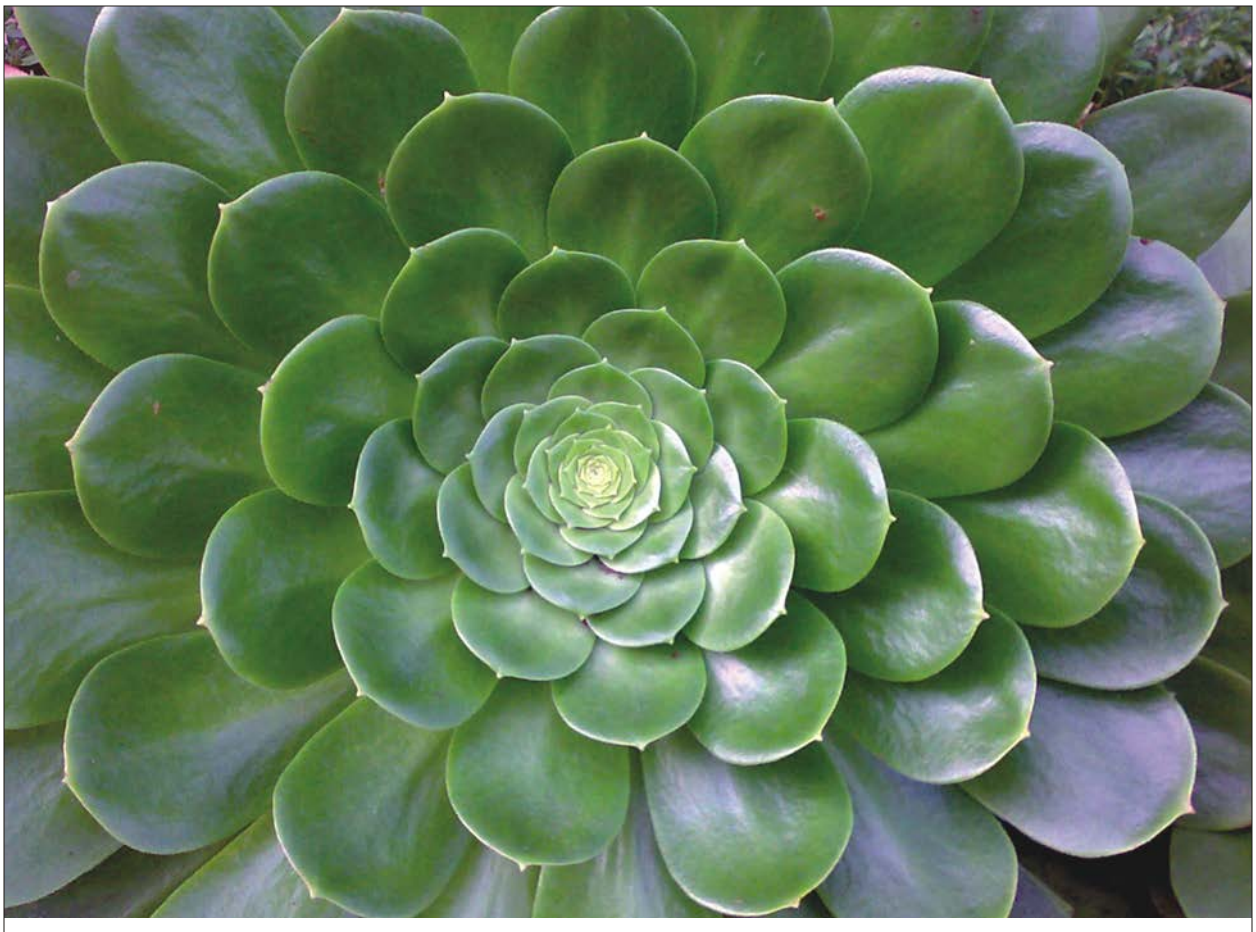

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Protocol Establishment for Micro propagation and *in vitro* Callus Regeneration of Maulavi Kachu (*Xanthosoma sagittifolium* L Schott.) From Cormel Axillary Bud Meristem

