Journal of Innovations in Applied Pharmaceutical Science (JIAPS)
e-ISSN: 2455-5177
www.jiapsonline.com

Research article

In vitro cloning of dang sam (*Codonopsis pilosula* (Franch) Nannf)

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Key words: Acclimatization, callus, multiple shoots, regeneration, sterilization.

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Abstract

Sterilization of Dang sam (*Campanumoea pilosula* (Franch) Nannf) cutting explants by $HgCl_2$ 0.1% in 5 minutes reach 58.3% survivals. The MS medium supplemented with 2.4D 2 mg/L and TDZ 0.1 mg/L was favored for callus induction on cutting surface after 30 days. Callus was proliferated on MS-1/2 medium supplemented with 2.4D 2 mg/L and TDZ 0.1 mg/L after 8 weeks. Callus cluster of 5 mm diameter were regenerated on MS medium supplemented with BA 2 mg/L and NAA 0.5 mg/L initiated multiple shoots after 60 days. Multiple shoots were divided into shoot clusters having diameter of 5 mm with 3-5 shoots/cluster. Shoot clusters were cultured on multiplication medium of MS supplemented with BA 2 mg/L and NAA 0.5 mg/L initiated to new shoots and the shoots were bigger after 20 days. Shoots were divided into 1 cm length cuttings with 2 leaves and cultured on MS medium supplemented with BA 0.5 mg/L and IBA 0.7 mg/L for regeneration to whole plantlets having 4-5 leaves, 6-10 roots, 4-6 cm root length reaching survivals of 85.07% and being ready for acclimatization environment. Sand substrate was favored for acclimatization in the first 10 days of ex vitro culture and mix-soil substrate was favored for plantlet growth and development afterward.

Introduction

Vietnamese named as Sam day or Dangsam, *Codonopsis* is a genus of flowering plant in the family Campanulaceae, was one of the valuable pharmaceutical materials in red book, classified in endanger V and limited harvest [1]. It is valuable drug in health enhancement and stress relief. Dang sam radix is the main part for drug. Radix containing saponins, sugar, lipid, vitamin and amino acid [2] that stabilize cholesterol levels in the blood and reduce the fat effectively; balance the blood pressure; prevent the thrombosis, the cardiovascular and brain complications, anti-aging; increase the appetite and good sleep; increases the immunity and inhibits the growth of tumors significantly [3]. Bioactive compounds in radix enhance biotransformation in human body.

Dang sam was distributed in some wards of East Truong Son Range belonging to districts of Tu Mo Rong, DakGlei, Kon Plong of Kon Tum province. Today, Dang sam has been significantly exploited and the regeneration decreased in nature.

With the reasons above, there was a mission of gene conservation and cloning capacities for development of material production regions of Kon Tum province. Cloning of dang sam (*Codonopsis* sp.) was necessary having significance in science and social impacts in poor regions.

Much of research focused on biological characteristics, chemical structure, physiological pharmaceuticals, clinical pharmaceuticals. Dang sam was cloned by seed or micropropagation. Studies in cloning have rarely been reported. Dang sam of Vietnam contains phytosterol as β-sitosterol, flavonoids as hesperidine and kaemferol-3-0-β-D-sophoroside, polyacetylene as diacetylenicpolyolefinic alcohol (lobetyol) [4].

Dang sam were cloned in vitro via callus of *Codonopsis pilosula* [5,6], embryogenesis of *Codonopsispilosula*[,7], *Codonopsis pilosula* Nannf [8], seed of *Codonopsis kawakami* Hatata [9] and internode cuttings of *Codonopsis tsinlinggensis* [10]. In this paper, we report the effects of plant growth regulators on Dang sam cloning via callus induction, shoot regeneration and plantlets acclimatization.

Experimental

Dang sam plants were collected from the local forest of MangRi and TeXang belonged to Tu Mo Rong district, Kon Tum province on 20 December 2010. The samples were determined by the Department of Biochemistry, International University.

Growth conditions: growth room temperature was 25 ± 2°C; photoperiod period was 12 h/day under light intensity 22.2–27.8 µmol/m²/s and media was sterilized by 121°C at 1 at in 25 minutes.

