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Full Length Research Paper

Direct multiple shoot induction and plant regeneration from dormant buds of *Codonopsis pilosula* (Franch.) Nannf.

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An efficient and reproducible protocol for *in vitro* plant multiplication system via direct organogenesis from dormant buds of *Codonopsis pilosula* Nannf was developed. Multiple shoots were induced at a frequency of 75% after nine weeks on Murashige and Skoog (MS) medium supplemented with BAP (1.0 mg/l), NAA (0.5 mg/l), 3% sucrose and 0.7% agar. Approximately, 15 to 18 shoots were formed at the base of each dormant bud. Higher concentrations of BAP and NAA resulted in callus formation. Further development of shoot elongation and multiplication were also studied. Well-grown shoots of 2.8 cm height and 3.8 proliferation coefficient were achieved by sub-culturing on MS medium supplemented with 0.2 mg/l BAP and 0.05 mg/l IBA. At higher concentrations, BAP (0.5 mg/l) promoted higher shoot proliferation coefficient (4.2); however, it negatively affected shoot elongation. Further, low NAA concentration was beneficial to shoot proliferation. All *in vitro*-derived shoots measuring 2.5 to 3 cm in length, rooted when grown on ½MS (half of all MS elements) basal medium containing 1.5 mg/l IBA within 3 weeks, 100% of shoots developed roots and test-tube seedlings grew stout.

Key words: *Codonopsis pilosula*, dormant bud, direct organogenesis, multiple shoot, shoot elongation, rooting.

INTRODUCTION

Codonopsis pilosula is a perennial native to Asia, which is especially abundant in the Shanxi and Gansu provinces of China. It is one of the traditional Chinese medicinal herbs with uses for over 2,000 years. Its dry roots are commonly used as Chinese herb drug, sometimes as a substitute for much expensive Panax ginseng. In recent years, the increasing demand of *C. pilosula* increased its market price which has inevitably led to destructive over - harvesting and depletion of its natural resources (Zhang, 1982). The extremely low viability of seedling (Zhang et al., 2008) of this plant has hampered its propagation by conventional means, and its seedling raising is also comparatively difficult. Moreover, in *C. pilosula* production, seedlings are raised the year before and transplanted in the next spring, which is labor intensive and time-consuming.

Many successful examples for micropropagation and cryopreservation using overground dormant buds can be found in garden plants (Jacobsen and Dohmen 1990; Mistretta et al., 1991). In fact, many medical plants form underground dormant buds in winter, while there are few reports using underground dormant bud via direct multiple shoots to regenerate plantlets. To our knowledge, only few studies have been reported to date regarding tissue culture of *C. pilosula* using stem segments and hypocotyls as explants and frequent callus formation as an interme-

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Abbreviations: MS medium, Murashige and Skoog medium; **BAP,** 6-benzylaminopurine; **NAA,** α-naphthaleneacetic acid; **IAA,** indole-3-acetic acid; **IBA,** 3-indole butyric acid; **$HgCl_2$,** mercuric chloride.

Table 1. Different composition of subculture medium.

Hormone concentration (mg/L)	Composition of media								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
BAP	0*	0.1	0.2	0.3	0.5	0.1	0.2	0.3	0.5
NAA	0	0.05	0.05	0.05	0.05	0.1	0.1	0.1	0.1

*The numbers in the column are the concentrations of BAP and NAA in culture medium (No.1 to 9).

diary phase just prior to somatic embryogenesis or regeneration to plantlet (Niu and Shao, 1991; Pan and Wang, 2005). Direct organogenesis without undesirable callus formation shortens the time period needed for regeneration and reduces the possibility of the occurrence of somaclonal variability (Hussey, 1977; Manzanera and Pardos, 1990; Polonca et al., 2004). Hence, in this study, direct multiple shoots induction and plant regeneration from dormant buds of *C. pilosula* is focused on in order to establish an efficient and rapid propagation system for *C. pilosula*.

