SILVER NITRATE ($AgNO_3$) BOOSTED HIGH-FREQUENCY MULTIPLE SHOOT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF OKRA (*ABELMOSCHUS ESCULENTUS* L.)

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Abstract. Okra (*Abelmoschus esculentus* L.) regeneration frequency to a large extent depends on its genetic structure and its recalcitricity. In present study, the influence of silver nitrate ($AgNO_3$) in combination with cytokinins such as 6-benzyl amino purine (BAP) and kinetin (KIN) to regenerate multiple shoots from cotyledonary node explants of *A. esculentus* were optimized. Cotyledonary node explants were excited from 10-days old okra *in vitro* seedlings and incubated on Murishage and Skoog (MS) medium supplemented with different concentration and the combination of BAP alone and in combination with KIN. The highest percentage of shoot bud regeneration (75%) was achieved on MS medium supplemented with 2.0 mgL^{-1} BAP. Highest percentage of shoot multiplication and proliferation (85%) with 6.75 shoots per explant having 3.75 cm length was obtained on MS medium containing 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN + 3.0 mgL^{-1} $AgNO_3$. On the other hand, non-containing $AgNO_3$, the combination of 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN, a maximum of 53% shoot multiplication with 2.3 shoots per explant having 3.75 cm length was recorded. Individual shoots were elongated on MS medium supplemented with 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} GA_3 (Gibberellic acid) (shoot length 5.60 ± 0.70^a cm) and rooted on half strength MS medium + 1.0 mgL^{-1} IBA (Indole 3-butiric acid) + 200 mgL^{-1} AC (Activated charcoal), with highest root formation 83% with 5.20 roots per shoot having 4.8 cm length was achieved. The current protocol will be useful for the development of okra transgenic plant as a result of genetic transformation.

Keywords: *growth regulators, shoot multiplication, organogenesis, cytokinins and auxins*

Abbreviations: $AgNO_3$ – silver nitrate, MS – Murishage and Skoog, ddH_2O – double distilled water, $HgCl_2$ – mercuric chloride, NaOH – sodium hydroxide, HCL – hydrochloric acid, PGRs – plant growth regulators, AC – activated charcoal, 2,4-D- 2,4 – dicholorophenoxyacetic acid, BAP – 6-benzyl aminopurine, KIN – kinetin, GA_3 – gibberellic acid, IBA – indole-3-butyric acid, NAA – α-naphthyl acetic acid, μmol m^{-2} s^{-1}– micromoles per meter square per second

Introduction

Okra (*Abelmoschus esculentus* L.) is a member of Malvaceae family, which is mostly known as lady's finger. It is an important vegetable crop owing to its palatability for human consumption. It is cultivated in sub-tropical and tropical areas throughout the world (Dhande et al., 2012). India is the leading country followed by Nigeria, Sudan, Mali and Pakistan with the production of 5.5 million tons and covers an areas of 0.48 million hectares in the world (FAOSTAT, 2016). It is tremendous source of balanced human food containing protein, vitamins, and minerals which provides important compounds for medical (Gemede et al., 2015; Düzyaman and Vural, 2001). Moreover, its regular consumption could prevents hepatitis, cancer, ulcers and diabetes (Kumar et al., 2009a). Plants from the Malvaceae family such as okra are particularly susceptible to various

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environmental stresses such as abiotic and biotic stresses which reduce crop growth, yield and cause economic loss (Venkataravanappa et al., 2015).

Development of plant transformation and genetic engineering made it capable to isolate different disease and pest's resistant genes from bacteria and plants. Then transfer these resistant genes into economically valuable crops which can be grown under stressed conditions (Manickavasagam et al., 2015). Effective regeneration system is the pre-requirement for transgenic plants (Michel et al., 2008). In addition, *in vitro* reproduction technique offers a powerful tool to protect genetic resources and mass-multiplication of endangered plant species (Choffe et al., 2000). However, okra is one of the most recalcitrant crops for genetic modification with the lack of an efficient *in vitro* regeneration system amongst the leading reasons. It is widely reported that okra regeneration protocol using different explants such as hypocotyl, cotyledon, shoot tips, cotyledonary nodes, cotyledonary axis and plumule from zygotic embryo (Mangat and Roy, 1986; Roy and Mangat, 1989; Irshad et al., 2017; Narendran et al., 2013; Dhande et al., 2012; Manickavasagam et al., 2015; Daniel et al., 2018). However, available okra regeneration protocols are not well-organized for the development of transgenic plants. The main reasons for poor regeneration protocols of okra are due to its recalcitrant nature which takes longer time and requires skills (Manickavasagam et al., 2015).

Shoot multiplication through direct organogenesis is a good alternative way of regeneration, which takes less time, has lower soma clonal variability and higher efficiency index than indirect regeneration system (Juturu et al., 2015). Additionally, it has the ability to overcome the limitation of genotype requirement in transformation system. Several efforts have been made for getting an efficient regeneration system of okra by using different protocols (Anisuzzaman et al., 2008; Dhande et al., 2012; Kumar et al., 2009a; Mangat and Roy, 1986; Narendran et al., 2013; Irshad et al., 2017; Kumar et al., 2015). Various factors including media composition, explants, culture conditions, carbon sources, browning of callus, phenolic secretion, culture vessels and slow *in vitro* response have been shown to influence *in vitro* regeneration of okra (Irshad et al., 2017; Kumar et al., 2015). Media composition plays an important role in the regeneration efficiency. Kumar et al. (1998) reported that the development and growth of cells cultured *in vitro* rely on existence of ethylene and phytohormones in culture environment. Different evidence's showed that the morphogenic response of adventitious bud induction can be improved by inhibition of ethylene biosynthesis and gaseous environment in the culture medium (Pua, 1999). Inhibition of ethylene biosynthesis is a good alternative to improve the adventitious bud induction.

