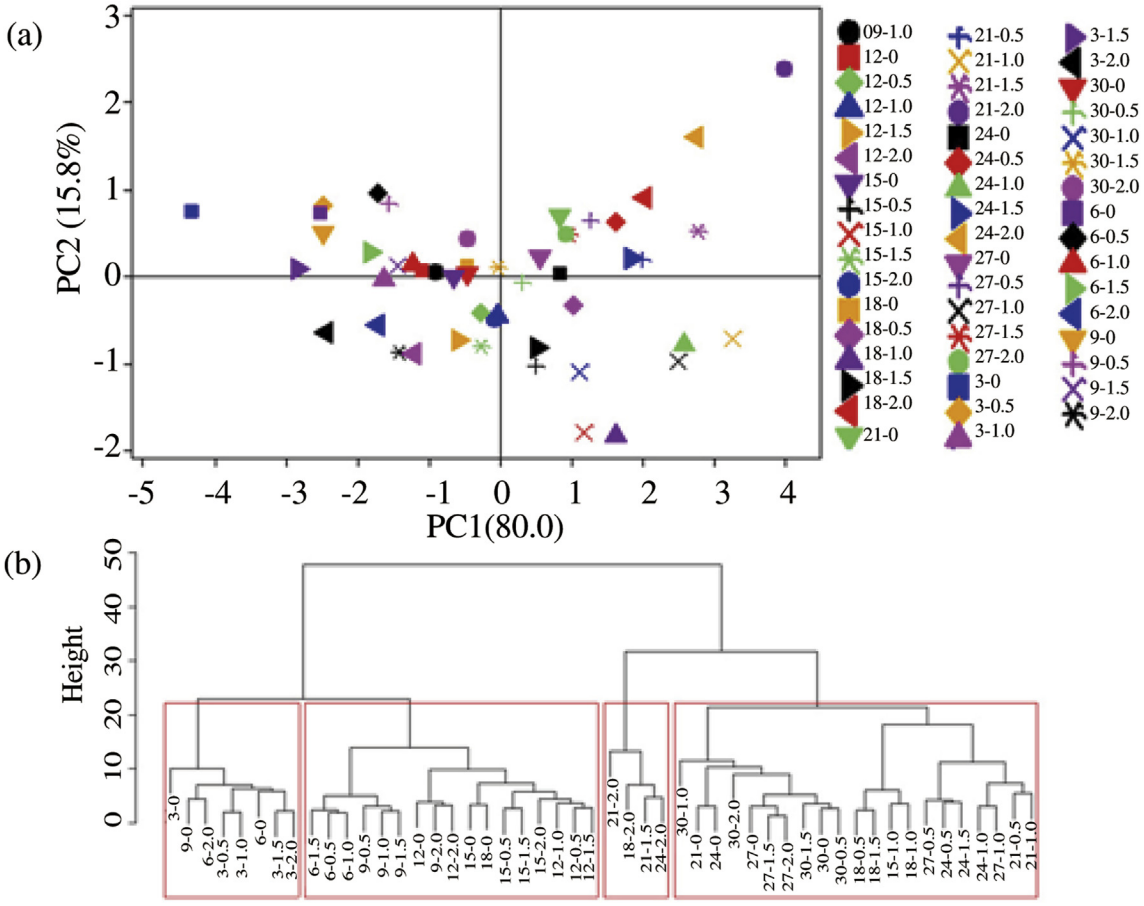

Figure 3. Principal component analysis (a) and hierarchical cluster analysis on principal components (b) of the cell biomass of *P. vietnamensis* suspension on MS supplemented with or not different concentrations (0.5, 1.0; 1.5 and 2.0 g/L) of elicitors (*i.e.*, YE and CH) from 3 to 30 d.
PCAs show the first (PC1) and second (PC2) principal components. Treatments were coded as follows: incubation period (days: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30) – concentration (0 (control), 0.5, 1.0, 1.5 and 2.0 g/L) of elicitors.

