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Full Length Research Paper

Organogenesis and Somatic Embryogenesis in various Cultivars of *Codiaeum Variegatum* (L.)

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Croton (Codiaeum variegatum) with its amazing colors and leathery leaves is regarded as a beautiful foliage plant. The plant is native of South East Asia. Generally crotons are multiplied vegetatively by means of cuttings and air layering. This process is slow in response and need to maintain large number of mother plants stock. Micropropagation is an alternative mean of propagation that can meet its high demand in relatively shorter time. An improved and enhanced method was established for the In vitro propagation of croton. A series of experiments were conducted for the optimization of shooting and rooting media. enhanced shoots and buds proliferation formation can be achieved by using the MS media with 2mg/L of KIN and BA. The higher concentration of these hormones (5mg/l each) resulted in shoot formation. The In vitro roots were successfully induced by 5.0 mg/L of 2, 4-D. The rooted plants were then effectively acclimatized with the potting mix of 80% sand and 20% farm yard manure, as a potting mix, was best suited for the acclimatization of plantlets.

Keywords: Tissue Culture, Somatic Embryogenesis, MS Media, Hormones, Acclimatization.

INTRODUCTION

Codiaeum variegatum, commonly known as Croton and sometimes called Joseph's Coat, belongs to the family Euphorbiaceae, is one of the most popular ornamental plants because of vivid foliage colors and varied leaf shapes. Croton with their colorful, glossy foliage and variation of leaf types are one of the most popular plants in India. It is an evergreen shrub, up to 6 m in height but usually maintained at 60-90 cm and grows well in areas having humid climate. More than 200 varieties of croton exist on the globe, available in different leaf sizes, shapes and color patterns. The agricultural strategy is now much on to the ornamental plants production for local and exportation. Croton (*Codiaeum variegatum*) belongs to the family Euphorbiaceae is one of the beautiful indoors and out door plants need extensive agriculture development. The leaves extracts of crotons are reported to have many medicinal properties including purgative, sedative, antifungal, antiamoebic and anticancerous activities (Deshmukh and Borle, 1975; Kupchan et al., 1976). The plant is also well reputed for the production of valuable secondary metabolites of alkaloids, terpenes and flavanoids in nature (Puebla et al., 2003; Maciel et al., 1998; Martins et al., 2002). Croton can be propagated by various methods such as cuttings, grafting, by seeds and air layering. From shoot tip cuttings, one mother/stock plant can yield only 20 plants per year (our own nursery experience). Due to its slow rate of conventional multiplication, the plant is very high in demand. Micropropagation is a relatively new technology and application of innovative method have served to overcome barriers to progress in the multiplication of elite

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Figure 1: Three different cultivars of *Codiaeum variegatum* var. **Pictum** (Plate I A), *Codiaeum variegatum* var. **Curly Boy** (Plate I B) and *Codiaeum variegatum* var. **Pictum spot** (Plate I C).

species and further improvements are anticipated. *In vitro* growth and development is considerably influenced by several factors like genotype, the age and size of mother plant and explant, the season, growth conditions, media composition, and various other physiological factors (Nasib et al, 2008). The simultaneous occurrence of both regeneration pathways is rare (Matsuoka and Hinata, 1979; Rajasekaran *et al.,* 1983; Barwale *et al.,* 1986; Reynolds, 1986; Tabei *et al.,* 1991), because the acquisition of totipotency by somatic cells is difficult (Dudits and Gyogyey, 1991). Moreover, the requirements for regeneration through each pathway are different and not yet well defined. Croton *(Codiaeum variegatum* (L.) Blume, *Euphorbiaceae)* is an ornamental woody shrub from east Asia, and is one of about 200 species in this genus. Cultivar "Corazon de Oro" has characteristic yellow leaves and is one of the most economically important genotypes of this species in Argentina. Literature on tissue culture of *Codiaeum* species is not common and, to date, includes only two reports on endosperm and embryo culture (Chikkannaiah and Gayatri, 1974; Chikkannaiah et al., 1976, Marconi and Radice, 1997)

MATERIAL AND METHODS

Collection of samples

Fresh shoot samples of the three cultivars of *C. variegatum* (Figure 1) were collected from the Germplasm Repository at Littile Flower Nursery and Organic Manures, Alappuzha (Reg. No. 0901019936). Identification was carried out from the compiled checklist maintained on the stock and verified by Systematists in the Department of Biotechnology, Marthoma College of Science and Technology (Affiliated by University of Kerala), Ayur, India. The plants were sprayed with the fungicide and insecticide 2-3 week prior to start initiation and over head watering was strictly avoided. Freshly grown shoot tips, with two to three nodes, were selected as an explant source. Shoot tips were washed in running tap water for 10 minutes to remove the dust or sand particles. The tips were surface sterilized by using 0.5% of Sodium hypochlorite for 20 minutes. Few drops of Tween-20 were also added as a surfactant. After 20 minutes the plant material was washed three times with sterile distilled water to remove the traces of bleach with gentle shaking under sterile conditions.

