DOI: 10.31276/VJSTE.63(4).11-16

Induction and evaluation of secondary metabolite and antioxidant activity in adventitious root of *Codonopsis javanica*

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Received 21 May 2021; accepted 9 July 2021

Abstract:

In this study, the effects of auxin (IBA, NAA), explants, and culture conditions (light/dark) on adventitious root induction of *Codonopsis javanica* were investigated. The results showed that dark conditions were more suitable for adventitious root induction than light conditions. All three types of explants (internodes, leaves, and nodes) induced adventitious roots, and the appropriate concentration of auxin was 0.5 mg/l IBA. After 4 weeks of incubation under dark conditions, the rooting percentage and number of roots/explant of internode, leaf, and node segments on media supplemented with 0.5 mg/l IBA were 100% and 33.87 roots, 97.78% and 23.48 roots, 100% and 25.20 roots, respectively. These adventitious roots were analysed for the presence of alkaloids, carbohydrates, saponin, fixed oils and fats, phenol, flavonoids, gum, and mucilage. The total polysaccharide content, total phenolic content, and the antioxidant activity (IC_{50}) of *C. javanica* adventitious root biomass were 16.98%, 1.876 (mg GAE/g DW), and 2.44 (mg/ml), respectively. These results indicate that the adventitious roots of *C. javanica* contain bioactive compounds, which can be used as a material source for multiplication in large-scale systems.

Keywords: **adventitious roots, antioxidant activity, auxin, *Codonopsis javanica*, explant, light condition, secondary metabolite.**

Classification number: **2.2**

Introduction

Genus *Codonopsis* belongs to the family *Campanulaceae*, which has 42 species distributed around the world but mainly in central, east, and south Asia [1]. Several *Codonopsis* species have been widely used in traditional medicine. According to He, et al. (2015), the root of the *Codonopsis* species contains main compounds such as pyrrolidine alkaloid (Codonopyrrolidiums A, B), phenylpropanoid (Tangshenoside I), and polyacetylene (Lobetyol, lobetyolin, lobetyolinin, and cordifolioidyne B) [2]. *Codonopsis javanica* is found in Vietnam. *C. javanica* is distributed quite widely from the northern region to the southern central provinces of Vietnam such as Kon Tum, Lam Dong, Lao Cai, Lang Son, Lai Chau... *C. javanica* and other *Codonopsis* species have been used to treat diabetes and other diseases. The extracts of *C. javanica* possess insecticidal action against *Aedes albopictus* [3]. Chen, et al. (2013) [4] studied the reduction of blood insulin and the antioxidant capacity of *C. javanica* root extract in an insulin-resistant mouse model. According to a survey by Nguyen, et al. (2014) [5], *C. javanica* is a traditional medicine plant used by the K'Ho people in the buffer zone of Chu Sang Sin, the national park in Vietnam. Nowadays, *C. javanica* has been used in high demand not only as medicine but also as a daily food supplement. Due to overexploitation and deforestation, the reserves of medicinal plants are decreasing. For many years, *C. javanica* has been included in Vietnam's Red Data Book and recognised as a priority target for conservation actions [6].

Nowadays, *Codonopsis* genus has not only been studied in terms of medicinal materials but also in terms of micropropagation and preservation. For example, Peng, et al. (2010) [7] succeeded in regenerating *C. lanceolata* plants. Wojciech, et al. (2011) [8] carried out the project of micropropagation of *C. pilosula* (Franch.) Nannf. from axillary buds. Besides, the compounds of *C. javanica* have mainly been found in the roots. Therefore, the adventitious root culture of *C. javanica* is a suitable solution for large-scale root production that is independent of natural conditions and an alternative to traditional methods. The formation and development of adventitious roots of *C. lanceolata* were studied by Krishna, et al. (2007) [9] and Ahn, et al. (2008) [10]. In 2012, Kim, et al. [11] studied adventitious rooting of *Codonopsis* species such as *C. lanceolata*, *C. pilosula,*

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and *C. ussuriensis*.

