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# High Frequency Plant Regeneration from Leaf, Petiole and Internode Explants of *Codonopsis lanceolata* Benth.

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**ABSTRACT : An efficient regeneration system was developed using leaf, petiole, and internode explants. Highly embryogenic callus was obtained following cultivation on MS basal nutrient supplemented with 2 mg/ℓ 2,4-D. Globular, heart, torpedo and cotyledon shaped somatic embryo were produced from the surface of embryogenic callus. Direct shoot regeneration without intermediate callus formation has been achieved on MS medium supplemented NAA and BAP. The percentage of response varies with different concentration of auxin and cytokinin treated individually or in combination. The best shoot regeneration response (54.28%) and number of shoot per explant (12.67) were achieved on the medium supplemented with 0.1 mg/ℓ NAA and 1 mg/ℓ BAP. The regenerated shoot transformed into young plant when cultured into elongation and root induction medium. More than 90% of *in vitro* propagated plants could survive when transferred to the greenhouse for acclimation. This optimized regeneration system can be used for rapid shoot proliferation and genetic transformation.**

***Key words*** : *Codonopsis lanceolata,* embryogenic callus, somatic embryogenesis, polyamines, histology, plant regeneration

## INTRODUCTION

Bounet bell flower, (*Codonopsis lanceolata)* is perennial climber and belongs to family Companulacea, grows naturally in moist places in woods, low mountains and hills (Hong *et al,* 1984). The flowers are hermophrodite and seeds ripen from September to October. Its root is widely used as herbal medicine for the remedy of depurative emmenagogue, galactogogue, dyspepsia, poor appetite, fatigue and psychoneurosis (Zhang, 1982), tonic, lungs abscesses, anticancer as well as a wild vegetable in Korea, China and Japan. The conventional propagation through seed is not sufficiently reliable and inadequate to meet its demand due to its poor rate for germination, low viability, delayed rooting of the seedlings (Kirtikar & Basu, 1935). Hence, the development of an efficient regeneration system was inevitable for this species. Plants tissue culture and development of regeneration system is influenced by several factors such as species variability, kinds of tissue, nutritional components of the medium, plant growth regulator and culture environment (Murashige & Skoog, 1962). Rapid regeneration of plants directly from explants minimizes somaclonal variations, culture duration, eliminates or minimizes callus formation in culture (Smith, 2006).

High frequency somatic embryogenesis and plant regeneration from leaf explants of *Codonopsis lanceolata* previously been reported by Min *et al.* (1992) and Shin *et al.* (2000). Similarly, Cho *et al.* (1999) reported the induction of embroyogenic calli on MS medium supplemented with 2,4-D and Zeatin. In the present study we report on the successful regeneration of shoots directly from cultured leaf, internode and petiole explants of *Codonopsis lanceolata.* The main objective of present work was to increase the efficiency and speed of regeneration and to compare their responses to different hormone concentration, media and culture conditions.

## MATERIALS AND METHODS

### Seed germination and explant preparation

Seeds of *Codonopsis lanceolata* were collected from Bioherb Research Institute, Kangwon National University, Korea. Seeds were hand separated from the pods and stored in plastic pot at room temperature under laboratory condition at 18-25 ℃. Seeds were sterilized in 70% ethyl alcohol for 1 minute followed by rinsing twice by sterilized deionized water and sub-

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sequently soaked in 5% NaOCl supplemented with 2 drops of £'ween 20 and agitated on mechanical shaker (130 rpm) for 30 minutes followed by 6 times rinsing by sterilized water. Surface sterilized seeds were planted in MS medium supplemented with 3 mg/ℓ $GA_3$. Seeds were incubated at 25-27 ℃ in 16 h photoperiod with 36 μmol $m^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes. After germination of seeds, plantlets were maintained in ½ MS medium.

*In vitro* grown seedling (4-5 weeks after germination) was used as sources of explants. Leaf, internode and petiole were segmented to 0.5 cm, 1 cm, 0.5 cm respectively and inoculated in each petridish (100 × 15 mm) containing regeneration medium. 15-30 explants were used per treatment and each experiment was repeated at least three times. Inoculated explants were placed at 25 to 27 ℃ and 16 h photoperiod provided by cool white fluorescent lights (36 μmol $m^{-2}s^{-1}$). Percentage of shoot initiation and average number of shoots per explants were scored after 4 weeks.

