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Standardization of a Suitable Protocol for *in Vitro* Clonal Propagation of *Acorus calamus* L. an Important Medicinal Plant in Bangladesh

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Abstract: The present investigation was carried out with a view to standardize an *in vitro* culture technique for mass propagation of important medicinal plant species, *Acorus calamus* L. Naturally grown rhizome tip was used as explants for initial culture. The explants were cultured on standard MS (Murashige and Skoog, 1962) medium supplemented with different concentration and combination of cytokinines and auxines for primary shoot proliferation. The best shoot proliferation was observed in MS medium containing 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA where 98.99 % of explants showed proliferation. For rooting of the micro shoots, MS medium supplemented with 1.0 mg l^{-1} IBA was showed the maximum 40.51% of root formation. It was also evident that 40 g l^{-1} sucrose of Merck Ltd., Mumbai showed the best results for shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus*. After acclimatization and transplantation, 80 % of the *in vitro* derived plants were healthy in *ex vivo* condition.

Key word: *Acorus calamus* L. · *In vitro* culture · Cytokinin · Auxin · MS medium

INTRODUCTION

The sweet flag *Acorus calamus* L (vernacular: Boch) is perennial herb belonging to the family Araceae. It is found in marshy land, shallow water andpond edges of the northern temperate, subtropical and warm regions of Indian subcontinent [1, 2]. In Bangladesh it is found in limited scale as wild habitat or cultivated. The rhizome (root) and leaves of *A. calamus* produce glycoside [3], oxalic acid [4] andvolatile oil [5]. The volatile oil is known as calamus oil that has been successfully employed in medicine since the old time and at present it is used for the preparation of perfume, flavors and medicine [5, 6]. It is very effective in bronchitis [7], affected heart, lungs [8], pain in the liver, Kidney [7] and gall bladder disorders [9]. The leaves of Acorus calamus also to have antifungal properties [10]. *In vitro* method of vegetative multiplication of *A. calamus* would have considerable benefits for the medicinal trade and germplasm conservation. At present, application of plant tissue culture offers valuable ways to overcome all the problems that's found in natural propagation. *In vitro* micropropagation of *A. calamus* was also achieved using different explants [11-13]. This study describes a standard method of micropropagation of *A. calamus* through shoot tip (rhizome bud) culture for obtaining large scale diseases free seedlings. This can ensure adequate supply of diseases free *A. calamus* to meet different purposes. Further investigation of molecular biotechnology could depend on this protocol.

MATERIALS AND METHODS

Rhizome tip of field growing *A. Calamus* plants were used as primary explants. The rhizome were washed thoroughly with fresh tap water followed by treated with 0.1 % $HgCl_2$ for 15 minutes and washed at least for 6-7 times with autoclaved distilled water to remove $HgCl_2$. The surface sterilized rhizomes were cultured on MS medium with different concentration and combination of BAP (6-benzyl amino purine), NAA (Naphthalene acetic acid), IAA (Indole-3-acetic acid) and KIN (2-furfurylamino purine) for shoot formation and proliferation. Each treatment consisted of three replications and for each replication; 10-12 explants were used. Data were recorded after 6 weeks of initial culture. As carbon sources, sucrose

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40 gm l^{-1} (4%) were added in different culture medium. The P^H was adjusted to 5.5 and solidified by 7 gm l^{-1} agar and dispensed into glass tube (conical flux). The medium was sterilized by autoclaving. After inoculation all the cultures were grown under a photoperiod of 12 hours light (with white fluorescent tubes) at a temperature of 25±3 ºC. Subcultures were carried out regularly after every four weeks. For rooting were excised and cultured on the MS medium with 4% sucrose with different concentration of NAA and IBA (Indole-3-butyric acid) alone and in combination. Effect of different types (Merck Ltd., Mumbai, India; *Phyto* Technology Laboratories, USA; BDH chemicals Ltd., England and local market sugar) and concentration (10, 20, 30, 40, 50 and 60 g l^{-1}) of sucrose on shoot proliferation was also studied on MS medium containing 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 3. Rooted plantlets were removed from the culture tube and agar was carefully washed away. The plant lets were then transplanted into small plastic pot containing garden soil and humus (3:1). For hardening they were kept in growth chamber where high humidity and under controlled atmosphere was maintained for two weeks. Once acclimatized and the plantlets were finally established in the field.

