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ORIGINAL ARTICLE

High frequency plant regeneration from cotyledonary node explants of *Cucumis sativus* L. cultivar 'Green Long' via adventitious shoot organogenesis and assessment of genetic fidelity by RAPD-PCR technology

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Abstract

Influence of cytokinins, silver nitrate ($AgNO_3$) and auxins on plant regeneration from cucumber was investigated. The cotyledonary node explants were cultured on MS medium augmented with various concentrations (0.5–2.5 mg l^{-1}) of 6-benzyl amino purine (BAP) and kinetin (KIN) for shoot bud induction. BAP at 1.5 mg l^{-1} was found to be the best concentration for induction of high frequency of multiple shoots (98.4%). Interestingly, maximum percent of multiple shoot regeneration (100%) as well as number of shoot buds (54.6 shoots/culture) was recorded on MS medium containing the combination of 4.5 mg l^{-1} $AgNO_3$ and 1.5 mg l^{-1} BAP. Multiple shoot bud regeneration frequency as well as the number of shoots was positively correlated with the concentrations of $AgNO_3$. Addition of silver nitrate in the medium not only enhanced the rate of multiple shoot bud regeneration but also elongation of shoot buds was observed. The highest percent of rooting (96.2%) was noticed on a medium containing the combination of indole 3-butyric acid (IBA), 1.5 mg l^{-1} and KIN 0.5 mg l^{-1}. Acclimatized plantlets were successfully established in the field where the survival rate observed was 72%. The RAPD profiles of in vitro regenerated plants were found to be highly monomorphic and identical banding pattern with mother plant. DNA fingerprinting results confirmed that the tissue culture plantlets were found to be true-to-type. The present study describes efficient protocol for high frequency plant regeneration via adventitious shoot organogenesis in cucumber.

Keywords Auxins · Cucumber · *Cucumis sativus* · Cytokinins · Multiple shoot bud regeneration · Random amplified polymorphic DNA

Abbreviations

BAP	6-Benzyl amino purine
KIN	Kinetin
IBA	Indole 3-butyric acid
IAA	Indole 3-acetic acid
NAA	α-Naphthalene acetic acid
MS	Murashige and Skoog
$AgNO_3$	Silver nitrate
RAPD	Random Amplified Polymorphic DNA

Introduction

Cucumber is an important agricultural as well as horticultural crop which belongs to the family Cucurbitaceae. *Cucumis sativus* L. is an important vegetable crop worldwide which grows both in tropical and subtropical regions. Cucumber is not only used for food dishes but also different parts such as leaf, fruit, and seeds have been explored for their therapeutic potentials such as cosmetics and wound healing activity. Most recently, leaves and callus tissues from cucumber plant were used for synthesis of biomolecules loaded metallic silver nanoparticles and its wound healing

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property was studied by preparation of nanodrug-based ointment in rat model (Venkatachalam et al. 2015). Therefore, there is a growing interest for enhancement of cucumber productivity worldwide due to its versatile applications. The ability of cucumber to grow under a wide range of agroclimate and soil conditions, disease and pest problems significantly affected the yield of this important crop (Vengadesan et al. 2005; Grozeva and Velkov 2014). Plant tissue culture technology is an important tool for genetic manipulation which is essential to overcome crop yield losses due to various biotic and abiotic stresses (Kumar et al. 2015). Somaclonal variation is considered as one of the important phenomena which is a potential factor detected in tissue culture of plants with reduced levels of desired secondary metabolites such as cafestol and kahweol in coffee (Sridevi and Giridhar 2014). Therefore, development of an efficient protocol for high frequency plant regeneration system with no risk of somaclonal variations is a major focus for genetic improvement of horticultural crops including cucumber. In vitro regeneration has many advantages, such as higher rates of multiplying clean (pest and disease-free) planting material and a little space is required to multiply large number of plants. Plant regeneration has been achieved via organogenesis in cucumber using different explants such as cotyledons (Gambley and Dodd 1990; Selvaraj et al. 2007), shoot tips (Vasudevan et al. 2004), embryonal axis (Vasudevan et al. 2007), hypocotyls (Selvaraj et al. 2006), nodal segments (Ahmad and Anis 2005; Kontas and Kintzios 2003). Somatic embryogenesis was also reported in cucumber by Mashayekhi et al. (2008). Plant regeneration from one cultivar may be different from that of another cultivar within the same species (Vasudevan et al. 2007). Therefore, efficient plant regeneration protocol should be developed for each cultivar (Walden and Wingender 1995). 'Green Long' is one of the most popular cucumber cultivars in India, especially in Tamil Nadu (Vengadesan et al. 2005). According to the earlier report, the regeneration rate was found to be low and it was largely dependent on the type of cultivar, nature of the explants and growth regulators (Selvaraj et al. 2007). There is an increasing interest to use the cotyledonary node which is a juvenile meristematic part from the seedlings, as alternative explant for in vitro regeneration in the recent past. Earlier, Singh et al. (2007) demonstrated that about 3–4 shoots were obtained with BAP at lower concentrations and multiple shoot regeneration was inhibited at higher concentrations of BAP. For breeding purpose, an efficient protocol for high frequency plant regeneration is still essential for development of cucumber transgenic plants with 'Green Long' cultivar.