### Culture condition

To examine the effect of culture media on shoot regeneration, different basal media such as MS (Murashige & Skoog, 1962), $B_5$ (Gamborg *et al.*, 1968), SH (Schenk & Hildebrandt, 1972) including various concentration of auxins (2,4-D, NAA, IAA), cytokinins (BAP, TDZ) and $GA_3$ were examined. In addition, the effect of polyamines were also tested for induction of callus and initiating shoots from different types of cultured explants. The pH of all media was adjusted to 5.8 prior to addition of 0.8% agar and autoclaving for 20 minutes at 120℃.

### Shoot elongation and rooting

Regenerated shoots of 2 to 2.5 cm in length were excised from explants and transferred to shoot elongation medium supplemented with 0.8% agar and 3% sucrose. Elongated shoots were excised from original explants and transferred into rooting media (MS media with different concentration of IAA or IBA) supplemented with 0.8% agar and 3% sucrose.

### Acclimatization

Regenerated plantlets having well developed roots were gently washed in tap water to remove attached medium from their roots and were then transferred to 100 × 85 cm diameter plastic pot containing sterilized bed soil (Hangnong Chongmyo, South Korea) and Perlite (3 : 1) mixture and soaked with 1/3 strength MS salts. Each pot covered with a polythene bags to maintain high humidity and were placed in a glass house in 16 h photoperiod under reduced light intensity 30 μmol $m^{-2}s^{-1}$ at 25 to 27 ℃. After two weeks, the humidity of sealed pot reduced by punching holes in the bags. The polythene bags were gradually opened and after 6weeks, the plantlets were shifted to shade (70%). Thereafter, they were acclimatized to greenhouse condition and then shifted to field after one month. Data were obtained after 6 weeks for the percentage of survival of plants.

### Histological study

For the histological study, calli with somatic embryo and regenerating shoot were fixed in FAA (formaldehyde, acetic acid, ethanol, 5 : 5 : 90, v/v/v ratio) for 24 h and dehydrated in the tertiary butyl alcohol series. They were embedded in paraffin wax at 58 ℃ and were cut into 8 μm thickness using a rotatory microtome (Yamato Kohki, Japan). The sections were stained with haemotoxylin and fast green. They were permanently mounted in entellan and observed under a microscope (Olympus CH 40, Japan).

### Statistical analysis

Each treatment was consisted of 15-30 explants. All experiments were repeated at least three times. The data shown represent the mean ±S.E. The data were statistically analyzed using one-way ANOVA.

## RESULTS

### Influence of plant growth regulator on callus induction

To investigate the effect of plant growth regulator on embryogenic callus induction, explants (leaf, petiole and internode) were transferred into the callus induction medium supplemented with different concentrations of auxins and cytokinins. Swelling and expansion of explants were observed 5 days after culture initiation. This was followed by the callus initiation from the cut end and margins of the explants within 3 weeks of inoculation in culture medium. The distal part of leaf segments was less regenerative than the segments closer to the petiole. Callus growth was rapid and the explants was covered with callus 3 weeks after culture. Two types of callus were observed on the basis of their color and texture; type A- nodular, yellowish green, compact form of organogenic callus, type B- friable, creamy white to light yellow coloured, embryogenic callus. Callus growth, size and colour was influenced by the age, type of explants and concentration of plant growth regulators used in the medium. However, all cultures of three explants showed callusing in basal medium supplemented with different anxins and cytokinins. Among the different test, 2 mg/ℓ 2.4-D demonstrated best result for producing morphogenic callus (94.33%). NAA alone or in combination with BAP at different concentrations yielded higher frequencies of callus induction (Table 1, 2). When the subculture calli

**Table 1.** The single effect of plant growth regulators on callus induction and shoot regeneration from leaf explant culture in *Codonopsis lanceolata*.