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Abstract: The effects of various growth regulators on organogenesis from cormel axillary bud meristems of cultivated species of Maulavi kachu (*Xanthosoma sagittifolium* (L). Schott. were investigated. Highest percentage (65%) direct regeneration takes place within 25 days of culture but significant number of shoot per plant and length of shoot did not found significantly. Highest percentage (90%) calluses were initiated from axillary buds on MS medium supplemented with 1 mg L^{-1} BAP+2.0 mg L^{-1} NAA. Light green, greenish friable or semi friable calluses were found. The highest fresh weight of callus was 11.60 g. These calluses were cultured into MS media containing with 3 mg L^{-1} Kn + 2 mg L^{-1} NAA to induce plantlets regeneration. Highest percentage (86%) regeneration was observed with single or aggregated green shoot buds. The average number of green shoot, daughter buds and plantlets were 2.60 and average length of shoots were 7.0 cm with the same medium. Highest percentage of root induction and proliferation were also found into media containing 0.4 mg L^{-1} IAA and number of roots and root length of regenerated plantlets were 7.90 No. and 6.90 cm, respectively. Plantlets were then established with normal and natural fertile soil on plastic pot showed best performance in acclimatization.

Key words: Callus, maulavikachu, organogenesis, meristem, acclimatization, regeneration, *Xanthosoma sagittifolium*

INTRODUCTION

Maulavi kachu is one of most important root and tuber crop world wide (Jennings, 1987; Onwueme and Charles, 1994) and cultivated in the tropics and subtropics as important root crops and are widely affected by dasheen mosaic viruses (Zettler *et al*., 1970). It is a neglected edible aroid crop in Bangladesh, mainly grown by small scale farmers and attempts to improvement have therefore been limited. *Xanthosoma sagittifolium* (L.) Schott commonly known as maulavi kachu in Bangladesh, belongs to the monocotyledonous family Araceae. The corm, cormels and green leaves of maulavi kachu are important source of carbohydrates for human nutrition, animal feed (Ndoumou *et al*., 1995; Nyochembeng and Garton, 1998) and of cash income for village farmers (Tambong, 1997). The crop is mainly cultivated by small scale farmers (Onwume and Charles, 1994) in Asia, Africa and Latin America (Wilson, 1984). In spite of its importance as a staple food in many countries, maulavikachu could receive very little research attention (Goenaga and Heperly, 1990) and is regarded as an under exploited and insufficiently studied crop (Nguyen and Nguyen, 1987; Giacometti and Leon, 1994; Watanabe, 2002).

In recent years biotechnology offers enormous opportunity for improvement of food crops which equally hold the potential for the improvement of aroids including maulavikachu (*Xanthosoma sagittifolium* (L.) Schott) (Kacker *et al*., 1993). Previous study on callus initiation in *Xanthosoma* sp. using shoot and/or meristem tips has been reported (Hartman, 1974; Strauss *et al*., 1979;

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Licha *et al*., 1982. Acheampong and Henshaw (1984) also reported development of protocorms directly from shoot tips in agitated media. Many researchers already reported some tissue culture research study on some species of aroids. Tissue culture techniques open up many possibilities for sustainable production and improvement of crops. Meristem culture of maulavi kachu have been reported (Tsala *et al*., 1996; Zok *et al*., 1998) and meristem derived plants perform better in terms of yield than virus infected *in vitro* plants (Reys *et al*., 2006). Meristem derived plants are also important for a safe exchange of germplasm between countries. Tissue culture also gives the possibility to generate new genotypes through somaclonal variation and a variety of morphological changes in callus derived maulavi kachu (*Xanthosoma sagittifolium* (L.) Schott) plants has been reported by Gupta (1985). Plant regeneration from tissues and cell culture is important in achieving rapid clonal multiplication, recovery of pathogen free plants, preservation of valuable germplasm and induction of chromosomal and genic variation (Murashige, 1974; Vasil and Vasil, 1980; Larkin and Scoweroft, 1981). The technique is particularly valuable in vegetatively propagated plants,where stem segments and tubers encourage the spread of many pathogens resulting in severe loss of plant vigor and productivity like maulavi kachu (*Xanthosoma sagittifolium* (L.) Schott). In this study we describe a complete *in vitro* protocol for Micro propagation, callus induction and their regeneration for maulavikachu offering the scopes for culturing virus free propagules and under taking genetic engineering research of this crop for its further improvement.

