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In Vitro Plant Regeneration and Genetic Transformation of Okra (*Abelmoscus esculentus* [L.] Moench)

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ABSTRACT

Regeneration of recalcitrant okra (*Abelmoschus esculentus* [L.] Moench) plants *in vitro* would permit their rapid propagation and also aid in genetic transformation. Explants (hypocotyls, cotyledonary nodal meristems, cotyledons and shoot tips) were excised from okra seedlings that were grown aseptically in Murashige and Skoog (MS) basal nutrient medium. The explants were cultured on MS medium supplemented with auxins, cytokinins, and different auxin-cytokinin combinations. More organogenic callus was obtained on medium fortified with MS nutrients with low concentrations of 2,4-D (dichlorophenoxy acetic acid), NAA (α-naphthalene acetic acid) and TDZ (thidiazuron), in combination. Shoots were produced on hypocotyls, cotyledonary nodes, and shoot tips, and calli were derived from leaf and cotyledon explants cultured in medium supplemented with BAP (6-benzylaminopurine) and TDZ. Both Kn (kinetin) and Zn (zeatin) proved ineffective in inducing either shoot buds or shoots. Roots were induced on elongated shoots using MS medium containing NAA or IAA (indole-3-acetic acid). Genetic transformation was carried out with *Agrobacterium tumefaciens* carrying the plasmid pBI121 with a selectable marker gene for *npt*II (neomycin phosphotransferase). Transformed cells were cultured on kanamycin (50 mg/L) and cefatoxime (300 mg/L). Proliferation of callus was achieved, with complete suppression of *Agrobacterium*. About 50-60% calli showed GUS (β-glucuronidase) expression, confirming transformation. Thus, genetic transformation of okra was successfully achieved by optimizing various parameters for regeneration and *Agrobacterium* infection. The regenerated plants were successfully hardened in earthen pots after adequate acclimatization.

Keywords: *Agrobacterium*, callus and MS medium, GUS

Abbreviations: BA, benzyl adenine; **BAP**, 6-benzyl amino purine; **2,4–D**, 2,4–dichlorophenoxy acetic acid; **DMF**, dimethyl formamide; **GA_3**, gibberellic acid; **GUS**, β-glucuronidase; **X-gluc**, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; **IAA**, indole-3-acetic acid; **Kn**, kinetin; **MS**, Murashige and Skoog (medium); **NAA**, α-naphthalene acetic acid; ***npt*II**, neomycin phosphotransferase II; **PPFD**, photosynthetic photon flux density; **TDZ**, thidiazuron; **Ti**, tumor inducing; **YEM**, yeast extract mannitol; **Zn**, zeatin

INTRODUCTION

Okra is an important annual vegetable crop in India. It is diploid (2n = 130), belonging to the *Malvaceae* family, and is grown from seed in tropical and sub-tropical parts of the world. The tender green fruits of okra are consumed as vegetables, generally marketed in a fresh state, and sometimes in canned or dehydrated forms. In India, it occupies an area of about 370,000 ha with a production of 3,530,000 tones and a yield of 9540 kg/ha (FAO statistical database, 2005; www.FAOstatistics.com). It is rich in B complex vitamins, vitamin C, calcium, potassium and other minerals. It was found to be useful in protection against genito-urinary disorders, spermatorrhoea and chronic dysentery (Kirtikar *et al*. 1984). Available techniques for the transfer of genes could significantly shorten the time required for conventional breeding procedures and overcome some of the agronomic and environmental problems. Plant tissue culture has long been recognized as an efficient tool for rapid clonal propagation. A high efficiency plant regeneration system is essential for genetic transformation. Exploitation of cell and tissue culture techniques and efficient plant regeneration of okra is a prerequisite for the development of new varieties. *In vitro* studies on Indian varieties of okra have not yet been undertaken in detail. Very few reports are available on the production of somatic embryos from callused okra hypocotyls. Morphogenic responses of hypocotyls, cotyledon, cotyledonary node and primary leaf explants in different media combinations were reported (Reynolds *et al*. 1984). Direct and indirect plant regeneration from hypocotyl explants was successfully achieved (Mangat *et al*. 1986). Studies on micropropagation of *Abelmoschus* were also reported previously (Cook *et al.* 1991; Ganesan *et al*. 2007; Kabir *et al*. 2008). Cook *et al.* (1991) used a callus initiation medium comprising of Murashige and Skoog (1962; MS) medium + 40 g/L sucrose + 1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) + 2 g/L phytagel. A simple and reliable protocol for regeneration of okra through somatic embryogenesis from suspension cultures was developed by Ganesan *et al.* (2007) in which embryogenic callus was obtained from hypocotyl explants cultured on media with MS salts, Gamborg (B5) vitamins, 2.0 mg/L 2,4-D, 1.0 mg/L 1-naphthaleneacetic acid (NAA), 25 mg/L polyvinylpyrrolidone and 30 g/L sucrose. Kabir *et al*. (2008) reported that MS medium supplemented with 6-benzyl amino purine (BAP) and NAA was most effective for callus induction, and that hypocotyl explants were good for producing high amounts of organogenic callus. They observed that combinations of BAP with NAA or indole-3 acetic acid (IAA) gave the most effective plant regeneration from callus.