In this study, the roles of several auxins such as IBA and NAA in the induction of adventitious root from different explants of *C. javanica* was studied. This adventitious root is then used to quantify several important groups of compounds such as polysaccharides, phenolic compounds, and antioxidant activity.

Materials and methods

Materials

Codonopsis javanica (Blume) Hook.f. & Thomson originating in Kon Tum were used as the subject of this study.

C. javanica plantlets (4 cm of height) cultured on the MS [12] medium without plant growth regulator for 4 weeks were used material for experiments in this study.

Methods

Effect of auxin and light condition and explants on the adventitious root induction: the explants (internode/node/leaf) were placed on the MS medium containing 8 g/l agar (Ha Long, Vietnam), 30 g/l sucrose, and IBA (Duchefa, Netherland) or NAA (Duchefa, Netherland) with different concentrations of 0.5; 1.0; 1.5; 2.0; 2.5; 3.0 mg/l and without auxin supplementation treatment was the control.

The internode and node explants were cut to 1.0 cm in length, and the leaf explants were cut to 0.5x0.5 cm in size.

All cultures were placed under white fluorescence (Philips, Vietnam) with a light intensity of 40±2 µmol m^{-2} s^{-1}, 12 h/day at 22±2°C and 60% relative humidity (RH), or dark condition. The treatments were repeated 3 times with 3 bottles/time and 5 explants/bottle.

The observation targets such as rate of adventitious root formation explants (%) and the average number of roots/explant were collected after 4 weeks of culture.

Evaluation quality of C. javanica adventitious root biomass: the quantification of total phenolic and polysaccharide contents and the antioxidant activity of *C. javanica* adventitious root biomass cultured in the optimal medium is given below.

Preparation of ethanol extracts of C. javanica adventitious root biomass: the dried samples of *C. javanica* adventitious roots were weighted, then separately macerated with 30 ml ethanol (4×30 ml for 24 h at room temperature). After filtration, the solvent was evaporated in vacuum at 50°C to obtain crude ethanol extract.

Quantification of total polysaccharide content of C. javanica adventitious root biomass: soluble sugar content was determined by the method of phenol - sulfuric acid. To calculate the content in the original sample, Eq. (1) is used [13]:

$$\text{Sugar content } (\%) = \frac{\text{Sugar concentration in PS powder (µg/ml)}}{\text{sample concentration PS (µg/ml)}} * \text{PS extract performance} \quad (1)$$

Quantification of total phenolic content of C. javanica adventitious root biomass: the total phenolic content was determined according to the Folin-Ciocalteu method [14]. Briefly, 1 mg of the crude extract is dissolved in 1 ml of distilled water and 0.1 ml of the diluted sample solution is placed into an Eppendorf tube. Then, 0.5 ml of 10% Folin-Ciocalteu solution is added and the solution is mixed well. Next, the mixture is incubated to react for 5 min, and then 0.4 ml 7.5% Na_2CO_3 solution was added and mix well. The solution was incubated at room temperature for 1 h, then the optical absorbance was measured at 765 nm.

Evaluation antioxidant activity and bioactive compound of C. javanica adventitious root biomass:

Qualitative chemical components: the explant powder was extract by ethanol. The ethanol and extracts were analysed for the presence of phytochemicals by using standard qualitative chemical procedures [15, 16]. The colour reaction was used to test the presence of common metabolite classes such as alkaloid, carbohydrate, glycosides, protein, saponin, phenolic compound, flavonoid, fixed oils, gum, and mucilage.

Antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity assays were used to determine the total antioxidant activity of the explant crude extract. The extract was initially diluted to concentrations varying from 0.0625 mg/ml to 1 mg/ml and assays were performed in a 96-well microplate. The reaction mixture in each well of the 96-well microplate consisted of 100 µl of DPPH solution (300 µM) and 100 µl of the sample. Ethanol and ascorbic acid (2 mg/ml) were used as negative and positive control, respectively. The plate was kept for 30 min at 37°C, and the absorbance was immediately recorded at 517 nm on a Bio-Rad Benchmark Plus Microplate Spectrophotometer (USA). The scavenging activity percentage was determined according to Mensor, et al. (2001) [17]:

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{control}) \times 100}{Abs_{control}}\right] \quad (2)$$

The radical scavenging activity of the extract was expressed in form of the IC_{50} values defined as the concentration of the sample required to decrease the absorbance at 517 nm by 50%.

Data analysis

Experiments were a completely randomized design and each treatment was repeated at least three times. All collected data were analysed by Minitab. All diagrams were drawn by Microsoft Excel® 2010.

Results and discussion

Results

Effect of auxin and light condition on the adventitious root induction from internode explant:

The results showed that in both light and dark conditions, IBA and NAA showed differential effects on adventitious root induction from internode explants of *C. javanica*. The data in Table 1 shows that the rooting percentage and number of roots per explants in the dark condition were higher than in the light condition. The rate of adventitious root induction was the highest on the medium supplemented with 0.5 mg/l IBA; the rooting percentage was 100% and the number of roots per explant was 33.87 roots. The formation of adventitious roots were totally absent in the culture containing higher concentration of NAA (3.0 mg/l) and the control in both light and dark conditions (Fig. 1).

Table 1. The effect of IBA, NAA and light condition on the adventitious root induction from internode explants after 4 weeks of incubation.

Auxin	Concentration (mg/l)	Light		Dark	
		No. of roots/ explant	*Rooting percentage (%)*	*No. of roots/ explant*	*Rooting percentage (%)*
IBA	0.0	0.00^{d}	0.00^{c}	0.00^{h}	0.00^{g}
	0.5	0.00^{d}	0.00^{c}	**33.87^{a}**	**100.00^{a}**
	1.0	11.99^{a}	60.00^{a}	19.59^{b}	75.56^{b}
	1.5	9.97^{ab}	51.11^{ab}	14.79^{c}	66.67^{bc}
	2.0	8.37^{b}	46.67^{ab}	11.70^{cde}	57.78^{cd}
	2.5	4.22^{c}	22.22^{cd}	11.42^{de}	57.78^{cd}
	3.0	2.00^{cd}	15.56^{de}	8.59^{ef}	31.11^{ef}
NAA	0.0	0.00^{d}	0.00^{c}	0.00^{h}	0.00^{g}
	0.5	0.00^{d}	0.00^{c}	12.06^{cd}	46.67^{de}
	1.0	8.03^{b}	35.56^{bc}	10.33^{de}	37.78^{ef}
	1.5	4.22^{c}	11.11^{de}	9.00^{def}	28.89^{f}
	2.0	0.00^{d}	0.00^{e}	6.11^{fg}	28.89^{f}
	2.5	0.00^{d}	0.00^{e}	3.17^{g}	28.89^{f}
	3.0	0.00^{d}	0.00^{e}	0.00^{h}	0.00^{g}

Means in the same column that are followed by different letters are significantly different (p≤0.05) using Duncan's Multiple Range Test.

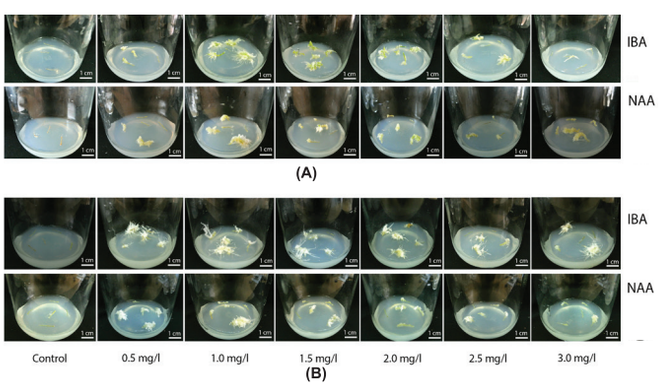

Fig. 1. Effect of IBA, NAA and light condition on the adventitious root induction from internode explants after 4 weeks of incubation. (A): light condition; **(B):** dark condition.

