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Vol. 10(52), pp. 4755-4761, 24 December, 2015
DOI: 10.5897/AJAR2015.10483
Article Number: 592F16D56474
ISSN 1991-637X
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African Journal of Agricultural Research

Full Length Research Paper

Effect of culture media on *in vitro* germination rate and shoot-forming potential of *Codonopsis javanica* (Blume) Hook f. & Thomson artificial seeds

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Received 3 October, 2015; Accepted 20 November, 2015

Being a tropical country, Vietnam has an exceptional diversity of medicinal plants, particularly in the highland provinces (for example, Lam Dong, Daklak and Kontum provinces). However, a list of many rare medicinal plants are recorded in the Vietnamese Red Data Book and most of them are globally threatened. In this study, the effect of the type of culture media and the concentration of phytohormones on germination, shoot height and shoot number derived from *Codonopsis javanica* (Blume) Hook f. & Thomson synthetic seeds were evaluated under laboratory conditions. The germination rate and shoot-formatting potential of *C. javanica* artificial seeds were greatly affected by the components of plant tissue culture media after 40 days of sowing. Particularly, germination rate of *Codonopsis* artificial seeds was maximum when they were sown in hormone-free Murashige and Skoog (MS) medium in comparison with other culture media (1/2 MS, 1/2 Schenk and Hide-brandt (SH), and SH). Addition of 1.0 mg l^{-1} N-6-benzyladenine (BA) and 0.1 mg l^{-1} naphthaleneacetic (NAA) into MS medium led to increased shoot height, shoot number and germination percentage of the synthetic seeds compared to other concentrations of these phytohormones. The height of shoots derived from the synthetic seeds was inhibited according to the increase of supplemented abscisic acid (ABA) concentration in MS medium. The best planting medium to use to germinate the artificial seeds of *C. javanica* and to increase their shoot-forming potential was the MS supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. The study is expected to provide new insights into the application of synthetic seeds in rapid multiplication of Vietnamese *C. javanica*, establishing the traditional herbal medicine resource for human demands.

Key words: Medicinal plant, *Codonopsis javanica*, artificial seed, plant tissue culture medium.

INTRODUCTION

Codonopsis javanica (Blume) Hook f. & Thomson is a herbaceous plant and a member of the Campanulaceae family (Ueada et al., 2002; He et al. 2015). This species called “Dang sam” in Vietnamese and ‘‘Dangshen’’ in

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Chinese, has been distributed in East Asia and China (Hoang et al., 2014). In Vietnam, it is mainly found in the northern mountainous region, and in the Highland Central like Langbian (Lam Dong province) and Ngoc Linh (Gia Lai province (Hoang et al., 2014; He et al., 2015). It has been documented that the fresh and dried root extract of this plant contains many medicinal compounds (for example, polysaccharides, phenylpropanoids, alkaloids and triterpenoids (Chen et al., 2013; Chung, 2014)). In traditional medicine, *C. javanica* has been used for improving appetite, remedying poor gastrointestinal function, strengthening the immune system, replenishing a deficiency of qi (vital energy) and decreasing blood pressure (Do, 2003; Dinh, 2010; Chen et al., 2013; He et al., 2015).

However, unplanned development and the over-exploitation of *Codonopsis* plants from unmanaged natural resources have not only caused a shortage of this plant, but they have also contributed to their extinction in nature. *C. javanica* plants were recorded in the Vietnamese Red Data Book 2007 and they are globally threatened. There is growing evidence that conservation of medicinal plant resources is considered a critical ecological, cultural and economic issue in Vietnam (Nguyen, 2009). The trade in herbal medicines has provided income for many people involved in surveying, processing, transporting and selling plants (Williams et al., 2007; van Andel et al., 2015). Taken together, a study on the status of *C. javanica* plants in their locations (that is,, Langbian and Ngoc Linh mountains) in terms of building the protocol of their propagation and conservation is required. In fact, an understanding of the best methods of propagating and conserving this herbal species is essential for their successful cultivation.