Silver Nitrate ($AgNO_3$) has been reported a potential ethylene inhibitor in various economically important plant species (Zhang et al., 2001; Jakubowicz et al., 2010; Kumar et al., 2016; Mookkan and Andy, 2014; Venkatachalam et al., 2017; Aylin Ozudogru et al., 2005). Addition of regeneration enhancer such as silver nitrate ($AgNO_3$) in growth medium plays a vital role in shoot multiplication and stated in various economically important plants such as *Vigna mungo* (Mookkan and Andy, 2014), *Gossypium hirsutum* (Kumar et al., 2016), *Arachis hypogaea* (Aylin Ozudogru et al., 2005), *Cucumis sativus* (Venkatachalam et al., 2018), *Catharanthus roseus* (Panigrahi et al., 2017) and *Prosopis cineraria* (Venkatachalam et al., 2017).

In this study, we reported an effective *in vitro* regeneration system to induce multiple shoots through cotyledonary node explants of *A. esculentus* by using various plant growth regulators with different combination, concentration and growth promoter e.g. silver nitrate ($AgNO_3$). The basic objectives of this study were (a) to improve direct regeneration

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protocol for okra by using cotyledonary node as explant (b) to examine the impact of cytokinins and auxins by using alone or in combination with $AgNO_3$ on shoot multiplication through *in vitro* seedling derived cotyledonary node explants. This alternative method will be beneficial for attaining transgenic plants of commercial okra genotypes.

Materials and methods

Silver nitrate ($AgNO_3$) stock solution preparation

For the preparation of stock solutions, chemicals were purchased from Solarbio® (China). A Stock solution of $AgNO_3$ (0.5 mgL^{-1}) was prepared with sterilized distilled water and stored in screw-capped bottles. The stock solution was kept at 4 °C until further use.

Plant material and seed germination

Abelmoschus esculentus "Xiang Fu" cultivar was used in the experiment. Seeds purchased from vegetable seed market (P. R. China, Fuzhou), were surface sterilized by washing with sterilized double distilled (ddH_2O) water 3-5 times and then treated by immersion in 0.1% weight per volume (w/v) $HgCl_2$ (mercuric chloride) for 3 minutes, following the 5 rinses with ddH_2O to remove the residual of $HgCl_2$, then treated with 70% ethanol for 30 seconds and washed 3-5 times with ddH_2O and immersed overnight in ddH_2O. The sterilized seeds were inoculated in half-strength Murishage and Skoog (1962) (MS) basal solid medium without addition of plant growth regulators in sterilized baby food jars (5×9 cm), 5-8 seeds per jar and was kept in dark condition.

Culture medium and conditions

The cotyledonary node explants were cultured on MS medium fortified with different concentration and combination of BAP, KIN, thidiazuron (TDZ), silver nitrate ($AgNO_3$), gibberellic acid (GA_3), indole 3-butiric acid (IBA), α-naphthalene acetic acid (NAA) and activated charcoal (AC). For the whole experiment, MS basal medium containing 2.5% sucrose (w/v), 0.8% agar (w/v) and pH was adjusted to 5.8 by the use of 0.5 N NaOH or HCl prior to autoclaving at 121 °C for 20 min. The culture conditions were maintained at 25 $\pm$ 2 °C with 70% relative humidity and 16:8 h light/dark photoperiod supplied by cool white fluorescent lamps (Philips, Beijing, China) at an intensity of 60 $\mu mol\ m^{-2}\ s^{-1}$.

Explant preparation and shoot induction

Cotyledonary node explants were excised aseptically from 2-week old *in vitro* raised seedlings (*Fig. 1A*) and cultured on MS medium augmented with different concentration of BAP (0.5, 1.0, 2.0, 2.5 mgL^{-1}) alone or in combination with KIN (0.5, 1.0, 2.0, 2.5 mgL^{-1}) for shoot bud induction. Consequently, the shoot buds were sub-cultured into same fresh MS medium for two weeks intervals to enhance the shoot bud development. Data on shoot proliferation percentage (%) and number of shoots per explant were measured after two weeks intervals.

Multiple shoots induction

To investigate the effect of $AgNO_3$ on shoot multiplication, two experiments were carried out containing $AgNO_3$ and non-containing $AgNO_3$. In the first experiment (containing $AgNO_3$), *in vitro* derived shoot buds were cultured on MS medium containing

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various concentration of BAP (0.5, 1.0, 2.0, 2.5 mgL^{-1}), KIN (0.5, 1.0, 2.0, 2.5 mgL^{-1}) and TDZ (0.5, 1.0, 2.0, 2.5 mgL^{-1}) in combination with $AgNO_3$ (0.5, 1.0, 2.0, 2.5 mgl^{-1}), as growth enhancer. In second experiment (non-containing $AgNO_3$), shoot buds were cultured on MS medium supplemented with BAP, KIN and TDZ at equimolar concentration. The shoot bunches were sub-cultured on same fresh shoot multiplication medium at every two weeks intervals for fast shoot growth with more number of shoot buds. Shoot initiation and growth characteristics were noticed per week, while data for shoot multiplication responses, mean number of shoot per explant and shoot length was noted after four weeks of inoculation.