| Plant growth regulators (mg/ℓ) | | No. of explant | No. of callus (Mean ± S.E) | Nature of callus† | No. of shoot (Mean ± S.E) | No. of root (Mean ± S.E) |
|---|---|---|---|---|---|---|
| 2,4-D | 0.1 | 30 | 22.7 ± 2.52 | A | 2.00 ± 1.00 | 0.00 ± 0.00 |
| | 1.0 | 30 | 26.0 ± 2.00 | A | 1.70 ± 0.58 | 0.00 ± 0.00 |
| | 2.0 | 30 | 28.3 ± 2.08 | A | 0.00 ± 0.00 | 0.00 ± 0.00 |
| NAA | 0.1 | 30 | 22.3 ± 1.53 | B | 0.70 ± 0.58 | 0.00 ± 0.00 |
| | 1.0 | 30 | 23.7 ± 2.52 | B | 0.00 ± 0.00 | 2.67 ± 1.20 |
| | 2.0 | 30 | 27.0 ± 2.65 | B | 2.00 ± 0.00 | 3.00 ± 0.58 |
| IAA | 0.1 | 30 | 21.3 ± 2.52 | C | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | 1.0 | 30 | 15.3 ± 2.08 | C | 0.00 ± 0.00 | 3.33 ± 1.33 |
| | 2.0 | 30 | 13.3 ± 2.31 | C | 0.00 ± 0.00 | 4.00 ± 0.58 |
| BAP | 0.1 | 30 | 3.70 ± 0.58 | C | 1.00 ± 0.00 | 0.00 ± 0.00 |
| | 1.0 | 30 | 7.70 ± 2.08 | C | 2.70 ± 0.58 | 0.00 ± 0.00 |
| | 2.0 | 30 | 10.3 ± 2.52 | C | 4.30 ± 1.15 | 0.00 ± 0.00 |
| TDZ | 0.1 | 30 | 11.3 ± 2.08 | C | 3.70 ± 1.15 | 0.00 ± 0.00 |
| | 1.0 | 30 | 9.30 ± 1.53 | C | 3.30 ± 0.58 | 0.00 ± 0.00 |
| | 2.0 | 30 | 8.30 ± 2.53 | C | 1.70 ± 0.58 | 0.00 ± 0.00 |
| $GA_3$ | 0.1 | 30 | 2.70 ± 1.53 | C | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | 1.0 | 30 | 2.30 ± 2.08 | C | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | 2.0 | 30 | 1.70 ± 0.58 | C | 0.00 ± 0.00 | 0.00 ± 0.00 |

† A: Friable, creamy white callus; B: Friable, yellowish green callus; C: Nodular, compact, yellowish green callus.

When three basal media (SH, B5 and MS) used to investigate the rate of callus induction, MS showed the better result than B5 and SH, producing morphogenic calli (Fig. 2). Similarly, different types and concentrations of polyamines were tested, in which spermine demonstrated the best result on callus formation among the rest (Fig. 3). To optimize the level of sucrose on the callus induction, 1-6% of sucrose was added in the basal medium. When 3% sucrose was supplemented in the MS medium, the largest growth yield of callus was achieved. In the absence of sucrose, callus was not induced (Table 3).

**Table 2.** The combination effect of plant growth regulators on callus induction and shoot regeneration from leaf explant culture in *Codonopsis lanceolata*

| Plant NAA | Growth BAP | Regulator TDZ | (mg/ℓ) $GA_3$ | No. of explant | No. of callus (Mean ± S.E) | Nature of callus† | % of explants producing shoot | No. of shoot/explant (Mean ± S.E) |
|---|---|---|---|---|---|---|---|---|
| 0.1 | 0.1 | | | 30 | 27.70 ± 2.52 | B | 20.98 | 2.30 ± 0.88 |
| 0.1 | 1.0 | | | 30 | 26.67 ± 0.67 | B | 54.28 | 12.7 ± 2.40 |
| 0.1 | 2.0 | | | 30 | 21.30 ± 2.08 | B | 8.99 | 7.00 ± 1.00 |
| 1.0 | 0.1 | | | 30 | 24.00 ± 2.08 | B | 24.31 | 3.33 ± 1.86 |
| 1.0 | 1.0 | | | 30 | 26.00 ± 1.15 | B | 12.32 | 6.00 ± 0.58 |
| 1.0 | 2.0 | | | 30 | 25.67 ± 2.40 | B | 22.31 | 6.33 ± 1.33 |
| 2.0 | 0.1 | | | 30 | 27.30 ± 2.52 | A | 12.32 | 1.33 ± 0.33 |
| 2.0 | 1.0 | | | 30 | 25.33 ± 2.60 | A | 12.32 | 6.00 ± 0.57 |
| 2.0 | 2.0 | | | 30 | 28.30 ± 1.15 | A | 50.95 | 3.00 ± 0.58 |
| 2.0 | 3.0 | | | 30 | 26.70 ± 1.53 | C | 37.63 | 5.67 ± 0.88 |
| 0.1 | | 0.1 | | 30 | 21.70 ± 1.53 | C | 26.64 | 4.33 ± 0.88 |
| 0.1 | | 2.0 | | 30 | 10.70 ± 2.08 | C | 5.66 | 5.00 ± 0.38 |
| 2.0 | | 0.1 | | 30 | 5.30 ± 1.53 | C | 8.99 | 3.00 ± 1.33 |
| 2.0 | | 2.0 | | 30 | 21.30 ± 1.15 | C | 15.65 | 3.33 ± 0.88 |
| 0.1 | | | 0.1 | 30 | 18.00 ± 1.73 | C | ND* | 0.00 ± 0.00 |
| 0.1 | | | 2.0 | 30 | 14.30 ± 1.15 | C | 2.33 | 1.00 ± 0.58 |
| 2.0 | | | 0.1 | 30 | 10.70 ± 2.08 | C | ND* | 0.00 ± 0.00 |
| 2.0 | | | 2.0 | 30 | 4.70 ± 1.53 | C | ND* | 0.00 ± 0.00 |