MATERIALS AND METHODS

C. pilosula seedlings were obtained from Weiyuan County Gansu Province in northwest China during March and November, 2009.

Preparation of explants

Contamination is one of the greatest problems in the tissue culture of *C. pilosula*, when underground dormant buds are used as explants. In order to control contamination, they were carefully sterilized. Dormant buds with 0.2 cm long root were washed in washing powder solution, rinsed in running tap water to remove dirt. After initial washing, they were surface-sterilized with 0.1% (w/v) $HgCl_2$ solution for 6, 8,10 or 12 min, then thoroughly rinsed with sterilized distilled water for 5 times. The scales were carefully removed from the buds and the roots at the base of the buds were cut away with a sterilized razor in laminar flow cabinet. Finally, the explants were inoculated onto MS medium. After two weeks, the percentage of contaminated explants was recorded.

Culture condition

Unless otherwise stated, all cultures were grown in a climatic chamber at 22 ± 1℃ and16/8h photoperiod with a photon flux density (PFD) of 34 μ/m^2s.

Culture initiation and maintenance

Sterile dormant buds were placed on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of BAP and NAA, 3% sucrose and 0.7% agar (pH 5.8) for multiple shoots induction.

Proliferation of shoots

The shoots obtained from primary culture were transferred on MS medium with lower concentration of BAP and NAA separately in order to study their effects on the shoots proliferation and elongation (Table 1). The other conditions of the medium were same as the primary culture. Shoots were subcultured at 4-week intervals.

Rooting induction

When the regenerated shoots became 2.5 to 3.0 cm long, they were excised and inoculated on either ½MS_1 (half-strength macro salts of MS), ½MS_2 (half-strength macro and micro salts of MS) or ½MS_3 (half of all MS elements) medium, supplemented with different concentration of NAA, IBA and IAA, 1.5% sucrose, 0.1% activated charcoal and 0.7% agar (pH 5.8) for root-induction.

RESULTS

Efficiency of sterilization with different time using $HgC1_2$

In preliminary experiment, high contamination rate was common and it was the most important reason for losses during *in vitro* culture of dormant buds growing underground. Consequently, different sterilizing time using $HgCl_2$ have been used for surface treatment of dormant buds. Sterilizing the explants for 6, 8, 10 and 12 min, the contamination rate was 62.5, 59.5, 34.0 and 31.2%, respectively. Longer exposure to the sterilant not only reduced the rate of contamination, but also the viability of the buds. As such, 10 min exposure significantly reduced contamination and maintained high viability (80.4%) (Figure 1).

Sampling time and hormone concentration

In this study, dormant buds were sampled in March and November separately, and inoculated onto three culture media supplemented with different concentrations of BAP and NAA. The result shows that sampling time and hormone concentration affected the induction, regeneration, number and form of *C. pilosula* regenerants. On the same medium with BAP (1.0 mg/L) and NAA (0.5 mg/L), the effects of culture initiation had significant differences between March and November on induction rate of shoots, the former was 50.5% and the latter 75.0%, but there was no obvious difference in the quantity of shoots per dormant and shoots' growth potential (Table 2). High concentration of hormone combination was inappro-