Shoot elongation

In order to obtained healthy shoots with appropriate length for rooting, well developed shoots (1-1.5 cm) in length were isolated from shoot bunches and cultured on MS medium containing different combination and concentration of BAP (1.0, 1.5, 2.0 mgL^{-1}) and GA_3 (1.0, 1.5, 2.0 mgL^{-1}) alone or in combination. The shoot length (cm) and shoot elongation frequency (%) were noticed after three weeks of culture.

Rooting of regenerated shoots and acclimatization

Elongated shoots (2.0-5.0 cm in length) were excised aseptically and cultured on half strength MS medium containing (100, 200 mgL^{-1}) AC with various concentration (0.5, 1.0, 1.5 mgL^{-1}) of IBA and NAA. Root formation frequency, root length and number of roots per shoot were recorded after four weeks of cultured. The plantlets with roots were cautiously detached from the culture medium and distilled water was used to remove the agar traces and rooting medium. Plantlets were shifted into plastic cups comprising of vermiculite and garden soil (1:1) and watered every second day. The plastic cups were covered with polyethylene bags for 5 days and kept in the growth chamber at 25 ± 2 °C with relative humidity (70%) and 16/8-h of light/dark photoperiod having light intensity of 80 µmol m^{-2} s^{-1} supplied by cool white fluorescent tubes. These plantlets were then transferred to greenhouse under natural conditions for further growth and development.

Statistical analysis

The experiments were carried out based on a CRD (complete randomized design) with n = 10, and each experiment was performed three times. MS medium without plant growth regulators was used as a control treatment at every step of experiment. One-way analysis of variance (ANOVA) was performed using SPSS version 17.0 version (SPSS Inc., Chicago, IL, USA) for all statistical analysis and difference among mean values were analyzed by using Tukey's test, p-value ≤ 0.05.

Results

Effects of plant growth regulators on shoot bud induction

Morphogenetic responses of cotyledonary node explants (*Fig.1B*) with various concentrations of BAP and KIN were given in *Table 1*. Cotyledonary nodal explants cultured on MS medium without PGRs (control) gave no response. However the response was quicker in the presence of cytokinins including BAP and KIN (*Fig.1C*). Various results were observed on medium supplemented with different type and concentration of

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cytokinins. Among the tested concentration of BAP and KIN, shoot induction percentage (75%), with 4.5 numbers of shoots, was observed on MS medium supplemented with 2.0 mgL^{-1} BAP alone was higher as compare to the combination of 2.0 mgL^{-1} BAP + 2.0 mgL^{-1} KIN, (4.15 number of shoots) with 70% response.

Table 1. *Effects of different concentration of plant growth regulators (PGRs) on shoot bud differentiation from cotyledonary node explants of okra.*

Culture media	Plant growth regulator (mgL^{-1})		Shoot proliferation %	No. of shoots/explant
	BAP	KIN		
M1	0	0	0.00 ± 0.00^{j}	0.00 ± 0.00^{d}
M2	0.5	-	43.00 ± 0.58^{g}	1.33 ± 0.17^{cd}
M3	1.0	-	52.00 ± 0.58^{e}	2.75 ± 0.13^{abc}
M4	2.0	-	**75.00 ± 0.76^{a}**	**4.50 ± 0.29^{a}**
M5	2.5	-	60.00 ± 0.58^{d}	3.80 ± 0.44^{a}
M6	-	0.5	58.00 ± 0.50^{d}	1.68 ± 0.23^{bcd}
M7	-	1.0	48.00 ± 0.29^{f}	2.55 ± 0.26^{abc}
M8	-	2.0	65.00 ± 0.76^{c}	3.50 ± 0.29^{ab}
M9	-	2.5	38.00 ± 0.87^{h}	2.90 ± 0.67^{abc}
M10	0.5	0.5	31.00 ± 0.58^{i}	1.40 ± 0.70^{cd}
M11	1.0	1.0	40.00 ± 0.76^{gh}	3.75 ± 0.25^{a}
M12	2.0	2.0	70.00 ± 0.64^{b}	4.15 ± 0.45^{a}
M13	2.5	2.5	28.00 ± 0.58^{i}	2.90 ± 0.59^{abc}

The labels M1-M13 (M1- Controlled) presents PGRs combination, values represented mean ± standard error; data were recorded after four weeks of culture on MS medium supplemented with BAP and KIN. Best results are indicated in bold, mean followed by the same letter within a column are not significantly different according to Tukey's test with p-value ≤ 0.05

Shoot multiplication and proliferation

Multiple shoot induction was not observed in medium without PGRs (control). Among the tested combinations medium containing $AgNO_3$, maximum shoot multiplication percentage (85%) was noticed on a medium fortified with 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN + 3.0 mgL^{-1} $AgNO_3$ with maximum number of shoots (6.75 shoots/ explants, 3.90 cm in length) followed by the other combination (1.75 shoots/ explants, 1.0 cm in length), with lowest multiple shoot percentage (37%), on a medium containing 2.0 mgL^{-1} TDZ + 4.5 mgL^{-1} $AgNO_3$ was recorded (*Table 2A*). Whereas highest percentage of shoot multiplication (51%) with maximum shoot numbers (3.80 shoots/explants, 2.30 cm in length) was observed on MS medium having the combination of 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN with non-containing $AgNO_3$ (*Table 2B*) (*Fig. 1D, 1E*).