It has been noted that propagation based on somatic embryogenesis and artificial seeds is the only way out for some plants like ornamental plants (Ravi and Anand, 2012). Indeed, synthetic seeds have offered powerful advantages for plant biotechnology scientists. They have a variety of applications in breeding and plant tissue culture such as large scale mass propagation, germplasm conservation, and breeding of plants which cannot propagate by normal seeds (Ravi and Anand, 2012; Reddy et al., 2012). Therefore, the procedure for the artificial seed productions of many plants (for example, ornamental plants, *Oxalis triangularis* and *Coffea arabica* L.) has been presented (Lambardi et al., 2006; Ravi and Anand, 2012; Reddy et al., 2012; Taha et al., 2013). In general, artificial seeds are defined as the synthetic encapsulation of somatic embryos, shoot buds or any other meristematic tissues, applied for sowing as seeds and they are possible to convert into whole plants under either *in vitro* or *in vivo* conditions. Especially, synthetic seed can keep the potential for their seed germination after storage (Ravi and Anand 2012; Reddy et al., 2012).

With respect to the propagation and conservation of Dang sam or *C. javanica* in Vietnam, studies on these topics have much less paid attention. Duc (2014) showed the results of propagation *in vitro* of *C. javanica* through callus culture and shoot regeneration by callus. Breeding techniques of this species from seed, cutting and shoot were successfully found by using 500 ppm indole-3-acetic acid (IBA) for micropropagation from shoots. To the best of this study knowledge, the shoot multiplication derived from *in vitro* artificial seeds of *Codonopsis* plants has not much studied. In previous study, the protocol of *C. javanica* (distributed in Langbian mountain, Lam Dong province, Vietnam) artificial seed production derived from the nodal segments, shoot tips and adventitious shoots isolated from *in vitro*-derived *Codonopsis* shoots as embryos were indicated (Phan et al., 2014). Here, the study paid attention on the effect of components of plant tissue culture media on the germination and shoot formation of *C. javanica* artificial seeds. This study is expected to provide new insights into the rapid multiplication of *C. javanica* in Vietnam towards either supplying the useful resource for human demands as well as scientific studies or conserving the powerful medicinal species.

MATERIALS AND METHODS

The artificial seeds derived from *C. javanica* nodal segments (2 to 3 mm length) observed from previous study (Phan et al., 2014) were used in this study (Figure 4). These synthetic seeds were stored into liquid solution at 4°C in dark condition within 60 days.

The components of culture media of *Codonopsis* synthetic seeds

Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Schenk and Hide-brandt (Schenk and Hildebrandt, 1972) media were used as culture media in this study. The supplementation of plant growth regulators (that is, BA, ABA and NAA) and nutrient elements (i.e., sucrose and agar) into culture medium was changed according to the specific experiments. pH was adjusted at 5.8. All experiments were carried out in a growth chamber with 25±2°C, 10L:14D (LED lighting, 34 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation during the light period) and 75±5% relative humidity, with the exception of the artificial seed conservation which was left at 4°C in dark room.

Effect of the components of plant tissue culture media on germination rate and shoot-forming potential of *C. javanica* artificial seeds

Type of culture media

The artificial seeds were sown in 1/2 MS; MS; 1/2 SH and SH media in addition with 30 g/l sucrose, 8 g/l agar and pH 5.8.

Plant growth regulators

BA and NAA combination

The synthetic seeds were sowed in MS medium with the combination of 0 (as the control); 0.5; 1.0; 1.5; 2.0 mg l^{-1} BA and

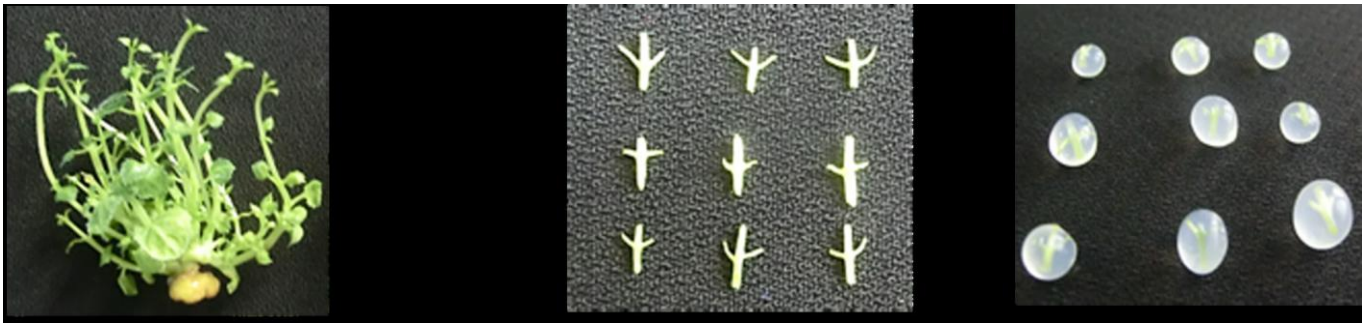


Figure 4. The artificial seed production of *C. javanica* derived from the nodal segments of *in vitro* plantlets (1 month-old) (adopted from Phan et al., 2014).