† A: Friable, yellowish green callus; B: Compact, yellowish green callus; C: Nodular, compact, yellowish green callus.
* ND: not detected.

were transferred on to the medium with NAA and BAP, numerous green patches appeared which later developed into adventitious shoot.

### Induction and development of the somatic embryo

Embryogenic calli were transferred into MS medium supplemented with various concentrations of sucrose and 2,4-D. On the medium added with 6% sucrose and 1 mg/ℓ 2,4-D, smooth, round structures appeared on the surface of embryogenic callus within 10 days and were identified as somatic embryo. The somatic embryos were initially globular but later grew into heart shaped structure. It was well consisted with the earlier report of Soh *et al.* (1992). A histological analysis of longitudinal section of somatic embryo revealed a shoot pri-

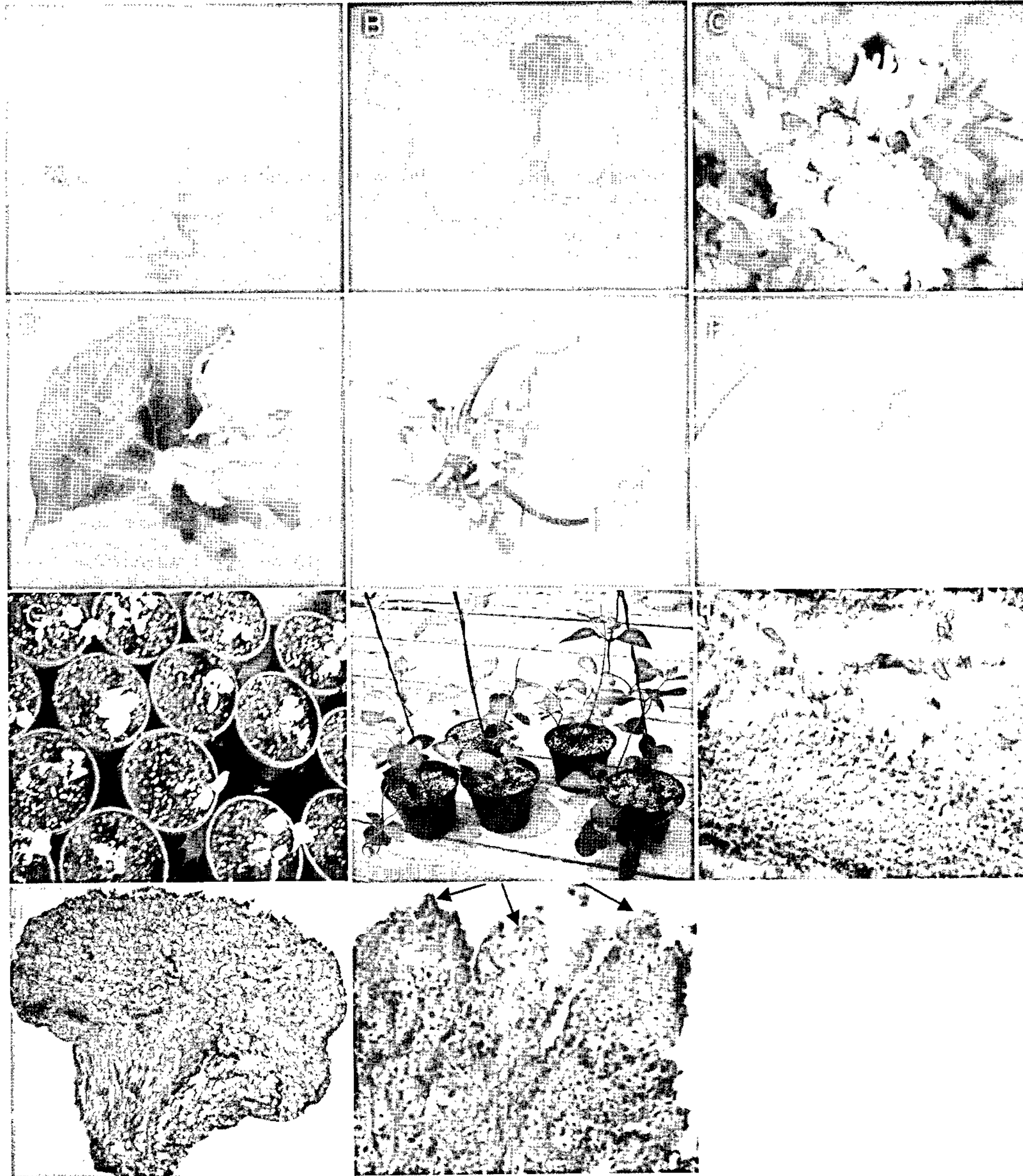

**Fig. 1.** Plant regeneration in *Codonopsis lanceolata*. (a) Embryogenic callus induction from leaf explant on MS medium containing 2.0 mg/ℓ 2,4-D. (b) Somatic embryos at different developmental stages. (c) Germination of somatic embryo. (d) Direct shoot regeneration from leaf explant on medium containing 0.1 mg/ℓ NAA and 1 mg/ℓ BAP. (e) Elongation