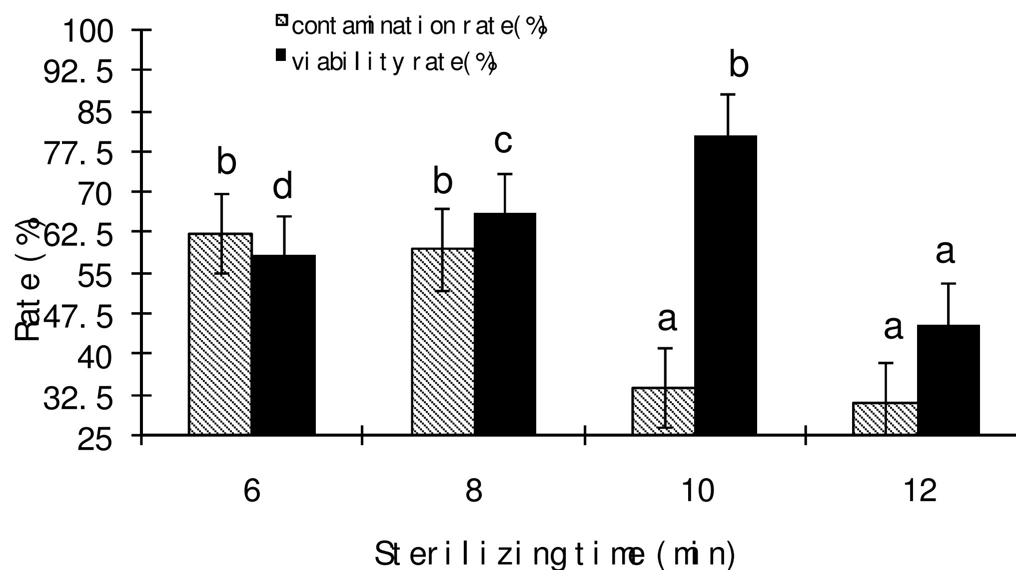

Figure 1. Effect of sterilizing time (min) on the rate of contamination and viability after inoculation for 2 weeks. Data represents mean values ± standard error of three replicates with at least 30 explants per treatment. Data having the same letter are not significantly different by Duncan's multiple comparison test (P<0.05).

Table 2. Effects of sampling season of material and exdogenous hormone on shoot clumps induction.

Sampling time	Hormone concentration (mg/L)		Induction rate (%)*		Visual observation
	BAP	NAA	Shoot	Callus	
March	2.0	1.0	0±0.00000^{a}	44.4±0.72188^{c}	Loo|Se, yellowish green
	1.0	0.5	75.0±0.60828^{c}	0±0.00000^{a}	15 to 18 multiple shoots per bud
	0.1	0.1	0±0.00000^{a}	0±0.00000^{a}	Single shoot
November	2.0	1.0	0±0.00000^{a}	23.0±0.55076^{b}	Loose, yellowish green
	1.0	0.5	50.5±0.61101^{b}	0±0.00000^{a}	13 to 17 multiple shoots per bud
	0.1	0.1	0±0.00000^{a}	0±0.00000^{a}	Single shoot

Each value represents the mean ± standard error of three independent experiments with at least 30 explants per treatment. Data having the same letter in a column were not significantly different by Duncan's multiple comparison test (P<0.05).

priate for direct shoot induction since the base of the bud in contact with the medium produced loose, yellowish green callus. In contrast, when the hormone concentration was too low, there was neither callus nor multiple shoots formation. Multiple shoots could only be generated by combining low concentrations of auxin and cytokinin with BAP (1.0 mg/L) and NAA (0.5 mg/L), being the most suitable. After culture for 6 weeks, approximately, 4 to 5 shoots were produced per dormant bud (Figure 3A). After 9 weeks of culture, 15 to 18 shoots were regenerated from each individual bud (Figure 3B).

Shoot proliferation and elongation

In order to improve the vigor of the shoots, reducing the concentration of BAP (0 to 0.5 mg/L) and NAA (0 to 0.1 mg/L) was trialed. Both BAP and NAA had a significant

