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Somatic embryogenesis and plant regeneration of *Abelmoschus esculentus* through suspension culture

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Abstract

A simple and reliable protocol for regeneration of okra through somatic embryogenesis from suspension cultures has been developed. Embryogenic callus was obtained from hypocotyl explants cultured on media with Murashige and Skoog (MS) salts, Gamborg (B5) vitamins, 2.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg dm^{-3} naphthaleneacetic acid (NAA), 25 mg dm^{-3} polyvinylpyrrolidone and 30 g dm^{-3} sucrose. More number and high frequency of healthy embryoids appeared individually in suspension culture containing MS salts, B5 vitamins, 2.0 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} kinetin. Formation of cell clusters from the single cells was clearly noticed during ontogeny. Matured embryos at the cotyledonary stage were transferred to agar solidified medium for germination. The best conversion of embrya into plantlets (67.3 %) was recorded on media with half strength MS salts, B5 vitamins, 0.2 mg dm^{-3} benzylaminopurine (BAP) and 0.2 mg dm^{-3} gibberellic acid (GA_3). The plantlets were transferred to soil and hardened in the plastic pots. After proper acclimatization, the plantlets regenerated through somatic embryogenesis were compared to seed grown plants to observe any variation.

Additional key words: auxins, cell aggregates, embryoids, liquid medium, okra.

Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] is considered to be an important vegetable. The okra seeds are a good source of vitamins, minerals, and medicinally important compound (*e.g.* Uda *et al.* 1997). Regarding okra tissue culture, only limited numbers of protocols were reported for shoot organogenesis and no regeneration protocol has previously been reported through embyogenic callus induction and somatic embryogenesis. Mangat and Roy (1986) reported that supplementation of naphthalene-acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) showed negative response for callus induction and shoot bud regeneration and they proved that shoot bud differentiation and regeneration of okra is possible through node and shoot tip explants by using cytokinins. Haider *et al.* (1993) also proved that shoot organogenesis was possible in okra and they successfully regenerated the whole plantlets through hypocotyls callus cultures by using combined treatment of benzylaminopurine (BAP) and NAA. Our experiments showed that there were several challenging difficulties to be overcome during somatic embryogenesis in okra including low percentage of embryo induction and plant regeneration, excretion of mucilage and phenolic compounds from the explants to medium, browning of callus within short period of culture, non embryogenic callus formation with variations in colour and texture, conversion of embryogenic callus into non-embryogenic callus within short period of culture, low frequency of embryoid induction and germination and abnormal embryo formation. Hence, our aim in this research was to achieve regeneration of okra without the difficulties listed above.

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Abbreviations: B5 - Gamborg *et al.* (1968) medium; BAP - benzylaminopurine; GA_3 - gibberellic acid; KIN - kinetin; MS - Murashige and Skoog (1962) medium; NAA - naphthaleneacetic acid; PVP - polyvinylpyrrolidone; SH - Schenk and Hildebrandt (1972) medium; 2,4-D - 2,4-dichlorophenoxyacetic acid.

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Materials and methods

Seed germination: Okra [*Abelmoschus esculentus* (L.) Moench] cv. Surabhi-10 seeds were collected from the National Seeds Corporation, Trichy, Tamil Nadu, India. Surface sterilization of seeds was achieved by the method of Ganesan and Jayabalan (2004). The surface sterilized seeds were inoculated on seed germination medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al*. 1968), BAP (0.2 - 2.0 mg dm^{-3}), 30 g dm^{-3} sucrose and 0.8 g dm^{-3} agar. The pH of the medium was adjusted between 5.7 - 5.8, using 0.1 M NaOH before auto-claving at 121 °C for 15 min. Initially the cultures were maintained in the dark for 2 d and all cultures were maintained at 25 ± 2 °C under a 16-h photoperiod with an irradiance of 75 μmol m^{-2} s^{-1}. Vitamins, such as thiamine-HCl and myo-inositol, were freshly added to the medium, as required, in the form of a solution in double-distilled water.

