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IN VITRO INDIRECT PLANTLET REGENERATION FROM HYPOCOTYL SEGMENTS AND COTYLEDONARY EXPLANT DERIVED CALLI IN LADY'S FINGER (*ABELMOSCHUS ESCULENTUS* L. MONECH)

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Abstract

An experiment was conducted to develop an efficient protocol for *in vitro* regeneration of plantlets from hypocotyl segments and cotyledonary explants of *Abelmoschus esculentus* L. Monech. In this investigation the cultivar OK-285 of *Abelmoschus esculentus* L. Monech was used. At first somatic embryogenic calli were induced using MS medium supplemented with different concentrations of auxin and cytokinin singly or in combination. The cotyledonary explants showed the best callus induction rate (87.9%) in MS medium containing 1.5mgl^{-1} 2.4-D + 0.1mgl^{-1} BAP hormonal concentration while hypocotyl segments showed 82.6% callus induction rate in the same medium. For shoot formation, calli were subcultured on MS solid medium and the hypocotyl segments showed the best result (72.1%) in MS medium containing 2.0 mgl^{-1} BAP + 0.1 mgl^{-1} IAA and the mean number of shoots per callus was recorded 4.2. For root induction from shoots MS and ½MS media were used. The highest 63.5% microshoots initiated roots in ½ MS + 0.1 mgl^{-1} IBA medium and the highest mean number of root was 4.8. Rooted shoots were acclimated and successfully established into soil under natural condition with 70% survival.

Key words: *Abelmoschus esculentus*, hypocotyl segments and cotyledon, embryogenic calli, regeneration.

Introduction

Lady's finger (*Abelmoschus esculentus* L. Monech) is an erect hispid herb. Its natural reproduction takes place by seeds. Varying chromosome numbers (about n=36; 33; 59 to 72) have been reported for *A. esculentus* (Joshi *et al.* 1974). It is an annual vegetable crop, in parts of the year (Joshi and Hardas 1976). Its tender green fruits are used as a vegetable and are generally marketed in the fresh state. It is used as a very common vegetable in many people's diet. The principal edible portion is pod and sometimes young leaves are also used as a fresh state. The fruit has high mucilage content and are used in many popular dishes. In the Indian subcontinent, the pods are boiled whole or sliced before cooking and then fried. Due to the presence of high mucilage content, it is used in soups and gravies and is one of the ingredients of Callaloo soup, a Trinidadian dish. The ripe seeds contain about 20% of edible oil. The stem of the plant is used for extraction of a fibre which is used in paper industry. The fruit is also used in baking industry as a substitute for part of the wheat flour and fat. Mucilage from the stem and roots is used for clearing sugarcane juice in India and Bangladesh and for sizing paper in China (Purseglove 1968).

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For further research into the biochemical compositions and potential food values of this plant, an efficient *in vitro* regeneration system for production of plants is required because field-grown plants may be subject to seasonal and somaclonal variations, infestations of bacteria, fungi and insects as well as environmental pollution that can affect the food values of the harvested tissues. In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species (Murch *et al*. 2000, Roja *et al*. 1991). The application of *in vitro* propagation techniques might offer the possibility of producing large numbers of uniform plants for further field culture. In nature, this species propagates through seeds. However, propagation protocols for this species *in vitro* have not yet been reported. Based on results from preliminary investigations on propagation via seed, we concluded that specific habitat conditions for seedling survival and growth are required. For this reason, the development of an *in vitro* protocol will be of great importance for production of planting material to conserve the species.

Materials and Methods

The experiment was conducted in Biotechnology Laboratory, Institute of Biological Sciences, University of Rajshahi from January 2006 to January 2007. The hypocotyl segments and cotyledonary explants of OK-285 cultivar of *A. esculentus* were used as experimental materials in this investigation. Seeds of this variety were collected from East-West Seed Company, Dhaka, Bangladesh and used for conducting different experiments. Seeds were washed thoroughly under running tap water for about 60-90 minutes and then treated with 1% Savlon and 2-3 drops of Tween-80 for about 12 minutes. This was followed by successive three washing with distilled water to make free the seeds from savlon and Tween-80, Surface sterilization was carried out with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes followed by gentle shaking. After this treatment, the seeds were rinsed 4-5 times in sterile distilled water to make free the seeds from $HgCl_2$. Sterilized seeds were aseptically germinated in glass bottle containing 50 ml of autoclaved (121°C temperature and 15 psi for 15 minutes) half strength MS (Murashige and Skoog 1962) medium. Germinating seeds were maintained at 25±2°C temperature and 60% RH in darkness. After germination of the seeds, seedlings were maintained under 16 and 8 hr light and dark respectively.