MATERIALS AND METHODS

Plant Materials

In the present study axillary bud meristem used as explants of Maulavikachu were collected from field grown (2004-2006) cultivated species from experimental research farm of Institute of Biological Sciences, Rajshahi University. Bangladesh.

Explants and Media Preparation

The collected axillary bud meristem attached with cormels washed under running tap water. Then terminal sprouts were excised from cormels and surface sterilized with 1-3 drops of tween-80 and Savlon per 100 mL water for 15 min followed by several washing with sterilized distilled water.Surface sterilization was carried by dipping 0.10% $HgCl_2$ by gentle shaking for 15 min. The sterilized materials were washed 4-5 times with sterile distilled water immediately to remove all traces of $HgCl_2$. The basic culture medium was MS (Murashige and Skoog, 1962) supplemented with various concentrations and combinations of IAA, 2, 4-D NAA, BAP and Kn and. The media were adjusted to pH 5.8 and subsequently were jelled with 0.8% agar and 3% sugar and autoclaved for 20 min at 121°C and 1.1 kg cm^2. Explants were inoculated longitudinally on to media containing culture bottle in a laminar flow cabinet. Cultures were incubated at 26+1°C under the warm in fluorescence light intensity varied from 2000-3000 lux. The photo period was maintained 16 h light and 8 h dark. Calluses were aseptically weighed and regenerated shoot lengths were measured.

RESULTS AND DISCUSSION

Direct Regeneration

For direct regeneration three auxins (NAA, IAA, 2, 4-D) and Cytokinins (BAP and Kn) were used in MS basal medium in different concentration s and combinations and their data are presented in Table 1. Highest percentage (65%) of plantlets regeneration was found with the number of shoot per plant (1.00) and shoot length (0.783 cm) in MS media supplemented with 0.50 mg L^{-1} BAP+1.0 mg L^{-1} IAA followed by media containing 0.50+1.50 mg L^{-1} with same number of

Table 1: Effect of different concentration and combination of BAP, KIN, IBA, NAA, 2, 4-D on direct shoot regeneration from axillary cormel bud explants of Maulavi kachu (*Xanthosoma sagittifolium* (L.) Schott. and Data were recorded after 4 weeks of culture