Somatic Embryonic Induction, Maturation of Somatic Embryos and Shoot Elongation

Initial cultures were established in Murashige and Skoog's 1962 (MS) media. Separate stock solution of macro and micro salts, vitamins and amino acids were prepared in double distilled water and stirred at 4 ± 1^{o} C. Stocks of plant growth regulators were also prepared and kept under refrigerating conditions. (Table 1). The prepared medium was supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 and gelled with 0.8% agar-agar (Hi media, India). The medium, complete with all ingredients was dispensed into autoclaved culture tubes and glass bottles. The tubes were plugged with non-absorbent cotton plugs wrapped in double layered cheese cloth and jars with parafilim (American Can corp.). Each explant was cultured on MS basal medium supplemented with various combination of hormones were used (Table 2).

The explants i.e., leaflets, stem segments, were inoculated in MS medium containing hormones like BA, KIN, IAA, NAA and 2,4D (1-6 mgl^{-1}) (Table 3). Callus obtained were visually analyzed for its growth rate. The

Table 1 MS medium (Murashige and Skoog, 1962) cited from George *et al.,* 2008

Macro salts		**Mgl^{-1}**	**Stock A [500 ml - 20x]**	**Qty ml/l medium**
1.	KNO_3	1900	16.5 g	50
2.	$NH_4.\ NO_3$	1650	19.0g	
3.	$CaCl_2.\ 2H_2O$	440	4.4g	
4.	$Mg\ SO_4\ 7H_2O$	370	3.7g	
5.	$KH_2\ PO_4$	170	1.7g	
Micro salts		**Mgl^{-1}**	**Stock B [500 ml - 200x]**	
6.	$MnSO_4.\ 4H_2O$	22.3	2.23g	5
7.	$Zn\ SO_4.\ 7H_2O$	8.6	860mg	
8.	$H_3\ BO_3$	6.2	620mg	
9.	KI	0.83	83mg	
10.	$CuSO_4.\ 5H_2O$	0.025	2.5mg	
11.	$Na_2\ MoO_4.\ 2H_2O$	0.25	25mg	
12.	$CoCl_2.\ 6H_2O$	0.025	2.5mg	
Iron salts		**Mgl^{-1}**	**Stock C [100 ml - 100x]**	
13.	$FeSO_4.\ 7H_2O$	27.85	278.5	10
14.	Na_2 EDTA $2H_2O$	37.25	372.5	
Vitamins & Amino acid		**Mgl^{-1}**	**Stock D [100 ml - 100x]**	
15.	Thiamine HCl	0.1	1mg	10
16.	Nicotinic acid	0.5	5mg	
17.	Pyridoxine HCl	0.5	5mg	
18.	Glycine	2	20mg	
19.	Inositol	100 mgl^{-1}	Added separately	
20.	Sucrose 3 %	30g	Added separately	
21.	Agar 0.8%	8g	Added separately	
	pH	5.8		
	Autoclaving	Pressure 15 lb/inc^2 & Temperature 121^{0}C (250^0 F) for 15-20 min		

Table 2 Preparation of Plant growth hormone stock

Hormones	**Abbr.**	**Qty (mg) ***	**Solvents**
AUXINS			
Indole-3-acetic acid	IAA	10	Ethanol/ 1N Na OH
1-Naphtheline acetic acid	NAA	10	1N Na OH
2, 4-dichloro phenoxy acetic acid	2,4-D	10	Ethanol/ 1N Na OH
Indole-3-butyric acid	IBA	10	Ethanol
CYTOKININS			
Benzyl adenine	**BA**	10	**1N Na OH**
6-Furfuryl aminopurine (Kinetin)	KIN	10	1N Na OH
GIBBERELLINS			
Gibberellic acid	GA_3	10	dd H_2O

Note: 10ml stock (Conc: 1 mgl^{-1}) Preparation: Dissolve 10 mg hormone in 0.5 ml of the respective solvents and gradually diluted to 10ml using distilled water.

callus obtained from different explants were sub cultured at regular intervals of 25 - 30 days either to the same fresh medium or to a medium supplemented with varying combination of hormones. The *In vitro* rooting in croton was studied using MS medium with different concentrations of IAA and IBA. All the media formulations for root induction had 2.5% sugar and 2.5 gm/L phytagel as a solidifying agent.