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Table 2A. *Effects of different concentration and combination of plant growth regulators containing $AgNO_3$ on shoot multiplication from cotyledonary node explant of okra*

Culture media	Plant growth regulator (mgL^{-1})				Shoot proliferation %	No. of shoots/explant	Shoot length (cm)
	BAP	KIN	TDZ	$AgNO_3$			
M1	0	0	0	0	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
M2	-	-	-	1.5	38.00 ± 0.58^{h}	3.80 ± 0.47^{bcd}	$1.750.38^{abc}$
M3	-	-	-	3.0	75.00 ± 0.29^{b}	4.00 ± 0.58^{bcd}	2.60 ± 0.31^{abc}
M4	-	-	-	4.5	50.00 ± 1.15^{fg}	3.33 ± 0.44^{bcd}	2.50 ± 0.29^{abc}
M5	1.0	-	-	1.5	45.00 ± 1.73^{g}	3.80 ± 0.44^{bcd}	1.75 ± 0.18^{abc}
M6	1.5	-	-	3.0	77.00 ± 1.15^{b}	5.50 ± 0.29^{ab}	3.40 ± 0.38^{ab}
M7	2.0	-	-	4.5	68.00 ± 0.58^{cd}	4.75 ± 0.14^{abc}	2.35 ± 0.62^{abc}
M8	-	1.0	-	1.5	50.00 ± 1.00^{fg}	2.90 ± 0.67^{cd}	1.50 ± 0.87^{abc}
M9	-	1.5	-	3.0	63.00 ± 0.58^{de}	3.83 ± 0.17^{bcd}	2.00 ± 0.29^{abc}
M10	-	2.0	-	4.5	67.00 ± 1.04^{cd}	3.60 ± 0.31^{bcd}	2.80 ± 0.42^{ab}
M11	-	-	1.0	1.5	60.00 ± 2.89^{e}	2.75 ± 0.38^{cd}	2.50 ± 0.50^{abc}
M12	-	-	1.5	3.0	49.00 ± 0.58^{fg}	3.00 ± 0.58^{cd}	1.80 ± 0.61^{abc}
M13	-	-	2.0	4.5	37.00 ± 0.76^{h}	1.75 ± 0.38^{de}	1.00 ± 0.50^{bc}
M14	1.0	1.0	-	1.5	53.00 ± 1.53^{f}	5.00 ± 0.58^{abc}	2.75 ± 0.38^{ab}
M15	1.5	1.5	-	3.0	**85.00 ± 0.58^{a}**	**6.75 ± 0.63^{a}**	**3.90 ± 0.49^{a}**
M16	2.0	2.0	-	4.5	49.00 ± 0.76^{fg}	4.33 ± 0.33^{bc}	2.55 ± 0.29^{abc}
M17	1.0	-	1.0	1.5	53.00 ± 0.58^{f}	3.10 ± 0.49^{bcd}	1.90 ± 0.95^{abc}
M18	1.5	-	1.5	3.0	48.00 ± 1.15^{fg}	2.90 ± 0.38^{cd}	1.75 ± 0.52^{abc}
M19	2.0	-	2.0	4.5	69.00 ± 1.00^{c}	3.75 ± 0.66^{bcd}	2.90 ± 0.38^{ab}

The labels M1-M19 represent PGRs combination, values represented mean ± standard error; data were recorded after four weeks of culture on MS medium supplemented with $AgNO_3$ in combination with BAP, KIN and TDZ. Best results are indicated in bold, mean followed by the same letter within a column are not significantly different according to Tukey's test with p-value ≤ 0.05

Table 2B. *Effects of different concentration and combination of plant growth regulators on shoot multiplication from cotyledonary node explant of okra with non-containing $AgNO_3$*

Culture media	Plant growth regulator (mgL^{-1})			Shoot proliferation %	No. of shoots/explant	Shoot length (cm)
	BAP	KIN	TDZ			
M1	0	0	0	0.00 ± 0.00^{i}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
M2	1.0	-	-	26.00 ± 0.58^{h}	2.10 ± 0.38^{ab}	1.00 ± 0.29^{ab}
M3	1.5	-	-	43.00 ± 1.53^{bcd}	2.75 ± 0.52^{a}	2.18 ± 0.43^{ab}
M4	2.0	-	-	33.00 ± 0.87^{fg}	2.38 ± 0.62^{ab}	1.80 ± 0.42^{ab}
M5	-	1.0	-	28.00 ± 1.15^{gh}	1.90 ± 0.49^{ab}	1.38 ± 0.31^{ab}
M6	-	1.5	-	36.00 ± 0.58^{ef}	2.00 ± 0.58^{ab}	1.85 ± 0.45^{ab}
M7	-	2.0	-	40.00 ± 1.53^{cde}	2.60 ± 0.38^{ab}	2.15 ± 0.45^{ab}
M8	-	-	1.0	35.00 ± 1.26^{ef}	1.75 ± 0.25^{ab}	1.50 ± 0.87^{ab}
M9	-	-	1.5	45.00 ± 0.29^{abc}	2.80 ± 1.17^{a}	2.00 ± 0.29^{ab}
M10	-	-	2.0	38.00 ± 1.53^{def}	1.70 ± 0.30^{ab}	1.00 ± 0.29^{ab}
M11	1.0	1.0	-	34.00 ± 2.08^{efg}	1.80 ± 0.42^{ab}	1.38 ± 0.19^{ab}
M12	1.5	1.5	-	**51.00 ± 1.15^{a}**	**3.80 ± 0.12^{a}**	**2.30 ± 0.35^{a}**
M13	2.0	2.0	-	47.00 ± 0.29^{ab}	2.60 ± 0.70^{ab}	1.00 ± 0.50^{ab}
M14	1.0	-	1.0	38.00 ± 2.52^{def}	2.15 ± 0.34^{ab}	1.60 ± 0.31^{ab}
M15	1.5	-	1.5	40.00 ± 1.73^{cde}	3.00 ± 0.58^{a}	1.92 ± 0.58^{ab}
M16	2.0	-	2.0	48.00 ± 0.50^{ab}	2.90 ± 0.38^{a}	1.40 ± 0.31^{ab}