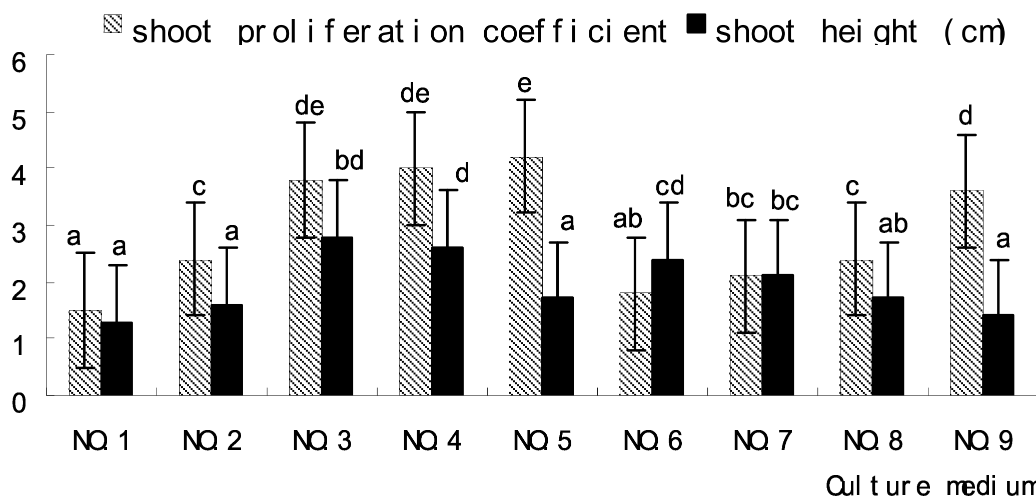

Figure 2. Effect of different culture medium (No.1 to No.9) on shoot height (cm) and shoot proliferation coefficient after inoculation for four weeks. Data represents mean values ± standard error of three replicates with at least 30 explants per treatment. Data having the same letter are not significantly different by Duncan's multiple comparison test (P<0.05).

effect on shoot elongation with NAA having a more pronounced effect over BAP. In the absence of both growth regulators, the average shoot height was only 1.3 cm. However, in the presence of low concentration of BAP and NAA in No. 2 medium, shoot height increased slightly to 1.6 cm. In No. 6 medium, with BAP concentration the same as in No. 2 medium, doubling of NAA concentration resulted in shoot elongation dramatically to 2.4 cm (Figure 2). With increasing BAP concentration from 0.2 to 0.5 mg/L, the shoot height decreased gradually (Figure 2). It would appear that a negative correlation existed between BAP concentration and shoot height. In all hormone combinations (BAP and NAA), shoots in No. 3 medium was the highest (2.8 cm), followed by No. 4 and No. 6 medium, where the height was 2.5 and 2.4 cm, respectively. In our experiment, the proliferation coefficient of shoots increased with the ascension of BAP concentration, the highest proliferation coefficient was up to 4.3 (Figure 2). However, in medium without hormone, its proliferation coefficient was only 1.5. In general, it can be said that the higher the concentration of BAP, the greater the proliferation coefficient of shoots, and at the same time, the lesser the elongation of shoots.

Apparently, lower concentration of BAP (0.2 mg/L) combined with low NAA (0.05 mg/L) were optimum for the proliferation and elongation of shoots with the proliferation coefficient reaching 3.8, per month and the height of shoot clumps reaching 2.8 cm (Figures 2 and 3C).

Effect of basal medium and auxin on root-induction

Robust multiple shoots were taken for rooting experiments. The factors, basal medium and auxin influencing rooting of shoots *in vitro* were studied. Shoots longer than 2 cm were separated and transferred to three different basal medium: (i) without hormone and containing either IBA or IAA, NAA at 0.5, 1.0, and 1.5 mg/L. Rooting rate, number and growth potential were considered together as the index of rooting culture. It was found that ½MS_3 was suitable for rooting since on this medium without any hormone, the root rate was 16.7%, while there were no roots on the other two media (Table 3). Consequently, higher concentrations of organic elements and micro-inorganic salts were unfavorable for root induction of *C. pilosula*. Hence, ½MS_3 (half of all MS elements) was used as basal medium for rooting. Adding different kinds and concentrations of auxins to the ½ MS_3 had significant differences in rooting rate, root numbers and growth potential.