Genetic improvement of crop plants can be achieved by transferring useful alleles at existing loci through conventional breeding or by adding new loci across diverse sources through genetic transformation (Miflin *et al*. 2000). For successful development of transgenic plants, identification of suitable target tissue and efficient gene transfer protocols are essential (Taylor *et al*. 1991). The need for genetic transformation of okra is paramount in order to introduce biotic stress resistance to improve yields and prevent post

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Table 1 Comparative morphogenetic responses of cotyledons, hypocotyls, cotyledonary nodal meristems and shoot tips of aseptically grown okra (*Abelmosschus esculentus*) seedlings cultured in MS medium supplemented with various PGRs.

MS media + PGR concentration (mg/L)	Cotyledons	Hypocotyls	Cotyledonary nodal meristems	Shoot tips
MS basal	NG	NG	NG	–
0.1 2,4-D	C	C	C	–
0.5 2,4-D	C	C	C	–
1.0 2,4-D	C^{+}	C^{+}	C	–
0.1 IAA	C	C	C	–
0.5 IAA	C + FR	C + FR	C	–
1.0 IAA	C + FR	C + FR	C	–
0.1 NAA	C + FR	C + FR	C + FR	–
0.5 NAA	C + R	C + R	C + FR	–
1.0 NAA	C + MR	C + R	C + R	R
0.1 BAP	C	C	C + Sh	Sh
0.5 BAP	C	C	C + Sh	Sh
1.0 BAP	C + Sh	CSh	C + M.Sh	C + M.Sh
2.0 BAP	C^{++}	C^{++}	C + M.Sh	C + M.Sh
3.0 BAP	C	C	C + Sh	C + Sh
4.0 BAP	C	C	C	C + Sh
1.0 KN	NG	NG	NG	NG
2.0 KN	NG	NG	NG	NG
1.0 Z	C	C	NG	NG
2.0 Z	C	C	NG	NG
1.0 BAP + 0.5 NAA	C + M.Sh	C + Sh + FR	C + M.Sh + FR	Sh + FR
1.0 BAP + 1.0 NAA	C + M.Sh+R	C + M.Sh + R	C + M.Sh + R	M.Sh + R
2.0 BAP + 0.5 NAA	C + M.Sh	C + M.Sh	C + Sh + FR	C + Sh +FR
2.0 BAP + 1.0 NAA	C + Sh + R	C + Sh + R	C + M.Sh + R	C + Sh +R
1.0 BAP + 0.5 NAA + 0.2 TDZ	C + Sh + FR	C + Sh + FR	C + M.Sh + FR	C + Sh + FR
1.0 BAP + 0.5NAA + 0.4 TDZ	C + M.Sh + R	C + M.Sh + FR	C + M.Sh + FR	C^{+}+ Sh + FR
1.0 BAP + 0.5 NAA + 0.8 TDZ	C + Sh + FR	C + Sh + FR	C + M.Sh + FR	C + Sh + FR
1.0 BAP + 1.0 NAA + 0.2 TDZ	C + M.Sh + R	C + M.Sh + R	C + M.Sh + R	C + Sh + R
1.0 BAP + 1.0 NAA + 0.4 TDZ	C + M.Sh + FR	C + M.Sh + R	C + M.Sh + R	C + M.Sh + R
1.0 BAP + 1.0 NAA + 0.8 TDZ	C + Sh + FR	C + M.Sh + FR	C + Sh + FR	C + Sh + R

BAP = 6-benzylamino purine; C = callus; C^{+} = Good callus; C^{++} = excellent callus; 2,4–D = 2,4–dichlorophenoxy acetic acid; FR = few roots; IAA = indole-3-acetic acid; KN = kinetin; MR = more roots; M.Sh = multiple shoots; NAA = α-naphthalene acetic acid; NG = no growth; PGR = plant growth regulator; R = roots; Sh = shoots; TDZ = thidiazuron; Z = Zeatin; – = not used for experiment.

harvest losses. The present investigation was therefore undertaken to address existing challenges with respect to *in vitro* regeneration and transformation of okra.

