STUDY ON APPLICATION OF THIN CELL LAYER CULTURE FOR *IN VITRO* PROPAGATION OF *CHRYSANTHEMUM INDICUM*

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SUMMARY

Chrysanthemum indicum L. is a common flower which can bring highly economic, *C. indicum* is on of the most important cut flower and pot plant. Thin cell layer (TCL) culture is a potential method for *in vitro* propagation of *C. indicum*. However, this method is still limited in Vietnam. After sterilization with $HgCl_2$ 0.1% solution for 6 minutes and being cultured on Murashige T. and Skoog F. (1962) (MS) medium supplemented with 6-benzyl amino purine (BAP) 0.5 mg/l, α-naphthaleneacetic acid (NAA) 0.2 mg/l, sucrose 30 g/l, agar 7 g/l, the cultured samples were recorded with survival percentage of 81%, shoots were generated after 4 weeks. Callus induction and shoot regeneration on MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l, NAA 0.2 mg/l were obtained with 82.2% and 80%, respectively. Shoots were generated after 20.33 day on average. Multi shoots were generated by culturing on MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l, NAA 0.1 mg/l, the result was indicated by multi shoot rate reaching 4.31 and the average length of the shoot being 4.91 cm. Shoots were green and healthy. Highest rooting rate (97.78%) was obtained on MS medium supplemented with IBA 0.2 mg/l, NAA 0.3 mg/l and root length reaching 6.97 cm after 4 weeks of culture.

Keywords: Callus, *Chrysanthemum indicum*, *in vitro,* propagation, thin cell layer.

I. INTRODUCTION

Chrysanthemum indicum L. are herbaceous perennial plants with deeply lobed leaves and flowers in wide range of colors and sizes. *C. indicum* is a popular ornamental plant which origins from China, Japan, and several European countries. *C. indicum* appeared in Vietnam in the 15th century and has been widely used for decoration and as a medicinal plant. According to Vietnam medicinal plant dictionary, *C. indicum* has many good effects on human health such as detoxification, headache treatment (Chi V. V., 2011). *C. indicum* is normally propagated by rooting of cuttings but the quality declines over generations. Unlike the cutting, the *in vitro* propagation technique by thin cell layer (TCL) could overcome this problem.

TCL has been developed for over 30 years, and applied successfully to many plant species (Da Silva et al, 2003) or generated transgenic plants (Nhut D.T. et al, 2001). Recently, TCL has been studied in Vietnam for propagation of some plants such as orchid (Thach N. Q. et al, 2000), pineapple (Thach N. Q. et al, 2004) and *Spilanthes acmella* (Singh et al, 2009), *Sesamum indicum* (Chattopadhyaya et al, 2010), *Lilium* (Nhut D.T. et al, 2001; 2002).

In this study, we presented the data showing the ability of TCL on *in vitro* propagation of *C. indicum* and further application for commercial production.

II. RESEARCH METHODOLOGY

2.1. Materials

Immature flower buds of *C. indicum* be removed were collected in Dao Duc village, Binh Xuyen district, Vinh Phuc province, Vietnam.

2.2. Methods

Sterilization of plant materials: after cleaning by soap solution for 3 - 4 times immature flower buds of *C. indicum* were sterilized sequentially with 70% ethanol for 1 min, and $HgCl_2$ 0.1% solution for the different times with shaking. Finally, these flower buds were rinsed several times thoroughly with sterilized water.

TCL: After sterilization, immature flower

buds were dried by laying on sterilized filter papers in ventilation box. Petals and pistils were removed, the only calyx remained. Using a knife to cut calyx into thin slides (0.5 - 1 mm), then the slides were cultured on MS medium supplemented with BAP 0.5 mg/l, NAA 0.2 mg/l, sucrose 30 g/l and agar 7 g/l. After 4 weeks, the percentage of sterile samples and shoot formation from immature flower buds were recorded.

Callus induction and shoot regeneration: Sterile samples were cultured on MS medium supplemented with BAP (0.5 – 1.5 mg/l), NAA 0.2 mg/l, kinetin 0.2 mg/l, sucrose 30 mg/l and agar 7 g/l. After 4 weeks, the number of samples generating callus, the number of samples generating shoots and time regeneration of shoots were determined.

Shoot multiplication: Shoots were cultured on MS medium supplemented with BAP (0.3 - 0.5 mg/l), NAA (0.1 - 0.3 mg/l), Kinetin 0.2 mg/l, sucrose 30 g/l and agar 7 g/l. After 4 weeks, the number of shoots and shoot lengths were recorded.