Addition of IAA or NAA had weaker effect on root induction of shoots than IBA. The frequency of rooting was significantly lower when compared with IBA treatment. Furthermore, the plantlets were thin and the root number ranged from 1 to 4. When the concentration of IBA was increased from 0.5 to 1.5%, root rate increased markedly. The shoots rooted on medium containing 0.5 mg/L IBA at a frequency of 50%. When 1.5 mg/L IBA was used, the percentage of rooted shoots increased to 100% and the roots were morphologically healthy in terms of their number and color (Figure 3D).

Although, shoots could spontaneously root on hormone-free ½MS_3, the application of auxin was necessary for high frequency of initiation. As a result, the optimum medium for root induction was agar-solidified ½MS_3 (half of all MS elements) containing 1.5 mg/L IBA, 0.1% activated charcoal and 1.5% sucrose, which made the

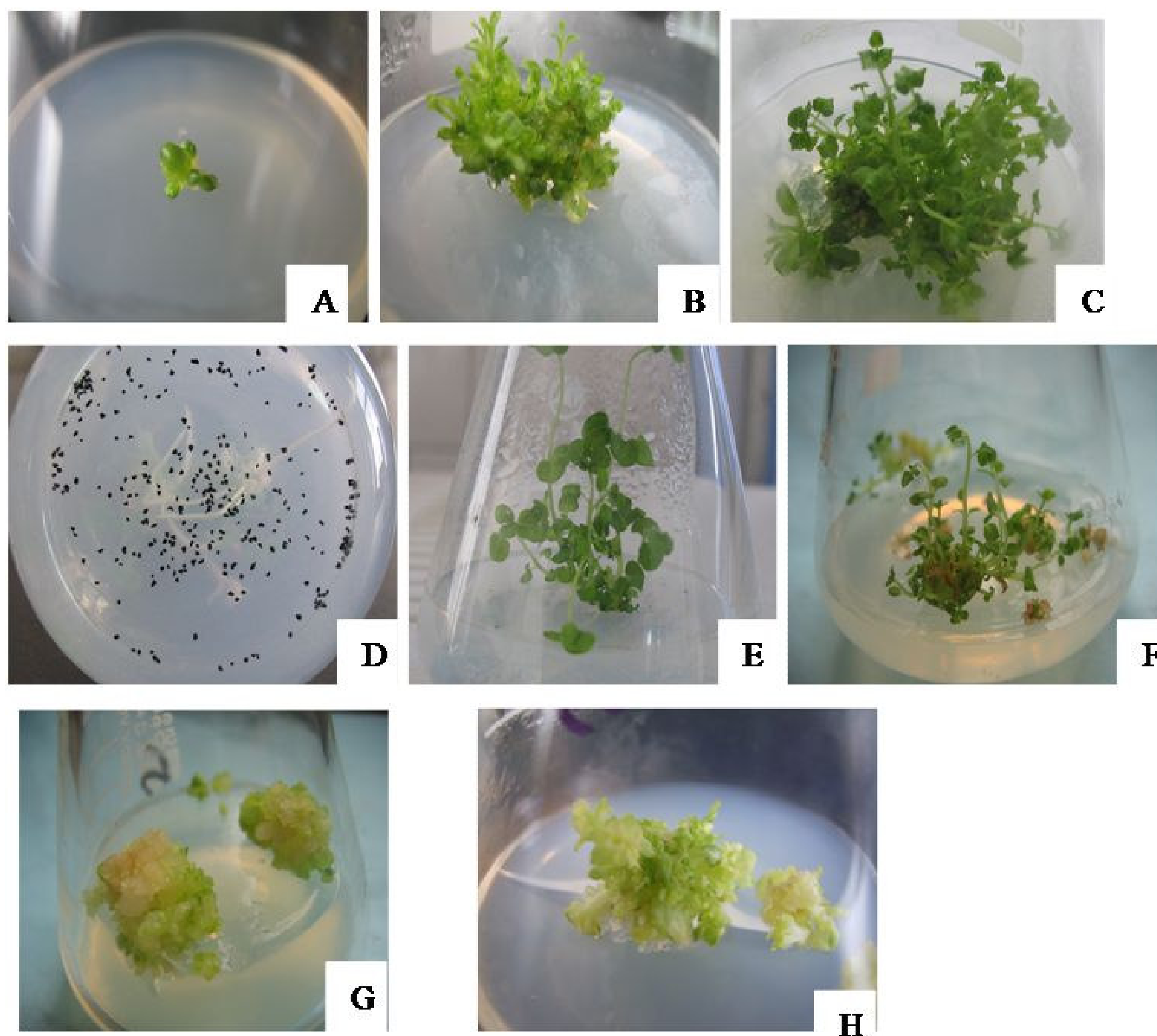

Figure 3. Shoot induction and plant regeneration of *C. pilosula*. A: Dormant bud sending out four new shoots after 6 weeks of incubation in MS containing 1.0 mg/l BAP and 0.5 mg/l NAA. B: Multiple shoots from dormant bud after 9 weeks in the same medium as A. C: Shoots in B were dissected and inoculated on the medium containing 0.2 mg/l BAP and 0.05 mg/l NAA for 4 weeks, and the shoots are grew up to 2.5 to 3 cm long. D: Root development after the shoots were transferred onto the root-induction medium (1/2 MS_3 (half of all MS elements) basal medium supplemented with 1.5 mg/l IBA, 1.5% sucrose and 0.7% agar, pH 5.8) for 3 weeks. E: Whole plantlets of *C. pilosula* from multiple shoots. F: Leaflike structures were inoculated on the MS medium for 2 weeks, which pushed many small shoots which look like seedlings from seed germination of *C. pi*losula. G: Callus formed in the leaf axil of stem segment in medium (MS supplemented with 2 mg/L BAP, 1.0 mg/L NAA, 3% sucrose and 0.7% agar, pH 5.8). H: Adventitious buds regenerated from callus.