MATERIALS AND METHODS

Source of seeds

Mature seeds of okra (*Abelmoschus esculentus* [L.] Moench) variety *Parbhani kranthi* were collected from the Agricultural Research Institute (ARI), Rajendranagar, Hyderabad.

Plant materials and explants preparation

Explant material, namely, hypocotyls, cotyledons, cotyledonary nodes, leaf segments, and shoot tips, were obtained from aseptically grown seedlings. Seeds were washed in running tap water to remove surface particles and then with 2.0% (v/v) Teepol detergent solution for 4 min followed by 70% ethanol for 1 min. They were rinsed in sterile distilled water, followed by disinfectant $HgCl_2$ (Anisuzzaman *et al*. 2008) for 6-8 min, and finally subjected to 4-6 rinses with sterile distilled water. Surface sterilized seeds were inoculated in test tubes containing moist cotton and germinated in the dark. After radical emergence, the tubes were transferred to a growth chamber maintained at 24 ± 2°C with a 16/8 h photoperiod (PPFD = 83.6 $\mu Em^{-2}S^{-1}$), using white fluorescent tubes.

Organogenic callus induction

Explants of cotyledons, cotyledonary nodes, hypocotyls and shoot tips were inoculated on MS medium containing 2% (w/v) sucrose, 0.8% agar, and different concentrations of IAA, NAA, BAP and 2,4-D (0.5-4.0 mg/L). Low concentrations of BAP + NAA combinations or BAP + NAA + TDZ (0.2-0.8 mg/L) combinations, were also used for callus induction (**Table 1**). The effect of these different combinations on callus induction was evaluated, and efforts were made to determine the appropriate combination for optimal callus growth. All the cultures were incubated at 25 ± 2°C under 16 h light (PPFD = 83.6 $\mu Em^{-2}S^{-1}$). Data on observations with respect to callus induction were recorded on the 12^{th}, 15^{th} and 18^{th} day after incubation.

Shoot induction

Explants (hypocotyls, cotyledons, cotyledonary nodes, shoot tips) were excised from plantlets that were two weeks old and 6-10 cm in height, aseptically grown on basal MS medium, and subjected to shoot induction by growing them on MS medium with different concentrations of cytokinins: BAP (0.1-4.0 mg/L), Kn (kinetin) and Zn (zeatin, 1.0-2.0 mg/L) and TDZ (thidiazuron, 0.2-0.8 mg/L). All the tubes were incubated at 25 ± 2°C under 16 h light (PPFD = 83.6 $\mu Em^{-2}S^{-1}$). Subsequently, shoot growth and development was assessed after 18 days.

Plant regeneration

Shoot buds and shoots derived from well-developed calli of cotyledons, cotyledonary nodes and hypocotyls were transferred to regeneration medium containing MS basal salts, B5 vitamins, 2% (w/v) sucrose and 0.8% (w/v) agar. Different concentrations of NAA (0.5-1.0 mg/L) in combination with 0.02-2.0 mg/L of cytokinins, BAP (1.0-2.0 mg/L) and TDZ (0.02-0.08 mg/L) were used for shoot bud regeneration (**Tables 2a-2c**). After shoot bud differentiation was observed (30-35 days), the calli were maintained on the same medium and regenerated shoot buds developed into plantlets. The influence of auxin (NAA) in combination with cytokinins (BAP and TDZ) on plantlet development was studied.

Root induction and acclimatization

Well-developed shoots, 4-5 cm in length, were transferred to MS basal medium containing 2% (w/v) sucrose, 0.8% (w/v) agar, different concentrations of NAA, IAA (0.1-0.5 mg/L) and TDZ

Table 2a Effect of combinations of BAP and NAA on cotyledonary nodal meristem and shoot tip development in okra (*Abelmosschus esculentus*).

PGR concentration (mg/L)	№ of shoots/explant		
	Cotyledonary nodal meristems	Hypocotyls	Cotyledons
MS basal	0.47 ± 0.54 a	0.67 ± 0.65 a	0.42 ± 0.55 a
1.0 BAP + 0.5 NAA	2.50 ± 1.31 b	1.80 ± 0.81 b	1.70 ± 0.64 b
1.0 BAP + 1.0 NAA	3.40 ± 1.30 c	3.38 ± 1.22 c	3.16 ± 0.70 c
2.0 BAP + 0.5 NAA	1.56 ± 0.73 d	1.45 ± 0.73 d	1.37 ± 0.52 d
2.0 BAP + 1.0 NAA	2.27 ± 1.06 e	1.93 ± 0.87 b	2.06 ± 0.30 e

Different letters indicate significantly different treatments (between rows) ($p < 0.05$, critical difference = 0.2144).
No significant differences were observed between explant types (between columns) for all treatments ($p > 0.05$).
Values represent mean ± S.D.
BAP = 6-benzylamino purine; NAA = α-naphthalene acetic acid.