Embryogenic callus induction and maintenance: Hypocotyl explants (0.4 - 0.6 cm in length) from 6-d-old seedlings were placed horizontally on medium comprising MS salts, B5 vitamins, 8.0 g dm^{-3} agar and 30 g dm^{-3} sucrose, 2,4-D (0.2 -5.0 mg dm^{-3}), NAA (0.2 - 2.0 mg dm^{-3}) and polyvinylpyrrolidone (PVP; 5 - 50 mg dm^{-3}). After 2 weeks of culture, pro-embryogenic callus was isolated and subcultured at weekly intervals on the same medium for another 2 weeks for the induction of embryogenic calli. The embryogenic callus was identified by means of anatomical studies on the basis of its creamy white colour and the presence of small, less vacuolated and densely filled cytoplasmic cells. Conversion of embryogenic callus into non-embryogenic callus within 10 d of culture is common. Hence, we subcultured the embryogenic callus every 8 d. During embryogenic callus induction the impact of four different carbon sources (sucrose, glucose, fructose and maltose) and concentrations were tested on okra embryogenic callus induction. The influence of various additives on the browning of embryogenic callus during embryoid induction was also observed [PVP (5 - 50 mg dm^{-3}), charcoal (100 - 1000 mg dm^{-3}) and ascorbic acid (10 - 100 mg dm^{-3})]. For callus induction, maximum of 75 explants were tested and these experiments were repeated for four times with six replicates.

Suspension cultures: Cell suspension cultures were initiated by placing 200 to 250 mg of callus into 50 cm^3 of MS liquid medium with different concentrations of 2,4-D (0.5 - 5.0 mg dm^{-3}), NAA (0.5 - 5.0 mg dm^{-3}), KIN (0.25 - 2.5 mg dm^{-3}), dispensed in 250 dm^{-3} Erlenmeyer flasks. The cultures were incubated on an orbital gyratory shaker at 120 rpm. During the first 25 d, single cells and small aggregates of cells released into the medium were subcultured. During each subculture half of the liquid medium was removed, and fresh medium with the desired amount of growth regulators were added. To achieve embryoid induction in suspension culture, weekly subculture was needed.

Embryoid induction and maturation: After 6 - 7 weeks of subculture, batches of pro-embryogenic tissues were formed, these tissues only induced pro-embryoid formation. The pro-embryoids were characterized by the multiple cell aggregate stage. After 6 weeks of subculture of pro-embryoids, whitish transparent, visible globular embryoids were formed. All the embryoids obtained were allowed to mature in the same medium. Matured torpedo embryoids were subcultured from the above liquid to semisolid medium (0.6 % agar) to complete maturation. During embryoid induction different concentrations of NH_4NO_3 and $MgSO_4$ were tested. During embryoid induction the experiments were repeated for 3 times with 6 replicates.

Plantlet regeneration and acclimatization: Matured green somatic embryos were identified at the cotyledonary stage (greenish white in colour) and transferred to embryoid regeneration medium: MS basal salts (half strength), B5 vitamins, 30 g dm^{-3} glucose along with GA_3 (0.1 - 0.5 mg dm^{-3}) and BAP (0.1 - 0.5 mg dm^{-3}). After complete regeneration of embryoids into plantlets (35 - 40 d), the regenerated plants were transferred to plastic pots containing sand and soil in a 1:1 ratio for hardening in the greenhouse. The plants were completely covered with plastic bags for 2 weeks to maintain the humidity and these were progressively removed to allow adaptation to normal environmental conditions. For the initial 10 d of hardening, plants were supplemented with 25 % MS liquid medium (MS basal salts + B5 vitamins) and the medium strength was progressively reduced to reach 0 % at the end of 30 d. Selected regenerated plants, adapted to normal environmental conditions, were transferred to earthen pots for further growth and development. Evaluation of various concentrations of B5, MS and SH vitamins were also carried out for enhanced somatic embryo germination.

Statistical analysis: Means and standard errors were used throughout the study and the values were assessed using a parametric moods median test (Snedecor and Cochran 1989). The data were analysed for variance by Duncan's multiple range test (DMRT) using the *SAS* programme (SAS Institute, Cary, NC, USA).