RESULTS AND DISCUSSION

An efficient and reliable system for the *In vitro* propagation of *Codiaeum variegatum* has been optimized. These set of experiments have produced valuable results which are discussed in the following sections:

Table 3 The Callus Induction medium Explants: Leaf segments

Hormones (mgl^{-1})				
BA	**NAA**	**KIN**	**IAA**	**2,4 - D**
1-6	-	1-6	-	-
1-6	1-6	-	-	-
-	-	-	1-6	-
-	1-6	1-6	-	-
-	-	1-6	-	1-6

Table 4 Response of Somatic Embryogenesis of leaf segments in Callus Induction medium

	Hormones (mgl^{-1})	Response		
		Codiaeum var. Pictum	*Codiaeum var. Curly Boy*	*Codiaeum var. Pictum spot*
1.	1 mgl^{-1} NAA & KIN	**+++**	**+**	**+**
2.	2 mgl^{-1} BA & 4 mgl^{-1} NAA	**++**	**+++**	**+++++**
3.	2 mgl^{-1} BA & 4 mgl^{-1} KIN	**++**	**++**	**+++**
4.	1 mgl^{-1} BA & KIN	**++**	**+**	**+**

** Data recorded after 45 days of incubation in respective medium*

Table 5 Response of Organogenesis in Callus Regeneration (Shoot & root formation)

	Hormones (mgl^{-1})	Response*					
		Codiaeum var. Pictum		*Codiaeum var. Curly Boy*		*Codiaeum var. Pictum spot*	
		shoot	**Root**	**shoot**	**Root**	**shoot**	**root**
1.	1 mgl^{-1} IAA	-	-	-	-	**2**	**-**
2.	5 mgl^{-1} 2,4-D & 1 mgl^{-1} KIN	**1**	**-**	**-**	**-**	**-**	**-**
3.	1 mgl^{-1} BA & 1 mgl^{-1} NAA		**2**	**-**	**-**	**2**	**-**
4.	2 mgl^{-1} 2,4-D & 1 mgl^{-1} KIN	**1**	**2**	**-**	**-**	**-**	**-**
5.	1 mgl^{-1} 2,4-D & 2 mgl^{-1} KIN	**6**	**2**	**-**	**-**	**1**	**-**
6.	2 mgl^{-1} BA & KIN	**2**	**-**	**3**	**1**	**-**	**-**
7.	1 mgl^{-1} 2,4-D & 3 mgl^{-1} KIN	**-**	**-**	**2**	**3**	**-**	**-**
8.	4 mgl^{-1} BA & KIN	**8**	**1**	**-**	**--**	-	-

* Data recorded after 45 days of incubation in respective medium

Table 6 Response of Somatic Embryogenesis/Organogenesis in Callus growth rate of cultivars

		Response (Callus weight in mg)	
		Mature leaf	**Young leaf**
1	*Codiaeum var. Pictum*	**0.471**	**0.662**
2	*Codiaeum var. Curly Boy*	**0.305**	**0.540**
3	*Codiaeum var. Pictum spot*	**0.219**	**0.458**

Codiaeum var. Pictum **1** mgl^{-1} **NAA & KIN**
Codiaeum var. Curly Boy **2** mgl^{-1} **B**A & **4** mgl^{-1} **NAA**
Codiaeum var. Pictum spot **1** mgl^{-1} **B**A & KIN

* Fresh weight differences after 30 days of exposure; Data recorded are mean of 7 replicates

Micropropagation

Leaf Explant segments were established in culture after 15 days on MS medium supplemented with various composition of BA, NAA and KIN. After this establishment period, the multiplication step was accomplished with axillary and adventitious buds that formed in the leaf axils and at the base of the shoot on the cut surface,

Induction of somatic embryos

Adventitious buds and somatic embryos were observed after the subculture on multiplication medium from expanded in the culture medium. A large callus proliferation was observed when leaf sections were cultured on media supplemented with auxin (BA, NAA and KIN).