The hypocotyl segments and cotyledonary explants were collected from 8 days old seedlings and used as explants. Explants (20 pieces) were cultured in 9cm Petri dish and placed horizontally in callus induction medium. The MS medium supplemented with 3% sucrose and different concentrations of 2,4-D, NAA, IAA, BAP and KIN singly or in combination were compared for the induction of embryogenic calli. The medium was adjusted to pH 5.8 and autoclaved (as described earlier). All the cultures were maintained as described earlier for aseptic seed germination. The data for callus initiation were scored after 28-30 days of culture. Induction frequencies for all types of calli were calculated as the percentage of cultured pieces of cotyledons and hypocotyl segments.

About 28-30 days after culture, calli were rescued aseptically on a sterile petridish and were cut into convenient size by a sterile scalpel and these were inoculated to a freshly prepared medium supplemented with same or different hormonal combinations for shoot formation. The regenerated shoots were very carefully rescued from the culture bottles and cut from the basal end of the shoots. Then each of the shoots was cultured in freshly prepared full and half strength MS medium containing different combinations of hormonal supplements for root initiation. After sufficient growth of shoot and root system, the plantlets were transferred soil.

Result and Discussion

Tissue culture techniques provide viable alternative methods of mass production of healthy plants with uniform characteristics. The indirect regeneration is a useful means of producing plantlets from young and mature plants with a lower risk of genetic instability than by other routes (Rao and Lee 1986).

Between two types of explants (cotyledon and hypocotyl segments) of *A. esculentus,* the best callus induction rate was observed in cotyledonary explants and it was 87.9% at the concentration of 1.5mgl^{-1} 2.4-D + 0.1mgl^{-1} BAP in MS medium. The color of calli was green and loose in nature in this case. The second best callusing rate was 82.6% in 1.5mgl^{-1} 2.4-D + 0.1mgl^{-1} BAP for hypocotyl segments. The calli were green in color and compact in nature (Table 1). Medora *et al.* (1979) demonstrated inhibitory effect of BAP in combination with 2,4-D on induction or growth of callus in papaya. But in the present investigation 2,4-D and BAP combination induced highest amount of callus from cotyledon which is not in agreement with Medora *et al.* (1979). In addition, Gita and Grover (1999) also used Kn and BAP with auxin for induction of callus and they got 85% callus induction frequency. It is concluded that combined action of auxin and cytokinin was more effective on callus induction than those of the medium containing auxin only.

Shoot formation: The calli obtained from cotyledon and hypocotyl segments were used for multiple shoots formation. Development of shoots occurred when the calli were subcultured in MS medium supplemented with BAP and Kn in combination with NAA, IAA or IBA. Such a combined effect has also been reported in *Petasites hubridus* of family Asteraceae (Wildi *et al*.1998). Significant improvement in shoot formation over control has previously been achieved with the addition of cytokinins like BAP and Kn in many composites. For example, Conchou *et al*. (1992), Le (1994), Nin *et al*. (1994), Fauconnier *et al.* (1996), Wildi *et al*. (1998) and Cuenca *et al.* (1999) used BAP and Kn in combination with different concentrations of NAA and IAA. In the present investigation different combinations of BAP with auxins were found to be better than combinations of KIN with different auxins. In case of morphogenic response of callus derived from hypocotyl segments, BAP + IAA combination was found to be more effective and maximum 72.1% of the callus cultures showed shoot initiation in medium containing MS + 2.0 mgl^{-1} BAP + 0.1 mgl^{-1} IAA and the mean number of shoots per callus was recorded 4.2. But in case of cotyledonary explants, the highest shoot formation rate was 61.2% in MS + 2.0 mgl^{-1} BAP + 0.1 mgl^{-1} IAA medium and the highest mean number of shoots per culture was 3.9(Table 2). It was observed that, higher cytokinin (BAP and KIN) concentrations and lower auxin concentrations (NAA, IAA and IBA) proved better for embryogenesis in callus. Ratio of cytokinin and auxin seems to play an important role (Murashige and Skoog 1962, Skoog *et al.*1967, Steward *et al.* 1969, Thomas and Street 1970) and interaction between growth hormones is a highly significant factor in inducing morphogenesis in cultured tissues (Thomas and Street 1970, Pareek and Chandra 1981, Beck and Caponetti 1983).