Results and discussion

Embryogenic callus induction: The surface sterilized seeds showed 85 % germination in the medium with MS salts, B5 vitamins and BAP (1.0 mg dm^{-3}). Hypocotyl explants alone were selected and cultured on the

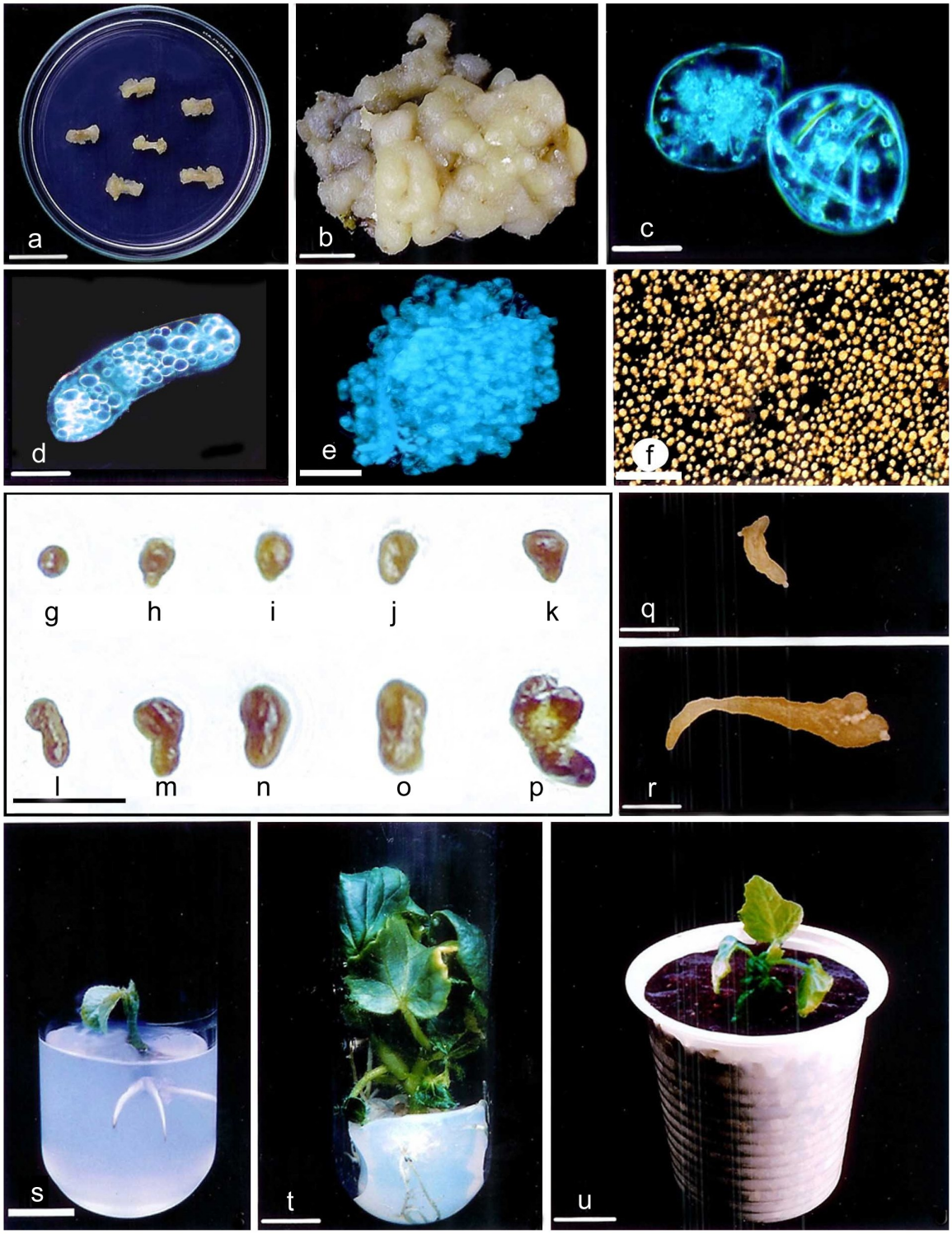

Fig. 1. Different developmental stages of okra through suspension culture: *a* - embryogenic callus induction from hypocotyl explants. (*bar* 2 cm), *b* - 4-week-old embryogenic callus (*bar* 5 mm), *c* - single cells observed in the suspension culture (*bar* 100 μm), *d* - double celled stage showing the important starch granules (light microscope with high contrast, *bar* 150 μm), *e* - embryogenic cell cluster observed in suspension culture (*bar* 300 μm), *f* - globular embryoids on suspension culture (*bar* 1 cm), *g* to *r* - various stages of somatic embryos from pro-embryoids to torpedo stage (*bar* 6 mm), *s* - germinated somatic embryo (*bar* 1 cm), *t* - regenerated plantlet (*bar* 1 cm), *u* - hardened plantlet in plastic pot (*bar* 1.5 cm).