RESULTS AND DISCUSSION

Naturally grown rhizome tip was used as explant to investigate shoot regeneration and multiplication. Regeneration of shoot from the explants was observed at all levels hormone treatment. The highest percentage of shoot proliferation was recorded at the treatment of BAP at 3.0 mg l^{-1} which was 95.86% and highest shoot length was 2.85 cm (Table 1). BAP showed better results compared to KIN. Multiple shoot formation was also found best in BAP with 4 mg l^{-1} [12]. Combination of BA and KIN was used at different concentrations in this investigation. The maximum percentage of shoot proliferation (98.99) was found at 2.0 mg l^{-1} KIN and 0.1 mg l^{-1} NAA (Figure 1D). The highest number of shoots (10.20) was observed at 2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA and the longest shoot length (12.21 cm) was found in 2.0 mg l^{-1} KIN + 0.05 IAA (Table 1). Shoot formation was also effective in combination of BAP and NAA (Table 1). Similar result was also reported by

Table 1: Effect of different concentrations of BA with KIN singly or in combination on shoot proliferation from shoot tip explants. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 6 weeks of culture

Growth regulators	Concentration (mg l^{-1})	% of explants showing proliferation	No. of shoots per culture	Average length of the longest shoot (cm)
BAP	0.5	70.03	4.43	2.49
	1	90.81	5.91	3.09
	2	90.56	6.00	3.31
	3	95.86	5.15	2.85
	4	60.20	3.92	2.38
KIN	0.5	75.51	3.60	2.23
	1	85.39	3.99	2.71
	2	90.11	4.85	3.15
	3	80.07	3.43	2.47
	4	65.39	2.91	2.19
BA+KIN	1.0+1.0	66.66	4.03	2.01
	2.0+1.0	50.00	3.30	2.09
	2.0+2.0	41.66	2.87	1.82
BA + NAA	1.0+0.1	60.90	4.12	2.37
	1.0+0.5	73.08	2.53	2.91
	1.0+1.0	60.11	4.66	3.25
	2.0+0.1	98.90	9.23	2.05
	2.0+0.5	95.18	10.20	2.12
	2.0+1.0	90.33	6.87	3.51
	3.0+0.1	86.56	7.99	2.65
	3.0+0.5	80.55	5.75	2.23
	3.0+1.0	66.39	4.36	1.90
BAP+IAA	1.0+0.1	40.99	3.77	2.14
	1.0+0.5	53.33	3.13	2.81
	1.0+1.0	33.33	1.36	1.49
	2.0+0.1	88.59	9.39	2.14
	2.0+0.5	83.33	8.38	2.50
	2.0+1.0	86.66	6.11	3.18
	3.0+0.1	80.13	7.10	2.48
	3.0+0.5	66.66	5.35	2.01
	3.0+1.0	53.33	3.87	1.85
KIN+NAA	1.0+0.05	46.66	3.11	2.03
	1.0+0.5	40.10	2.81	2.66
	1.0+1.0	53.33	1.10	1.35
	2.0+0.05	98.99	8.34	1.44
	2.0+0.5	86.66	7.35	2.81
	2.0+1.0	80.00	5.15	3.50
	3.0+0.05	73.33	6.19	2.23
	3.0+0.5	60.01	4.58	1.97
	3.0+1.0	53.63	3.16	1.78
KIN + IAA	1.0+0.05	40.60	2.71	1.75
	1.0+0.5	33.13	2.45	2.43
	1.0+1.0	26.76	1.18	1.29
	2.0+0.05	97.33	7.53	12.21
	2.0+0.5	73.36	6.99	2.69
	2.0+1.0	80.53	4.75	2.48
	3.0+0.05	66.61	5.62	2.02
	3.0+0.5	53.34	4.10	1.81
	3.0+1.0	46.61	2.64	1.55