Table 2b Effect of combinations of 1.0 mg/L BAP, 0.5 mg/L NAA and TDZ on cotyledonary nodal meristem and shoot tip development in okra.

PGR concentration (mg/L)	№ of shoots/explant		
	Cotyledonary nodal meristems	Hypocotyls	Cotyledon
1.0 BAP + 0.5 NAA + 0.02 TDZ	1.67 ± 0.55 a	1.59 ± 0.60 a	1.47 ± 0.65 a
1.0 BAP + 0.5 NAA + 0.04 TDZ	2.38 ± 0.55 b	2.23 ± 1.04 b	1.84 ± 0.81 b
1.0 BAP + 0.5 NAA + 0.08 TDZ	2.98 ± 0.64 c	2.57 ± 0.88 c	2.26 ± 0.55 c

Different letters indicate significantly different treatments (between rows) ($p < 0.05$, critical difference = 0.2144).
No significant differences were observed between explant types (between columns) for all treatments ($p > 0.05$).
Values represent mean ± S.D.
BAP = 6-benzylamino purine; NAA = α-naphthalene acetic acid; TDZ = thidiazuron.

Table 2c Effect of combinations of 1.0 mg/L BAP, 1.0 mg/L NAA and TDZ on cotyledonary nodal meristem and shoot tip development in okra.

PGR concentration (mg/L)	№ of shoots/explant		
	Cotyledonary nodal meristems	Hypocotyls	Cotyledon
1.0 BAP + 1.0 NAA + 0.02 TDZ	1.71 ± 0.55 a	1.61 ± 0.70 a	1.63 ± 0.64 a
1.0 BAP + 1.0 NAA + 0.04 TDZ	4.14 ± 0.96 b	3.13 ± 0.81 b	2.84 ± 0.84 b
1.0 BAP + 1.0 NAA + 0.08 TDZ	1.70 ± 0.66 a	1.66 ± 0.62 a	1.64 ± 0.30 a

Different letters indicate significantly different treatments (between rows) ($p < 0.05$, critical difference = 0.2144).
No significant differences were observed between explant types (between columns) for all treatments ($p > 0.05$).
Values represent mean ± S.D.
BAP = 6-benzylamino purine; NAA = α-naphthalene acetic acid; TDZ = thidiazuron.

(0.02-0.08 mg/L) and a low concentration of BAP (0.1-0.5 mg/L) for root induction. After root development was observed (10-15 days), the plantlets with well developed roots were rinsed in water to remove adhering agar medium and grown in 1:1:1: mixture of Soilrite®, farmyard manure and sand, in the plastic pots. The pots were covered with a plastic cover with four holes for aeration. Since all the cultured plantlets grown in a controlled environment need gradual acclimatization for their survival in the field, plantlets were left for 15 days in the plastic pots at a controlled temperature (24 ± 2°C) with 60% relative humidity. After four weeks acclimatization, all the plants were shifted to a greenhouse.

Transformation with *Agrobacterium tumefaciens* strain EHA105

A. tumefaciens strain EHA 105 carrying a disarmed Ti plasmid (pBI121) with a selectable marker gene, *npt*II, that confers resistance to the antibiotic kanamycin (Kan; Sigma, St. Louis, USA) in transformed cells, along with a reporter gene (*uidA*) encoding GUS, was used for genetic transformation of okra. A single loop of *A. tumefaciens* culture was inoculated into 5 ml of YEM (Yeast Extract Mannitol) medium containing 50 mg/L Kan. The culture was incubated in a 100 ml sterile conical flask which was placed overnight (~17 hours) on a shaker (180 to 220 rpm) at 28°C. The density of the culture was checked using a UV-visible spectrophotometer at a wavelength of 600 nm and the optical density was adjusted to an absorbance of 2.0 with liquid MS. This bacterial culture was used for infection of the explants. Cotyledons and hypocotyls were cut into small pieces (~0.5-1.0 cm^2). Explants were infected with *Agrobacterium* culture for 30 min, blotted using sterile filter paper and transferred to the co-cultivation medium (MS medium with BAP 1.0 mg/L + NAA 0.5 mg/L) without antibiotics. The culture was kept at room temperature (28°C) for 2 days.

