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Callus Induced Organogenesis in Okra (*Abelmoschus esculents* L. Moench.)

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Abstract: A viable protocol has been developed for indirect shoot organogenesis of okra. To establish a stable and high-frequency plant regeneration system, leaf disc and hypocotyl explants were tested with different combinations of α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA),iIndole-3-butyric acid (IBA), thidiazuron (TDZ) and 6-benzylaminopurine (BAP). Morphogenic callus induction was observed in highest frequency from hypocotyl explant by culturing in MS medium supplemented with 2.0 mg L^{-1} NAA plus 0.5 mg L^{-1} TDZ. The highest percentage of shoot regeneration and highest mean number of shoot per callus mass was obtained with 2.0 mg L^{-1} BAP plus 0.1 mg L^{-1} IBA. Root formation was observed from callus induced in medium containing 1.5 mg L^{-1} NAA. Morphogenic difference due to explant type is clear for the studied *in vitro* traits. About 80% of regenerated plantlets were survived and showed new leaves development under *ex vitro* condition. This protocol would be useful to create somaclonal variation and to utilize transgenic approach for varietal improvement of okra.

Key words: *Abelmoschus esculentus*, callus, *in vitro*, Malvaceae, okra, organogenesis

INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench.), often called lady's finger in England or gumbo in USA, belongs to the family Malvaceae, is an important vegetable crop throughout the tropical and warm temperate regions. It is valued for its immature green fruits and leaves. Okra seeds are a good source of vitamins, minerals and medicinally important compound (Uda *et al*., 1997). One of the mucilagenous preparation from the pod can be used as a plasma replacement or blood-volume expander (Chopra *et al*., 1986). Protein content of the seed is 20% or more and oil content 14% or more. Therefore, okra is considered as a potential protein and oil crop (Martin *et al*., 1981). Considering its tremendous importance in world's vegetable supply conventional breeding and agronomic practices have been adopted to improve the yield and quality of this crop (Martin *et al*., 1981; Ariyo, 1987; Ali *et al*., 2000; Shoba and Mariappan, 2007; Gulsen and Abak, 2007). However, use of modern biotechnology to improve this crop is limited. Recognizing its economic importance, still there is a vast scope to utilize modern biotechnology for further improvement of this valuable crop. Success of utilizing such technology, such as transformation and somaclonal variation, largely rely on efficient *in vitro* regeneration techniques. Regarding *in vitro* culture of okra, only limited numbers of protocols were reported for shoot organogenesis. Ganesan *et al*. (2007) has reported protocol for plant regeneration through somatic embryogenesis. Recently, we have developed protocol for mass *in vitro* propagation using meristem culture for disease-free plant production (Anisuzzaman *et al*., 2008). Here, reported a simple and reproducible organogenic regeneration protocol from leaf disc and hypocotyl via callus phase.

MATERIALS AND METHODS

Plant materials and culture conditions: One of the high yielding okra cultivar, BARI Dherosh-1 was used in the experiments. Hypocotyl explants were collected from *in vitro* germinated seeds. Seeds were surface sterilized using 70% ethanol and 0.1% $HgCl_2$ and germinated on hormone-free MS medium (Murashige and Skoog, 1962) following the earlier report (Anisuzzaman *et al*., 2008). Leaf disc explants were collected from meristem derived *in vitro* plantlets as described earlier (Anisuzzaman *et al*., 2008). Besides growth regulators,

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MS medium were supplemented with 3% sucrose and vitamins. After adjusting the pH to 5.7±0.01 prior to gelledwith 0.8% agar (w/v), (BHD, England) the medium was sterilized by autoclaving at 121°C for 20 min (1.06 kg cm^{-2}). All cultures were maintained in a growth chamber at 25±1°C under a 16/8 h (light/dark) photoperiod with a light intensity of 28-30 mol/m/sec (supplied by cool-white fluorescent lamps). Similar cultural condition was maintained in the entire experiment.

Callus induction, plant regeneration and rooting: Leaf disc and excised hypocotyls were placed on MS medium supplemented with different concentration of plant growth regulators to induce callus (Table 1). After callus induction, calli were subcultured in fresh medium in a 3-4 weeks of interval for further proliferation. Watery, spongy brown and dead portion of calli were discarded during subcultures and only friable, nodular calli were maintained to develop organogenic nature. For plant regeneration, calli were transferred to regeneration medium having different auxin-cytokinin combinations (Table 2). When the regenerated shoots were elongated up to around 2 cm, shoots were excised and transferred to MS medium fortified with different concentrations of NAA or IAA for root induction.

Acclimatization and transfer to soil: Plantlets, with a well-developed root system, were washed carefully to remove agar and then transferred to the pots containing sterile vermiculite. After watering, plantlets were maintained in a growth chamber at 27±1°C under 16 h illumination (45 mol m/sec) with fluorescent lamps. After 3 weeks of acclimatization had been completed, plantlets were transferred to large pots for further growth.