Analysis of in vitro derived plants for genetic variability via clonal fidelity is one of the prerequisites to ensure the desired superior genotypes for agronomically important traits including good growth, tolerance to both biotic and abiotic stresses (Kumar et al. 2015). Though various molecular marker techniques were applied to detect the genetic fidelity of in vitro derived clones, the Random Amplified Polymorphic DNA (RAPD) technique has proven to be effective in detecting genetic variability and it was successfully applied for identification of genetic similarities among micropropagated plants until recently (Williams et al. 1990; Hussain et al. 2008; Martins et al. 2004; Thiyagarajan and Venkatachalam 2012; Kumar et al. 2015).

In view of the above, the development of an efficient regeneration protocol is one of the prerequisites for production of uniform cucumber plants that could be used for genetic manipulation of this crop. The present study was focused to develop an efficient protocol for high frequency plant regeneration using different growth regulators and silver nitrate via adventitious shoot organogenesis from cotyledonary node explants of commercially important cucumber cultivar 'Green Long'. In addition, the genetic fidelity of the cucumber regenerants was assessed using RAPD-PCR technology.

Materials and methods

Plant material and seed germination

Matured certified seeds of cucumber (*Cucumis sativus* L., cv. 'Green Long') were collected from the Agricultural farm, Salem, India. The seed coats were removed and soaked overnight in sterile distilled water. Then, the seeds were washed with 10% (v/v) Tween 20 for 10 min, surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 5 min and washed with sterile distilled water for 5 times to remove the mercuric chloride traces. Sterilized seeds were placed on half-strength MS medium for germination and kept in the dark condition.

Culture medium and conditions

The cotyledonary node explants were cultured on MS (Murashige and Skoog 1962) medium supplemented with various concentrations and combinations of 6-benzyl amino purine (BAP), kinetin (KIN), indole 3-acetic acid (IAA), indole 3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and silver nitrate ($AgNO_3$). The pH of the medium was adjusted to 5.7 prior to adding 0.7% (w/v) agar and medium (15 ml) was aliquoted into each culture tubes (25 × 150 mm). Then, the media were autoclaved at 121 °C for 20 min with 15 lbs pressure. All the cultures were maintained at 25 ± 2 °C with a photoperiod of 16/8 h (light/dark) at 60 µE m^{-2} s^{-1} light provided by cool white fluorescent tubes.

Shoot bud initiation

Initially, the cotyledonary node explants from 5-day-old in vitro grown seedlings were excised by removing the epicotyls as well as cotyledons and used for plant regeneration. For shoot bud induction, the cotyledonary node explants were cultured on MS medium containing different concentrations of BAP and KIN (0.5–2.5 mg l^{-1}) alone. The shoot cultures were transferred into same fresh medium at 2 weeks interval. For each experiment, ten cotyledonary node explants were used and at least repeated thrice.

Multiple shoot bud regeneration and elongation

In vitro raised adventitious shoot buds from the cotyledonary node explants grown on MS medium containing 2.0 mg l^{-1} BAP were selected for multiple shoot bud regeneration. The selected in vitro adventitious shoot clumps were dissected out and transferred onto MS medium supplemented with various concentrations of BAP (0.5–2.5 mg l^{-1}) alone and/or in combination with 0.5 mg l^{-1} NAA/IBA for multiple shoot bud development. The adventitious shoot cultures were subcultured onto the same media combinations for further growth and development at 2 weeks interval. After 2 subcultures, the regenerated shoot buds were transferred onto MS medium containing different concentrations of $AgNO_3$ (1.5–6.0 mg l^{-1}) alone and/or in combination with 1.5 mg l^{-1} BAP for enhancement of multiple shoot bud development. The regenerated shoot buds were elongated quickly on the same media. For multiple shoot bud regeneration, minimum seven regenerants were tried per treatment and each experiment was repeated three times.