Growth regulator (mg L^{-1})	Days to shoot proliferation	Percentage of shoot forming from explant	No. of shoot per plant	Shoot length (cm)
NAA			Mean±SE	Mean±SE
0.5	20-25	9.00	1.00±0.000	0.2833±0.003
1.0	20-25	14.00	1.00±0.000	0.4667±0.004
1.5	20-25	31.00	1.33±0.2108	0.5833±0.007
2.0	20-25	45.00	1.33±0.2108	0.9000±0.005
2.5	20-25	26.00	1.00±0.0000	0.5000±0.002
BAP+NAA				
1.00+0.50	20-25	16.00	1.00±0.0000	0.1167±0.001
1.00+1.00	20-25	35.00	1.00±0.0000	0.1833±0.002
1.00+1.50	20-25	43.00	1.00±0.0000	0.2667±0.003
1.00+2.00	20-25	33.00	1.00±0.0000	0.2167±0.002
1.00+2.50	20-25	27.00	1.00±0.0000	0.1833±0.001
BAP+2,4-D				
0.50+0.50	20-25	08.00	1.00±0.0000	0.1167±0.001
0.50+1.00	20-25	11.00	1.00±0.0000	0.1667±0.002
0.50+1.50	20-25	28.00	1.00±0.0000	0.2000±0.002
0.50+2.00	20-25	17.00	1.00±0.0000	0.1333±0.002
0.50+2.50	20-25	17.00	1.00±0.0000	0.1167±0.016
BAP+IAA				
0.50+0.50	20-25	24.00	1.00±0.0000	0.3000±0.003
0.50+1.00	20-25	65.00	1.00±0.0000	0.7833±0.003
0.50+1.50	20-25	48.00	1.00±0.0000	0.6833±0.003
0.50+2.00	20-25	40.00	1.00±0.0000	0.4833±0.003
0.50+2.50	20-25	22.00	1.00±0.0000	0.2000±0.002
KIN+NAA				
2.00+0.50	20-25	8.00	1.00±0.0000	0.1333±0.002
2.00+1.00	20-25	13.00	1.00±0.0000	0.2167±0.003
2.00+1.50	20-25	24.00	1.00±0.0000	0.2333±0.002
2.00+2.00	20-25	40.00	1.00±0.0000	0.3167±0.003
2.00+2.50	20-25	20.00	1.00±0.0000	0.2167±0.003
KIN+IAA				
2.00+15.00	20-25	21.00	1.00±0.0000	0.2000±.0.000
2.00+20.00	20-25	26.00	1.00±0.0000	0.2500±0.002
2.00+25.00	20-25	43.00	1.00±0.0000	0.4333±0.003
2.00+30.00	20-25	25.00	1.00±0.0000	0.2500±0.002
2.00+35.00	20-25	14.00	1.00±0.0000	0.1500±0.002

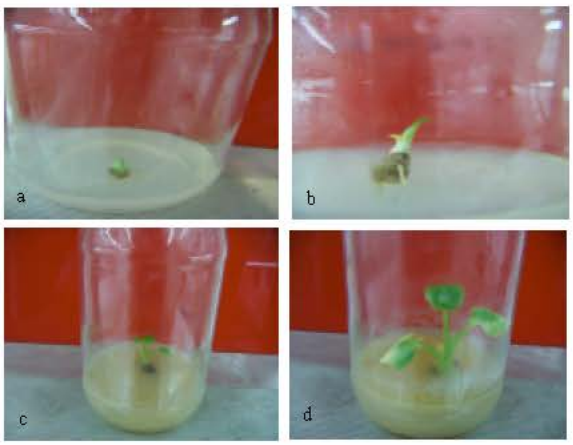

Fig. 1: Showing different stages of direct regeneration in *Xanthosoma sagittifolium* (L.) Schott, (a) Insertion of fresh axillary cormel bud into the MS medium supplemented with 0.5 mg BAP L^{-1} + 1.0 mg IAA L^{-1}, (b) Shoot regeneration after 20 days from insertion to the medium containing with 0.5 mg BAP L^{-1} +1.0 mg IAA L^{-1}, (c) Shoot regeneration after 25 days from insertion to the medium with same medium and (d) After 30 days observation

shoot and shoot length. In most observation the and regeneration percentage is slow and low. Data was recorded Four weeks after from culturing date (Table 1, Fig. 1). Reys (2006) acheived the highest percentage of regenerating explants from NG,cocoyam (83%) using 1.0 mg L^{-1} IAA in combination with 0.5 mg L^{-1} BAP to the medium. Faria and Illg (1995) reported that the addition of 10 μM BA along with 5 μM indole-3 acetic acid (IAA) or 5 μM NAA induces a high rate of shoot proliferation of *Zingiber spectabile*. They also noticed that the number of shoots/explant depends on concentration of the growth regulators and the particular genotype. Plant regeneration was obtained by (Tsala *et al*., 1996) from shoot tip explants cultured in MS medium devoid of any Phythormone after twenty days.