Data recording: To test the efficiency of callus induction medium, percentage of callus induction was noted. For plant regeneration, regeneration frequency and number of shoot per callus were calculated. Same parameters were noted for rooting. Each experiment contained at least 12 replications and the whole experiment was repeated three times.

RESULTS AND DISCUSSION

Callus was initiated within 7-10 days directly on the cut surfaces in all hormone supplemented medium, but not in hormone-free medium (Table 1). However, callusing rate was markedly affected by the type of primary explant and type of growth regulator used. Different cytokinin and auxin combinations and concentrations had a distinct effect on callus induction. Hypocotyl was found better source of explant for callogenesis (95%) in MS medium containing 2.0 mg L^{-1} NAA plus 0.5 mg L^{-1} TDZ (Fig. 1A). Both color and texture of the callus were varied derived from two explants. Callus derived from hypocotyls was mostly friable and creamy in color, whereas, leaf derived calli were soft and pale brown in color and found relative less potential for further organogenesis.

Table 1: Effect of different concentrations of plant growth regulators on organogenic callus induction from leaf disc and hypocotyl of okra. Data were recorded after 3 weeks of inoculation in callus induction medium

PGR	Frequency (%)	
(mg L^{-1})	Leaf disc	Hypocotyl
NAA		
0.5	10.5	12.5
1.0	18.1	20.0
2.0	33.3	27.5
3.0	22.5	35.7
5.0	17.5	16.1
NAA+BAP		
1.0+0.5	33.3	45.2
1.0+1.0	45.0	58.3
2.0+0.5	55.2	65.3
3.0+0.5	80.2	87.5
3.0+1.0	60.3	45.2
NAA+TDZ		
1.0+0.1	30.0	33.3
1.0+5.0	50.0	55.5
2.0+0.5	87.5	95.0
2.0+1.0	55.6	60.0
3.0+0.5	20.0	22.5

In vitro callus induction and plant regeneration depends on the endogenous concentration of plant growth regulator as well as exogenously supplied growth regulator. 2,4-D is among the most widely used auxin used for *in vitro* callus induction in a wide range of plant species (Pal *et al*., 2007). However in present study 2,4-D was not found effective for callus induction in okra (data not shown). A combination of BAP and NAA was found to be suitable for induction of callus and shoot differentiation. This cytokinin-auxin combination has been widely used for indirect organogenesis in various protocols developed for the species of Malvaceae family (McLean *et al*., 1992; Haider *et al*., 1993; Ikram-ul-Haq, 2005) and as well as in other species (Kumari *et al*., 2008; Lima *et al*., 2008). Nevertheless, the most encouraging results were observed by adding TDZ with NAA. Using this combination in the nutrient medium, both the explant type formed potentially embryogenic callus within several weeks of culture. TDZ is believed to be involved in the regulation of purine cytokinin metabolism and may act directly as a cytokinin or in combination with auxin. It has also been suggested that the biological activity of TDZ is higher than, or comparable to that, of the most active adenine type cytokinins (Mok *et al*., 2000; Khawar *et al*., 2004). Present result justifies the earlier reports (Gill and Ozias-Akins, 1999; Nobre *et al*., 2001). Data from the

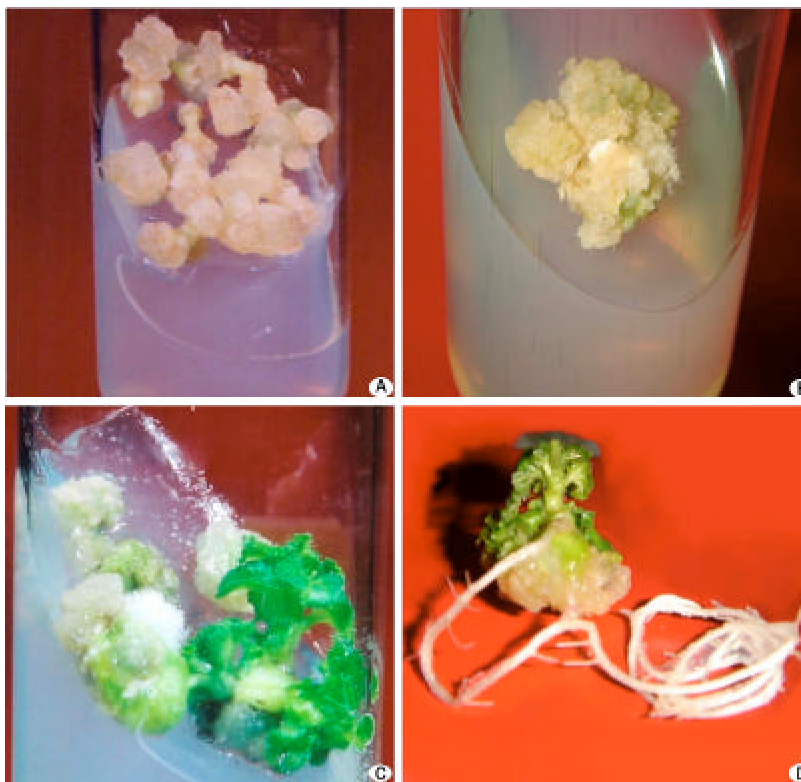

Fig. 1: Representative picture of shoot differentiation from callus through organogenesis in okra, (A) initiation of callus from hypocotyl explant, (B) organogenic calli showing green points, (C) development of plantlets from callus and (D) regenerated plantlets with developed roots

current study provide the evidence that growth regulator requirements for callus induction vary depending on the source of the explant. Such responses have been established previously (Nikam and Shitole, 1998).