Plate I

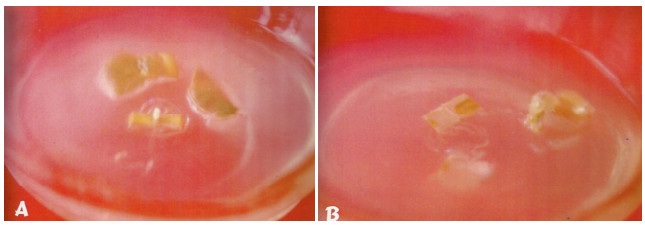


Plate II

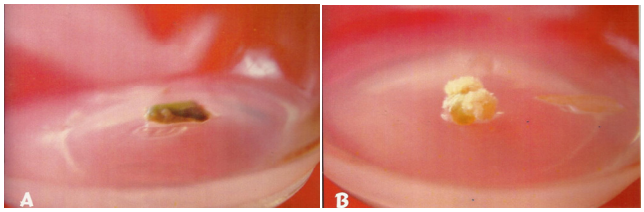


Plate III

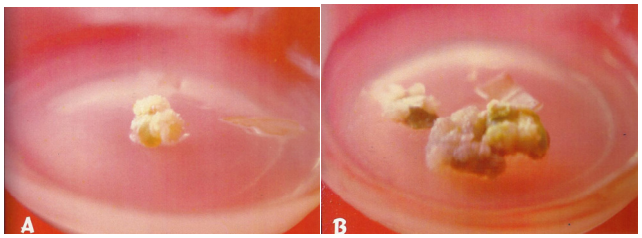
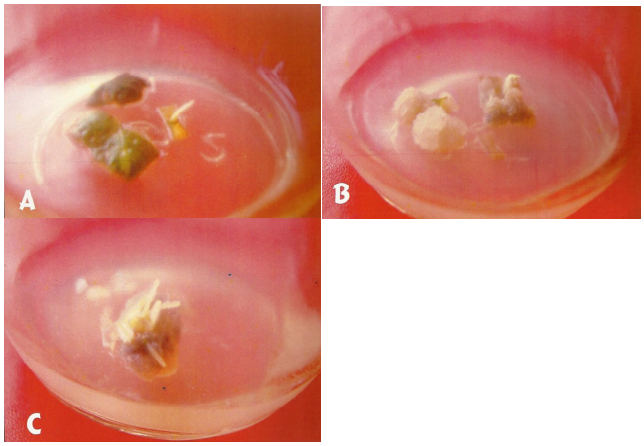


Plate IV



Callus initiation and Proliferation

Though leaf and stem explants of the three plants were used for callus induction, only leaf explants responded consistently. Hence for the current study leaf explants were consistently used. Properly trimmed explants were inoculated in MS medium containing various hormonal milieus as shown in Table 4-6. Two basal salt mixtures (high and low), Woody Plant Media (Lloyd and McCown, 1980) and MS Media (Murashige and Skoog Media, 1962), were used initially to study the effect of basal media on shoot multiplication.

The Leaf explants of the three cultivars were cultured in MS medium fortified with varying hormonal milieu. Initial response of the cultivars in MS medium was more or less similar and indistinguishable. Pictum culivars cultured in 1mg/l NAA and KIN containing medium showed explant swelling with 10 days of inoculation (Plate I A and B). Though apparent signs of callusing was observed in Curly boy (Plate II A and B) and Pictum spot (Plate III A