Table 2: Effect of different concentration and combination of NAA and IBA on *in vitro* root formation from micro cuttings of *A. calamus*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 5 weeks of culture

Growth regulators	Concentration (mg l^{-1})	% of root formation	No. of roots per cutting	Average length of the longest root (cm)	Days to root formation	Degree of Callus formation
NAA	0.1	-	-	-	-	+++
	0.5	25.53	2.26	2.15	13-Oct	+++
	1.0	20.33	2.12	2.15	12-Oct	+++
	1.5	18.23	2.01	2.03	12-Oct	++
	2.0	-	-	-	-	-
IBA	0.1	-	-	-	-	+
	0.5	35.81	3.11	2.28	7-10	+
	1.0	40.51	3.15	2.25	7-10	++
	1.5	32.56	2.93	2.11	7-10	++
	2.0	14.60	1.56	1.67	7-10	+
NAA+IBA	0.5	30.39	2.35	2.15	7-10	+
	1.0+0.5	30.69	2.15	2.00	7-10	-
	1.0+1.0	25.59	2.36	2.12	7-10	-
	0.5+0.5	25.32	2.21	2.10	7-10	+
	0.5+1.0	21.89	1.92	1.90	7-10	+

+, ++, +++, indicate slight, considerable, profuse callusing, respectively. - indicates no response.

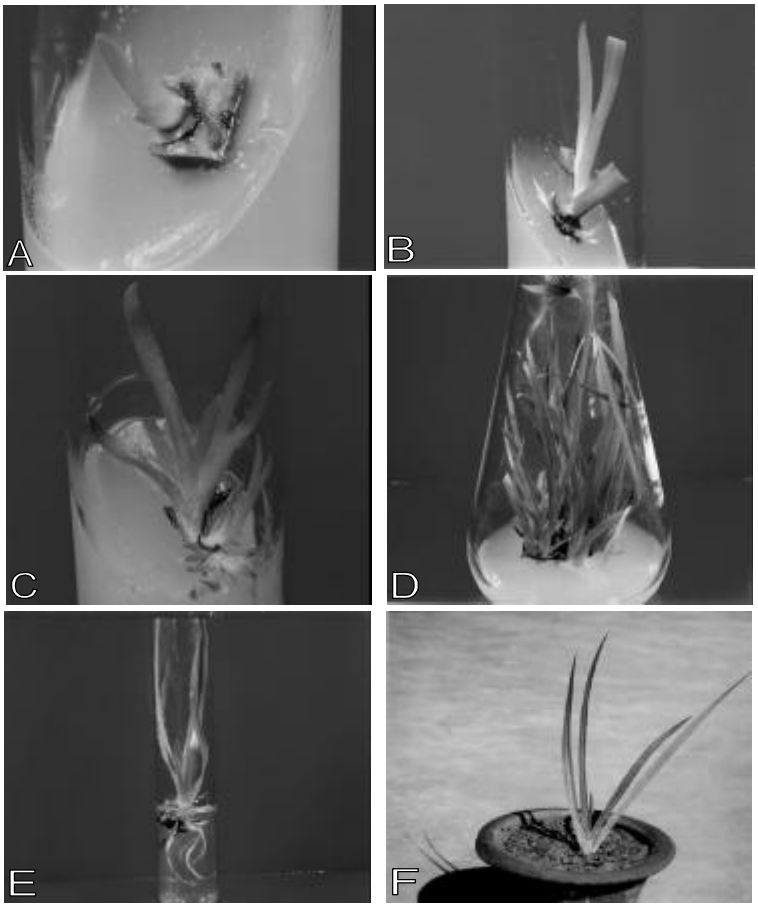

Fig. 1: *In vitro* organogenesis in *Acorus calamus* using rhizome tip explant. Shoot proliferation from Rhizome tip explants of field grown *Acorus calamus*. Plant cultured on MS medium containing 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA after 2 weeks (A), 4 weeks (B) and 6 weeks (C) respectively. Regenerated plantlets on MS medium containing 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA (D). Rooted shoots on MS medium supplemented with 1.0 mg l^{-1} IBA (E). Plantlets acclimatized after 5 weeks of transplantation (F).