After several subcultures, the putative organogenic calli were transferred to regeneration medium containing auxin-cytokinin combination (Table 2). After 3-4 weeks of culture on regeneration medium, some green spots (Fig. 1B) and followed by development of several shoots were observed gradually from single callus mass (Fig. 1C). The types and concentrations of the plant growth regulators in the culture medium affect successful induction of organogenesis (Oggema *et al*., 2007). Generally, shoot initiation was observed after fourth weeks of inoculation on regeneration medium. The shoot-like structures could be distinguished by the presence of green, opaque and compact nodules. Over a period of 7 weeks, around 65% of callus produced shoots, with a highest average of 7 shoots per explant in the medium containing 2.0 mg L^{-1} BAP plus 0.5 mg L^{-1} IBA. Whereas, 2.5 mg L^{-1} BAP plus 0.1 mg L^{-1} NAA also exhibit good number of shoot regeneration event. Similar results were observed in cotton (Nobre *et al*., 2001; Tripathy and Reddy, 2002).

Table 2: Effect of different concentrations of plant growth regulators on organogenic regeneration from callus. Data were recorded after 7 weeks of inoculation in regeneration medium

PGR	Regeneration frequency (%)		Shoots/Callus ($\bar{X}$ ±SE)	
(mg L^{-1})	Leaf disc	Hypocotyl	Leaf disc	Hypocotyl
BAP+NAA				
1.0+0.1	25.0	34.4	2.56±0.85	2.20±0.58
1.0+0.5	33.3	37.5	2.61±0.32	2.42±0.45
2.0+0.1	47.5	52.5	5.26±1.05	6.81±0.25
2.0+0.5	31.5	30.3	1.70±0.33	1.82±0.56
BAP+IBA				
1.0+0.1	36.7	41.5	1.90±0.25	2.10±0.53
1.0+0.5	42.5	50.0	3.71±0.25	4.98±0.28
2.0+0.1	53.3	65.0	6.70±0.34	7.01±0.36
2.0+0.5	30.4	38.2	2.55±0.13	2.30±0.48

The regenerated shoots were elongated to about 2 cm in 8 weeks of culture. These plantlets continued to develop and formed roots upon transfer to the rooting medium. Roots were observed as early as 2 weeks after placing the microshoots (2-3 cm) on rooting medium. Most of the shoots developed roots by week 4 (Fig. 1D). Highest number of root was induced in MS supplemented

Table 3: Effect of different concentrations of plant growth regulators on root induction. Data were recorded 4 weeks after transfer in rooting medium

PGR (mg L^{-1})	Rooting frequency (%)	Root/shoot ($\bar{X}$ ±SE)	Mean length of root (cm) ($\bar{X}$ ±SE)
NAA			
0.5	55.0	6.50±0.35	3.2±1.11
1.0	65.0	8.50±0.53	4.2±1.22
1.5	80.0	10.56±0.66	4.5±0.33
2.0	62.5	7.80±0.15	3.8±0.56
3.0	50.0	4.50±0.30	2.9±0.58
IAA			
0.5	52.5	5.50±0.33	3.1±1.21
1.0	57.5	6.70±0.16	4.1±0.78
1.5	70.0	7.16±0.21	4.5±1.51
2.0	68.5	4.16±0.28	3.7±1.01
3.0	50.0	3.60±0.34	2.9±0.79

with 1.5 mg L^{-1} NAA (Table 3). NAA is widely used plant growth regulator for root induction in a number of species including Malvaceae members (Tripathy and Reddy, 2002), while IAA is also used (Kathiravan *et al*., 2006). In some cases, rooting in growth regulator free medium during organogenesis has been also reported (Hazra *et al*., 2000). Rooting responses variation may be due to genotype and different pre culture conditions of shoots prior to root induction. The *in vitro* propagated plantlets were transferred to large soil pots after 2 weeks of initial hardening under culture-room conditions. Almost 80% of these regenerants were survived and showed normal growth and development.

The importance of developing callus-mediated regeneration has increased over the years to widen the opportunity of genetic transformation. The present callus induction regeneration system would be important for genetic transformation and, also has considerable potential to explore somaclonal variation as an alternative means of conventional hybridization.

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