Effect of auxin and light condition on the adventitious root induction from leaf explant:

In this experiment, NAA affected root induction more effectively than IBA in light conditions. The rooting percentage and number of roots per explant in NAA-added medium (the rate ranged from 37.78-80% and 3.89-17.90 roots/explant, respectively) were higher than IBA-added medium (the rate ranged from 17.78-60% and 1.37-6.81 roots/explant, respectively). After 4 weeks of incubation in the light condition, the highest rooting percentage and number of adventitious roots per explant were 80% and 17.90, respectively, on the medium supplemented with 0.5 mg/l NAA.

Table 2 shows that the dark condition also enhanced root formation better than the light condition. In the dark condition, the rate of adventitious root induction was the highest on the medium supplemented with 0.5 mg/l IBA (the rooting percentage and the number of roots per explant were 97.78% and 23.48 roots, respectively) after 4 weeks of culture. The adventitious root induction decreased with increasing IBA concentrations from 1 to 3 mg/l. Fig. 2 shows that the leaf explants produced adventitious roots from the cut ends, and the roots formed directly from the leaf sample and not through the callus. The leaf explants did not produce adventitious root on the control under both incubation conditions.

Table 2. The effect of IBA, NAA and light condition on the adventitious root induction from leaf explants after 4 weeks of incubation.

Auxin	Concentration of auxin (mg/l)	Light		Dark	
		No. of roots/ explant	*Rooting percentage (%)*	*No. of roots/ explant*	*Rooting percentage (%)*
IBA	0.0	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{e}
	0.5	0.00^{g}	0.00^{g}	**23.48^{a}**	**97.78^{a}**
	1.0	2.33^{fg}	17.78^{fg}	17.28^{b}	80.00^{ab}
	1.5	6.81^{cde}	60.00^{abc}	11.50^{c}	71.11^{bc}
	2.0	4.61^{def}	42.22^{bcde}	8.73^{de}	60.00^{bcd}
	2.5	3.67^{efg}	31.11^{def}	7.77^{def}	46.67^{cd}
	3.0	1.37^{fg}	20.00^{efg}	6.72^{ef}	37.78^{d}
NAA	0.0	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{e}
	0.5	17.90^{a}	80.00^{a}	12.62^{c}	80.00^{ab}
	1.0	13.52^{b}	64.44^{ab}	9.98^{cd}	75.56^{ab}
	1.5	9.07^{c}	60.00^{abc}	8.17^{def}	71.11^{bc}
	2.0	7.66^{cd}	51.11^{bcd}	7.54^{def}	64.44^{bc}
	2.5	6.13^{cde}	42.22^{bcde}	6.90^{ef}	62.22^{bcd}
	3.0	3.89^{ef}	37.78^{cdef}	5.43^{f}	48.89^{cd}

Means in the same column that are followed by different letters are significantly different (p≤0.05) using Duncan's Multiple Range Test.

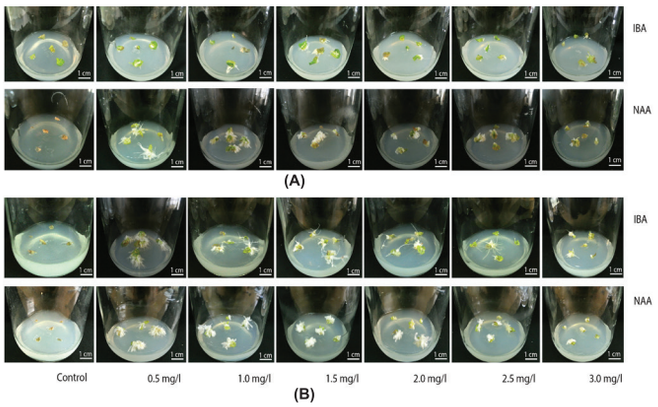

Fig. 2. Effect of IBA, NAA and light condition on the adventitious root induction from leaf explants. (A): light condition; **(B):** dark condition.