Medium nutrients was MS (Murashige-Skoog, 1962) supplemented with 2.4D (2.4-dichlorophenoxyacetic acid), TDZ (thiadiazuron), BA (6-benzylaminopurine), NAA (α-naphthalenacetic acid), activated charcoal, agar 8 g/L, sucrose 30 g/L.

Experiments were designed in randomize complete block (RCB) by cultivation of 5 samples per flask, 3 flask per replication, and 3 replications. Data were collected and ANOVA analyzed by MSTAC software.

Experimental designs

Experiment 1: Sterilization of explants: internode cuttings used as materials were cultured on MS medium without plant growth regulators. Explants were sterilized by $HgCl_2$ in 3-4-5-6-7 minutes. Determination of survival after 10 days.

Experiment 2: Callus induction: internode samples were cultured on the MS medium supplemented with 2.4D (0-2 mg/L), TDZ (0-0.3 mg/L). Determination of quantity and quality of callus induction after 30 days.

Experiment 3: Effects of mineral nutrients on callus proliferation: each callus collected from exp.2 was divided into 2-4 clusters, each cluster was cultured on the media with minerals varied MS, MS1/2 (1/2 x macro), 1/2MS (1/2 macro and 1/2 micro) supplemented with 2.4D 2 mg/L, TDZ 0.1 mg/L. Determination of rate and quality of callus after 8 weeks.

Experiment 4: Regeneration of callus: callus with diameter of 5 mm were cultured on MS medium supplemented with BA (0-3 mg/L) and NAA (0-0.5 mg/L). Determination of shoots number per callus cluster after 60 days.

Experiment 5: Shoot multiplication: Single shoot was cultured on MS medium supplemented with BA (0-3 mg/L), NAA (0-0.5 mg/L), and activated charcoal 1 g/L. Determination of multiple rate after 20 days.

Experiment 6: Regeneration of the whole plantlets: internode explants were cultured on MS medium supplemented with BA (0-0.5 mg/L), IBA (0-1 mg/L). Determination of percentage of rooting, root length, root number, leaf number, and shoot height after 15 days.

Experiment 7: Effects of substrates on plantlet survivals: plantlet with shoots of 6-7 cm height, 4-5 leaves, 8-10 roots were cultivated in a substrate of coconut fiber, sand, mix-soil and covered by 50% lighting shading. Mix-soil was composted for 2-3 months by mixing 1 part of coconut fiber, 1 part of organic manure and supplemented with 0.5% NPK (16:16:8+TE) and 2% CaO_2. Determination of survival rate after 10 days.

Experiment 8: Effects of substrates on plantlet growth and development: Plantlets from exp. 7 were cultivated on 3 substrates: coconut fiber, soil and mix-soil. Spraying plantlets with leaf-fertilizer Gibber-TB (0.5 g/L, 1 time/week). Planting 50 plantlets per replication and prepare 3 replication. Data was collected after 2 weeks. Determination of root length (mm), plant height (cm), stem diameter (mm), leaf diameter (mm), root number, leaf number after 8 weeks.

Project was carried out at Kon Tum Center for Scientific Application and Technology Transfer, Kon Tum City in 2010.

Results and Discussion

Sterilization of samples

Internode cuttings were cultivated on MS medium without plant growth regulators. They were sterilized by HgCl2 0.1% in 5 minutes to reach the highest survival rate 58.3% after 10 days (Table 1). Time of treatment was lower at 4 minutes to reach 13.3% and higher at 6 minutes to reach 25% survivals and leading explants browning.