After co-cultivation, explants were washed three times with sterile distilled water. Cleaned explants were blotted dry using a sterile filter paper and cultured on the selection medium consisting of MS with 300 mg/L cefotaxime, and 50 mg/L Kan to inhibit further *Agrobacterium* growth. Explants not cultured with *A. tumefaciens* were plated on selection medium and used as a negative control. A regeneration control was also maintained along with transformed plants, without antibiotics, and was used as the positive control. The Petri dishes were incubated at 24 ± 2°C under a 16-h photoperiod (PPFD = 83.6 $\mu Em^{-2}S^{-1}$) provided by white fluorescent tubes, and sub-cultured every 15 days. Control and non-transformed explants showed hyperhydric growth on the selection medium. After 15 days of incubation in selection medium, calli were transferred to fresh selection medium. Following three cycles of selection, resistant embryogenic calli were transferred to regeneration medium with added Kan. For assessing transient expression histochemically, a few transformed calli were incubated overnight at 37°C in a X-gluc solution (10 mg/L X-gluc in 100 µl DMF, 10 ml 1 M sodium phosphate pH 7.0, 1 ml Triton X-100, 10 ml H_2O).

Molecular characterization was also done to assess the presence of the selectable marker *npt*II gene in transformed okra plants, to confirm transformation with GUS reporter gene. Amplification of *npt*II sequences was achieved using a primer pair (*nptII* F: 5′ GAG GCT ATT CGG CTA TGA CGT; and *nptII* R: 3′ ATC GGG AGC GGC GAT ACC GTA), which amplified a 700-bp sequence from the *npt*II gene in the T-DNA of constructs (Robbins *et al*. 1998). Genomic DNA was isolated by the modified CTAB method (Saghai-Maroof *et al*. 1984). PCR analysis was done using 2 µl of genomic DNA, 2.0 µl of 10X PCR buffer, 2.0 µl of 2 mM dNTPs, 0.6 ml of 50 mM $MgCl_2$, 1.0 µl of 10 µM forward and reverse *npt*II primers, and 0.2 µl of *Taq* DNA polymerase (Sigma), made up to 20 µl with distilled water. The thermal cycler (Eppendorf mastercycler gradient EP) was programmed for an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1.0 min, and again kept at 72°C for 10 min and a hold temperature of 14°C. The presence of the *npt*II gene in the PCR product was detected by agarose gel electrophoresis using a 1% agarose gel, and a 100 bp DNA ladder (New England Biolabs Inc., USA) for identification of the expected band size of 700 bp.

Statistical analysis was conducted to study the effects of different concentrations of auxins and cytokinins using two way analysis of variance test (ANOVA) at 5% level of significance, and results are reported in **Tables 2a-2c**.

RESULTS AND DISCUSSION

Organogenic callus induction

Cotyledons (i.e., cotyledonary nodal meristems, hypocotyls and shoot tips) were inoculated on MS medium supplemented with different concentrations of plant growth regulators (PGRs). All explants became swollen and callus initiation occurred, initially at the cut ends, and then at the surface, of the 25-day old explants. The calli were light greenish, friable, greenish, compact and white, on medium containing auxin alone (2,4-D, NAA, and TDZ) (**Fig. 1A**), while a combination with cytokinin (BAP) induced greenish compact nodular calli (**Fig. 1B**). High frequency of callus