Root formation: Regenerated shoots need to root formation for their healthy growth. So, an experiment was conducted with basal MS medium and ½ strength MS medium supplemented with different types of auxins (NAA, IBA and IAA). Auxins were used singly or in combination with other auxins in various concentrations. Among different auxins, IBA was found to be the best for root induction and maximum 63.5% of the cultured shoots induced roots in ½ MS + 0.1 mgl^{-1} I BA and the highest mean number of roots per shoot was 4.8 (Table 3).

Table 1. Effect of different concentrations of auxin (2, 4-D, NAA and IAA) and cytokinin (BAP and KIN) in combination employed in MS medium on callus induction.

Growth Regulators	Concentration of growth regulators (mg/L)	Hypocotyl segments		Cotyledons	
		% of explants responded	Nature of callus	% of explants responded	Nature of callus
NAA+ BAP	1.0 + 0.5	45.8	GL	29.1	GC
	1.5 + 0.5	50.7	GL	37.5	GC
	2.0 + 0.5	60.4	GL	40.3	GC
	3.0 + 0.5	72.8	GWL	50.5	GC
	5.0 + 0.5	70.8	GWL	52.3	GC
	0.5 + 1.0	51.2	GC	60.5	GWC
	1.0 + 1.0	58.3	GC	67.6	GW
2, 4-D + BAP	0.5 + 0.1	50.2	GL	50.5	GC
	1.0 + 0.1	62.5	WL	54.1	GC
	1.5 + 0.1	82.6	GL	87.9	GC
	2.0 + 0.1	65.3	GL	60.2	GC
	3.0 + 0.1	48.4	WL	58.3	GC
	5.0 + 0.1	30.2	GL	50.0	GC
	0.5 + 0.2	8.3	WL	20.	GC
IAA + BAP	1.0 + 0.5	45.8	WGC	55.3	GC
	1.5 + 0.5	50.3	WGC	58.5	GC
	2.0 + 0.5	63.1	WGC	60.3	GC
	3.0 + 0.5	70.3	WGC	65.1	GC
	5.0 + 0.5	62.2	WGC	56.3	GC
	0.5 + 1.0	30.6	GC	54.1	GC
	0.5 + 0.2	20.8	WL	54.1	GC
IBA + BAP	0.5 + 1.0	29.1	WL	25.0	WC
	1.0 + 1.0	54.1	WL	50.0	WC
	1.5 + 1.0	27.5	WL	62.5	WC
	2.0 + 1.0	65.9	WL	70.8	WC
	3.0 + 1.0	61.6	WL	66.6	WC
	5.0 + 1.0	52.2	WC	62.5	WC
	0.5 + 1.5	20	WC	20.8	WC
NAA + KIN	0.5 + 1.0	16.6	GW	20.3	GW
	1.0 + 1.0	41.6	GW	41.6	GW
	2.0 + 1.0	58.3	GW	54.1	GW
	3.0 + 1.0	62.5	GW	70.8	GW
	5.0 + 1.0	54.1	GW	62.5	GW
	0.5 + 1.5	20.8	GW	16.6	GW
IBA + KIN	1.0 + 1.5	20.8	WC	41.6	WC
	2.0 + 1.5	45.8	WC	41.6	WC
	3.0 + 1.5	45.8	WC	45.8	WC
	5.0 + 1.5	41.6	WC	37.5	WC
	0.5 + 2.0	20.8	WC	29.1	WC
	1.0 + 2.0	25.0	WC	33.3	WC

GL=Green-Loose, WC=White-Compact, WGC=White-Green-Compact, GW=Greenish-white,WL=White-Loose

Table 2. Effect of different concentrations of cytokinin (BAP and KIN) with auxin (NAA, IAA and IBA) in combination employed in MS medium on shoot formation from hypocotyl and cotyledonary explants derived embryogenic calli.