Plate V

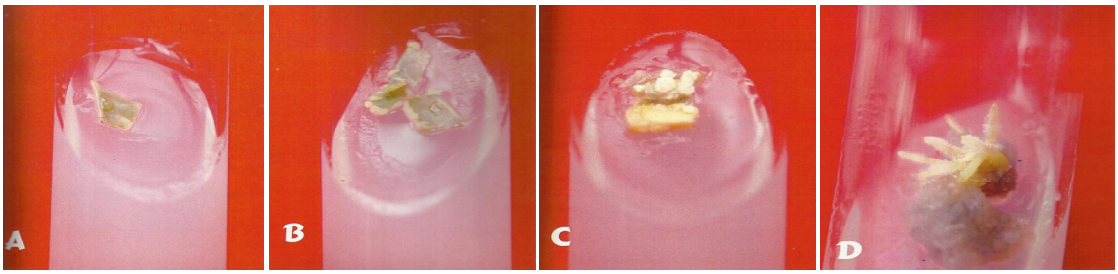


Plate VI

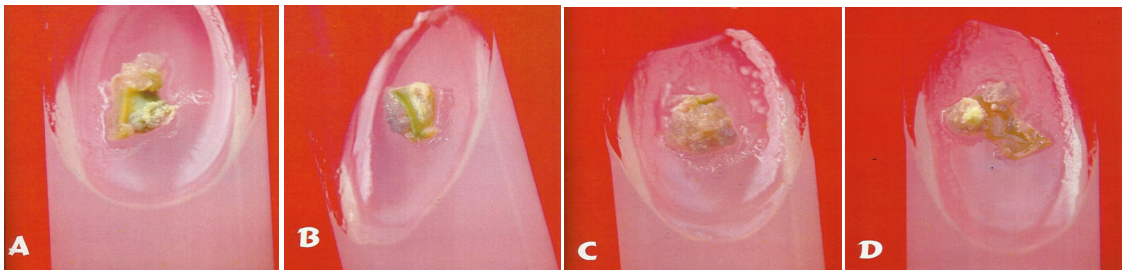


Plate VII

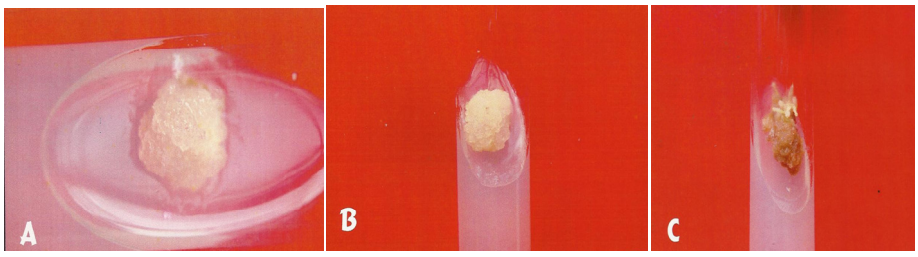


Plate VIII

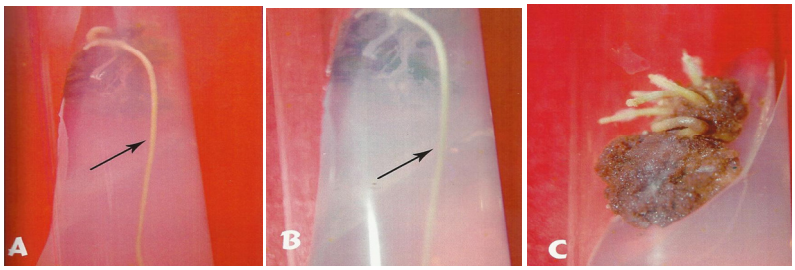


Plate IX

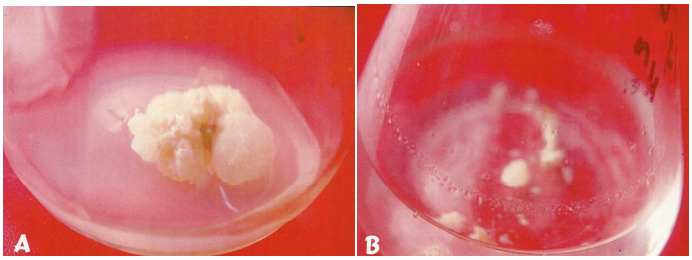


Plate X

Figure 2 (Plate I –Plate IX): Various phases of Somatic Embryogenesis, Organogenesis and acclamatization in different cultivars of *Codiaeum variegatum.*