Table 1. Effects of $HgCl_2$ on explants sterilization

Time of treatments (minutes)	Survivals rate (%)
3	0.0
4	13.3
5	58.3
6	25.0
7	0.0

Callus induction

Callus formation was not induced by MS medium without plant growth regulators. MS medium supplemented with combination of 2.4D 1 mg/L and TDZ 0.1-0.2 mg/L, callus were induced least and not stable, fresh weight was increased 0.0235 g/cluster and 0.0263 g/cluster respectively; with 2.4D 1 mg/L and TDZ 0.3 mg/L, callus was induced more with callus weight 0.0836 g/cluster; with 2.4D 2 mg/L and TDZ 0.1 mg/L, callus were induced strongly and fresh weight increased to 0.1168 g/cluster after 30 days (Table 2 and Figure 1). Callus had creamy color. The over concentration of 2.4D and TDZ combination leading the fresh weight decreased to 0.0991 g/cluster. Thus, 2.4D 2 mg/L and TDZ 0.1 mg/L was optimal for *Codonopsis* sp. callus induction. Research results were same as Niuet. al. [8] and Zhang et. al. [10].

Figure 1. Dang sam callus having creamy color was induced after 30 days.

Table 2. Effects of 2.4D and TDZ on callus induction

2.4D (mg/L)	TDZ (mg/L)	Callus fresh weight (g/cluster)	Quality of callus
0	0.0	Mẫu không tạo callus	
1	0.1	0.0235 ± 0.0033	++
1	0.2	0.0263 ± 0.0046	++
1	0.3	0.0836 ± 0.0030	+++
2	0.1	0.1168 ± 0.0117	+++
2	0.2	0.0991 ± 0.0015	+++
2	0.3	0.0278 ± 0.0048	+

** Note: +++ (good) ++ (average) + (not good, abnormal)*

Effects of mineral nutrients on callus proliferation

Results after 8 weeks of callus cultivation showed that in MS medium with full macro and micro, the rate of callus was 1.30; in MS medium with a half of macro nutrients, the rate was 3.83; and MS medium with a half macro and micro nutrients, the rate was 1.87 (Table 3). MS medium with a half of macro nutrients and full micro nutrients was favored. A half of macro nutrients were favored for callus proliferation.

Table 3. Effects of nutrients concentration on callus proliferation

Concentration of nutrients	Proliferation rate of callus (time)	Quality of callus
MS	1.30 ± 0.10	+++
MS1/2	3.83 ± 0.12	+++
1/2MS	1.87 ± 0.05	++

** Note: +++ (good), ++ (average)*

Regeneration of callus

Callus clusters with 5 mm diameter were cultured on MS medium supplemented with BA (0-3 mg/L) and NAA (0 and 0.5 mg/L). The MS medium without plant growth regulators did not initiate shoots; but the MS medium supplemented with the combination of BA 1 mg/L and NAA 0.5 mg/L initiated 3.37 shoots/cluster; with BA 3 mg/L and NAA 0.5 mg/L initiated 4.40 shoots/cluster; and with BA 2 mg/L and NAA 0.5 mg/L gave the best results of 7.50 shoots/cluster after 60 days (Table 4 and Figure 2). The results were the similar to the findings reported by Niuet. *et. al.* [8] and Zhang *et. al.* [10].

Table 4. Effects of BA and NAA on shoot regeneration

BA (mg/L)	NAA (mg/L)	Shoots/cluster
0	0.0	0.0
0.5	0.5	2.37 ± 0.15
1	0.5	3.37 ± 0.17
2	0.5	7.50 ± 0.30
3	0.5	4.40 ± 0.36

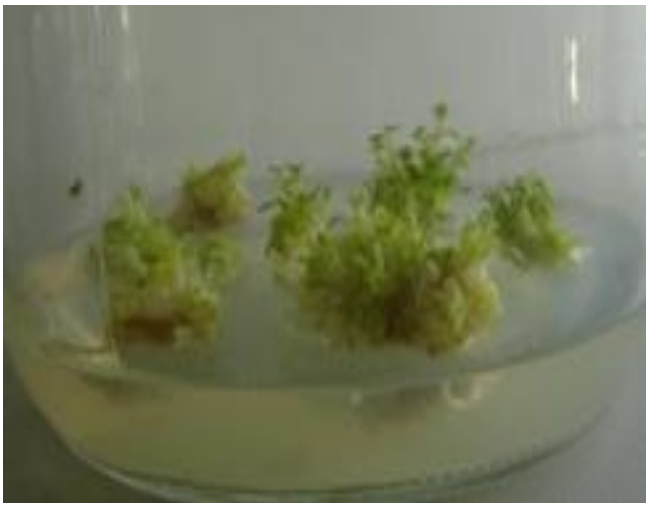

Figure 2. Shoots were initiated from callus after 60 days of cultivation.