Growth Regulators	Concentration of growth regulators (mg/L)	Hypocotyl segments		Cotyledons	
		% of calli regenerated shoot	Mean no. of shoot/callus	% of calli regenerated shoot	Mean no. of shoot/callus
BAP + NAA	0.5 + 0.1	13.5	2.6±0.58	10.6	2.1±0.72
	1.0 + 0.1	18.7	3.2±0.56	16.5	2.6±0.45
	1.5 + 0.1	39.3	3.8±0.88	32.5	2.8±0.57
	2.0 + 0.1	38.1	3.5±0.85	37.2	3.5±0.74
	3.0 + 0.1	25.0	3.5±0.85	22.6	3.3±0.47
	0.5 + 0.2	18.6	3.1±0.63	16.7	3.2±0.54
	1.0 + 0.2	22.1	2.9±0.75	20.3	3.0±0.72
	1.5 + 0.2	28.5	2.6±0.71	25.8	2.8±0.72
BAP + IAA	0.5 + 0.1	19.6	2.8±0.81	15.6	2.5±0.45
	1.0 + 0.1	30.8	3.1±0.87	26.8	2.8±0.74
	1.5 + 0.1	46.9	3.8±0.69	48.9	4.1±0.38
	2.0 + 0.1	72.1	4.2±0.79	61.2	3.9±0.47
	3.0 + 0.1	53.8	4.0±0.55	48.6	3.7±0.48
	0.2 + 0.5	20.6	2.1±0.32	23.5	3.1±0.54
	0.2 + 1.0	31.5	3.6±0.53	35.1	3.4±0.45
	0.2 + 1.5	20.2	3.8±0.80	26.5	3.6±0.74
BAP + IBA	0.5 + 0.1	11.2	2.5±0.70	11.4	2.3±0.45
	1.0 + 0.1	8.6*	1.8±0.88	9.7	2.0±0.64
	1.5 + 0.1	12.5	3.1±0.72	11.5	2.6±0.52
	2.0 + 0.1	13.7	2.7±0.71	12.8	2.3±0.57
	3.0 + 0.1	15.3	2.0±0.68	13.7	1.8±0.77
	0.5 + 0.2	10.8	1.7±0.62	11.1	1.4±0.54
	1.0 + 0.2	9.6	2.6±0.69	10.3	2.5±0.47
	1.5 + 0.2	18.2	2.8±0.65	16.6	2.3±0.43
KIN + NAA	0.5 + 0.2	10.8	3.8±0.73	12.6	3.2±0.83
	1.0 + 0.2	16.8	2.6±0.54	15.8	2.1±0.63
	1.5 + 0.2	14.1	1.9±0.75	12.8	1.6±0.59
	2.0 + 0.2	5.3	2.8±0.64	8.7	1.9±0.47
	3.0 + 0.2	4.2	1.6±0.79	6.3	1.2±0.54
	0.5 + 0.5	18.6	2.3±0.65	14.6	2.1±0.74
	1.0 + 0.5	13.6	3.2±0.75	12.7	3.2±0.68
	1.5 + 0.5	8.2	2.4±0.74	9.2	2.4±0.36
KIN + IAA	0.5 + 0.2	29.1	2.8±0.84	27.5	2.3±0.65
	1.0 + 0.2	14.2	2.6±0.82	11.7	2.1±0.83
	1.5 + 0.2	16.3	3.1±0.58	14.3	3.1±0.63
	2.0 + 0.2	12.5	2.6±0.74	9.5	2.3±0.56
	3.0 + 0.2	6.2	1.2±0.38	4.6	1.5±0.75
	0.5 + 0.5	20.1	2.5±0.55	12.6	2.7±0.67

Table 3. Effects of different concentration of NAA, IBA and IAA singly or in combination in MS or ½ MS medium on root induction from regenerated shoot cuttings obtained from different explants.