of shoot. (f) Rooting of regenerated shoot on MS medium containing 3.0 mg/ℓ IAA. (g) Plantlets transferred to pot covered with a polythene bags to maintain high humidity (h) Potted plants in the greenhouse. (i) Cross section of callus. (j) Longitudinal section of somatic embryo. (k) Multiple shoot induction with multiple shoot primordia.

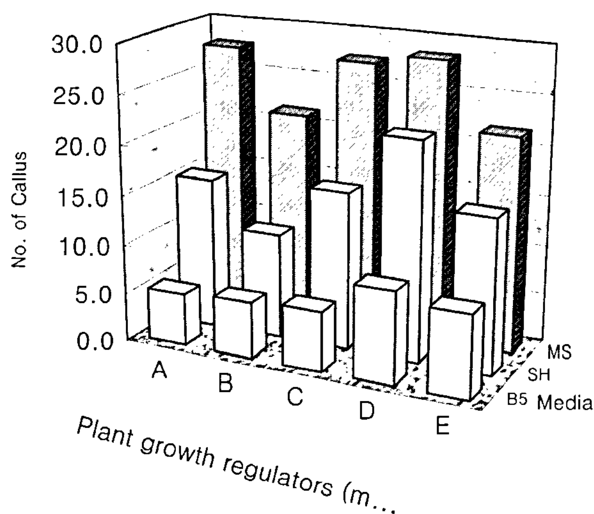


**Fig. 2.** The effect of medium on callus induction from leaf tissue in *Codonopsis lanceolata*. A. NAA 0.1 mg/ℓ + BAP 0.1 mg/ℓ, B. NAA 0.1 mg/ℓ + BA 2.0 mg/ℓ, C. NAA 2.0 mg/ℓ + BAP 0.1 mg/ℓ, D. NAA 2.0 mg/ℓ + BAP 2.0 mg/ℓ, E. NAA 0.1 mg/ℓ + TDZ 0.1 mg/ℓ.

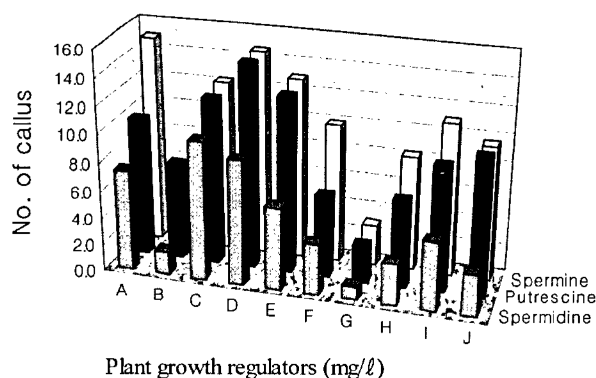


**Fig. 3.** The effect of polyamines on callus induction from leaf tissue culture in *Codonopsis lanceolata*. A. NAA 0.1 mg/ℓ + BAP 0.1 mg/ℓ, B. NAA 0.1 mg/ℓ + BA 2.0 mg/ℓ, C. NAA 2.0 mg/ℓ + BAP 0.1 mg/ℓ, D. NAA 2.0 mg/ℓ + BAP 2.0 mg/ℓ, E. NAA 0.1 mg/ℓ + TDZ 0.1 mg/ℓ.

mordium enclosed with a pair of cotyledons and a distinct root primordium (Fig. 1-k). There was no vascular connection between the parent explant and the developing embryos. Embryo maturation and plantlets recovery was achieved by transferring the clusters of somatic embryo into MS basal medium.