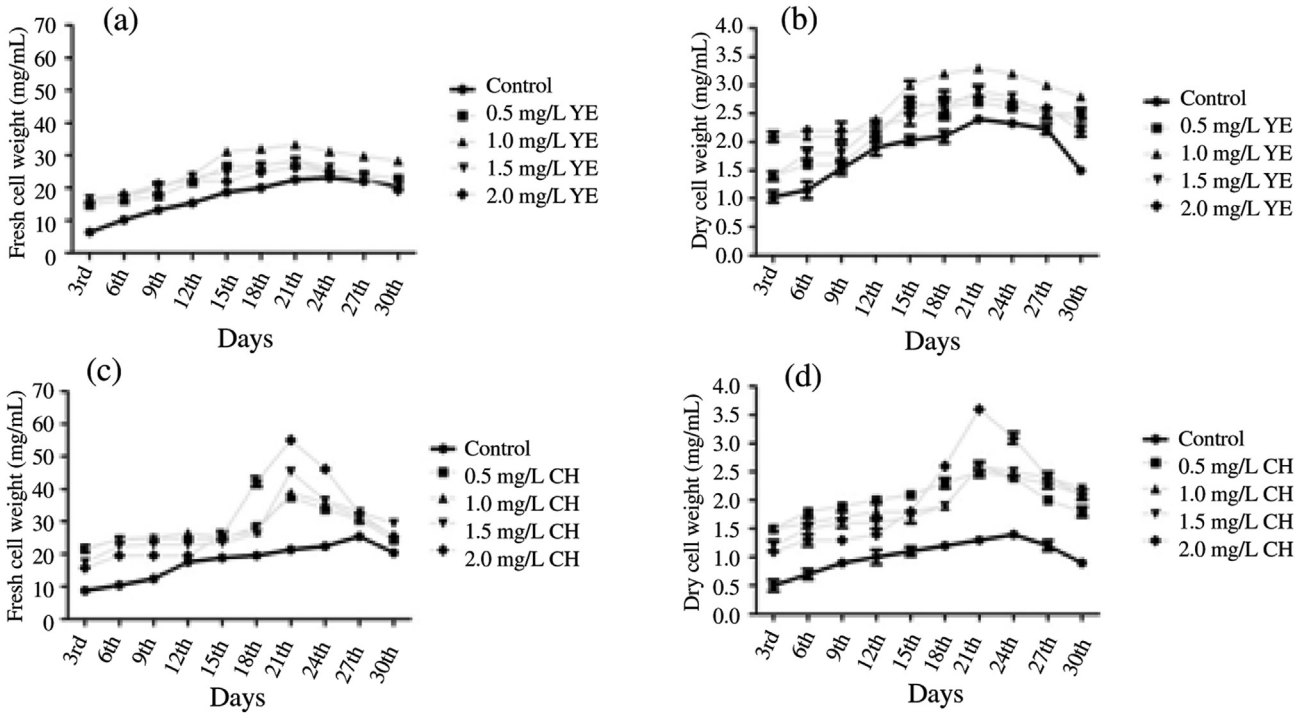

Figure 4. Effect of various concentrations (0.5, 1.0, 1.5 and 2.0 g/L) of elicitor (YE or CH) on fresh (a, c) and dry (b, d) weight of *P. vietnamensis* cell suspension from 3 to 30 d.

The elicitor-free MS basal medium was considered as the control treatment. Results are the mean of three replicates ± SD ($P < 0.05$). *Panax* cell derived callus on MS supplemented or not with YE (a, b) and CH (c, d).

weight increased almost 2-fold compared to control cells (Figure 4b). The addition of higher concentrations of this elicitor (1.5 and 2.0 g/L) generally inhibited the accumulation of biomass over the culture period. The fresh and dry weight of *P. vietnamensis* cells significantly decreased over 27–30 d (Figure 4a and b).