Callus Formation

The basal media only MS (Murashige and Skoog,1962) was used in callus initiation. Shoot tip explants were cultured on MS media supplemented with different concentrations and combinations of 2,4-D, Kn, BAP, NAA and IAA. Data were recorded at the induction of the calluses,cultures were maintained under 16 h light and 8 h dark regime and results obtained (Table 2 and Fig. 2). Callus

Table 2: Effect of phytohormones on induction of calluses from axillary bud explants of Maulavi kachu (*Xanthosoma sagittifolium* L. Schott)

Treatments (mg L^{-1})	Days to callus initiation	Callus formation (%)	Callus colour	Texture of callus	Fresh weight of callus (g)	Organogenic response		Intensity of callus growth
NAA					Mean±SE	Root	Shoot	
1.0	70-85	15.00	LG	C	3.80±0.583	-	-	*
1.5	70-85	16.00	LG	F	4.40±0.244	-	-	*
2.0	70-85	29.00	LG	F	6.10±0.331	-	-	*
2.5	70-85	21.40	LG	F	4.40±0.509	-	-	*
3.0	70-85	15.80	G	Spongy	3.30±0.200	-	-	*
BAP+NAA								
1.0+0.5	70-85	50.00	LG	C	7.30±0.300	-	+	**
1.0+1.0	70-85	57.00	LG	C	8.80±0.583	-	+	**
1.0+1.5	70-85	77.00	LG	F	10.20±0.406	-	+	**
1.0+2.0	70-85	90.00	G	F	11.60±0.187	-	+	***
1.0+2.5	70-85	70.00	G	C	9.30±0.300	-	+	**
BAP+NAA								
2.0+0.5	70-85	34.00	G	F	7.35±0.358	-	+	*
2.0+1.0	70-85	59.00	G	C	7.95±0.145	-	+	**
2.0+1.5	70-85	58.00	G	F	10.60±0.187	-	+	**
2.0+2.0	70-85	44.00	G	F	8.55±0.4359	-	+	*
2.0+2.5	70-85	40.00	LG	C	8.20±0.7089		+	*
BAP+NAA								
3.0+0.5	70-85	79.00	LG	F	10.40±0.244	-	+	**
3.0+1.0	70-85	56.00	LG	C	6.55±0.508	-	+	*
3.0+1.5	70-85	36.00	LG	C	6.25±0.237	-	+	*
3.0+2.0	70-85	36.00	LG	C	7.15±0.331	-	+	*
3.0+2.5	70-85	26.00	LG	F	5.30±0.398	-	+	*
KIN+NAA								
2.0+0.5	70-85	35.00	LG	F	6.10±0.187	-	-	*
2.0+1.0	70-85	53.00	LG	F	7.30±0.570	-	-	*
2.0+1.5	70-85	59.00	LG	F	7.95±0.365	-	+	**
2.0+2.0	70-85	76.00	LG	F	9.80±0.255	-	+	***
2.0+2.5	70-85	52.00	LG	F	8.10±0.331	-	+	**
KIN+NAA								
3.0+0.5	70-85	34.00	LG	F	6.05±0.320	-	+	*
3.0+1.0	70-85	55.00	LG	F	6.70±0.255	-	+	**
3.0+1.5	70-85	60.00	LG	F	8.60±0.1871	-	+	**
3.0+2.0	70-85	85.00	G	F	8.60±0.291	-	+	***
3.0+2.5	70-85	60.00	LG	F	7.20±0.374	-	+	**

-indicates no response, * Indicate poor growth, ** Indicates moderate growth, *** Indicates profuse growth. - No. of root /shoot growth, + root/shoot (1-3)/callus, LG = Light Green, DG = Dark Green, C = Compact, F = Friable