### Direct shoot regeneration

Plant explants (leaf, petiole and internode) were cultured on MS medium with different combination of auxins and cytokinins to optimize the shoot regeneration. Interestingly, adventitious buds developed directly from cut surface, which initially resembled knob like structure and later developed into new plant (Fig. 1-d). The kind of plant growth regulator as well as their concentration and combination influenced the frequencies of shoot regeneration. Among the different combination of plant growth regulators, MS medium supplemented with 0.1 mg/ℓ NAA in combination with 1 mg/ℓ BAP demonstrated the best result with higher frequency of shoot regeneration (54.28%) and the number of shoot per explants (12.67). In the absence of NAA or BAP, low frequency of shoot regeneration was observed, implying that these plant growth regulators are critical in shoot regeneration in *Codonopsis lanceolata* (Table1, 2). Similarly, addition of 0.1mg/ℓ of TDZ in combination with 0.1mg/ℓ NAA enhanced the direct shoot regeneration. Whereas, no significant enhance in the shoot regeneration observed when $GA_3$ was added in the MS basal medium. Hence, among the cytokinin used, BAP in combination with NAA proved to be efficient for shoot initiation.

Shoot bud induction occurred when adaxial surface of the leaf explant was in contact with the medium. Among the different type of carbohydrate sources tested, sucrose proved to be the most effective for shoot development (data not shown). To find the optimal level of sucrose on shoot regeneration, MS medium was supplemented with different concentration of sucrose (1-6%), among which 3% of sucrose proved to be the most reproducible for shoot development. In the absence of sucrose, shoot regeneration was reduced (Table 3).

Histological observation of regenerating shoots revealed that direct development of shoot occurred from the surface of the responsive explants without intermediate callus formation (Fig. 1-k). The frequency of direct shoot organogenesis was varied among the types of explant. Among the three types of explant tested, leaf explant demonstrated higher frequency of shoot regeneration and number of shoot per explants (Fig. 4).

### Shoot elongation

Healthy regenerated shoots were excised from explants culture and transfer into the different elongation medium supplemented with various cytokinins. An efficient elongation of shoots was obtained by using 1/2 MS medium . Similarly, addition of $GA_3$ in the medium promoted the elongation of shoot (Table 4).

### Rooting of shoot

Well developed and elongated shoots of height 1.5 to 2 cm were excised from elongation shoot culture and transfer into the rooting medium supplemented with various concentration of IAA or IBA. Root initiation occurred within 8 days and good root system developed after four weeks of culture.

**Table 3.** The effect of sucrose on callus induction and shoot regeneration from leaf explant culture in *Codonopsis·lanceolata*

| Sucrose (%) | Plant growth regulator (mg/ℓ) NAA | BAP | No. of explant | No. of callus (Mean ± S.E) | No. of shoot (Mean ± S.E) |
|---|---|---|---|---|---|
| | 0.1 | 0.1 | 30 | 2.70 ± 1.53 | 0.30 ± 0.58 |
| | 0.1 | 2.0 | 30 | 2.70 ± 1.15 | 0.00 ± 0.00 |
| 1 | 2.0 | 0.1 | 30 | 5.00 ± 2.65 | 0.70 ± 0.58 |
| | 2.0 | 2.0 | 30 | 8.30 ± 1.53 | 3.00 ± 1.73 |
| | 0.1 | 0.1 | 30 | 5.70 ± 2.08 | 1.30 ± 0.58 |
| | 0.1 | 0.1 | 30 | 27.7 ± 2.52 | 6.70 ± 0.58 |
| | 0.1 | 2.0 | 30 | 21.3 ± 2.08 | 2.30 ± 2.08 |
| 3 | 2.0 | 0.1 | 30 | 27.3 ± 2.52 | 3.30 ± 1.53 |
| | 2.0 | 2.0 | 30 | 28.3 ± 1.15 | 16.0 ± 2.65 |
| | 0.1 | 0.1 | 30 | 21.7 ± 1.53 | 8.30 ± 1.53 |
| | 0.1 | 0.1 | 30 | 9.70 ± 2.52 | 2.70 ± 1.15 |
| | 0.1 | 2.0 | 30 | 6.70 ± 1.15 | 1.00 ± 0.00 |
| 6 | 2.0 | 0.1 | 30 | 11.3 ± 1.53 | 3.30 ± 2.08 |
| | 2.0 | 2.0 | 30 | 14.7 ± 0.58 | 5.30 ± 1.15 |
| | 0.1 | 0.1 | 30 | 12.7 ± 2.52 | 4.00 ± 0.58 |