0.1 mg l^{-1} NAA, 30 g/l sucrose, 8 g/l agar, pH 5.8.

Abscisic acid (ABA)

The synthetic seeds derived from stem segments were sowed in MS medium with the addition of 0 (as the control); 0.5; 1.0; 1.5; 2.0 mg l^{-1}ABA, 30 g l^{-1}sucrose, 8 g l^{-1}agar and pH 5.8. Three biological replicates were conducted, and 40 synthetic seeds were sown for each experiment. The shoot height (cm), the number of shoots and the germination percentage of artificial seeds were collected after 40 days of sowing.

Statistical analysis

Principal component analysis (PCA) was performed on a dataset containing the mean of shoot height (cm), shoot number and the germination percentage of *Codonopsis* artificial seeds sown in different types of culture media after 40 days. One-way ANOVA and subsequent *post hoc* Tukey's test were used to evaluate the variation in the shoot height, shoot number and the germination percentage of *Codonopsis* artificial seeds cultivated in different culture media. These tests (PCA and one-way ANOVA) were conducted with Minitab® 16.2.2 software (State College, Pennsylvania, USA).

RESULTS

Effect of the components of plant tissue culture media on germination rate and shoot-forming potential of *C. javanica* artificial seeds after 40 days of sowing

To reveal the patterns of the shoot height, the shoot number derived from *C. javanica* artificial seeds and their germination percentage among different types of culture media, a principal component analysis (PCA) using mean data was performed. The results showed that the first two components explained 96.6% of the observed variation, that is, PC1 63.3 and PC2 33.3%, forming two main groups:

1. The synthetic seeds were sown in culture media without plant growth regulators, the addition of highest N-6-benzyladenine (BA) and naphthaleneacetic (NAA) concentration into culture medium was included in this group and the artificial seeds were sown in culture media with the supplementation of either BA, NAA or abscisic acid (ABA) with different concentrations (Figure1). The correlation between variables on two first principal components (PCs) revealed that PC1 was positively correlated with the germination percentages.
2. The shoot number derived from *Codonopsis* artificial seeds negatively correlated with the height of shoots. The germination percentages and the shoot number were positively correlated with PC2, whereas the shoot height was negatively correlated.

Type of culture medium of *C. javanica* artificial seeds

The formation of shoots of *C. javanica* synthetic seeds varied according to the type of used culture medium. Table 1 showed that although the number of shoots did not change significantly ($p = 0.57$) between the artificial seeds sown in different culture media (that is,, 1/2 Murashige and Skoog (MS) (1/2 MS, MS, 1/2 Schenk and Hide-brandt (SH), and SH), the height of shoots was significantly different ($p = 0.05$). In addition, the highest germination percentage of these artificial seeds was observed when they were sown in culture MS medium.

Addition of combined BA and NAA into culture medium of *C. javanica* artificial seeds

The supplementation of combined BA and NAA in MS medium led to significant changes in shoot height, shoot number and germination rate of *C. javanica* synthetic seeds ($p < 0.001$; Table 2 and Figure 2). In particular, the highest number of shoots was observed under the combination of 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA in culture medium of *Codonopsis* artificial seeds (2.69±0.10 cm). Similarly, the number of shoots and the percentage of germination were maximum when the synthetic seeds were sown into these medium conditions.