Table 1. Effect of different concentrations of 2,4-D, NAA and KIN on mean number of proembryoids formed after 6 weeks, embryoids formed after 8 weeks and matured somatic embryos formed after 10 weeks from hypocotyl derived embryogenic callus cultured on the liquid medium with MS salts and B5 vitamins. Values are means ± standard error of three repeated experiments. Each treatment consisted of six replicates (0.5 dm^3). Means within a column followed by the same letters are not significant at $P = 0.05$ according to DMRT.

Growth regulators	[mg dm^{-3}]	Number of pro-embryoids	Number of embryoids	Number of somatic embryos	Somatic embryo maturation [%]
2,4-D	0.5	30.2 ± 0.45l	12.0 ± 0.22ij	4.4 ± 0.08m	33.3f
	1.0	38.4 ± 0.25k	18.1 ± 0.18j	8.2 ± 0.95k	44.4e
	1.5	42.7 ± 0.52ij	22.8 ± 0.33h	17.2 ± 0.12h	77.2b
	2.0	66.0 ± 0.45g	38.1 ± 0.15f	31.4 ± 0.23f	88.5a
	2.5	56.1 ± 0.40h	35.4 ± 0.17g	24.1 ± 0.11g	68.5c
	3.0	43.7 ± 0.38i	20.0 ± 0.21i	12.0 ± 0.14j	60.0d
	3.5	39.3 ± 0.36jk	12.2 ± 0.11ij	5.1 ± 0.11lm	41.6ef
NAA	2.0	12.2 ± 0.36o	5.2 ± 0.16l	2.5 ± 0.13mn	40.0a
2,4-D+KIN	2.0 + 0.25	75.3 ± 0.75f	73.7 ± 1.11ef	30.4 ± 1.18fg	41.0c
	2.0 + 0.50	89.2 ± 1.20c	85.4 ± 0.97c	46.4 ± 1.52d	54.1b
	2.0 + 0.75	98.4 ± 0.92b	96.1 ± 1.05b	69.6 ± 2.15b	71.9ab
	2.0 + 1.00	125.0 ± 1.15a	125.0 ± 0.87a	98.0 ± 2.75a	78.4a
	2.0 + 1.25	85.1 ± 0.98d	83.1 ± 0.95d	61.2 ± 1.85c	52.5bc
	2.0 + 1.50	80.2 ± 0.85e	80.4 ± 1.10e	42.4 ± 1.52e	25.0d
NAA+KIN	2.0 + 0.25	15.8 ± 0.52n	8.2 ± 0.41k	5.1 ± 0.07l	62.5cd
	2.0 + 0.50	21.0 ± 0.45mn	11.7 ± 0.27j	8.2 ± 0.09k	72.7c
	2.0 + 0.75	29.1 ± 0.48lm	18.7 ± 0.36h	16.7 ± 0.11ij	88.8b
	2.0 + 1.00	41.1 ± 0.46j	28.4 ± 0.24gh	28.2 ± 0.77fg	100.0a
	2.0 + 1.25	22.2 ± 0.66m	15.2 ± 0.28i	12.2 ± 0.55j	86.6bc

embryogenic callus induction medium. Okra embryogenic callus induction, somatic embryo induction and germination showed an auxin dependent response. Among the different concentrations of auxins tested, the combined effect of 2.0 mg dm^{-3} 2,4-D with 1.0 mg dm^{-3} NAA gave the best response for embryogenic callus induction on the media with MS salts, B5 vitamins, 30 g dm^{-3} sucrose and 0.8 % agar. At this concentration, only creamy white friable embryogenic callus was obtained (Fig. 1*a-c*). Usually, embryogenic callus induction has been effectively achieved by the combined treatment with auxin and cytokinin (Bhansali *et al*. 1990, Aly *et al*. 2002, Ma and Xu 2002, Hernandez *et al*. 2003, Nath and Bugagohain 2005). In the present study, the individual effect of 2,4-D and NAA showed a poor response for the induction of embryogenic callus (Table 1). In contradiction to the above, individual or combined treatment of auxins produced embryogenic callus and somatic embryos (Chalupa 1990, Wachira and Ogada 1995, Kim *et al*. 2003). These results proved that 2,4-D is the essential plant growth regulator for the induction of embryogenic callus (Maureen *et al*. 1990, Haider *et al*. 1993). During embryogenic callus induction, a very slow response of the explants was observed. The required mass of embryogenic callus was only observed after 2 months of culture. For the induction of embryogenic callus at high frequency, 7 to 8 d subculture was regularly followed otherwise browning of the embryogenic callus and medium could not be controlled.