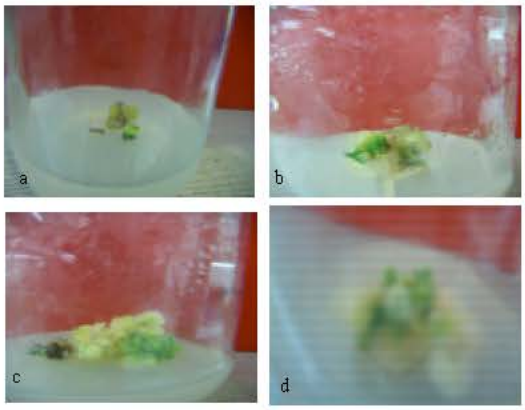

Fig. 2: Different stages of Callus induction in *Xanthosoma sagittifolium* (L.) Schott, (a) Insertion of axillary bud to medium supplementing 1 mg L^{-1} BAP+2.0 mgL^{-1}NAA, (b) Advance stage of Callus proliferation media containing with 1mg L^{-1} BAP+ 2.0 mg L^{-1} NAA (c) and Callus proliferation after 70 days, Callus formation after 85 days observation Ms media supplemented with supplemented with 1 mg L^{-1} BAP+ 2.0 mg L^{-1} NAA

proliferation was not noticed in all media formulations. But there was a wide variations in morphological maturity and percentage of callus formation among them. Callus initiation occurred within 70-80 days depending upon the concentration and combination of hormones. Frequency of callus formation ranged from 15-90%. Highest percentage (90%) of callus formation occurred in MS medium containing 1 mg L^{-1} BAP+2 mg L^{-1} NAA (Fig. 2) and followed by 85% in MS medium containing 3 mg L^{-1} Kn +2 mg L^{-1} NAA. The lowest percentage of callus (15%) formation was observed in media having 1 mg L^{-1}NAA. In most cases calli were green, light green and dark green and texture of calluses were compact and friable. The optimum callus growth in terms of fresh weight was 11.60 g. in 1 mg L^{-1} BAP+2 mg L^{-1} NAA and followed by 10.40 g in 3 mg L^{-1} BAP+0.5 mg L^{-1}NAA. The minimum weight was 3.30 g in 3 mg L^{-1} NAA. Most of calluses were responded with shoot positively and some calluses showed adventive root formation. Tsala *et al.* (1996) also showed that in vitro plantlets were obtained when shoot apices were cultured in MS medium supplemented with 10^{-5} M 2 mg L^{-1} Kn, Stem apices of *Xanthosoma sagittifolium* developed fast growing calluses and were transferred to medium without growth regulators after 12-16 weeks (Gomez *et al*., 1992). NAA and 10^{-6} Kn M.MS medium supplemented with 5 mg L^{-1} NAA, 100 mL^{-1} coconut milk and 2 mg L^{-1} Kn produced compact green calluses and organogenesis occurred within eight weeks (Gupta, 1985). After maintenance for eight weeks in MS medium supplemented with 25 mg L^{-1} IAA and Nyochembeng LM and Garton S (1988) reported calluses were initiated from shoot tips and petiole explants and proliferated on medium containing 1.36 μM dicamba. He also reported that thidiazuron (0.045 μM) enhance callus production when dicamba (13.5 μM) was used and was more favourable to petioles that shoot tips.