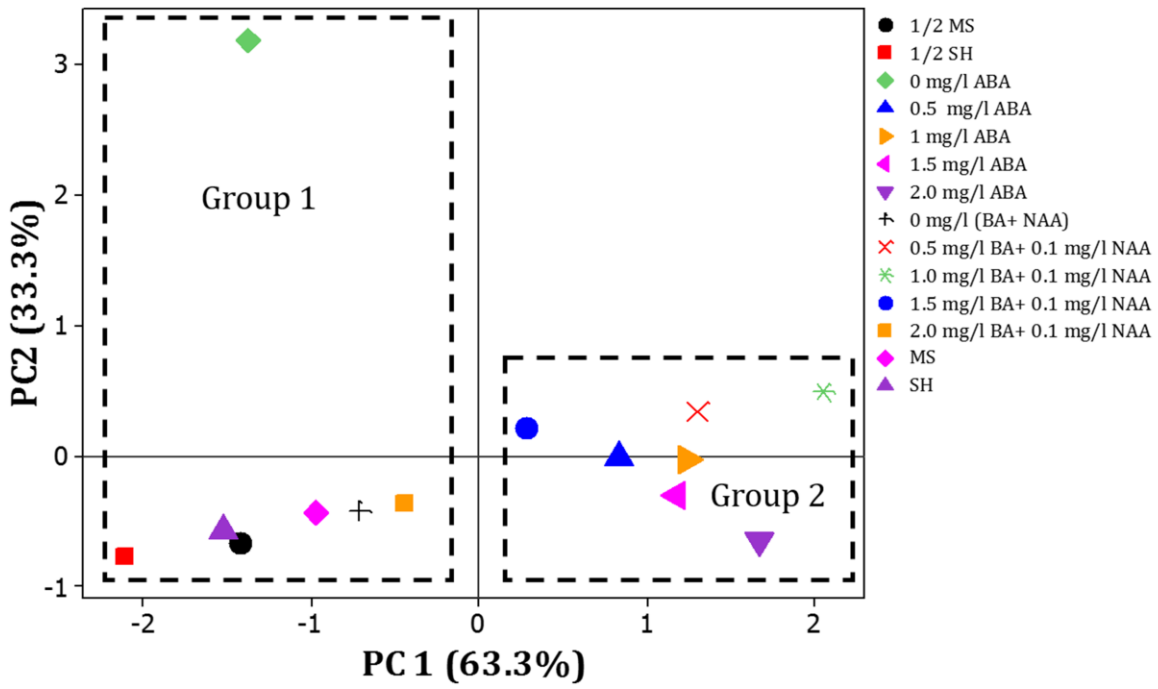


Figure 1. Principal components analysis (PCA) of the germination rate of *C. javanica* artificial seeds and the shoot-forming potential of explants derived from such synthetic seeds after 40 days of sowing in different types of culture media *in vitro*.

Table 1. Variation in the germination rate of *C. javanica* artificial seeds and the shoot-forming potential of explants derived from such synthetic seeds after 40 days of sowing in different types of culture media.

Culture medium	Shoot height (cm)	Shoot number per one artificial seed	Germination percentage (%)
1/2 MS	1.39±0.14^{b}	1.00±0.01^{a}	57.50±2.50^{b}
MS	1.61±0.20^{a}	1.11±0.03^{a}	65.00±3.07^{a}
1/2 SH	1.39±0.15^{b}	1.00±0.04^{a}	45.00±4.30^{c}
SH	1.56±0.15^{ab}	1.09±0.06^{a}	55.00±2.80^{b}

Data presented mean percentage ± SD of values of three replicates. Means followed by the different letter are significantly different ($p < 0.05$, one-way ANOVA, post hoc Tukey's HSD test).

Abscisic acid (ABA)

The germination rate of artificial seeds of *C. javanica* and the shoot-forming potential of explants derived from such synthetic seeds were strongly influenced by the supplementation of ABA into culture medium (Table 3 and Figure 3). In fact, after 40 days of sowing, the height of shoots significantly decreased and the study observed the highest shoots in culture medium without ABA ($p < 0.001$). In contrast, the highest number of shoots were obtained from the explants derived from synthetic seeds sown into medium with supplemented 2.0 mg l^{-1} ABA ($p < 0.001$), whereas the maximum germination rate was observed in culture medium containing 1.0 mg l^{-1} ABA ($p < 0.05$).

DISCUSSION

On the basic of micropropagation of most plants, culture media take an important role in germination, shoot- and root-forming potential (Thawaro and Te-chato, 2010). Evidence indicates that germination rate of seeds strongly changes according to the components of culture media (Qin et al., 2008; Rizzardi et al., 2009; Ghaderi-Far et al., 2010). In addition, changes in the type of culture medium and the plant growth regulators led to either increased or decreased the seed viability and shoot-forming potential of explants (Namli et al., 2010; Patil et al., 2012; Yaacob et al., 2014).