plantlets to grow stout (Figure 3E).

DISCUSSION

The toxicity effect of $HgCl_2$, reported by Manzanera and Pardos (1990) were also observed in this study. Therefore, sterilizing efficiency should be considered not only in terms of sterilizing effect but also in terms of viability (Romano and Martins-Loucao, 1992). The result shows that sterilizing efficiency by 10 min is the best, the contamination rate was 34% and the viability rate was 80.4%.

Explants sampled in March had higher induction rate than in November. So seasonal factors may influence the physiological state of dormant buds, maybe explants gathered in March contained more endogenous hormone that those gathered in November. Cultures could be readily

Table 3. Effects of different basal medium and auxin concentration on root induction.

Basal medium	Hormone concentration (mg/L)			Root number	Root rate (%)	Growth potential
	IBA	IAA	NAA			
½MS1	0	0	0	0.0±0.00000[a]	0.0±0.00000 [a]	+++
½MS2	0	0	0	0.0±0.00000[a]	0.0±0.00000[a]	++
½MS3	0	0	0	1.0±0.10000[b]	16.7±0.40415[c]	+++
	0	0.5	0	2.0±0.33830[c]	12.5±0.26034[b]	++
	0	1.0	0	3.3±0.11547[f]	50.0±1.06927[f]	++
	0	1.5	0	2.0±0.11547[cd]	25.0±0.37859[d]	+
	0	0	0.5	0.0±0.00000[a]	0.0±0.00000[a]	+
	0	0	1.0	4.0±0.05774[f]	33.3±0.08819[e]	+++
	0	0	1.5	2.5±0.11547[e]	25.0±0.50332[d]	++
	0.5	0	0	2.2±0.05774[de]	50.0±0.52915[f]	+++
	1.0	0	0	6.0±0.15275[g]	57.0±0.40415[g]	++++
	1.5	0	0	6.3±0.06667[g]	100.0±0.00000[h]	++++

Each value represents the mean ± standard error of three independent experiments with at least 30 explants per treatment. Data having the same letter in a column were not significantly different by Duncan's multiple comparison test ($P<0.05$).With the increasing of "+", the growth potential of plantlet is better.

initiated from explants gathered in March.