Organogenesis

Calli derived from the cormel bud meristematic tissues were transferred to cytokinin enriched media for organogenesis. For shoot differentiation light green compact calli were sub cultured on MS medium supplemented with different combinations and combinations of BAP and Kn alone and in combination with different concentration of NAA, 2, 4-D, IBA and IAA.Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3 and Fig. 3). Calli sub cultured with different concentration of IAA, BAP, Kn alone failed to differentiate any callus shown (data not shown). Calli

Table 3: Effect of phytohormones on regeneration of calluses from axillary bud explants of Malavi kachu (*Xanthosoma sagittifolium* L.schott)

Treatments (mg L^{-})	Days to callus regeneration	Callus regeneration (%)	Callus colour	Texture of callus	Fresh weight of callus with shoot	Orgagenic response	Intensity of callus	Average no. shoot per culture	Average length of shoot per culture
BAP+NAA					Mean±SE	Shoot		Mean±SE	Mean±SE
1.0+1.0	100-110	54.00	LG	F	5.30±0.530	+	*	1.2±0.200	1.8±0.374
1.0+1.5	100-110	66.00	LG	F	7.1±0.331	+	**	2.40±0.244	3.20±0.20
1.0+2.0	100-110	89.00	DG	FC	12.65±0.336	+	***	2.60±0.244	7.00±0.316
1.0+2.5	100-110	73.00	DG	F	8.40±0.509	+	**	1.40±0.244	2.80±0.374
1.0+3.0	100-110	53.00	LG	F	6.00±.0.3162	+	**	1.80±0.374	1.40±0.244
BAP+NAA									
2.0+1.0	100-110	69.00	DG	F	7.60±0.244	+	*	2.00±0.316	1.80±0122
2.0+1.5	100-110	78.00	DG	F	9.60±0.244	+	***	2.20±0.200	1.90±0.244
2.0+2.0	100-110	66.00	DG	F	8.40±0.291	+	**	2.40±0.244	2.70±.122
2.0+2.5	100-110	56.00	DG	F	7.00±0.353	+	*	1.60±0.244	2.30±0.230
2.0+3.0	100-110	49.00	DG	F	6.6±0.187	+	*	2.00±0.316	1.70±0.200
KIN+NAA									
3.0+1.0	100-110	49.00	LG	FC	6.60±0.187	-	*	2.00±0.316	1.70±0.200
3.0+1.5	100-110	73.00	LG	F	7.10±0.374	+	**	1.40±0.244	1.70±0.374
3.0+2.0	100-110	86.00	DG	FC	12±0.353	+	***	1.80±0.374	1.80±0.122
3.0+2.5	100-110	69.00	LG	F	9.44±0.400	+	**	1.40±0.244	1.70±0.200
3.0+3.0	100-110	36.00	LG	F	6.40±0.509	+	*	1.20±0.200	1.30±0.200

-: Indicates no response,*Indicate poor growth,** Indicates moderate growth, *** Indicates profuse growth. –No. of root /shoot growth, + root/shoot (1-3)/callus, LG = Light Green, DG = Dark Green, C = Compact, F = Friable

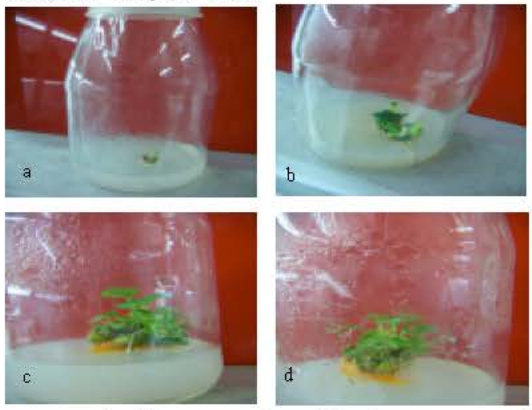

Fig. 3: Showing different stages of callus regeneration of *Xanthosoma sagittifolium* (L.) Schott, (a) Callus insertion to the medium supplementing with 3 mg Kn L^{-1} +2 mg L^{-1} NAA, (b) Proliferation or regeneration of callus growing shoots with same medium after 25 days of Culturing, (c) Multiple shoot production after 30 days and (d) Multiple plantlets produce after 30-40 days with same medium