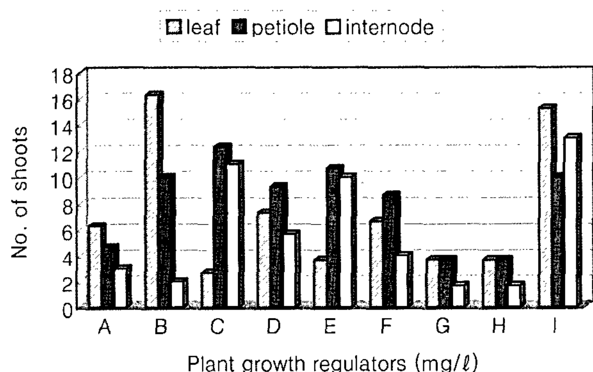


**Fig. 4.** Comparative analysis of shoot regeneration from leaf, petiole and internode explants. A. NAA 0.1 mg/ℓ + BAP 0.1 mg/ℓ, B. NAA 0.1 mg/ℓ + BA 1.0 mg/ℓ, C. NAA 0.1 mg/ℓ + BAP 2.0 mg/ℓ, D. NAA 1.0 mg/ℓ + BAP 0.1 mg/ℓ, E. NAA 1.0 mg/ℓ + BAP 1.0 mg/ℓ, F. NAA 1.0 mg/ℓ + BA 2.0 mg/ℓ, G. NAA 2.0 mg/ℓ + BAP 0.1 mg/ℓ, H. NAA 2.0 mg/ℓ + BAP 1.0 mg/ℓ I. NAA 2.0 mg/ℓ + BAP 2.0 mg/ℓ.

Among the auxin tested, IAA was more effective than the rest for induction of root. However, the root length showed only minor differences among the various auxins treated for root induction. Growth of elongated shoot in medium containing different concentration IAA resulted in callusing at the base of the shoots. The best rooting was observed at 3mg/ℓ IAA, on which 100% of regenerated shoot developed roots with an average number of 10.67 roots per shoot (Table 5).

**Table 4.** Effect of salts strength and plant growth regulators on shoot elongation in *Codonopsis lanceolata*

| Media | Plant growth regulator (mg/ℓ) | No. of shoot tested | Shoot length (cm) (Mean ± S.E) | Base Callusing* |
|---|---|---|---|---|
| ½ MS | | 20 | 3.60 ± 0.60 | ND |
| MS | | 20 | 2.73 ± 0.76 | ND |
| ¼ MS | | 20 | 2.33 ± 0.89 | ND |
| MS | $GA_3$ 3 | 20 | 4.27 ± 0.77 | ND |
| MS | $GA_3$ 5 | 20 | 5.70 ± 0.90 | ND |

* ND: Not detected.

## Acclimatization

Healthy rooted plantlets on rooting medium were separated, washed for removing agar and transferred to pots and covered with polyethylene bags to maintain high humidity. Subsequently, the humidity was reduced by making holes in the polythene. Among the different type of artificial soil tested, vermiculite and perlite 1:1 proved to be the most suitable for hardening of *Codonopsis lanceolata.* More than 90% of plants survived when transferred to glass house. After hardening in the glass house, the plants were transferred to the field. The morphology of the plantlets was uniform to the mother plant.

**Table 5.** Effect of salts strength and plant growth regulators on root induction in *Codonopsis lanceolata*

| Media | Plant regulators IAA | Growth (mg/ℓ) IBA | No. of shoot tested | No. of root/shoot (Mean ± S.E) | Rootlength (cm) (Mean ± S.E) | Base callusing* |
|---|---|---|---|---|---|---|
| ½MS | – | – | 15 | 3.33 ± 0.67 | 1.13 ± 0.12 | ND |
| MS | – | – | 15 | 2.67 ± 0.88 | 1.27 ± 0.17 | ND |
| ¼MS | – | – | 15 | 2.00 ± 0.58 | 1.57 ± 0.32 | ND |
| MS | 1 | – | 15 | 6.67 ± 1.20 | 2.33 ± 0.23 | C |
| MS | 2 | – | 15 | 7.00 ± 0.58 | 2.70 ± 0.20 | C |
| MS | 3 | – | 15 | 10.67 ± 1.33 | 3.10 ± 0.23 | C |
| MS | – | 1 | 15 | 5.67 ± 1.20 | 2.40 ± 0.10 | C |
| MS | – | 2 | 15 | 5.00 ± 1.53 | 2.83 ± 0.50 | ND |
| MS | – | 3 | 15 | 4.00 ± 0.58 | 3.00 ± 0.46 | ND |

* ND: Not detected; C: Callusing.