Effect of auxin and light condition on the adventitious root induction from node explant:

The rate of adventitious root induction, as well as the average number of roots/explant on the medium containing IBA and NAA with levels from 0.5 to 3.0 mg/l, were significantly different after 4 weeks of incubation under both light and dark conditions. IBA was more effective than NAA for adventitious root induction from node explants of *C. javanica* in dark conditions, but NAA was quite more effective than IBA in light condition. Table 3 shows that the root induction decreased with the increasing IBA and NAA concentrations (from 1 to 3 mg/l) in both conditions. The root induction was the highest on the medium supplemented with 0.5 mg/l IBA in the dark condition (the rate and the number of roots per explant were 100% and 25.20 roots, respectively). The number of adventitious roots per explant under dark conditions were higher than light conditions.

In the control, the node segments did not induce adventitious root, but instead formed shoots. This was because the node segment contained auxiliary buds that were not inhibited in the medium without auxin supplementation, and shoots regenerated (Fig. 3).

Table 3. The effect of IBA, NAA and light condition on the adventitious root induction from node explants after 4 weeks of incubation.

Auxin	Concentration (mg/l)	Light		Dark	
		No. of roots/ explant	*Rooting percentage(%)*	*No. of roots/ explant*	*Rooting percentage (%)*
IBA	0.0	0.00^{g}	0.00^{e}	0.00^{h}	0.00^{j}
	0.5	4.44^{f}	24.44^{d}	**25.20^{a}**	**100.00^{a}**
	1.0	10.60^{bc}	57.78^{ab}	$18,87^{b}$	88.89^{ab}
	1.5	8.01^{cde}	51.11^{bc}	13.50^{cd}	82.22^{abc}
	2.0	6.27^{def}	51.11^{bc}	9.98^{def}	75.56^{bcd}
	2.5	4.66^{ef}	46.67^{bc}	8.94^{def}	62.22^{cdef}
	3.0	3.87^{f}	35.56^{cd}	6.47^{fg}	57.78^{defg}
NAA	0.0	0.00^{g}	0.00^{e}	0.00^{h}	0.00^{j}
	0.5	15.83^{a}	73.33^{a}	17.61^{bc}	71.11^{bcde}
	1.0	12.90^{ab}	60.00^{ab}	12.18^{de}	51.11^{efgh}
	1.5	11.06^{bc}	53.33^{abc}	10.28^{def}	46.67^{fghi}
	2.0	9.91^{bc}	51.11^{bc}	8.44^{ef}	37.78^{ghi}
	2.5	8.30^{cd}	46.67^{bc}	5.86^{fg}	28.89^{hi}
	3.0	6.47^{def}	33.33^{cd}	2.44^{gh}	26.67^{i}

Means in the same column that are followed by different letters are significantly different (p≤0.05) using Duncan's Multiple Range Test.

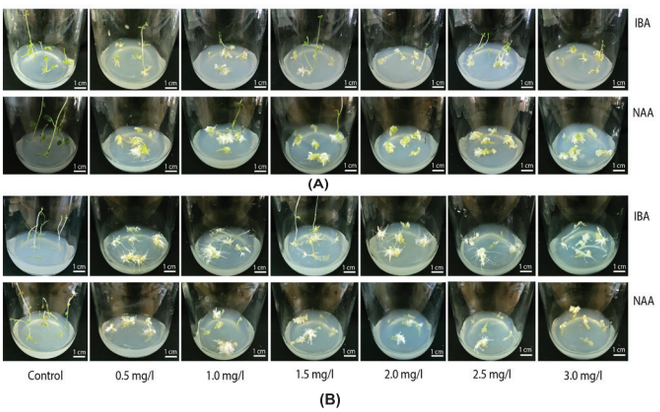

Fig. 3. The effect of IBA, NAA and light condition on the adventitious root induction from node explants. (A): light condition; **(B):** dark condition.