Root formation: The shoots about 3 - 5 cm in length were transferred to another culture medium for root induction which included MS medium supplemented with IBA (0.2 - 0.5 mg/l), NAA (0.3 - 0.5 mg/l), sucrose 30 mg/l, agar 7 mg/l. After 4 weeks, root length, number of roots and other features were evaluated in order to select a suitable medium for root formation.

Plantlet acclimation: plantlets in the flasks were grown under natural light and temperature for 1 weeks. Subsequently, plants were transferred to the soil mediauma (garden soil, rice husks, sand at 2:1:1 ratio) containers and supplied water twice per day.

The pH of all culture media were adjusted to 5.8 before autoclaving at 118^{o}C for 17 min. All cultures were incubated at 25 ± 2^{o}C under 14 hours of photoperiod should be 2,000; 2,500 or 3000 lux, not in the wide range of photoperiod with fluorescence tubes.

The experiments were randomly designed with three replications and more than 30 samples per replication. Data were obtained and analyzed by excel program and Should have article in references.

III. RESULTS AND DISCUSSION

3.1. Plant materials

Sterilized flowers buds of *C. indicum* with $HgCl_2$ 0.1% solution for 4 minutes showed low survival rate of samples (33.67%). However, an increase in sterilization time for 6 minutes resulted in significantly increase in survival rate of samples (81%) and reduced necrosis. Comparatively, when increasing the time of $HgCl_2$ 0.1% solution treatment to 8 minutes (KT_3) and 10 minutes (KT_4) the higher survival percentages, 88.67%, and 93%, were obtained, but samples became worse. This can be explained by the toxicity of $HgCl_2$ 0.1% solution which can toxify plant tissues after sterilizing for a long time (Trang N. Q. et al, 2013; Jaime A. et al, 2015). Altogether, sterilization of *C. indicum* calyx by $HgCl_2$ 0.1% solution for 6 minutes showed the most efficient results (table 1).

Table 1. The influence of $HgCl_2$ 0,1% treatment on disinfection of samples

Media	Time of sterilization (minutes)	Survival rates (%)	Sample characteristics
KT_1	4	33.67	Yellow
KT_2	6	81.00	Green
KT_3	8	88.67	Yellow
KT_4	10	93.00	Black

3.2. Callus induction and shoot regeneration

Kinetin, BAP, and NAA can induce the proliferation of plant cells, particularly affecting on shoot regeneration (Ket N. V. et al, 2010).

The result (table 2) revealed that callus formation and shoot regeneration time increased linearly while shoot regeneration rate was decreased when increasing BAP concentration (0.5 - 1.5 mg/l). Poor results in callus formation, percentage and time of shoot regeneration were recorded on media without supplement of kinetin or NAA (MC_{1-6}). A high rate of callus formation, from 82.22% (MC_7) to 92.22% (MC_9) was obtained, the shoot regeneration was also decreased, reaching 20.33 - 24 days on average, when using culture media supplemented with BAP, kinetin, and NAA (MC_{7-9}). In general, the best results in callus formation percentage (82.22%), shoot formation percentage (80.0%) and shoot formation time (20.33 days) were recorded on MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l, NAA 0.2 mg/l.

Table 2. The influence of growth regulators on callus and shoot formation of *C. indicum*

Media	Growth regulators (mg/l)			Callus induction (%)	Shoot regeneration (%)	Shoot formation time (days)
	BAP	Kinetin	NAA			
MC_1	0.5	0.2	-	76.67	75.56	22.33
MC_2	1.0	0.2	-	80.00	67.78	26.00
MC_3	1.5	0.2	-	82.21	65.56	29.33
MC_4	0.5	-	0.2	72.22	70.00	23.67
MC_5	1.0	-	0.2	78.89	60.00	26.67
MC_6	1.5	-	0.2	80.00	58.89	29.33
MC_7	0.5	0.2	0.2	82.22	80.00	20.33
MC_8	1.0	0.2	0.2	87.78	73.33	22.67
MC_9	1.5	0.2	0.2	92.22	67.78	24.00

3.3. Shoot multiplication

The multiple shoot formation is crucial for the efficiency and speed of *in vitro* propagation. With the aim to proliferate shoots of *C. indicum*, we used the modified MS medium supplemented with BAP, kinetin, and NAA at different concentrations.

Table 3. The influence of growth regulators on multiple shoot formation of *C. indicum*

Media	Growth regulators (mg/l)			number of shoot per plantlet /per shoot	Shoot length (cm)	Shoot charateristics
	BAP	Kinetin	NAA			
NC_1	0.3	0.2	-	3.38	4.67	+++
NC_2	0.5	0.2	-	3.18	4.63	++
NC_3	0.3	0.2	0.1	4.31	4.91	+++
NC_4	0.5	0.2	0.1	3.48	4.70	+++
NC_5	0.3	0.2	0.2	3.76	4.63	++
NC_6	0.5	0.2	0.2	3.71	4.53	++
NC_7	0.3	0.2	0.3	3.62	4.53	++
NC_8	0.5	0.2	0.3	3.53	4.37	++

Note: +++: Shoots were long, big, dark green and healthy; ++: Shoots were short, small, light green and necrotic.