Rooting and acclimatization

Elongated shoots (> 2 cm in length) obtained after completion of shoot bud multiplication cycles were dissected out individually and placed on half-strength MS medium augmented with various concentrations of IBA and NAA (0.5–2.0 mg l^{-1}) alone and/or in combination with 0.5 mg l^{-1} KIN for rooting. Seven elongated shoots were placed in each dose and all experiments were repeated thrice. The rooted plantlets were carefully removed from the culture tubes and gently washed under running tap water to remove traces of agar without damaging the root system. Then, the plantlets were transplanted into plastic cups containing sterile sand and soil in the ratio of 1:1 and kept at 25 ± 2°C with 80% relative humidity under 16/8 h of light/dark cycle provided with light intensity at 60 µE m^{-2} s^{-1} by cool white fluorescent tubes. After 2 weeks, acclimatized plantlets were subsequently established in the field.

Genomic DNA extraction and PCR amplification

Clonal fidelity of the in vitro raised plantlets that were isolated from 1.5 mg l^{-1} BAP and 4.5 mg l^{-1} $AgNO_3$ combination was analyzed using RAPD markers. For PCR analysis, a total of 10 in vitro regenerated plantlets which were acclimatized in the field conditions were selected randomly and the RAPD banding pattern was compared with the control plant (mother plant derived via seedling). Total genomic DNA was extracted from the control as well as in vitro regenerated plantlets by modified CTAB method (Doyle and Doyle 1990). Leaves were weighed (0.1 g) and ground well and homogenized with 1.0 ml of 2× CTAB buffer [(2% hexadecylcetyl trimethyl ammonium bromide), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris–HCl (pH 8.0), 1% (w/v) polyvinyl poly pyrolidone (PVPP), 1.0% (v/v) β-mercaptoethanol] and incubated the extract in a water bath at 65 °C for 20 min. Then, the extract was allowed to cool at room temperature and added 400 µl of saturated phenol, 400 µl of chloroform and 20 µl of β-mercaptoethanol, mixed well and centrifuged at 8000 rpm for 10 min. Aqueous phase was transferred into the fresh tube and the DNA was precipitated with 0.6 volume of ice-cold isopropanol. The DNA pellet was washed with 70% (v/v) ethanol and air dried. Then, it was dissolved in sterile distilled water and quantified spectrophotometrically.

For initial screening of DNA samples, 100 random decamer primers from Operon Inc., USA were used. PCR amplification was carried out in a volume of 20 µl containing 2 µl 1× PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$], 2 µl 1.5 mM dNTPs (dATP, dGTP, dCTP and dTTP), 250 nM primers (1 µl), 0.5 units of Taq DNA polymerase, 2 µl genomic DNA (15 ng) and 13 µl of sterile water. DNA amplification was performed in a thermal cycler machine (Cyber Lab, USA) using the following PCR conditions with initial denaturation at 94 °C for 4 min, followed by 40 cycles consisted of denaturation at 94 °C for 1 min, annealing at 37 °C for 1.30 min and extension at 72 °C for 2 min and a final extension at 72 °C for 7 min. After completion of PCR cycles, the amplicons were stored at 4 °C until further use.

Electrophoretic analysis of PCR amplicons

The PCR products mixed with appropriate volume of DNA loading buffer (10×) were analyzed on 1.5% (w/v) agarose gel electrophoresis containing 0.5 µg/ml ethidium bromide in 1× TAE buffer. Electrophoresis was performed at 50 V for 2 h until the bromophenol blue dye front migrated to the bottom of the gel. The molecular standard used was the lambda DNA double digested by *Eco*RI/*Hin*dIII. The gels were visualized under UV light and photographed with gel documentation system (Alpha Inotech Gel documentation system, USA) for further analysis.