Preliminary phytochemical screening:

After induction of roots from different explant sources, the adventitious roots of *C. javanica* were initially cultured to proliferate on liquid media and incubated under shaking conditions at 120 rpm. The results showed that the roots proliferated 3.87 times in the MS medium supplemented with 0.5 mg/l IBA, 30 g/l sucrose (data not shown). After 4 weeks of incubation in the liquid medium, adventitious root samples

Table 4. Results of qualitative test for phytochemicals.

Test/reagents		*C. javanica* adventitious root biomass
Alkaloid	Mayer's	-
	Wagner's	+
Carbohydrate	Mollish's	+
	Fehling's	+
Saponin	Foam test	+
Fixed oils and fats	Spot test	+
	Saponification test	+
Tanin	$FeCl_3$	-
Phenolic compounds	Ferric chloride test	+
Flavonoid	Alkaline	+
Gum and mucilage	Absolute alcohol	+

were used to quantify some important compounds. Some specific reactions and colour change reactions were used to screen phytochemicals from the ethanol extract of sample powders. The result showed that *C. javanica* adventitious root biomass contains alkaloid, carbohydrate, saponin, phenolic compounds, flavonoid, fixed oil and fats, gum, and mucilage (Table 4).

Total phenolic, polysaccharide content and the antioxidant activity of C. javanica adventitious root biomass:

After screening some important bioactive compounds of the adventitious roots, the explant was also used to quantify two important bioactive compounds such as polysaccharide and polyphenol content. Results showed that the adventitious root biomass presented polysaccharides (16.98%), polyphenol (1.876 mg GAE/g DW), and the half-maximal inhibitory concentration (IC_{50}) value determined by the 2,2-diphenyl-1-picrylhydrazyl was 2.44 mg/ml (Table 5).

Table 5. Total polysaccharide and polyphenol contents, and the antioxidant activity of the adventitious root biomass.

***C. javanica* of adventitious root biomass**	
Polysaccharide content (%)	16.98
Polyphenol content (mg GAE/g DW)	1.876
IC_{50} (mg/ml)	2.44

Discussion

Auxin is one factor that plays an important role in organogenesis and especially in adventitious root induction. The responses of each species to the type and concentration of auxin were different. Therefore, the determination of the appropriate auxin type and concentration is essential. In this study, IBA and NAA were used to investigate the induction of adventitious root and the result showed that IBA and NAA significantly affected the process of adventitious root induction of *C. javanica*. According to Basra (2004) [18], IBA and NAA were more effective than IAA in other research. The optimal concentration of IBA or NAA for the adventitious root induction of *C. javanica* was relatively low as the concentration varied in the range of 0.5 mg/l to 1.0 mg/l. This result is also consistent with the conclusions of Kim, et al. (2012) [11] when studying adventitious root induction in species of the genus *Codonopsis*. The authors demonstrated that culture media supplemented with 0.5 mg/l NAA were suitable for root induction from the root segment of *C. lanceolata* and leaves of *C. ussuriensis*. Meanwhile, in the medium supplemented with higher auxin concentration, the adventitious root formation was less effective [11]. Explants did not induce adventitious root on both IBA and NAA-free medium, which reaffirmed the important role of auxin in the process of adventitious root induction. The results in Tables 1, 2, and 3 showed that IBA was the most suitable auxin to induce adventitious root induction of *C. javanica* in vitro. According to Krishna, et al (2007) [9], the rate of *C. lanceolata* adventitious root formation on the medium containing IBA was highest with a rate of 100%.

As mentioned above, the adventitious root formation of *C. javanica* results directly from the explant without callus induction phase. Praveen, et al (2009) [19] showed that adventitious root of *Andrographis paniculata* was formed directly from leaf explants without the callus induction phase. In the study of Baque, et al. (2010) [20], 1.0 mg/l IBA was proven as the best auxin source for adventitious root induction of *M. citrifolia* without the callus phase. However, in some case, adventitious roots dedifferentiated to form calli [20]. According to Gao, et al. (2005) [21], *Panax notoginseng* adventitious root were formed from callus on the IBA-supplemented medium.