Influence of various additives and sugars on embryogenic callus induction: Our results showed that addition of PVP (25 mg dm^{-3}) reduced the phenolic compound secretion from explants to medium. Further experiments showed that addition of different concentrations of charcoal and ascorbic acid reduced the frequency of okra embryogenic callus induction and did not control the phenolic exudation from explants to medium (data not shown). PVP is a common additive used for the control of phenolic oxidation during embryogenic callus and embryoid induction (Saxena and Dhawan 1999). The influence of different concentrations of carbon sources like glucose, fructose, sucrose and maltose were tested for their effect on the frequency of embryogenic callus induction. Finally we confirmed that 30 g dm^{-3} of sucrose was an efficient carbon source for the embryogenic callus induction (Fig. 2). Sucrose-mediated high frequency embryogenic callus induction was also observed in black iris (Shibli and Ajlouni 2000). With the other concentrations and forms of carbon sources tested, variations in colour, texture and a delayed response for callus induction was observed. For the successful regeneration of plantlets from the somatic embryos, induction of good quality of embryogenic callus is essential (Ganesan and Jayabalan 2004).

Somatic embryo induction and maturation: Somatic embryo induction from hypocotyl derived callus of okra was achieved on liquid medium fortified with MS salts,

B5 vitamins, 30 g dm^{-3} sucrose, 2.0 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} KIN (Table 1). In this medium, cell suspensions with small cell aggregates were formed after 3 weeks of culture. Weekly subculture was followed as in other studies (Bais *et al*. 2000). Formation of cell clusters from the callus derived single cells was clearly observed during the ontogeny (Fig. 1*d-f*). The numerous cell clusters obtained were again subcultured in the same medium for the induction of embryoids. The embryogenic nature of the cell cluster was confirmed by the procedure of Tsay and Huang (1998). A similar tendency was found with cell suspension-cultures of carrot (Bayliss 1977) and tobacco (Evans and Gamborg 1982) and these authors confirmed that cell cluster formation positively influenced the somatic embryo induction and formation (Toonen *et al*. 1996). After 8 weeks of culture, globular embryoids were noticed and 125 globular embryoids were formed. The individual effect of varying concentrations of 2,4-D and NAA treatments showed a very low frequency of embryoid induction and sometimes browning of the cell suspension within 48 h was seen. Combinations of different concentrations of NAA with KIN gave a very low percentage of embryoid induction, maturation and plant regeneration when compared with the 2.0 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} KIN combination. In the case of *Capsicum annuum*, a similar trend was observed for the somatic embryo induction (Kintzios *et al*. 2001) and 9 μM 2,4-D with 12.9 μM BAP was used for the induction of somatic embryos at high frequency. In some cases, supplementation of 2,4-D alone was used for the induction of somatic embryos (Milivojević *et al.* 2005). Further subculture of globular embryoids in the same medium for another 2 weeks yielded matured green somatic embryos. By using the formation of heart and torpedo shaped embryos, we confirmed the embryo maturation process (Fig. 1*G-R*) (Varisai *et al*. 2004). During maturation, light yellowish green globular embryos turned into big, greenish brown cotyledonary embryos. From 125 globular somatic embryos, 98 cotyledonary stage embryos were selected as matured embryoids, *i.e*. 78.4 % embryo maturation was recorded using the above concentration of growth regulators.

During embryoid induction and maturation, combination of NH_4NO_3 (500 mg dm^{-3}) and $MgSO_4$ (1.0 g dm^{-3}) showed best response for enhanced production of embryoids and 178 somatic embryos per 500 cm^3 culture were observed after 10 weeks of culture. At the same time high frequency of somatic embryo maturation was also observed in the above said concentrations. Maximum of 167 (93.8 %) matured somatic embryos per 500 cm^3 culture were obtained at the 10^{th} week of culture. These results proved that addition of NH_4NO_3 highly influenced the somatic embryo induction and maturation percentage. In this concentration, 93.8 % of somatic embryo maturation was observed and in the case of control cultures only 78.4 % somatic embryo maturation was observed. This type of enhanced percentage of embryoid induction was noticed in cotton by the supplementation of NH_4NO_3 and $MgSO_4$ (Ganesan and Jayabalan 2004). Like our results, in apple rootstock, addition of N-sources like KNO_3 and MH_4NO_3 along with MS medium showed best response for the *in vitro* culture studies (Sotiropoulos *et al.* 2005).