Growth Regulators	Concentration of growth regulators (mg/L)	Medium (L)	No. of shoot inoculated to develop	% of shoots induced to develop root	Mean no. of roots per shoot.
NAA	0	MS	15	26.6	2.8±0.63
	0	½ MS	15	33.3	2.6±0.34
	0.1	MS	12	16.6	2.1±0.45
	0.1	½ MS	14	42.8	3.1±0.73
	0.2	MS	13	23.0	2.9±0.53
	0.2	½ MS	17	35.2	3.2±0.57
	0.5	MS	15	13.3	1.8±0.34
	0.5	½ MS	15	20.0	1.9±0.57
IBA	0.1	MS	17	52.9	3.5±0.53
	0.1	½ MS	18	63.5	4.8±0.57
	0.2	MS	20	20.0	1.8±0.84
	0.2	½ MS	16	37.5	3.0±0.56
	0.5	MS	14	21.4	2.9±0.84
	0.5	½ MS	13	30.7	2.8±0.64
	1.0	MS	18	11.1	1.3±0.62
	1.0	½ MS	20	10.0	1.3±0.82
NAA + IBA	0.1 + 0.1	MS	15	46.6	3.8±0.65
	0.1 + 0.1	½ MS	18	55.5	3.8±0.56
	0.1 + 0.2	MS	16	18.7	2.6±0.68
	0.1 + 0.2	½ MS	15	33.3	2.9±0.58
	0.1 + 0.5	MS	17	17.6	2.5±0.63
	0.1 + 0.5	½ MS	19	21.0	1.8±0.69
	0.2 + 0.1	MS	13	23.0	3.0±0.53
	0.2 + 0.1	½ MS	20	30.0	3.1±0.59
NAA + IAA	0.1 + 0.1	MS	18	16.6	1.8±0.68
	0.1 + 0.1	½ MS	14	28.5	2.1±0.63
	0.1 + 0.2	MS	20	15.0	1.3±0.68
	0.1 + 0.2	½ MS	18	33.3	2.5±0.65
	0.1 + 0.5	MS	12	-	-
	0.1 + 0.5	½ MS	13	-	-
	0.2 + 0.1	MS	20	15.0	1.9±0.68
	0.2 + 0.1	½ MS	19	26.3	2.5±0.55
IBA + IAA	0.1 + 0.1	MS	11	37.2	4.3±0.65
	0.1 + 0.1	½ MS	14	57.7	5.5±0.66
	0.1 + 0.2	MS	18	14.5	3.0±0.99
	0.1 + 0.2	½ MS	11	18.1	3.2±0.58
	0.1 + 0.5	MS	13	-	-
	0.1 + 0.5	½ MS	15	-	-
	0.2 + 0.1	MS	18	22.2	2.8±0.64