Shoot multiplication

The multiple shoots from exp.4 were divided into 3-5 shoots/cluster and cultured on MS supplemented with BA 2 mg/L, NAA 0.5 mg/L and activated charcoal 1 g/L; the shoots were bigger and initiated new shoots with multiplication rate of 3 after 20 days (Table 5 and Figure 3). The medium supplemented with BA 2 mg/L and NAA 0.5 mg/L resulted in the multiplication rate of 1.50 and with 3 mg/L and NAA 0.5 mg/L reached 1.80.

Table 5. Effects of BA and NAA on multiplication rate

BA (mg/L)	NAA (mg/L)	Multiplication rate (time)
0	0	1.10 ± 0.06
0.5	0.5	1.20 ± 0.03
1	0.5	1.50 ± 0.15
2	**0.5**	**3.00 ± 0.28**
3	0.5	1.80 ± 0.33

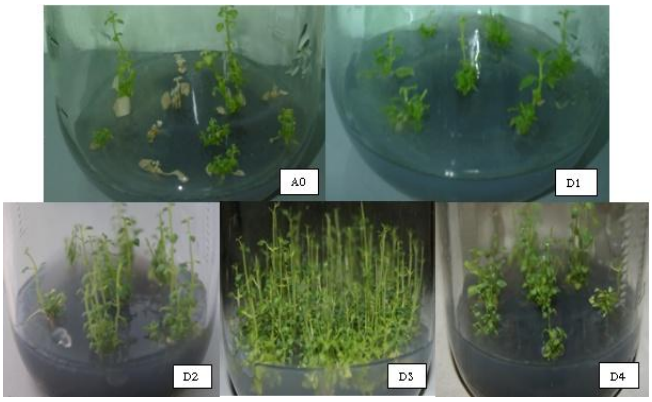

Figure 3. Effects of BA and NAA on multiplication rate

Regeneration of the whole plantlets

The shoots were divided into 1 cm internode cutting having 2 leaves and cultured on MS medium without plant growth regulators which initiated plantlets without root, low plant height and having 4.33 leaves. Medium supplemented with BA 0.5 mg/L and IBA 0.5 mg/L

resulted in plantlet with 5.30 cm plant height, 3.70 root numbers and 5.23 mm root length; with BA 0.5 mg/L and IBA 1 mg/L had 4.66 cm plant height, 4.66 root numbers and 4.07 mm root length; with BA 0.5 mg/L and IBA 0.7 mg/L gave the best results in regeneration of the whole plantlets that had 7.13 cm plant height, root number 8.60 and root length 7.39 mm after 15 days (Table 6 and Figure 4). Results were same as Niuet. al. [8] and Zhang et. al. [10].

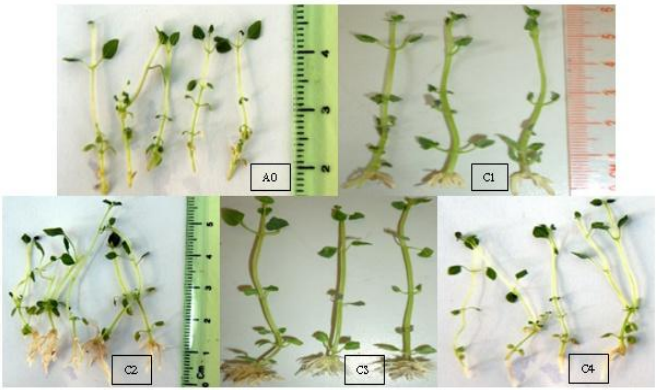

Figure 4. Effects of BA and IBA on regeneration of the whole plant

Effects of substrates on plantlet survivals

Plantlets having 4-5 leaves, plant height 6-7 cm and root number 8-10 were cultivated on the 3 types of substrates. The results showed that the substrate of soil was favored for plantlets acclimatization with survivals of 85.07% after 10 days (Table 7). The substrates of coconut fiber and mix-soil were not favored to natural conditions with low survivals 33.73% and 29.50% respectively. It could be due to the high humidity of coconut fiber and much of pathogen born in mix-soil.