## DISCUSSION

Callus formation was significantly influenced by the type and concentrations of growth regulator. In the present study, suitable concentration of auxin and cytokinin was essential for the induction of calli. In most dicotyledons, the addition of a low concentration of cytokinin to media containing auxin tend to increase the growth rate of embryogenic callus (George, 1996). MS medium supplemented with 2 mg/ℓ 2,4-D was most effective for the morphogenic callus induction from the leaf explants. The frequency of callus induction from leaf explant was higher than petiole and internode. Similar variations between explants for callus induction was reported for other plant species such as, in strawberry (Passey et al., 2003), in *Alliums* (Myeors and Simon; 1998) in *Cuphea ericoides* (Rita and Floh; 1995) in red pepper (Christopher and Rajam; 1996). Whereas, medium containing 2 mg/ℓ NAA and 2 mg/ℓ IAA supported the induction of callus as well as development of excessive roots from the explants (Table 1). Majority of calli were induced from the cut edges of the explants. Sarwar and Skirvin (1997) reported that the cut edges provided a way for nutrients and growth regulators to be absorbed efficiently form the medium. Similarly, frequency of callus induction and shoot regeneration was higher when adaxial side of the explants were in contract with medium.

Among the different auxins tested, 2 mg/ℓ 2,4-D proved to be the best to induce somatic embryo. The fundamental role of the exogenous application of auxins, mainly 2,4-D which considered to be one of the main inductive factors for somatic embryogenesis (Ammirato, 1993). Increasing sucrose concentration from 3% to 6% enhanced induction of somatic embryo. Litze (1986), May & Trigians (1991) reported that the effect of high concentration of sucrose on the osmotic potential of the cells during somatic embryogenesis. The successful induction of embroygenesis depends on the tissue culture environment, e.g. condition for osmosis, concentration of sucrose, amino acids and salts and hormone balance (Emons, 1994). Similar result was also reported for other plant species such as in Asparagus (Levi & Sink, 1991) and in soybean (Ranch *et al.*, 1985).

The shoot regeneration frequency and the number of shoots per explant varied in leaf, petiole and internode explants. Most of the regeneration of adventitious shoots occurred from the midrib of the leaf explants. This may be explained by the presence of vascular tissue in the mid rib of leaf explants (Kumar *et al.*, 1998). However, the leaf base near to petiole showed higher frequency of shoot regeneration. An increased density of vascular tissue and level of phytohormone and metabolites near the petiolar region of the explants might be responsible for the increase in shoot regeneration (Karam & Al-majathoub, 2000). Single hormone treatment showed lower rate of shoot regeneration, whereas, auxin such as NAA in combination with BAP and TDZ showed significant rise in the percentage of shoot regeneration frequencies. Furthermore, induction of shoot per explant was higher when the concentration of BAP used was higher than 1 mg/ℓ. The frequencies of shoot regeneration reduced in the absence of cytokinin. The presence of a cytokinin in the induction medium is essential for adventitious shoot formation (Makunga, 2005).

However, NAA alone showed low frequencies of regeneration of shoot. Kane (1996) reported that cytokinin is the most crucial growth regulator for the suppression of apical dominance, a processes which leads to axillary shoot formation. 3%

sucrose was found to be most effective in the induction of adventitious shoot. However, an increase or decrease in the sucrose levels reduced the number of shoots per explants. Similar result was reported in cashew by Boggetti *et al.* (1999).

Among the auxins tested for rooting of shoot, 3 mg/ℓ IAA showed the best result. However, frequency of root induction and average root length without plant growth regulator were considerably higher. Similar results were obtained by Tawfik and Noga (2001), Ebrahimie *et al.* (2003) in cumin and Anzidei *et al.* (2000) in fennel. The use of low salts MS medium for rooting of the *in vitro* induced shoots is a very common practice (Mohamed 1992). This may be due to the need for only a small amount of total nitrogen for rooting (George & Sherington, 1984). The morphology of the acclimatized plantlets was similar to the mother plants. However, apparent genomic variations were detected in randomly tagged, phenotypically normal plants of *Codonopsis lanceolata* (Guo & Liu, 2006).

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