and B) in the same medium better initiation and callus development was observed in MS medium supplemented with 2mg/l BA and 4mg/l NAA. The nature of the callus developed was whitish nodular in all the three varieties. Callus regeneration was attempted in all the varieties. Among the various hormonal compositions tried 1mg/l 2,4-D along with 1mg/l KIN (Plate IV A and B) and 2mg/l BA and KIN (Plate IV C) containing medium was conducive for shoot proliferation in Pictum cultivers. Curly leaf segments of cultivar curly boy were inoculated in MS medium augmented with 2mg/l BA and KIN. Swelling along the cut end was visible after 10 to 15 days of inoculation (Plate V A and B). Explants cultured in medium containing 1mg/l 2,4-D and 3mg/l KIN (Plate V C) produced translucent watery callus. This callus upon transferred to cytokinin rich medium (MS 2mg/l BA + KIN) resulted in shoot proliferation (Plate V D). Approximate 3-4 shoots were obtained in this medium. However higher concentration of these hormones (5mg/l each) resulted in vitrification of shoots. Leaf explants of variety Pictum spot was inoculated in medium supplemented with 1mg/l BA and NAA. Profuse callusing with occasional shoot sprouting was observed (Plate VI A, B, C) callusing rate increased in 1mg/l 2,4-D and 2mg/l KIN containing medium (Plate VI D). Incorporation of 2, 4- D to the tune of 5mg/l resulted in the formation of highly watery sponge like callus (Plate VII A-C). Easily separable callus clumps was observed in this medium. Prolonged exposure in this medium resulted in *de novo* root formation from the callus surface (Plate VIII A-C). Translucent callus obtained in 4 mg/l 2,4- D (Plate VII A) containing medium was used to initiate suspension culture (IX A). The medium was supplemented with 2mg/l KIN to promote early organogenesis. Few globular shaped structures were observed in this medium after a period of 30 days. Separated clumps were later plated in MS medium fortified with 2mg/l BA and KIN (Plate IX B) as shown in Figure 2. Adventitious shoots and somatic embryos were simultaneously induced on the same explant for *Codiaeum variegaturn cv.* These two morphogenic processes have previously been observed to occur simultaneously in leaf cultures of various woody species [i.e. Olea (Rugini, 1988); *Populus nigra* X P. maximowiczii. (Park and Son, 1988); *Rubus* (Fiola et al., 1990); *Camellia* (San Jose and Vieitez, 1993)]. Woody plant explants from juvenile specimens usually respond more readily than those from mature trees (San Jose and Vieitez, 1993). Thus, we could explain the differences observed between leaves taken from greenhouse plants and micropropagated buds in this study. A common feature of earlier protocols for adventitious shoot regeneration has been the use of high cytokinin and low auxin concentrations in the media (Toonen et al., 1996). In addition, it seems probable that leaf explants have a high level of endogenous auxins and need an exogenous cytokinin to induce embryo development (Oliveira and Pals, 1992).

Acclimatization

The rooted plants were then transferred to the green house for hardening. The potting mix used in this study comprised of 80% sand and 20% farm yard manure. The transferred plants were monitored after every week for at least 6 weeks as Shown in Fig 2 (Plate X).

Plate I: A and B *Codiaeum variegatum* var. Pictum cultivars cultured in 1mg/l NAA and KIN containing medium showing explant swelling.

Plate II: A and B *Codiaeum variegatum* var. Curly boy cultured in 1mg/l NAA and KIN containing medium showing explant swelling.

Plate III: A and B *Codiaeum variegatum* var. Pictum spot cultured in 1mg/l NAA and KIN containing medium showing explant swelling.

Plate IV: Callus Regeneration *Codiaeum variegatum* var. Pictum

A. Early stages of callusing in *Codiaeum variegatum* var. Pictum cultivars cultured in MS +1mg/l 2, 4-D and 1mg/l KIN

B. Shows bud sprouting

C. Shoot proliferation in MS+2mg/l BA and KIN

PLATE V: Callus Regeneration *Codiaeum variegatum* var. Curly boy

A and B Leaf segments showing callusing along the cut ends in MS+2mg/l BA and KIN

C. Leaf explants cultured in MS+1mg/l 2, 4-D and 3mg/l KIN

D. Callus regeneration in MS+2mg/l BA and KIN. Note the shoot proliferation

Plate VI: Callus Regeneration *Codiaeum variegatum* var. Pictum Spot

A-C Leaf explants of variety Pictum spot inoculated in medium supplemented with 1mg/l BA and NAA showing profuse callusing with occasional shoot sprouting.

D Increased callusing rate in MS+1mg/l 2,4-D and 2mg/l KIN

PLATE VII: A-C Formation translucent callus in medium containing 5mg/l 2,4- D

Plate VIII: A-C *De novo* root formation from the callus surface in MS+ 5mg/l 2,4- D

PLATE IX: A Translucent callus obtained in MS+4 mg/l 2,4- D and B Suspension culture

PLATE X: The rooted plants were then effectively acclimatized with the potting mix

CONCLUSION

The protocol here in described is very much efficient for the *In vitro* multiplication of *Codiaeum variegatum*. In the light of our results, it can be suggested that enhanced shoots and buds proliferation formation can be achieved by using the MS media with 2mg/L of KIN and BA. The higher concentration of these hormones (5mg/l each) resulted in vitrification of shoots exponentially increase the axillary shoots formation. The *In vitro* roots were successfully induced by 5.0 mg/L of 2,4-D. The rooted plants were then effectively acclimatized with the potting mix of 80% sand and 20% farm yard manure (v/v).