In our study, medium complemented with 1.0 mg/L BAP and 0.5 mg/L NAA caused direct regeneration. Medium augmented with 2.0 mg/L BAP and 1.5 mg/L NAA proved to be inappropriate for vegetative propagation of *C. pilosula* from dormant buds as there was callus formation which was deemed unfavorable for genetic stability. In preliminary experiment, BAP combined with 2,4-D had a negative effect on induction as many dormant buds turned necrotic. In the study of *C. lanceolata* tissue culture, using immature seeds as explants and MS as basal medium supplemented with 2 mg/l BAP, 0.1 mg/l NAA, a large population of *C. lanceolata* regenerated plants from callus (Guo et al., 2006, 2007). Further, genomic variations as revealed by ISSR and RAPD analysis are apparent in the regenerated plants. Therefore, higher concentration of BAP (2.0 mg/L) induces callus easily and indirect organogenesis can result in high frequency of somaclonal variation of regenerants. In order to improve genetic instability of regenerated plantlets, it is necessary to avoid callus production for fine varieties breeding.

Previous study on *in vitro* rapid propagation using stems with and without axillary bud from *C. pilosula* as explants was also conducted (unpublished data). Our test shows that using stems with axillary bud as explants, leaf-like structures formed in the leaf axil of stem segment on MS medium supplemented with 1 mg/L BAP, 0.5 mg/L NAA. When leaf-like structures were transferred into hormone-free MS medium for two weeks, they outnumbered the young shoots which looked like seedlings of *C. pilosula* (Figure 3F). But when cultured for three weeks, the shoots began to die. On the contrary, on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA, a little callus formed in the leaf axil of stem segment became hard and showed signs of differentiation following twice subculture (Figure 3G). When they were subcultured into MS medium with low concentration of BAP and NAA, it differentiated into abundant off-type adventitious buds (Figure 3H).

When using hypocotyls, leaf and stem without axillary bud as explant, callus was induced initially followed by organgenesis (Niu and Shao, 1991; Pan and Wang, 2005). Hence, using dormant buds as explants and plant regeneration via multiple shoots is an ideal way of *in vitro*

rapid propagation of *C. pilosula* while minimizing variation. It can be concluded from these results that forming callus or direct multiple shoots depended largely upon (i) hormone concentration and (ii) type of explants.

Our research shows that IBA, NAA and IAA could be applied to initiate root formation but IBA was the most appropriate one for *C. pilosula* with rooting rate reaching up to 100% and promoting healthy growth of plantlets. In the case of vegetative material, tissue with existing meristematic activity was used where possible; normally apical or lateral buds with surrounding tissues (Tokuhara and Mii, 1993; Roy and Banerjee, 2003). Dormant buds are latent organ with meristematic regions and their morphology has already been established. Therefore, it had a strong ability of regeneration and adaptability to the changes in the culture environment. In addition, these explants are highly prolific, genetically stable and can withstand a sudden drop in temperature such as snap freezing (Matsumoto et al., 2001; Oka et al., 1991). In many garden plants, overground dormant buds have been used for micropropagation and cryopreservation (Ai and Luo, 2003; Suzuki et al., 1997). Therefore, it is very meaningful to study medical plants tissue culture.

Now, medical plants are favorably considered by most countries and as such, the world's leading international pharmaceutical companies had strengthened the research and development of medical plants which caused great pressure on medicinal plant resources (Fay, 1992). Hence, the progress made on the regeneration of whole plantlets of *C. pilosula* via direct organgenesis using dormant buds as explants would enhance medicinal plants cultivation and *in vitro* conservation which could remedy the inability to store the seeds in the gene bank due to the seed's recalcitrance to germination, also reduce the breeding cycle of slow growing plants and promote germplasm movement of pathogen-free medical plantlets.

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