The labels M1-M16 represent PGRs combination, values represented mean ± standard error; data were recorded after four weeks of culture on MS medium supplemented with BAP, KIN and TDZ (Thidiazuron). Best results are indicated in bold, mean followed by the same letters within a column are not significantly different according to Tukey's test with p-value ≤ 0.05

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Shoot elongation

Well-proliferated shoots were isolated from the shoot bunches and transferred to the MS medium supplemented with different concentration of BAP and GA_3 for shoot elongation. Media without PGRs showed a negligible result of shoot length (0.5 cm) with 10% shoot elongation. On the other hand, Media containing GA_3 and BAP showed significantly effects on plant height with maximum shoot length (5.6 cm) and highest shoot elongation percentage (80%) in medium supplemented with 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} GA_3 following the lowest shoot length (2.50 cm) was measured on MS medium comprising 2.0 mgL^{-1} GA_3 alone. Among the tested PGRs, medium supplemented with GA3 alone was not effective for shoot elongation (*Table 3, Fig. 1F& 1G*).

Table 3. *Effects of MS medium supplemented with GA_3 and BAP on elongation of isolated shoots of okra*

Culture media	Plant growth regulator (mgL^{-1})		Shoot length (cm)	Shoot elongation %
	BAP	GA_3		
M1	0	0	0.50 ± 0.29^{c}	10.00 ± 1.00^{e}
M2	1.0	-	2.85 ± 0.45^{b}	34.00 ± 0.58^{d}
M3	1.5	-	3.57 ± 0.54^{ab}	40.00 ± 1.53^{d}
M4	2.0	-	4.20 ± 0.42^{ab}	63.00 ± 0.76^{bc}
M5	1.0	1.0	3.75 ± 0.43^{ab}	65.00 ± 1.53^{b}
M6	1.5	1.5	**5.60 ± 0.70^{a}**	**80.00 ± 1.32^{a}**
M7	2.0	2.0	4.50 ± 0.29^{ab}	57.00 ± 0.76^{c}
M8	-	1.0	2.60 ± 0.31^{b}	40.00 ± 1.04^{d}
M9	-	1.5	3.88 ± 0.19^{ab}	58.00 ± 1.53^{c}
M10	-	2.0	2.50 ± 0.29^{bc}	60.00 ± 2.31^{bc}

The labels M1-M10 represent PGRs combination, values represented mean ± standard error; data were noted after four weeks of inoculation on MS medium containing GA_3 and BAP. Best results are indicated in bold, mean followed by the same letters within a column are not significantly different according to Tukey's test with p-value ≤ 0.05

In vitro rooting and acclimatization

Well-developed, elongated shoots were excised from the *in vitro* regenerated shoots and cultured on half strength MS medium supplemented with different concentration of IBA, NAA along with AC for root induction. Highest rooting frequency (83%) as well as normal roots production (5.20 roots/shoot, 4.80 cm in length) was observed on MS medium supplemented with 1.0 mgL^{-1} IBA + 200 mgL^{-1} AC combination. While lowest rooting response (38%) was observed on medium fortified with 0.5 mgL^{-1} IBA alone (*Table 4, Fig. 1H*). Before transferring to the green house, plantlets with well-developed root system were transferred to the plastic cups having vermiculite and garden soil (1:1) and they were raised in the growth chamber for two weeks at 25 ± 2 °C with 70% relative humidity and consequently grown in the field with an of 87% survival rate (*Fig. 1I*).

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Table 4. *Effects of half strength MS medium supplement with different concentration of activated charcoal and auxins on rooting of okra*

Culture media	Plant growth regulator (mgL^{-1})			Root proliferation %	No. of root/explant	Root length (cm)
	IBA	NAA	AC			
M1	0	0	0	36.00 ± 1.53^{k}	1.20 ± 0.42^{e}	0.80 ± 0.44^{c}
M2	0.5	-	-	38.00 ± 1.00^{jk}	1.90 ± 0.26^{de}	2.50 ± 0.29^{abc}
M3	1.0	-	-	43.00 ± 0.29^{ijk}	2.70 ± 0.35^{cde}	3.10 ± 0.67^{abc}
M4	1.5	-	-	39.50 ± 0.29^{jk}	4.00 ± 0.50^{abc}	2.80 ± 0.42^{abc}
M5	0.5	-	100	45.00 ± 0.87^{ij}	3.75 ± 0.43^{abcd}	2.50 ± 0.29^{abc}
M6	1.0	-	100	54.00 ± 2.65^{fgh}	3.20 ± 0.61^{abcde}	2.70 ± 0.35^{abc}
M7	1.5	-	100	68.50 ± 0.76^{b}	4.80 ± 0.20^{ab}	3.50 ± 0.29^{ab}
M8	0.5	-	200	63.00 ± 1.53^{bcde}	3.10 ± 0.40^{bcde}	2.50 ± 0.29^{abc}
M9	1.0	-	200	**83.00 ± 1.76^{a}**	**5.20 ± 0.20^{a}**	**4.80 ± 0.35^{a}**
M10	1.5	-	200	60.00 ± 1.26^{cdef}	4.00 ± 0.29^{abc}	2.80 ± 0.20^{abc}
M11	-	0.5	-	50.00 ± 0.58^{ghi}	4.10 ± 0.10^{abc}	3.10 ± 0.46^{abc}
M12	-	1.0	-	48.00 ± 0.50^{hi}	2.50 ± 0.29^{cde}	1.80 ± 0.42^{bc}
M13	-	1.5	-	57.00 ± 0.76^{efg}	3.10 ± 0.49^{bcde}	2.50 ± 0.29^{abc}
M14	-	0.5	100	65.00 ± 2.89^{bcd}	2.80 ± 0.57^{bcde}	3.00 ± 0.58^{abc}
M15	-	1.0	100	58.00 ± 1.15^{def}	3.33 ± 0.33^{abcd}	2.10 ± 0.67^{bc}
M16	-	1.5	100	67.00 ± 1.00^{bc}	4.00 ± 0.58^{abc}	3.00 ± 1.15^{abc}
M17	-	0.5	200	54.00 ± 1.53^{fgh}	3.80 ± 0.15^{abcd}	2.80 ± 0.20^{abc}
M18	-	1.0	200	70.00 ± 1.04^{b}	4.88 ± 0.40^{ab}	3.70 ± 0.15^{ab}
M19	-	1.5	200	68.00 ± 1.44^{b}	3.90 ± 0.38^{abcd}	3.00 ± 0.58^{abc}