Table 3: Effect of different brands and concentration of sucrose on proliferation and growth of axillary shoots from *in vitro* proliferated shoots on MS medium containing 2.0 mg l^{-1} KIN + 0.05 mg l^{-1} NAA. Data were recorded after 6 weeks of culture

Different brands of sucrose	Sucrose concentration (g l^{-1})	% of explants showing proliferation	No. of shoots per culture	Average length of the longest shoot (cm)
Mark Ltd. Mumbai	10	46.67	4.24	2.05
	20	66.67	5.80	3.53
	30	93.33	8.20	4.91
	40	97.93	12.53	5.20
	50	80.00	6.10	4.69
	60	53.33	5.39	2.95
Phyto Technology Laboratories™, USA	10	40.53	3.85	2.04
	20	60.10	5.44	3.25
	30	46.68	9.05	2.45
	40	93.33	9.20	2.85
	50	73.33	6.81	3.89
	60	46.67	5.39	2.85
BDH Chemical Ltd. England	10	33.33	2.85	1.89
	20	46.67	3.94	2.85
	30	80.00	6.99	3.01
	40	86.67	8.12	2.25
	50	66.67	5.25	3.21
	60	40.00	3.10	2.21
Local market sugar	10	33.34	2.80	2.05
	20	53.33	3.10	2.55
	30	80.00	5.50	2.50
	40	93.34	7.95	2.12
	50	60.00	4.65	4.01
	60	33.37	2.58	2.55

Dheeranupattana *et al*. [12] and Anu *et al*. [11]. The highest percentage of shoot proliferation was observed at 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (98.90%). In comparison, combination of KIN and NAA showed better response than that of BAP and NAA or BAP and IAA. Best rooting was observed within 10-13 days of culture on rooting medium with 1.0 mg l^{-1} IBA (40.51%) followed by 0.5 mg l^{-1} IBA (Table 2, Figure 1E). Effect of different brands and concentration of sucrose on proliferation and growth of axillary shoots was also investigated. Results revealed that 40 g l^{-1} sucrose of Merck Ltd., Mumbai showed the best results for shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* on MS medium containing 2.0 mg l^{-1} KIN + 0.05 mg l^{-1} NAA (Table 3). The highest percentage of explants showing proliferation were noticed 97.93% in Merck Ltd., Mumbai; 93.33% in *Phyto* Technology Laboratories, USA, 86.67% in BDH chemicals Ltd., England and 93.34% in local market sugar were observed at 40 g l^{-1} of sucrose (Table 3). On average, among the four brands Merck Ltd., *Phyto* Technology Laboratories gave the highest percentage of explants showing proliferation and it was 73.33% and in other three brands equal response was observed.

CONCLUSIONS

The main objective was to establish a standard protocol for the *in vitro* culture of *A. calamus* L. From the present study it was found that the combination of cytokinin BA or KIN with auxin NAA and IAA responded better in shoot formation than separately used cytokinin or auxin.The best combination was 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA which showed 98.99 % of shoot proliferation. Rooting was found best on MS medium containing 1.0 mg l^{-1} IBA. Sucrose treatment showed different results on shoot proliferation according to type and level of sucrose. Results revealed that 40 g l^{-1} sucrose of Merck Ltd., Mumbai showed the best results for shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* on MS medium containing 2.0 mg l^{-1} KIN and 0.05 mg l^{-1} NAA (Table 3). So, here a suitable protocol was established for the *in vitro* shoot proliferation of *A. calamus* which can be cased for mass propagation of disease free healthy *A. calamus* plant.

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