The treatment of CH (0.5, 1.0, 1.5, and 2.0 g/L) to *P. vietnamensis* suspension cell culture led to a significant increase in cell weight over the experimental time period relative to control media ($P < 0.001$, Figure 4c and d). In particular, the largest biomass of *P. vietnamensis* cells was obtained in MS media supplemented with 2.0 g/L CH for 21 d [(55.0 ± 0.9) mg/mL FW and (3.6 ± 0.0) mg/mL DW] (Figure 4c and d). Although the addition of high concentrations of CH (1.5 and 2.0 g/L) to MS media inhibited the growth of *P. vietnamensis* biomass during the first 3–18 d relative to other tested concentrations (0.5 and 1.0 g/L), cell weights strongly increased between 21 and 30 d (Figure 4c and d).

4. Discussion

Cells in suspension culture can exhibit greater rates of cell division than cells in callus culture [12,29–32]. To optimize biomass production of an important medicinal plant, *P. vietnamensis*, we studied the impact of adding factors such as the PGRs (*i.e.*, KN, BA, and NAA), and the elicitors (i.e., YE and CH) on the growth of biomass of *P. vietnamensis* in cell suspension culture at 3-day intervals over 1 month.

With respect to the influence of plant hormones on *P. vietnamensis* biomass, the highest result for cell induction was obtained in MS media supplemented with 2.0 mg/L KN after 24 d. Treatment with either 1.0 mg/L BA or 1.5 mg/L NAA resulted in the highest levels of biomass from *P. vietnamensis* cell suspension cultures after 24 d. The addition of different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of KN, BA and NAA to MS media mostly led to significant increases in *P. vietnamensis* biomass accumulation over the course of the 30 d experiment when compared to cells raised in control media. In our cultures, we observed that the growth in biomass of *P. vietnamensis* was slightly increased at the beginning of the culture period (3 d) and had mean fresh cell weights ranging from 1.2 to 7% of final biomasses, but 15–30 d of culturing resulted in significantly higher biomasses ($P < 0.001$). This is consistent with the findings of Gurel *et al.* [33]. With regard to plant hormone factors, the maximum biomass yield of *P. vietnamensis* from cell suspension culture achieved after 24 d in MS media containing 2.0 mg/L KN was (57.0 ± 0.9) mg/mL FW and (3.1 ± 0.1) mg/mL DW, in MS media containing 2.0 mg/L BA was (29.1 ± 0.1) mg/mL FW and (1.6 ± 0.0) mg/mL DW, and in MS media containing 2.0 mg/L NAA was (34.9 ± 0.5) mg/mL FW and (1.9 ± 0.1) mg/mL DW. Nguyen and Paek found that MS media containing 2,4-D is optimal for culturing *P. ginseng* in cell suspension culture [34]. Thus, the results obtained in the present study are interesting because most cell suspension cultures of ginseng cells previously documented required 2,4-D, which is unsuitable for pharmaceutical and food industrial use due to its potency as an herbicide and a carcinogen. Generally, cytokinins and auxins promote cell division and cell expansion in plant cell suspension cultures [24,35–37]. Jang *et al.* reported that auxin and cytokinin are key regulators of plant secondary growth [38]. A study by Schmülling *et al.* also demonstrated the essential role of cytokinins in the cell cycle and primary metabolite formation in crop plant cell suspension cultures [39]. In contrast, the addition of an auxin and a cytokinin to half-strength MS media did not improve biomass accumulation of *P. vietnamensis* in