The labels M1-M19 represent PGRs combination, values represented mean ± standard error; data were recorded afterward the culture of four weeks on half strength MS medium composition with auxins IBA and NAA in combination with AC. Best results are indicated in bold, mean followed by the same letter within a column are not significantly different according to Tukey's test with p-value ≤ 0.05

Discussion

Direct *in vitro* regeneration system has the potential to improve the phyto-pharming genetics under the influence of internal and external factors, used for genetic transformation. In present study, our main purpose was to establish, an efficient regeneration system in okra through shoot multiplication by cotyledonary node as explant. Shoot multiplication was enhanced from cotyledonary node explants which varied with the type of growth regulator, concentration, and combination used. In general, cotyledonary nodes was found more effective for shoot initiation and multiplication explants source. Till date, regeneration through cotyledonary node explants have been found in various plant species including *Abelmoschus esculentus* (Irshad et al., 2018), *Pisum sativum* (Jackson and Hobbs, 1990), *Punica granatum* (Naik et al., 2000), *Gossypium hirsutum* ((Kumar et al., 2016), *Vigna mungo* (Mookkan and

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Andy, 2014), *Lathyrus sativus* (Tesfaye et al., 2017), *Prosopis cineraria* (Venkatachalam et al., 2017) and *Cucumis sativus* (Venkatachalam et al., 2018).

Cytokinins play a key role in shoot organogenesis. BAP and KIN used alone or in combination showed different responses. When BAP was used alone, at low level of 0.5 mgL^{-1} BAP showed only (43%) shoot induction with an average of (1.33) number of shoots, but highest shoot induction (75%), with (4.5) number of shoots was observed at 2.0 mgL^{-1} BAP. Similar observation was reported previously in *Prosopis cineraria* (Venkatachalam et al., 2017; Sangeetha and Venkatachalam, 2014), *Dendrocalamus strictus* (Goyal et al., 2015) and *Tinospora cordifolia* (Panwar et al., 2018). Whereas shoot induction slightly decreased (60%, 3.80 number of shoots) with increasing of BAP concentration up to 2.5 mgL^{-1} (*Table 1*). Increased concentration of cytokinins suppressed the initiation of shoot buds. Similar results have been reported in various plant species *Enicostema axillare* (Sasidharan and Jayachitra, 2017) and *Prosopis cineraria* (Venkatachalam et al., 2017).