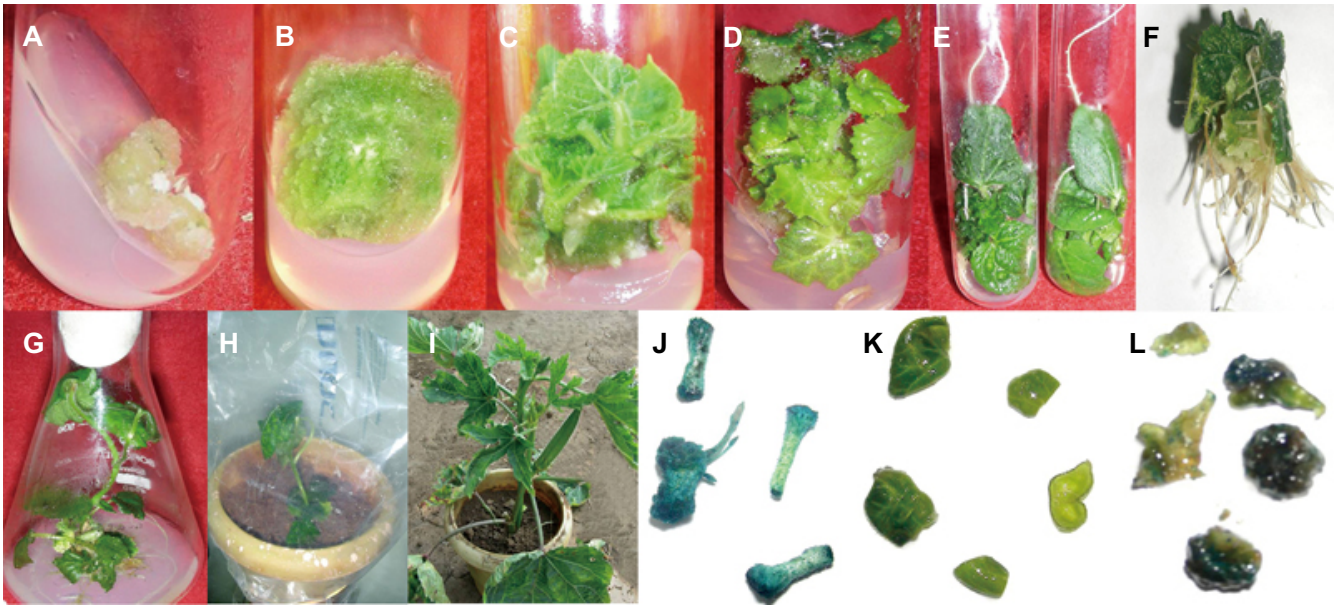

Fig. 1 Regeneration of okra var 'Parbhani Kranthi'. (**A**) Whitish compact callus on hypocotyls cultured on MS medium supplemented with TDZ (0.4 mg/L); (**B**) Greenish compact callus on cotyledonary nodal meristem cultured on MS medium supplemented with BAP (2.0 mg/L); (**C**) Multiple shoot development from cotyledonary nodal meristems on MS medium with BAP (1.0 mg/L); (**D**) Shoot elongation cotyledonary nodal meristems on MS medium with BAP (1.0 mg/L), NAA (1.0 mg/L) and 0.04 mg/L TDZ; (**E**) Induction of roots from cotyledonary nodal meristems on MS medium with BAP (1.0 mg/L); (**F**) Profuse rooting in cotyledons cultured on MS medium with NAA (1.0 mg/L); (**G**) Acclimatization of okra regenerants in culture room (temp. 24 ± 2°C, 16 hr photoperiod, 60% RH); (**H**) Acclimatization of okra regenerants in greenhouse; (**I**) Transgenic plant; (**J**) GUS expression in transformed hypocotyls; (**K**) GUS Expression in transformed leaf; (**L**) GUS expression in transformed callus.

Table 3 Comparison of different studies with respect to growth media and PGRs on callus, shoot and root development in okra.

	Basal media used	Most effective PGR combinations (mg/L)	Organogenic outcome
Present study	MS basal salts, B5 vitamins, 2% (w/v) sucrose	1.0 BAP + 1.0 NAA + 0.04 TDZ	C + M.Sh + R
Mangat and Roy 1986		1.0 BAP + 1.0 NAA	C + M.Sh
Cook *et al*. 1991	MS basal salts, B5 vitamins, 40 g/L (w/v) sucrose	1.0 2,4-D + 2.0 g/L phytagel	C^{++}
Ganesan *et al*. 2007	MS basal salts, B5 vitamins, 30 g/L (w/v) sucrose	1.0 2,4-D + 1.0 KN	C^{++}
		0.2 BAP + 0.2 GA_3	M.Sh + R
Anisuzzaman *et al.* 2008	MS basal salts, B5 vitamins, 3% (w/v MS basal	2.0 NAA + 0.5 TDZ	C^{++}
	salts, B5 vitamins, 2% (w/v) sucrose) sucrose	2.0 BAP + 0.1 IBA	C^{++} + M.Sh
		1.5 NAA	R
Kabir *et al*. 2008	MS basal salts, B5 vitamins, 2% (w/v) sucrose	0.5 BAP + 2.0 NAA	C^{++}
		2.0 BAP + 0.1 IAA	C^{++} + M.Sh
		2.0 BAP + 0.5 NAA	C^{++} + M.Sh
		2.0 IBA	R

BAP = 6-benzyl amino purine; C = callus; C^{++} = excellent callus; 2,4–D = 2,4–dichlorophenoxy acetic acid; FR = few roots; GA_3, = gibberellic acid; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; Kn = kinetin; M.Sh = multiple shoots; NAA = α-naphthalene acetic acid; PGR = plant growth regulator; R = roots; Sh = shoots; TDZ = thidiazuron

proliferation was observed at 20-30 days after inoculation. The frequency of callus induction ranged from 40.0 to 92.8% and was higher at 1.0 mg/L NAA, 0.4 mg/L TDZ with 1.0 mg/L BAP (**Table 1**), while increasing the NAA concentration above this level reduced the callusing frequency and induced rhizogenesis. The combination of higher auxin and lower cytokinin was more effective for callus induction. However, in other studies, the concentration of PGRs varied for the induction of organogenic callus depending on the explant used (**Table 3**).