In this study, all three types of explants (internode, nodes, and leaf) induced adventitious root (Fig. 4). Depending on the type of auxin as well as the culture conditions, the root formation rate, and the number of roots per explant were different. Evaluating the possibility of generating adventitious roots from three different types of explants such as node, internode, and leaf, it was found that the number of adventitious roots per internode explant of *C. javanica* was higher than node and leaf explant. Ahn, et al. (2008) [10] studied the induction of adventitious root of *C. lanceolata* and indicated that the number of adventitious roots per stem explant was higher than leaf and root explant. In the process of *C. javanica* adventitious root induction, most internode explants induced adventitious root under dark control. This could be explained that dark incubation enhanced the accumulation of endogenous IAA in the internode explant during adventitious root induction [22]. However, the result in this study was contrary to that of Baque, et al. (2010) [20] as fluorescent light was suitable for adventitious root induction of *M. citrifolia*. This showed that adventitious root formation was not only dependent on genotype, species, and growth regulator concentration, but also type of tissue, organ, age, and stage of plant development [23].

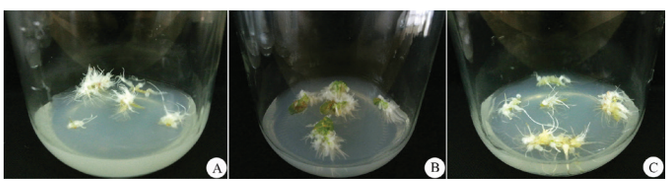

Fig. 4. Three types of explants [internode (A), leaf (B), and node (C)] produced adventitious root on the MS medium supplemented with 0.5 mg/l IBA under dark conditions.

The adventitious root biomass presented polysaccharides (16.98%). This result is similar to some species of the genus *Codonopsis* such as *C. pilosula* and *C. lanceolata* [1]. The content of a polyphenol in the *C. javanica* root was 1.876 mg GAE/g DW, and the IC_{50} value was 2.44 mg/ml. Compared to the results of Tri, et al. (2020) [24], the content of polyphenol

and the antioxidant activity of adventitious root were less than the natural plant-derived root. According to [20], the total phenolic content of *C. javanica* extract derived from this root was 2.9 mg GAE/g DW, and IC_{50} values determined by DPPH tests of the *C. javanica* root extract were 1042.3 µg/ml [24]. This can be explained by the short adventitious root culture period resulting in low accumulation of secondary compounds. Besides, under *in vitro* conditions, we can control the medium culture as well as use some elicitors to stimulate the increase in the content of bioactive compounds. In this study, the adventitious root contained an alkaloid, saponin, flavonoid, fixed oil and fats, gum, and mucilage in the *in vitro* condition. According to Lim (2015) [25], *C. javanica* natural root also contained glucose, essential oil, fatty substances, and alkaloids. The presence of these important compounds in the *in vitro* explants suggested that these explants could be used as a potential material source in traditional treatment against various diseases affecting humans and animals. More research about bioactivities needs to be done to analyse this potential.

Conclusions

All three types of explant (internode, nodes, and leaf) initiated adventitious root induction. The optimal concentration of IBA or NAA for the adventitious root induction of *C. javanica* was relatively low with the concentration varying in the range of 0.5 mg/l to 1.0 mg/l. The number of adventitious roots per internode explant of *C. javanica* was higher than node and leaf explant.

C. javanica adventitious root biomass contains alkaloid, carbohydrate, saponin, phenolic compounds, flavonoid, fixed oil, fats, gum, and mucilage. The adventitious root biomass presented polysaccharides (16.98%), polyphenol (1.876 mg GAE/g DW), and the IC_{50} value was 2.44 mg/ml.

ACKNOWLEDGEMENTS

The authors would like to thank the HCM-FOSTED, Department of Science and Technology for supporting this study under number 13/2019/HD-QPTKHCN.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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