Somatic embryo regeneration: The somatic embryos formed were transferred to media fortified with different concentrations of BAP or/and GA_3 for germination. Simultaneous development of shoot and root from the matured somatic embryos was achieved after 4 weeks of

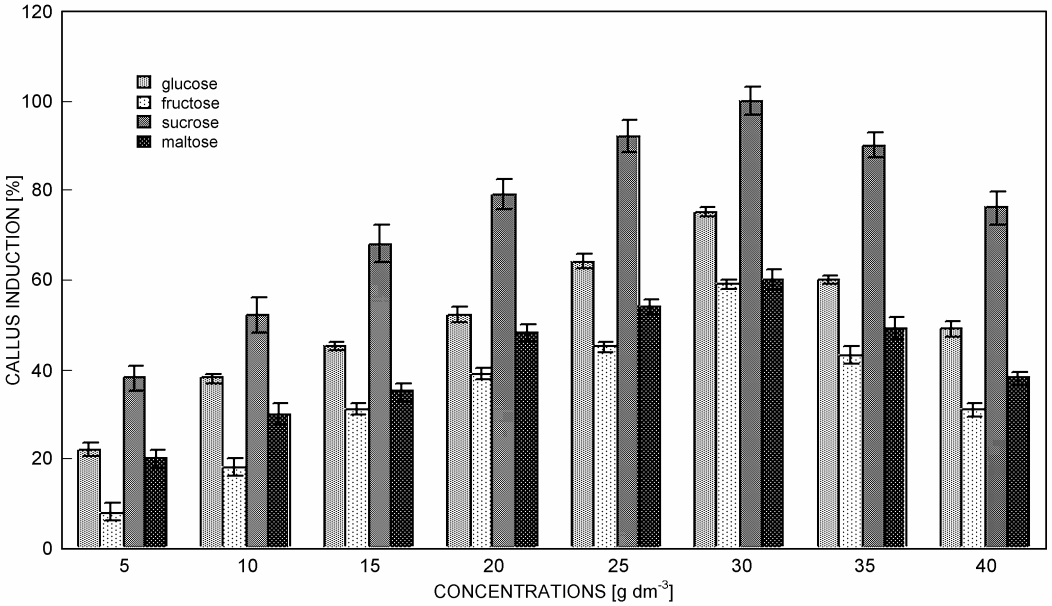

Fig. 2. Effect of different concentrations of sugars on embryogenic callus induction. Means ± SE of three repeated experiments. Each treatment consisted of six replicates.

Table 2. Influence of different concentrations of BAP and GA_3 on regeneration of somatic embryos cultured on the medium with half strength MS salts and B5 vitamins. Means ± SE of three repeated experiments. Each treatment consisted of five replicates. Means within a column followed by the same letters are not significant at $P = 0.05$ according to DMRT.

Growth regulators	[mg dm^{-3}]	Number of embryos tested	Number of regenerated plantlets	Regenerated plantlets [%]	Number of abnormal embryos
BAP	0.05	24	2.5 ± 0.35d	10.2d	12.4 ± 0.42b
	0.10	26	3.5 ± 0.40c	13.2cd	11.6 ± 0.45c
	0.15	22	4.0 ± 0.20b	18.2bc	8.2 ± 0.21de
	0.20	22	4.5 ± 0.55a	20.0a	4.5 ± 0.18e
	0.25	24	4.5 ± 0.25a	18.7b	8.3 ± 0.34d
	0.30	25	3.5 ± 0.45c	14.0c	11.6 ± 0.19c
	0.35	26	1.5 ± 0.35e	5.6e	20.4 ± 0.25a
BAP+GA_3	0.2+0.05	23	4.5 ± 0.45de	19.5g	8.8 ± 0.11bc
	0.2+0.10	23	5.5 ± 0.45d	23.9f	5.2 ± 0.08d
	0.2+0.15	25	12.0 ± 0.22b	47.0c	3.4 ± 0.18
	0.2+0.20	24	16.5 ± 0.64a	67.3a	2.7 ± 0.14f
	0.2+0.25	21	11.5 ± 0.25bc	54.8b	5.9 ± 0.15c
	0.2+0.30	22	8.5 ± 0.45c	38.6d	9.4 ± 0.16b
	0.2+0.35	24	07.5 ± 0.32cd	31.3e	11.7 ± 0.21a