Plant regeneration

Greenish compact nodular calli, obtained from all the explants listed above, were selected for regeneration studies. Calli were cut into 2-4 pieces and subcultured. After subculture on regeneration medium, shoot buds initiated between 14-16 days. The shoots were maintained on the same medium for 26-28 days to allow for sufficient growth (**Fig. 1C**). Regeneration frequency ranged between 26.0 and 72.8%. Significantly higher frequency of shoot regeneration was observed on medium containing 1.0 mg/L BAP, 1.0 mg/L NAA (68.2%) and 0.04 mg/L TDZ (**Table 2c**). Greenish compact nodular calli were suitable for plant regeneration. These calli were maintained on parental medium for four weeks (or) sub cultured on parental medium where they induced shoot buds between 16-28 days, following which, the shoots were elongated (**Fig. 1D**) and established into plantlets. Differences were observed in shoot regeneration frequency among different concentrations and combinations of auxin and cytokinin. The BAP, TDZ and NAA combination was more successful than any other treatment. During callus induction, there was no significant change in callus morphology and all the calli obtained using NAA (1.0 mg/L), BAP (1.0 mg/L) and TDZ (0. 04 mg/L) showed a green, compact nature. The maximum regeneration frequency was 92.6, 90.0 and 86.0 for cotyledonary node, hypocotyl, and cotyledon explants, respectively. Calli from explants of shoot tips were not used for further evaluation of plant regeneration, as their growth was not as good as calli from other explants (**Table 1**). Regeneration frequency was very low in treatments using lower concentrations of auxins/cytokinins. High concentration of auxins reduced the shoot regeneration frequency. A combination of different concentrations of cytokinins (1.0 mg/L NAA, 1.0 mg/L BAP and 0.04 mg/L TDZ) in the regeneration media resulted in higher shoot regeneration from different explants (**Table 2c**). Other studies (Haider *et al*. 1993; Anisuzzaman

et al. 2008) reported successful induction of shoot buds from cultured hypocotyl callus using NAA and BAP combinations. With an increase in BAP concentration, a corresponding increase in shoot regeneration was observed. Mangat *et al*. (1986) obtained high level of regeneration using 1.0 mg/L NAA in combination with 1.0 mg/L BAP for producing shoots or buds.

Root development of regenerants and acclimatization

The well developed shoots were transferred to rooting medium containing different concentrations of NAA. Shoots from calli derived from hypocotyls, cotyledons, and cotyledonary nodal meristems were isolated and transferred to root induction medium. Considerable rhizogenesis was observed in these explants i.e., 76, 74, and 78% at 1.0 mg/L NAA (**Fig. 1E, 1F**). Root induction took place between 18 to 24 days after transfer to rooting medium and rooting frequency was high in all these explant regenerants. In general, higher levels of NAA showed low frequency of root induction. Similar results were reported by Mangat *et al*. (1986), Haider *et al*. (1993), and Kabir *et al*. (2008) (**Table 3**).

Acclimatization

After acclimatization in the culture room at a temperature of 24 ± 2°C, 16 hr photoperiod (PPFD = 83.6 $\mu Em^{-2}S^{-1}$), 60% RH (**Fig. 1G**), the well developed rooted plantlets were first transferred to plastic pots having 1:1:1 mixture of Soilrite®, farmyard manure and sand. The pots were covered with a plastic cover with 4 holes for aeration (**Fig. 1H**), at 25 ± 2°C under a 16-h photoperiod (PPFD = 83.6 $\mu Em^{-2}S^{-1}$). After 10 to 12 days of hardening, these plantlets were transferred to the earthen pots (**Fig. 1I**) and then to the field. The hardened plants showed a 78.2 ± 2.0% survival rate under field conditions.

Based on this protocol the genetic transformation of okra plantlets using the GUS reporter gene was to be standardized prior to development of transgenic okra with introduction of *cry* genes for resistance to fruit and stem borer caused by lepidopteron pests.

Transformation with *A. tumefaciens* EHA105

The T-DNA of *Agrobacterium* can be engineered to contain gene/s or DNA sequences of interest that can be transferred into the host plant cells and integrated into the plant genome. *Agrobacterium* mediated transformation is attractive because of the ease of the protocol, coupled with minimal equipment costs. Moreover, transgenic plants obtained by this method often contain a single copy of T-DNA integrations. *Agrobacterium* mediated transformation and the subsequent regeneration of transgenic plants carrying inserted genes were described by Murai *et al.* (1983) and Fraley *et al.* (1983). Dodueva *et al.* (2007) reviewed plant tumorigenesis and stated that the cause for all types of plant tumorigenesis is deviations in the metabolism and/or signaling of two main groups of phytohormones - cytokinins and auxins, and examined different ways in which the cytokinin/auxin balance shifts for some types of plant tumors. Factors affecting *Agrobacterium*-mediated transformation of plants were described by Karami (2008). The factors described include bacterial strains and cell density, plant species and genotype, plant growth regulators and antibiotics, explant, explant wounding, light and temperature. It was stated that before attempting stable transformation of any new species, it is useful to optimize the factors influencing transformation efficiency, as this can reduce future costs in labor and materials.