REFERENCES

ad'ale UB, Kems HR, Widholm JM (1986). Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167:473-481.

Chikkannaiah PS, Gayatri MC (1974).Organogenesis in endosperm tissue cultures of *Codiaeum variegatum* Blume. Curr. Sci. 43:23-24.

Chikkannaiah PS, Nataraja K, Gayatri MC (1976). Responses of seeds and isolated embryos of *Codiaeurn variegatum in vitro.* Curt. Sci. 45:196- 197,

Deshmukh SD and MN Borle (1975). Studies on the insecticidal properties of indigenous plant products. *Ind. J. Ent.,* 37(1):11-18.

Dudits D, Bogre L, Gyogyey J (1991). Molecular and cellular approaches to the analysis of plant embryo development from somatic ceils *in vitro. J.* Cell Sci. 99:475-484.

Fiola JA, I-Iassan MA, Swartz HJ (1990). Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tissue Organ Cult. 20:223-228.

George EF, Hall MA and Klerk JD (2008). Plant propagation by tissue culture. Volume 1. The Background. Springer, pp. 65-75.

Kupchan SM, Uchida I, Branfman AR, Dailey RC and Fei BY (1976). Antileukemic principles isolated from euphorbiaceae plants. *Sci.,* 191:571-572.

Lloyd G and McCown B (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Proc. Intl. Plant Prop. Soc*., 30: 421-427.

Maciel AM, Pinto AC, Brabo SN and Silva MN (1998). Terpenoids from Croton cajura. *Phytochem.,* 49: 823-826.

Marconi PL and Radice S (1997). Organogenesis and Somatic Embryogenesis in Codiaeum Variegatum (L.) Blume Cv. "Coraon De Oro".

Martins AP, Salgueiro LR, Conclaves MJ, Vila R, Tomi F, Adzet T, Cunha AP, Canigueral S and Casanova J (2002). Antimicrobial activity and chemical composition of bark oil of Croton stellulifer. *Planta Medi.*, 66: 647-652.

Matsuoka M, Hinata K (1979). NAA-induced organogenesis and embryogenesis in hypocotyl callus of *Solanum melongena* L. J. Exp. Bot. 30 (116):363- 370.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*., 15: 473-497.

Nasib A, Ali K and Khan S (2008). *In Vitro* Propagation of Croton (*Codiaeum Variegatum*). *Pak. J. Bot*., 40(1): 99-104

Oliveira MM, Pals MS (1992). Somatic embryogenesis in leaves and leaf-derived protoplast *ofActinidia deliciosa* var. ddiciosa cv. Hayward (kiwi fruit). Plant Cell Rep. 11:314-317.

Park YC, Son SH (1988). *In vitro* organogenesis and somatic embryogenesis from punctured leaves *of Populus nigro X P: maximmviczii*. Plant Cell Tissue Organ Cult. 15:95-105.

Puebla P, Lopez JL, Guerrero M, Carron R, Martin ML, Roman LS and Feliciano AS (2003). Neo-clerodane diterpenoids from Croton schiedeanus. *Phytochem.,* 62: 551-554.

Rajasekarart K, Mullins MG, Nair Y (1983). Flower formation *in vitro* by hypocotyl explants of cucumber *(Cucumis sativLLz* L.) Ann. Bot. 52:417420.

Reynolds TL (1986). Somatic embD'ogenesis and organogenesis from callus cultures of *Solanum carolinense*. Am. J. Bot. 73:914-918.

San Jose MC, Vieitez AM (1993). Regeneration of *Camellia* plantlets from leaf explain cultures by embD'ogenesis and caulogenesis. Sci. Hortic. 54:303-315.

Tabei Y, Kanno T, Nishio T (1991). Regulation of organogenesis and somatic embryngenesis by auxin in melon, *Cucumis meto* L. Plant Cell Rep. 10:225-229.

Toonen MAJ, Schmidt EDL, De Vries SC (1996). Cell tracking as a tool to study initial processes in somatic embryogenesis to development. Plant Tissue Culture Biotech. 2(1):3-10.