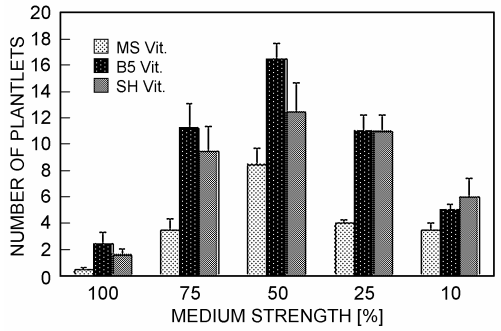

Fig. 3. Influence of vitamins and medium strength on somatic embryo germination. Means ± SE of three repeated experiments. Each treatment consisted of two replicates.

culture on the media with MS salts (half strength), B5 vitamins (half strength), BAP (0.2 mg dm^{-3}) and GA_3 (0.2 mg dm^{-3}) (Fig. 1*S*, Table 2). Negative influence of other vitamin sources (MS and SH vitamins) was clearly noticed during somatic embryo germination. Our studies also proved that medium strength and vitamin sources are essential for somatic embryo germination and variations in the concentrations of media and vitamins highly affected the somatic embryo germination (Fig. 3). From the different concentrations tested, half strength MS salts and B5 vitamins showed best response for somatic embryo germination. BAP and GA_3-mediated somatic embryo germination was also observed in cotton (Ganesan and Jayabalan 2004). During germination, the matured cotyledonary staged embryos turned completely green and then the embryos produced shoot and root simultaneously. In total, 16.5 plantlets were regenerated from the total of 24.5 matured somatic embryos tested. About 67.3 % plant conversion was observed under the above conditions (Table 2). Somatic embryo conversion was noticed in all the combinations of BAP and GA_3 tested. BAP and GA_3 alone led to a very slow response for embryo conversion and at the same time a high percentage of abnormal embryo formation was also noticed. Regenerated somatic embryos (Fig. 1*T*) were hardened in plastic pots with sand and soil in 1:1 ratio (Fig. 1*u*). The hardened plants were covered with plastic bags for proper acclimatization and these were removed after 5^{th} leaf formation. Before hardening, the germinated embryos were kept undisturbed for 8 weeks in the ordinary culture conditions. During this period, formation of secondary and tertiary roots was noticed. The advantage of secondary and tertiary root formation from the regenerated plantlets is that it leads to active growth after hardening. After proper acclimatization, the regenerated plantlets showed a 100 % survival rate in the greenhouse conditions. The regenerated plantlets exhibited all the parental characters without any phenotypic variation. The protocol described here is simple and easy and it will form a useful basis to improve the germplasm of okra.

References

Aly, M.A.M., Rathinasabapathi, B., Kelley, K.: Somatic embryogenesis in perennial statice *Limonium bellidifolium, Plumbaginaceae*. - Plant Cell Tissue Organ Cult. **68**: 127-135, 2002.

Bais, H.P., Travis, S., Walker, J.J., McGrew, V.J.M.: Factors affecting growth of cell suspension cultures of *Hypericum*

perforatum L. (St. John's wort) and production of hypericin. - *In Vitro* cell. dev. Biol. Plant **38**: 58-65, 2002.

Bayliss, M.W.: Factors affecting the frequency of tetraploid cells in a predominantly diploid suspension of *Daucus carota.* - Protoplasma **92**: 109-115, 1977.

Bhansali, R.R., Driver, J.A., Durzan, D.J.: Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis. - Plant Cell Rep. **9**: 280-284, 1990.

Chalupa, V.: Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). - Plant Cell Rep. **9**: 398-401, 1990.

Evans, D.A., Gamborg, O.L.: Chromosome stability of cell suspension cultures of *Nicotiana* species. - Plant Cell Rep. **1**: 104-107, 1982.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Ganesan, M., Jayabalan, N.: Evaluation of haemoglobin (erythrogen): for improved somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L. cv. SVPR 2). - Plant Cell Rep. **23**: 181-187, 2004.