In *Agrobacterium* transformation, adjustment of bacterial cell density is very important. In the previous reports varied optical densities (ODs) at different wave lengths such as 0.25 at 600 nm (Sangwan *et al*. 1993) 0.9 to 1.2 at 600 nm, 1.0 at 550 nm (Hiei *et al*. 1994), and 2.0 at 600 nm (Datta *et al*. 1996) were tried. In the present investigation, three OD levels *viz*., 1.0×10^8 cells/ml, 2.0×10^8 cells/ml and 3×10^8 cells/ml were tested. Among these densities, 2×10^8 cells/ml gave highly significant stable GUS positive and kanamycin-resistant calli.

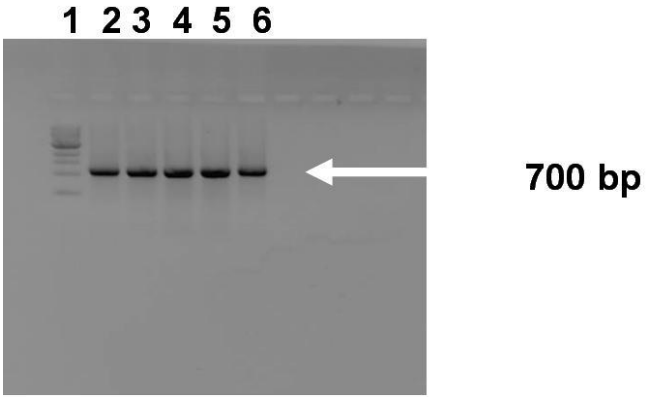

Fig. 2 Agarose gel electrophoresis showing amplified gene of selectable marker *npt*II in pBI121 vector used for transformation of okra. Lane 1: 100 bp marker; Lanes 2-6: Amplified 700 bp *npt*II gene.

Table 4 Transient *gus* expression in two explants of okra.

Explant	№ of calli incubated	№ of Gus^+ calli
Hypocotyl	10	6.6
Cotyledon	10	5.4
Cotyledonary nodal meristems	10	6.4

Effect of infiltration time on *A. tumefaciens* infection was studied by several researchers. According to Rashid *et al*. (1996), the calli of indica rice variety, soaked in bacterial suspension for 3 min resulted in optimum infection of *A. tumefaciens*. Mohanty *et al.* (1999) immersed embryonic calli generated from indica rice variety in bacterial suspension for 30 min. In the present study, infiltration periods of 15, 30, 45 and 60 min were attempted, 30 min being effective as it resulted in less growth of *A. tumefaciens* during selection. Variation in infection of *A. tumefaciens* might be due to genotypic differences, as observed in callus induction.

When transformed explants were sub-cultured on MS medium containing kanamycin (50 mg/L) and cefatoxime (300 mg/L) in the present study, a whitish compact callus was induced after 15 days of inoculation. Proliferation of callus was achieved in this selection medium and *A. tumefaciens* was suppressed completely.

Increasing the duration of pre-culture and co-culture period enhanced the transient GUS expression in transformed explants, leaves and calli up to a threshold level of 55-65% after overnight incubation with X-gluc. For assessing transient expression, putatively transformed calli (25-days old) were picked up randomly and incubated in X-gluc solution overnight. The percentage of transient GUS expression was scored for both hypocotyls and cotyledons. About 55-65% (**Table 4**) of calli showed GUS expression, confirming transformation. Control explants which were not co-cultivated with *Agrobacterium* did not show any GUS activity. In the present study, hypocotyls showed GUS^+-calli compared to cotyledons when used as explants (**Fig. 1J-L**).

Following PCR an expected 700 bp band was amplified for the *npt*II gene (**Fig. 2**). Future studies could adopt this protocol for genetic transformation of okra in order to incorporate various traits like resistance to *Yellow vein mosaic virus* and fruit and shoot borer (*Earias fabae*). *Yellow vein mosaic virus* of okra, which is caused by a complex consisting of the monopartite begomovirus, *Geminiviridae* and a small satellite DNA beta component, is one of the major biotic constraints limiting the production okra (Handa *et al.* 1993; Dhankar *et al.* 2005).

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