produced shoots only when NAA was combined with cytokinins. Highest percentage (89%) shoot regeneration was recorded in 1 mg L^{-1} BAP+2.0 mg L^{-1} NAA (Fig. 2) and followed by 86% in media having 3 mg L^{-1} Kn+2.0 mg L^{-1} NAA. Lowest percentage (36.00%) Callus regeneration was observed in media with having 3 mg L^{-1} Kn+3 mg L^{-1} NAA. The highest percentage of shoot per callus was recorded 2.60 in media having 1 mg L^{-1} BAP+2.0 mg L^{-1} NAA. The lowest number of shoots 1.20 per callus was recorded in media having 1 mg L^{-1} BAP+1 mg L^{-1} NAA and 3 mg L^{-1} Kn+3 mg L^{-1} NAA. Highest length of shoots 7.00 cm was recorded in 1 mg L^{-1} BAP+2 mg L^{-1} NAA and followed by 3.20 cm in media containing 1 mg L^{-1} BAP+1.5 mg L^{-1} NAA and the lowest length of shoot was 1.30 cm recorded in 3 mg L^{-1} BAP+3 mg L^{-1} NAA. Fresh weight of callus with shoot was highest in media having 1 mg L^{-1} BAP+2.0 mg L^{-1} NAA followed by 12.00 g. in media supplemented with 3.0 mg L^{-1} Kn+2.0 mg L^{-1} NAA and the lowest weight (5.30 g) was recovered in media having 1 mg L^{-1} BAP+1 mg L^{-1} NAA. Nyochembeng and Garton (1988) also reported that callus regeneration or producing single or aggregated shoot buds sub cultured into media containing 0,0.049 and 0.49 μM 2 iso pentenyladenine.

Fig. 4: Root induction, Ms medium supplementing with 0.4 mg L^{-1} IAA

Table 4: Effect of different Concentration of Auxin on root induction from the axillary bud explant derived plants of Maulavi kachu (*Xanthosoma sagittifolium* L. Schott)

Growth regulators (mg L^{-1})	Days to root proliferation	Percentage of root proliferation	No. of root per plant	Root length (cm)
			----------Mean± SE----------	
IBA-0.2	-	-	-	-
IBA-0.3	7-10	10.00	1.30±0.1528	1.50±0.1167
IBA-0.4	07-10	5.00	1.10±0.1795	0.70±0.0081
IBA-0.5	07-10	-	-	-
IBA-0.6	07-10	-	-	-
IAA-0.2	07-10	45.00	4.00±0.2108	4.00±0.2108
IAA-0.3	07-10	75.00	6.50±0.3416	5.90±0.2333
IAA-0..4	07-10	90.00	7.90±0.3786	6.90±0.2211
IAA-0.5	07-10	80.00	6.30±0.3000	2.90±0.1533
IAA-0.6	07-10	40.00	2.30±0.3000	2.05±0.1384

- = Not response

Induction of callus and subsequent organogenesis were by a two step system for plant regeneration. By the manipulation of growth regulating substances, it was possible to regenerate functional plants in a one step system. Plantlets possessed well develop shoot and root systems were regenerated from the excised meristems within eight weeks of culturing.

Plants free from specific pathogens, through tissue culture techniques have been reported in various vegetatively propagated crops e.g., Potato, Sugercane and asparagus (Murashiege and Skog, 1978). In the present studies dasheen mosaic virus and other pathogen free plants were regenerated from the meristematic domes of *Xanthosoma sagittifolium* (L.) schott and subsequently the regenerants were showed good performance in growth on natural growing soil medium.

Root Induction

Regenerated plants were then cultured on to a rooting medium containing different concentration and combination of growth regulators of which only 0.4 mg L^{-1} IAA showed best performance for root induction and profuse root proliferation occurred within 7-10 days from culture (Table 4 and Fig. 4).

Acclimatization

Plantlets with rooting were removed from the culture vessels and then washed with running distilled water or running tap water and then transferred to wet fertile loamy soil contained plastic pots for hardening. Within one months or before the plantlets showed best performance for acclimatization.

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