The results from this study indicated that hormone-free MS medium was the most suitable among the culture

Table 2. Variation in the germination rate of *C. javanica* artificial seeds and the shoot-forming potential of explants derived from such synthetic seeds after 40 days due to the combination of BA and NAA in culture media.

Plant growth regulator concentration		Shoot height (cm)	Shoot number per one artificial seed	Germination percentage (%)
BA ($^{mg\ l-1}$)	NAA ($^{mg\ l-1}$)			
0.0	0.0	1.61±0.09^{c}	1.30±0.48^{d}	67.50±3.70^{d}
0.5	0.1	2.52±0.10^{b}	3.40±0.70^{ab}	82.50±5.48^{b}
1.0	0.1	2.69±0.09^{a}	4.20±0.79^{a}	87.50±8.90^{a}
1.5	0.1	2.50±0.08^{b}	2.60±0.51^{bc}	72.50±9.50^{c}
2.0	0.1	1.72±0.10^{c}	2.00±0.82^{cd}	65.00±7.82^{d}

Data presented mean percentage ± SD of values of three replicates. Means followed by the different letter are significantly different ($p < 0.05$, one-way ANOVA, post hoc Tukey's HSD test).



Figure 2. Changes in the height of shoot of explants derived from *C. javanica* artificial seeds sown in the combination of different concentrations of BA and 0.1 mg l^{-1} NAA into culture media after 40 days of sowing.

media tested (1/2 MS, MS, 1/2 SH, and SH) and was sufficient for the germination and shoot formation of artificial seeds of *C. javanica* produced from nodal segments *in vitro* (Table 1). Indeed, MS medium is offered as a basal culture medium of many plants *in vitro* (Murashige and Skoog, 1962; George et al., 2008; Thawaro and Te-chato, 2010). It has been documented that the hormonal control of germination of seeds involves a balance of their inhibitory and stimulating compositions (Pedroza-Manrique et al., 2005; Patil et al., 2012). Moreover, the phytohormones strongly influence the shoot-forming potential of plantlets (Giladi et al., 1977; Azizi et al., 2015). These phenomena have been confirmed in the present study. Combination treatment of cytokinins and auxins led to either induced or inhibited germination rate of *Codonopsis* artificial seeds and shoot formation of explants derived from such synthetic seeds corresponding to the tested phytohormone concentration (Tables 2, 3 and Figure 2).

Particularly, addition of different concentrations of BA and NAA in MS medium led to significant changes in germination and shoot formation of *C. javanica* artificial seeds (Table 2 and Figure 2). Germination rate and shoot-forming potential were maximum when synthetic seeds were sown in MS medium supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA. This results is similar to the observation of Ma et al. (2011) on germination rate of artificial seeds of *Pseudostellaria heterophylla* in 1/4 MS supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA. Shoot height formed from artificial seeds significantly decreased according to the increase of ABA concentration in MS medium (Table 3 and Figure 3). Data shows that ABA mainly induced the proliferation of callus and root branching and root hair formation (Giladi et al., 1977; Biddington and Dearman, 1982). In contrast to shoot height, germination rate of *Codonopsis* synthetic seeds sown in MS supplemented ABA greatly increased in comparison with ABA-free medium. This is totally in agreement with the review of Nambara et al. (2010) who reported that the present of ABA can regulate seed dormancy and germination.

Duc (2014) observed the highest shoot-forming potential of the *C. javanica* callus in MS medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA in the cultured chamber. In this study, the optimum medium for the highest germination rate shoot-forming potential

Table 3. Variation in the germination rate of *C. javanica* artificial seeds and the shoot-forming potential of explants derived from such synthetic seeds after 40 days due to the supplementation of different ABA concentrations into MS media.

ABA (mg l^{-1})	Shoot height (cm)	Shoot number per one artificial seed	Germination percentage (%)
0.0	7.10±1.37^{a}	1.20±0.42^{c}	62.50±9.00^{d}
0.5	2.05±0.17^{b}	2.80±0.79^{b}	80.00±5.00^{c}
1.0	1.95±0.21^{b}	3.00±0.82^{b}	85.00±6.90^{a}
1.5	1.58±0.13^{bc}	3.10±0.88^{b}	82.50±9.50^{bc}
2.0	1.08±0.17^{c}	4.10±0.89^{a}	80.00±6.05^{c}

Data presented mean percentage ± SD of values of three replicates. Means followed by the different letter are significantly different ($p < 0.05$, one-way ANOVA, post hoc Tukey's HSD test).