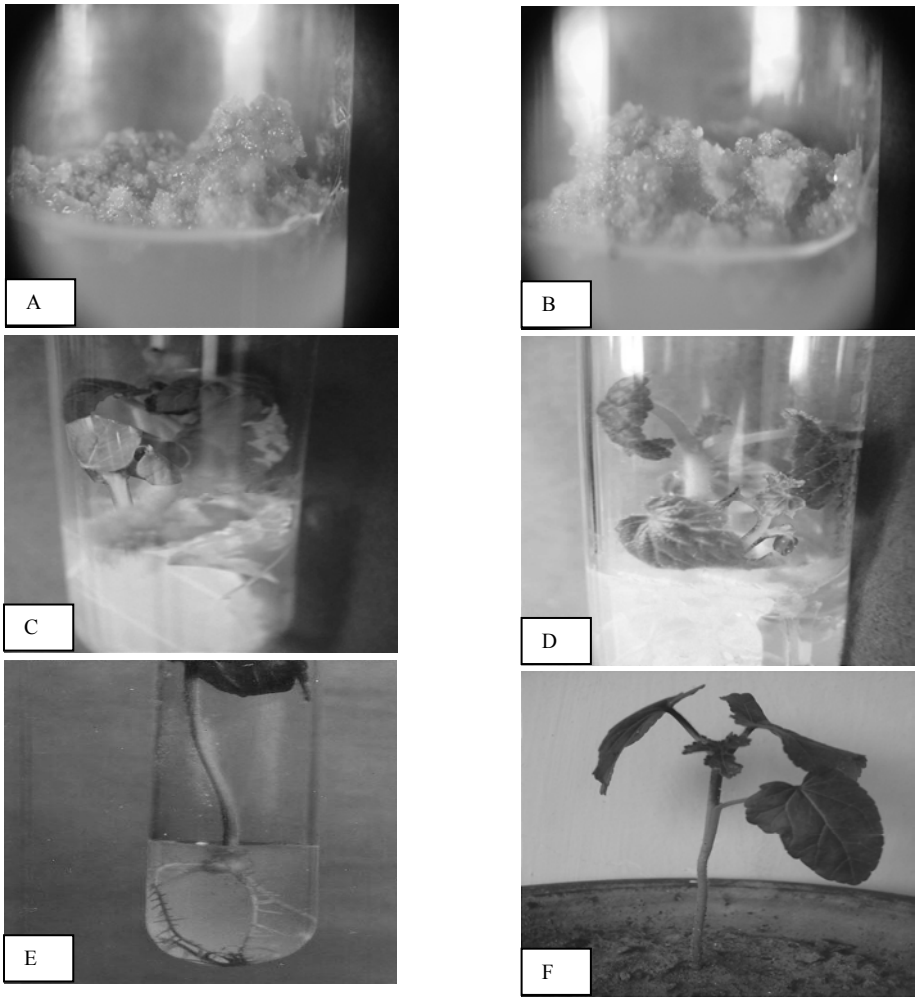

Fig 1. Callus induction, multiple shoot and root formation from *in vitro* grown hypocotyl segments and cotyledonary explants of lady`s finger(*Abelmoschus esculentus* (L.) Monech). **A-B:** Callus induction from *in vitro* hypocotyl segments and cotyledonary explants respectively after 21 days of culture. **C-D:** Multiple shoot formation from hypocotyle segments and cotyledonary explants derived calli on MS medium supplemented with 2.0 mgl^{-1} BAP + 0.1 mgl^{-1} IAA after 4 weeks of culture. **E:** Root formation on ½ MS medium supplemented with 0.1 mgl^{-1} IBA after 4 weeks of culture. **F:** Aclimatized plant

Most of the plants require the presence of auxin for efficient root regeneration. Herbaceous plants require lower concentrations of auxins for efficient rooting. In the present findings, ½ MS + 0.1 mgl^{-1} IBA was proved most efficient for rooting. The most efficient auxins for rooting are definitely IBA and NAA (Pierik 1987) and this information corroborates with the present findings. Efficiency of IBA in root induction was also observed in grape (Chakravorty 1986). The present findings also showed similarities with the respect of Roy and De (1986, 1990), in *Calotropis gigantea;* Agarwal *et al*. (1989), in *Capsicum annum* and Mante *et al.* (1989) in *Prunus* sp., as they obtained root only with IBA. Roy and De (1986) and Mante *et al*. (1989) also reported faster growth of roots in *C. gigantea* and *Prunus* sp. respectively. Almost for all the cases, ½ strength of MS salt proved better than full strength of MS in rooting purpose. This report is an agreement with Lu *et al*. (1982).

After sufficient development of roots, plantlets were successfully transplanted in pots and finally established to the field condition (Plate1 D). The survival rate of the transplanted plantlets was 70% which was similar to the findings of Roy and De (1986) in *C. gigantena,* Mante *et al.* (1989) in *Prunus* sp. and Karim (1991) in *Aegle marmelos*. In conclusion, we report an efficient and easy to handle protocol for micropropagation of *A. esculentus*. This protocol provides a successful and rapid technique that can be used for *ex situ* conservation. As a part of domestication strategy, these plants can be grown and further cultivated in fields. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of the valuable flora of Bangladesh.

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