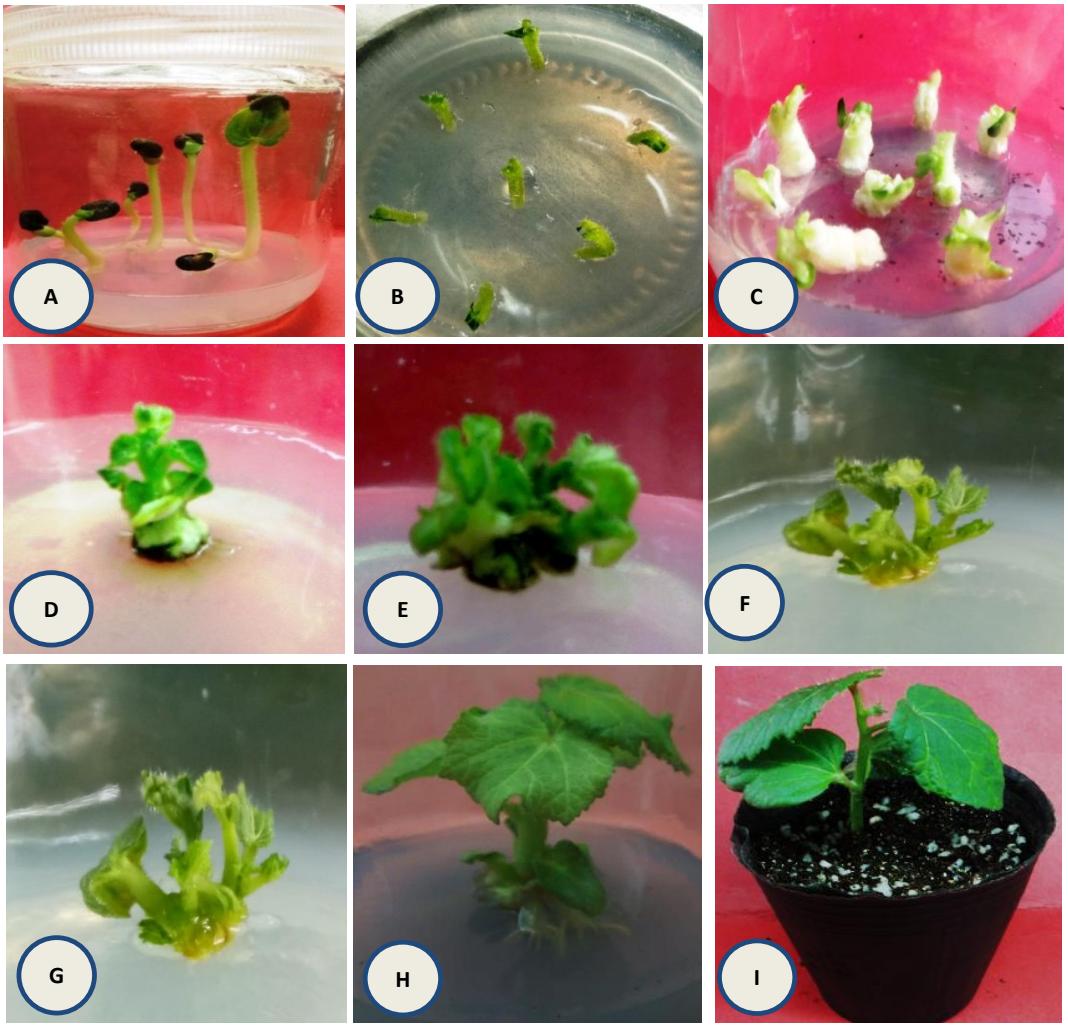

Figure 1. *Direct in vitro regeneration of okra (Abelmoschus esculentus L.) through cotyledonary node explant and the effect of $AgNO_3$ on multiple shoot formation. (A) 10-days old okra seedling. (B) Cotyledonary node explants. (C) Vigorous cultures after two weeks in culture media. (D) Multiple shoots inductions from nodal regions. (E) Multiple shoots formation and development. (F, G) Proliferation and elongation of multiple shoot. (H) Rooting of elongated shoots. (I) Regenerated plant*

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KIN showed less potential to shoot initiation then BAP. At lowest concentration of 0.5mgL^{-1}, it showed 58% with 1.68 shoots/explant and 65% with 3.50 shoots/explant at 2.0 mgL^{-1} BAP respectively. But the number of shoots per explant were significantly decreased at 2.5 mgL^{-1} BAP producing only 2.90 number of shoots with 38% response. Our results are in contrast with (Sivakumar et al., 2014). Rathore et al. (2016) also reported that KIN showed fewer shoot induction results in *Withania coagulans*. Fewer responses were observed at the lowest and highest plant growth regulator concentrations then intermediate level. Bakrudeen et al. (2011) in *Catharanthus roseus* preferred the use of BAP and KIN alone but not in combination. Our finding matches with the earlier reports of *Colocynthis citrullus* (Ntui et al., 2009), *Catharanthus roseus* (Bakrudeen et al., 2011) and *Prosopis cineraria* (Venkatachalam et al., 2017) in which BAP demonstrated positive influences on regeneration of shoot bud than to KIN.

Ethylene produced in culture medium, is known as an inhibitor of plant development and morphogenesis relying on the species and culture stage (Mohiuddin et al., 1997). $AgNO_3$ can induce variable responses *in vitro* by the inhibition of ethylene action. Kumar et al. (2009b) and Zhang et al. (2001) reported that the shoot multiplication is owing to the action of silver ions as a competitive ethylene inhibitor level by the crosstalk of ethylene and hormones rather than the ethylene biosynthesis inhibition. Use of regeneration enhancer in culture medium has been reported to be useful for shoot multiplication. Among the various compounds that have been widely used, $AgNO_3$ showed a positive effect on plant tissue cultures (Zhang et al., 2001). At 1.0 mgL^{-1} and 4.5 mgL^{-1} $AgNO_3$ induced 3.8 and 3.33 shoots/explant with 38 and 50% responses. But highest number of shoots/explant (4.0) was recorded at 3.0 mgL^{-1} $AgNO_3$ with 75% response. At 1.5 mgL^{-1} BAP + 3.0 mgL^{-1} $AgNO_3$ induced highest number of shoots 5.5 shoots/explant with 77% response but response decreased (68%) with increasing the concentration and produced 4.75 number of shoots/explant at 2.0 mgL^{-1} BAP + 4.5 mgL^{-1} $AgNO_3$, our results are similar with the finding of (Mookkan and Andy, 2014; Balkhande et al., 2013).