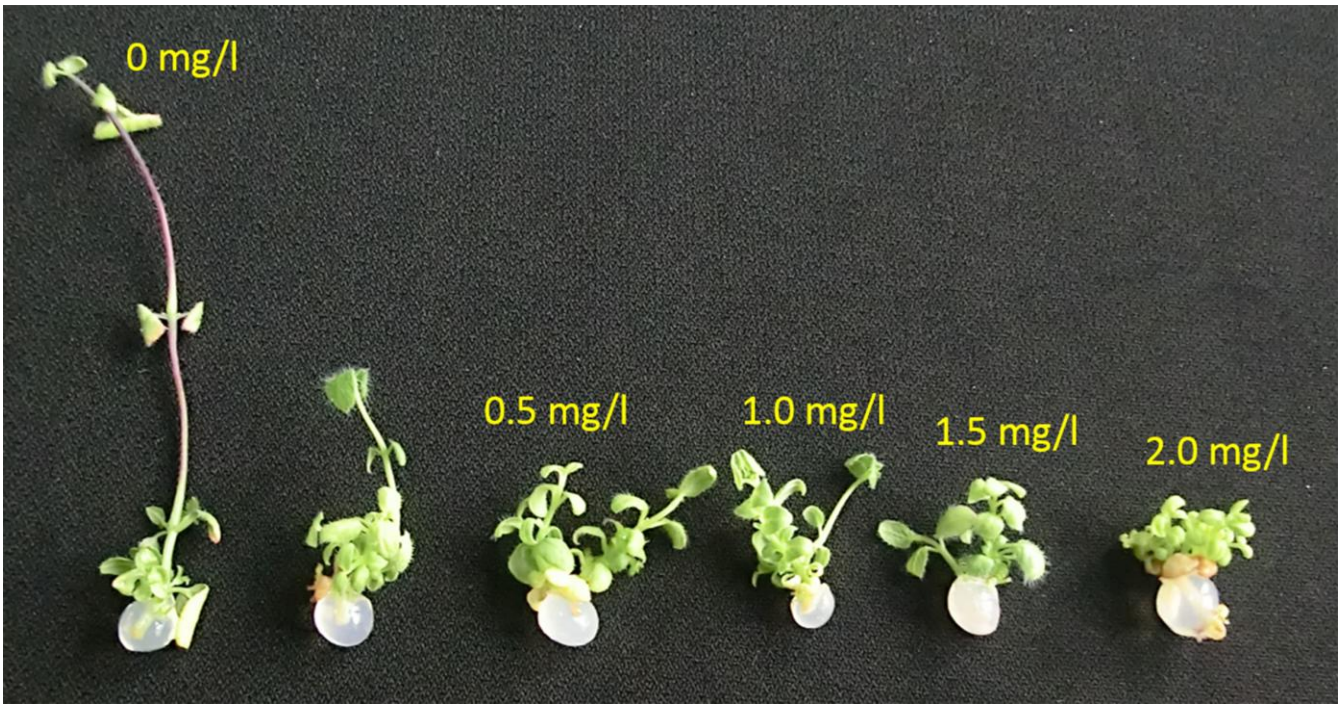


Figure 3. Changes in the height of shoots of explants derived from *C. javanica* artificial seeds due to the supplementation of various concentrations of ABA into MS medium after 40 days of sowing.

of the artificial seeds of *C. javanica* was the MS medium supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA under growth chamber conditions.

Conclusion

The results of this study are expected to provide new insights into the application of synthetic seeds in rapid multiplication of vietnamese *C. javanica*, establishing the traditional herbal medicine resource for human demands. In further experiments, the effects of environmental factors (for example, temperature, relative humidity and light intensity) should be investigated to supply more information to rapid multiplication of *C. javanica* artificial seeds *in vitro* and *ex vitro*.

ACKNOWLEDGEMENTS

The authors would like to thank the Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology for supporting materials and chemical compounds used in the study.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

ABA, Abscisic acid; **BA,** N-6-benzyladenine; **MS,** Murashige and Skoog; **NAA,** Naphthaleneacetic; **PCA,** Principal component analysis; **SH,** Schenk and Hide-brandt.

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