The minimum shoot number (3.38 in NC_1 and 3.18 in NC_2) was observed when the culture media supplemented only with BAP and kinetin. However, even on media supplemented BAP, kinetin, and NAA the gradual decreases in shoot number, length and condition were recorded with an increase in NAA concentration. This phenomenon can be due to the inhibiting effect of high concentration of NAA on the multi shoot formation. Overall, the optimum medium for the multi shoot generation was modified MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l and NAA 0.1 mg/l which gave the highest number of shoots (4.31), the length of shoots (4.91 cm), as well as green and healthy shoots.

3.4. Root formation

Shoots about 3 - 5 cm in length were transferred to root induction medium. Auxins are known as a useful growth regulator affecting positively on induction and development of roots (Han et al, 2009). Different concentrations of IBA (0.2 - 0.5 mg/l) and NAA (0.3 - 0.5 mg/l) were used to stimulate the root formation (table 4). High percentages of root formation (78.89 - 98.89%) were recorded in all root induction media. Among those media, RC_5 containing IBA 0.2 mg/l and NAA 0.3 mg/l showed a better rooting formation (97.78%), root number (7.03), root length (6.97 cm). Most of the roots were healthy and good quality. The minimum rooting percentage was seen in the medium without supplementation of NAA (RC_1).

After 4 weeks, the plantlets having healthy root set were transferred to the soil mediauma (garden soil, rice husks, sand at 2:1:1 ratio). After 2 weeks, the planets adapted to natural conditions produced/formed new leaves.

Table 4. Effect of growth regulator on rooting *C. indicum*

Media	Growth regulators (mg/l)		Root formation (%)	number of roots per plantlet	Root length (cm)	Root quality
	IBA	NAA				
RC_1	0.3	-	78.89	5.13	3.97	++
RC_2	0.5	-	88.89	5.18	4.33	++
RC_3	-	0.3	91.11	5.29	4.47	++
RC_4	-	0.5	93.33	5.66	4.23	++
RC_5	0.2	0.3	97.78	7.03	6.97	+++
RC_6	0.2	0.5	98.89	7.10	4.43	++

Note: +++: Roots were long, white with large number; ++: Roots were short, green with small number.

IV. CONCLUSION

Application of TCL for *in vitro* propagation of *C. indicum* was successfully conducted with the following results:

- Sterilization of calyx by $HgCl_2$ 0.1% solution for 6 minutes gave around 81% survival percentage of samples and reduced necrosis.

- Callus formation and shoot regeneration were induced on the modified MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l, NAA 0.2 mg/l. The percentage of callus formation and shoot regeneration reached 82.22% and 80%, respectively. Shoots were generated after 20.33 days on average.

- Multi shoot formation was recorded on the modified MS medium supplemented with BAP 0.5 mg/l, kinetin 0.2 mg/l, NAA 0.1 mg/l with good quality shoots, the average number of shoots per slide being 4.31 and shoot length being 4.91 cm.

- The optimal medium for root formation is

the modified MS medium supplemented with IBA 0.2 mg/l, NAA 0.3 mg/l. The percentage of root formation reached 97.78%, an average number of roots per shoot was 7.03, root had 6.97 cm in length and good quality.