Haider, S.A., Islam, R., Kamal, A.H.M., Rahman, S.M., Joarder, O.I.: Direct and indirect organogenesis in cultured hypocotyl explants of *Abelmoschus esculentus* (L.) Moench. - Plant Tissue Cult. **3**: 85-89, 1993.

Hernandez, L., Celestino, C., Toribio, M.: Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. - Plant Cell Rep. **21**: 759-764, 2003.

Kim, S.W., In, D.S., Kim, T.J., Liu, J.R.: High frequency somatic embryogenesis and plant regeneration in petiole and leaf explant cultures and petiole-derived embryogenic cell suspension cultures of *Hylomecon vernalis*. - Plant Cell Tissue Organ Cult. **74**: 163-167, 2003.

Kintzios, S., Drossopoulos, J.B., Lymperopoulos, C.: Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from leaves of chilli peper. - Plant Cell Tissue Organ Cult. **67**: 55-62, 2001.

Ma, G., Xu, Q.: Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. - Plant Cell Tissue Org. Cult. **70**: 281-288, 2002.

Mangat, B.S., Roy, M.K.: Tissue culture and plant regeneration of okra [*Abelmoschus esculentus* (L.) Moench]. - Plant Sci. **47**: 57-62, 1986.

Maureen, M., Fitch, M., Manshardt, M.: Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). - Plant Cell Rep. **9**: 320-324, 1990.

Milivojević, S., Mitrović, A., Ćulafić, L.: Somatic embryogenesis in *Chenopodium rubrum* and *Chenopodium murale in vitro.* - Biol. Plant. **49**: 35-39, 2005.

Murashige, T., Skoog, F.: A revised medium for rapid growth and biosynthesis with tobacco tissue culture. - Physiol. Plant. **15**: 474-497, 1962.

Nath, S., Buragohain, A.K.: Establishment of callus and cell suspension cultures of *Centella asiatica.* - Biol. Plant. **49**: 411-413, 2005.

Saxena, S., Dhawan, V.: Regeneration and large-scale propagation of bamboo (*Dendrocalamus strictus* Nees.) through somatic embryogenesis. - Plant Cell Rep. **18**: 438-443, 1999.

Schenk, R.U., Hildebrandt, A.C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. - Can. J. Bot. **50**: 199-204, 1972.

Shibli, R.A., Ajlouni, M.M.: Somatic embryogenesis in the endemic black iris. - Plant Cell Tissue Organ Cult. **61**: 15-21, 2000.

Snedecor, G.W., Cochran, W.G.: Statistical methods. 8th Ed. - Iowa State University, Ames 1989.

Sotiropoulos, T.E., Mouhtaridou, G.N., Thomidis, T., Tsirakoglou, V., Dimassi, K.N., Therios, I.N.: Effects of different N-sources on growth, nutritional status, chlorophyll content, and photosynthetic parameters of shoots of the apple rootstock MM 106 cultured *in vitro*. - Biol Plant. **49**: 297-299, 2005.

Toonen, M.A.J., Schimidt, E.D.L., De Vries, S.C.: Cell tracking as a tool to study initial processes in somatic embryo development. - Plant Tissue Cult. Biotechnol. **2**: 3-10, 1996.

Tsay, H.S., Huang, H.L.: Somatic embryo formation and germination from immature embryo derived suspension cultured cells of *Angelica sinensis* (Oliv.) Diels. - Plant Cell Rep. **17**: 670-674, 1998.

Uda, Y., Price, K.R., Williamson, G., Rhodes, M.J.C.: Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells *in vitro* by flavonoids. - Cancer Lett. **120**: 213-216, 1997.

Varisai, M.S., Wang, C.S., Thiruvengadam, M., Jayabalan, N.: *In vitro* plant regeneration via somatic embryogenesis through cell suspension cultures of horsegram [*Macrotyloma uniflorum* (LAM.) Verdc.]. - *In Vitro* cell. dev. Biol. Plant **40**: 284-289, 2004.

Wachira, F., Ogada, J.: *In vitro* regeneration of *Camellia sinensis* (L.) O. Kuntze by somatic embryogenesis. - Plant Cell Rep. **14**: 463-464, 1995.