In case of 1.0 mgL^{-1} KIN + 1.5 mgL^{-1} $AgNO_3$ showed 50% response with 2.90 number of shoots per explant, which slightly increase with increasing the concentrations of 1.5 mgL^{-1} KIN + 3.0 mgL^{-1} $AgNO_3$ 50% response, 3.83 shoots/explants and at 2.0 mgL^{-1} KIN + 4.5 mgL^{-1} $AgNO_3$ highest response 67% with decreasing number of shoots (3.60 shoots/explant) was recorded. Our findings are in line with (Mookkan and Andy, 2014). Whereas in comparison, at lowest concentration of 1.0 mgL^{-1} TDZ + 1.5 mgL^{-1} $AgNO_3$ showed higher response 60% with 2.75 shoots/explants, but at higher dose of 2.0 mgL^{-1} TDZ + 4.5 mgL^{-1} $AgNO_3$ presented lowest response 37% with 1.75 shoots /explants (*Table 2A*). Results clearly indicate that TDZ and $AgNO_3$ at low concentration favored multiple shoot induction while higher dose shows negative results. Same findings were observed by Ouma et al. (2004) in cotton. Among the combinations evaluated, maximum number of shoot percentage (85%) was observed at a medium 3.0 mgL^{-1} $AgNO_3$ + 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN by inducing (6.75 shoots/explant) which was comparatively higher than earlier reports but at higher dose of 4.5 mgL^{-1} $AgNO_3$ + 2.0 mgL^{-1} BAP + 2.0 mgL^{-1} TDZ, consequently decreased the number of shoots (4.33 shoots/explant), with (49%) shoot induction response (*Table 2A*). Whereas, lower number of shoots (2.90 shoots/explant) were observed at a medium containing 3.0 mgL^{-1} $AgNO_3$ + 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} TDZ having 48% response with (2.90 shoots/explant) followed by the lowest response 37% with 1.75 shoots/explant was observed at 4.5 mgL^{-1} $AgNO_3$ + 2.0 mgL^{-1} BAP (*Table 2A*, *Fig. 1E*). In general BAP

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was found to be most efficient cytokinins in comparison to TDZ and KIN, similar phenomenon was reported by (Venkatachalam et al., 2018) and the addition of $AgNO_3$ along with other cytokinins enhanced the multiple shoot induction was also previously reported by (Venkatachalam et al., 2017; Mookkan and Andy, 2014; Naik et al., 2000).

The results achieved from our study suggests that $AgNO_3$ could be used as a worthy improver or regeneration enhancer for *in vitro* regeneration of okra through shoots multiplication, meanwhile the responses may vary with the plant species. Fasolo et al. (1989) reported that cytokinins have the feature to stimulate the shoot proliferation but inhibit the elongation. Whereas, shoot elongation is important in plant tissue culture, which varies with the medium type, concentration and combination of PGRs used in different plants. Highest response of shoot elongation (80%) was achieved with 5.60 cm shoot length on optimal concentration of 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} GA_3 (*Fig. 1F, 1G*) followed by the lowest shoot length (2.50 cm) which was recorded at a medium containing 2.0 mgL^{-1} GA_3 (*Table 3*). Our results indicated that use of BAP and GA_3 enhance the shoot elongation. Similar results of BAP and GA_3 combinations were reported by (Selvaraj et al., 2006) in *Cucumis sativus*, (Gonbad et al., 2014) in *Camellia sinensis* and (Kumar et al., 2016) in *Gossypium hirsutum*.

Well-developed rooting is essential for successful *in vitro* regeneration in many plants and the use of half strength MS medium promotes the rooting (Rahman et al., 1992; Oakes et al., 2016). The addition of AC enhances the root formation due to the large surface area which can adsorb many types of substances leadings to improve the cell growth and development (Ghazzawy et al., 2017). IBA at 1.0 mgL^{-1} produced the 2.70 roots/shoot with 43% response, which varies on higher and lower concentration. While in combination with 100.0 mgL^{-1} AC + 1.5 mgL^{-1} IBA produced 4.80 roots/shoot with 68.50% response, which increases with increasing the concentration of AC and showed the highest response 83% with 5.20 roots/shoot on medium containing 200.0 mgL^{-1} AC + 1.0 mgL^{-1} IBA. In case of NAA, the highest responses 70% with 4.88 roots/shoots on a medium containing 200.0 mgL^{-1} AC + 1.0 mgL^{-1} NAA were observed. Our results are in agreement with previous reports of (Şan et al., 2015; Irshad et al., 2017; Kumar et al., 2016). The rooted plantlets were hardened by transferring into plastic cups containing vermiculite and garden soil (1:1) mixture. They were then kept in a growth chamber for two weeks at 25 ± 2 °C with 70% relative humidity and consequently established in the field condition with 87% survival rate. This protocol will be useful for multiple shoot induction, direct plant regeneration, shoot elongation, rooting and hardening of other relevant plant species.

Conclusion

In this study, an efficient *in vitro* plant regeneration protocol was established using cotyledonary node explants of *A. esculents* "Xiang Fu" cultivar. BAP at 2.0 mgL^{-1} was found to be most effective for shoot bud initiation from cotyledonary nodal explants. Shoot multiplication frequency was enhanced by the addition of silver nitrate ($AgNO_3$) in the medium and the highest shoot multiplication frequency was achieved on MS medium fortified with 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} KIN + 3.0 mgL^{-1} $AgNO_3$ with maximum number of shoots (6.75 shoots/explants, 3.90 cm in length). Highest shoot elongation (80%) was observed in a medium containing 1.5 mgL^{-1} BAP + 1.5 mgL^{-1} GA_3 with maximum shoot length 5.6 cm. Highest rooting frequency (83%) with (5.20 roots/shoot, 4.80 cm in length) was observed in MS medium supplemented with 1.0

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mgL^{-1} IBA + 200 mgL^{-1} AC. Furthermore, the whole protocol from seedling establishing to regenerated plants was completed in only 3.5 to 4.0 months. This efficient protocol for high-frequency plant regeneration through shoot multiplication in okra (*Abelmoschus esculentus* L.) will be a valuable tool for further genetic transformation.

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