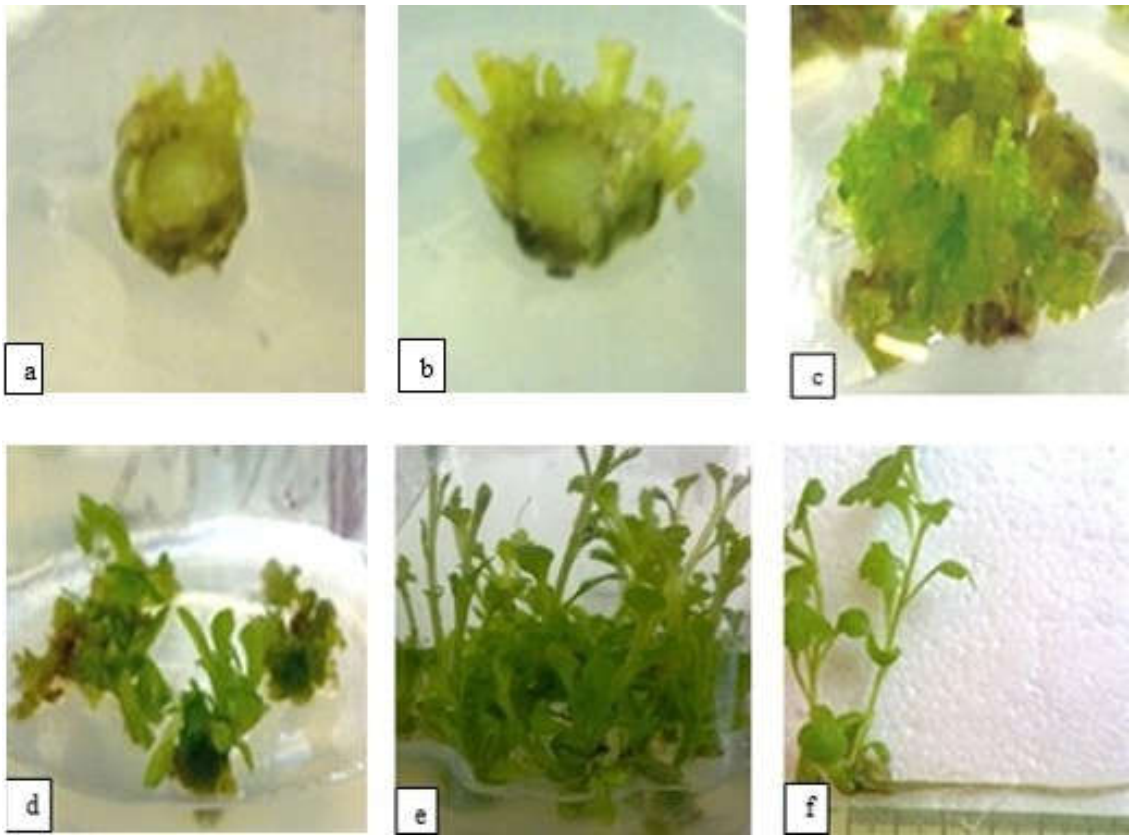

Figure 1. Stages of *in vitro* propagation of *C. indicum* by TCL

Note: (a) Calyx; (b) Calyx after 3 days; (c) Callus formation; (d) Shoot regeneration; (e) Plantlets cultured on NC_3 medium after 4 weeks; (f) Root formation.

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ỨNG DỤNG PHƯƠNG PHÁP NUÔI CẤY LÁT MỎNG TẾ BÀO TRONG NHÂN NHANH *IN VITRO* HOA CÚC VÀNG (*CHRYSANTHEMUM INDICUM* L.)

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TÓM TẮT

Hoa cúc vàng (*Chrysanthemum indicum* L.) là loài hoa phổ biến có thể mang lại giá trị kinh tế cao. Nhân giống *in vitro* thông qua nuôi cấy lớp mỏng tế bào là một phương pháp tiềm năng cho phép tạo ra lượng lớn cây con có năng suất và chất lượng tốt. Tuy nhiên, phương pháp này vẫn còn khá hạn chế ở Việt Nam. Kết quả nghiên cứu cho thấy khử trùng bằng dung dịch $HgCl_2$ 0.1% trong 6 phút và nuôi cấy trên môi trường Murashige T. và Skoog F. (MS) bổ sung 0.5 mg/L 6-benzylaminopurine, 0.2 mg/l α-naphthaleneacetic acid (NAA), 30 g/l sucrose và 7 g/L agar cho tỉ lệ sống là 81% sau thời gian 4 tuần nuôi cấy. Cảm ứng tạo mô sẹo và tái sinh chồi trên môi trường MS bổ sung 0,5 mg/l BAP, 0,2 mg/l kinetin, 0,2 mg/l NAA cho tỷ lệ mẫu tạo mô sẹo 82,22%, mẫu tái sinh chồi đạt tỷ lệ 80% với thời gian tái sinh 20,33 ngày. Cảm ứng tạo đa chồi trên môi trường MS bổ sung 0,5 mg/l BAP, 0,2 mg/l kinetin, 0,1 mg/l NAA cho hiệu quả nhân nhanh và kích thích tăng trưởng chồi tốt nhất, hệ số nhân chồi đạt 4,31 lần, chiều cao chồi đạt 4,91 cm, chồi mập, khỏe và có màu xanh đậm. Chồi ra rễ 97,78% và chiều dài rễ trung bình 6,97 cm khi nuôi trên môi trường MS bổ sung 0,2 mg/l IBA, 0,3 mg/l NAA sau 4 tuần nuôi cấy.

Từ khóa: Hoa cúc vàng, mô sẹo, nhân giống, nuôi cấy in vitro, nuôi cấy lát mỏng.

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