Table 7. Effects of coconut fiber, sand and mix-soil substrates on plantlets survivals in acclimatization environment

Substrates	Survivals (%)
Coconut fiber	33.73 ± 3.31
Sand	85.07 ± 3.20
Composted mix-soil	29.50 ± 3.67

Effects of substrates on plantlet growth and development

The substrates were affected on plantlets growth and development strongly. Table 8 showed that the mix-soil was favored to plantlet growth and development having plant height of 18.1 cm, stem diameter of 1.88 mm, leaf number of 6.2, leaf diameter of 20.4 mm, root number of 16.5, root length of 4.9 cm (Figure 5). The result parameters were higher than those on the substrates of fiber and soil. It could explain by the poor nutrients of coconut fiber and poor air and water exchange of soil substrates.

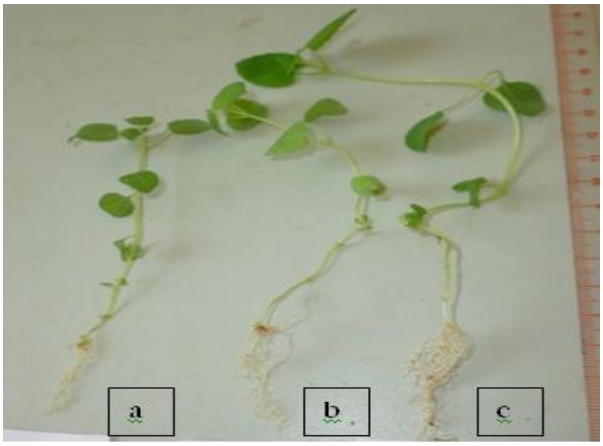

Figure 5. Growth and development of plantlets on substrates (a) coconut fiber (b) soil (c) mix-soil.

Table 6. Effects of BA and IBA on regeneration of the whole plantlets

BA (mg/L)	IBA (mg/L)	Root survivals (%)	Plant height (cm)	Root number (number)	Root length (mm)	Leaf number (number)
0.0	0.0	0	2.73 ± 0.22	0.0	0.0	4.33± 0.58
0.5	0.3	17	3.87 ± 0.33	3.10 ± 0.20	3.13 ± 0.31	4.83± 0.29
0.5	0.5	48	5.30 ± 0.20	3.70± 0.22	5.23 ± 0.25	6.00 ± 1.32
0.5	0.7	93	7.13 ± 0.30	8.60 ± 0.35	7.39 ± 0.36	4.83± 0.76
0.5	1.0	63	4.66 ± 0.24	2.10 ± 0.31	4.07 ± 0.25	4.00 ± 0.50

Table 8. Effects of substrate on plantlets growth and development

Substrate	Plant height (cm)	Stem diameter (mm)	Leaf number (number)	Leaf diameter (mm)	Root number/plant (number)	Root length (cm)
Fiber	11.2± 1.4	1.18±0.1	5.8±0.4	14.6±2.4	6.6±0.9	2.2±0.1
Soil	14.8±1.6	1.29±0.1	5.9±0.3	15.4±2.3	8.9±1.1	4.1±0.3
Mix-soil	18.1±1.7	1.88±0.1	6.2±0.3	20.4±2.8	16.5±1.0	4.9±0.3

Conclusion

Sterilization of cutting explants by $HgCl_2$ 0.1% in 5 minutes reached 58.3% survivals. The MS medium supplemented with 2.4D and TDZ was favored for callus induction and proliferation; with BA and NAA for shoot regeneration and multiplication; with BA and IBA for whole plantlets regeneration readily to be acclimatized. Sand was substrate for acclimatization environment and mix-soil was favored to plantlet growth and development.

Acknowledgement

Project was grantby Department of Science and Technology of Kon Tum province

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