cell suspension culture [40]. In a previous study, Te-chato *et al.* found that addition of KN promoted the growth of oil palm cells in cell suspension culture over 7–8 d, whereas BA was not effective [31]. This can be attributed to the different effects of various types of cytokinins on the induction of cell derived callus from plants. Auxins also play an important role in the adventitious root cell suspension culture of *P. ginseng* [34,41,42]. For example, Zhang *et al.* demonstrated that the growth rate of *P. ginseng* in culture increased significantly in MS media containing 2.0 mg/L NAA and 0.25 mg/L IAA [42]. The IBA treatment was more effective for induction and growth of roots than NAA treatment in *P. ginseng* adventitious root cultures [41]. Smirnova *et al.* detected a 5–6 fold increase in biomass of *P. ginseng* grown in cell suspension culture on MS media supplemented with NAA [43]. In this study, we observed a significant increase (1.6 and 1.8 fold in FW and DW, respectively) in biomass of *P. vietnamensis* cell suspension culture grown in MS media with 1.5 mg/L NAA compared to growth in MS media without NAA. It has been demonstrated that NAA is an essential factor for prolific growth of *Ophiorrhiza mungos* cells derived from friable calluses in cell suspension culture [44].

Besides PGRs, YE and CH are considered to be the most complex additives to plant tissue culture media [21,45]. YE and CH are widely applied as elicitors to enhance the production of plant secondary metabolites, particularly in plant cell or hairy root cultures [21,30,46]. YE is the water-soluble portion of autolyzed yeast and it can provide essential vitamins, nitrogen, amino acids, peptides and carbohydrates [47]. CH is the product of hydrochloric acid hydrolysis of casein and contains amino nitrogen and free amino acids [48]. In the present study, we probed the influence of YE and CH on cell growth of *P. vietnamensis* in cell suspension culture. The results illustrate that the addition of YE and CH (0.5, 1.0, 1.5, and 2.0 g/L) to cell suspension cultures leads to incremental increases or even significant enhancements in the growth in biomass of *P. vietnamensis*. In particular, treatment with YE led to a 1.4–2.4 fold increase in *P. vietnamensis* biomass compared to that from the non-YE treated cells ($P < 0.001$). MS media supplemented with CH induced a 1.8–2.6 fold increase in biomass of *P. vietnamensis* cell suspension culture relative to that of non-treated cells. The largest biomass values were obtained in MS media containing 1.0 g/L YE [(33.3 ± 0.8) mg/mL FW and (3.30 ± 0.06) mg/mL DW], whereas 2.0 g/L CH in MS media exhibited the largest cell weights [(55.00 ± 0.90) mg/mL FW and (3.60 ± 0.03) mg/mL DW] on day 21. Results also revealed changes in the growth of *P. vietnamensis* biomass depending on the time of sample harvest during the experimental period. The *P. vietnamensis* biomass increased from 3 to 21 d and its growth seemed to peak at 24 d. This is in line with a study by Rahman *et al.* [49], where cell growth of *Abrus precatorius* in cell suspension culture reached its peak between 6 and 8 days. Deepthi and Satheeshkumar found a significant increase in the biomass of *Ophiorrhiza mungos* grown in cell suspension culture in media supplemented with YE and $AgNO_3$, and growth depended on the YE concentration, incubation time and feeding time [44]. Martin was able to induce somatic embryos of *Andrographis paniculata* culture in media supplemented with 1.0, 1.5, 2.0, and 3.0 g/L CH [50]. A comparison of the influences of YE, CH and coconut water on the induction of *Oryza sativa* androgenic callus revealed that media supplemented with 0.1 g/L YE was optimal [51]. Experiments with *P. ginseng* cell suspension cultures grown with YE demonstrate a significant increase in biomass as well as secondary metabolite production [20,21,52,53]. In a study by Rahimi *et al.*, the induction of secondary metabolites in *P. ginseng* cell suspension culture by YE was related to *FPS* gene expression and could be mediated by reactive oxygen species signaling and jasmonic acid signal transduction [20].

Overall, this study demonstrated that the growth of *P. vietnamensis* was enhanced by augmenting MS media with plant growth factors (*e.g.*, KN or CH). It provides new insights to improve cell suspension culture of the important medicinal plant *P. vietnamensis*.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank the Ministry of Science and Technology, Vietnam for financial support.

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