Statistical analysis

All the experiments were set up in a completely randomized block (CRB) design and each experiment had three replicates. Based on the visual observation of the cultures, the percentage of cultures showing shoot bud induction, multiple shoot bud differentiation and rooting was recorded and used for statistical analysis. The analysis of variance (ANOVA) was performed using SAS (Statistical Analysis Software) programme. The differences among mean values were determined by Student–Newman–Keuls test at 5% ($p > 0.05$) significance level.

Results

Initiation of adventitious shoot buds

The morphogenetic responses of cotyledonary node explants to different concentrations of BAP and KIN (0.5–2.5 mg l^{-1}) are summarized in Table 1. After 5 days of culture, the adventitious shoot buds initiated directly from the cotyledonary node explants cultured on MS medium fortified with BAP, while no shoot bud development was noticed on medium containing KIN. It is noteworthy to mention that shoot bud initiation (100%) was recorded at all BAP concentrations used (Fig. 1a). Among the different concentrations of BAP tested, the highest percent of shoot bud regeneration (100%) with maximum number of shoots (6.4 shoots/explant) was noticed on MS medium containing 2.0 mg l^{-1} BAP followed by 1.5 mg l^{-1} BAP. It is interesting to note that no shoot bud regeneration was noticed on medium containing KIN, but induced roots from the cultured cotyledonary node explants. The number of shoot buds was increased with increasing the concentrations of BAP up to 2.0 mg l^{-1}; thereafter, it was decreased with further increase in the BAP concentration in the medium (Table 1).

Table 1 Effect of different concentrations of BAP and KIN on induction of adventitious shoot buds from cotyledonary node explants of cucumber

Cytokinin conc. (mg l^{-1})	Percent of shoot bud induction (mean ± SE)*	Number of shoots/explant (mean ± SE)*
BAP		
0.5	100	1.2 ± 1.09^{d}*
1.0	100	2.2 ± 0.30^{c}
1.5	100	5.2 ± 0.20^{b}
2.0	100	6.4 ± 0.22^{a}
2.5	100	4.0 ± 0.90^{b}
KIN		
0.5	Root development	–
1.0	Root development	–
1.5	Root development	–
2.0	Root development	–
2.5	Root development	–

*Means followed by same letter within a column are not significantly different at ($p \leq 0.05$). Percent of shooting response and number of shoots per explant were recorded after 2 weeks of culture

Development of multiple shoot buds

The results on percent of multiple shoot bud regeneration are presented in Table 2. Among the different concentrations and combinations used, BAP at 1.5 mg l^{-1} was found to be the best concentration for maximum number of multiple shoot bud development (19.85 shoots/culture) (Fig. 1b). The highest percent of multiple shoot bud regeneration obtained was 98.4% on MS medium fortified with 1.5 mg l^{-1} BAP alone and it was statistically significant at 5% level (Table 2). Of the two auxin combinations tested, maximum percent of multiple shoot bud development (97.6%) with 10.6 shoots/culture was noticed on MS medium augmented with 1.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA combination, followed by 1.5 mg l^{-1} BAP and 0.5 mg l^{-1} IBA combination in which the percent of shoot bud regeneration noticed was 96.4% with 11.4 shoots/culture. Increased rate of multiple shoot bud induction as well as the number of shoot buds was noticed with increasing the concentrations of BAP up to 1.5 mg l^{-1}, while the multiple shoot regeneration frequency was declined when the BAP dose was increased beyond the optimum level. Results suggest that if auxin (IBA/NAA) was added into the medium along with BAP, the number of multiple shoot bud development was significantly decreased due to the formation of callus at the basal part of the differentiated shoot buds. The present study clearly revealed that multiple shoot bud development was suppressed at higher BAP concentrations.

To enhance the shoot bud multiplication rate, different concentrations of $AgNO_3$ (1.5–6.0 mg l^{-1}) alone and/or in combination with 1.5 mg l^{-1} BAP were added into the regeneration medium. It is important to mention that the stunted shoot growth was overcome by addition of $AgNO_3$ in the medium and also promoted the shoot bud elongation in the same media composition (Fig. 1c). The frequency and number of multiple shoot bud regeneration increased with increasing the concentrations of $AgNO_3$ in the medium up to 4.5 mg l^{-1} (83.4%, with 32.2 shoots/culture), but it was slightly decreased at higher doses. Maximum percent of multiple shoot bud regeneration (100%) with 54.6 shoots/culture was recorded on MS medium fortified with 4.5 mg l^{-1} $AgNO_3$ in combination with 1.5 mg l^{-1} BAP (Table 3).

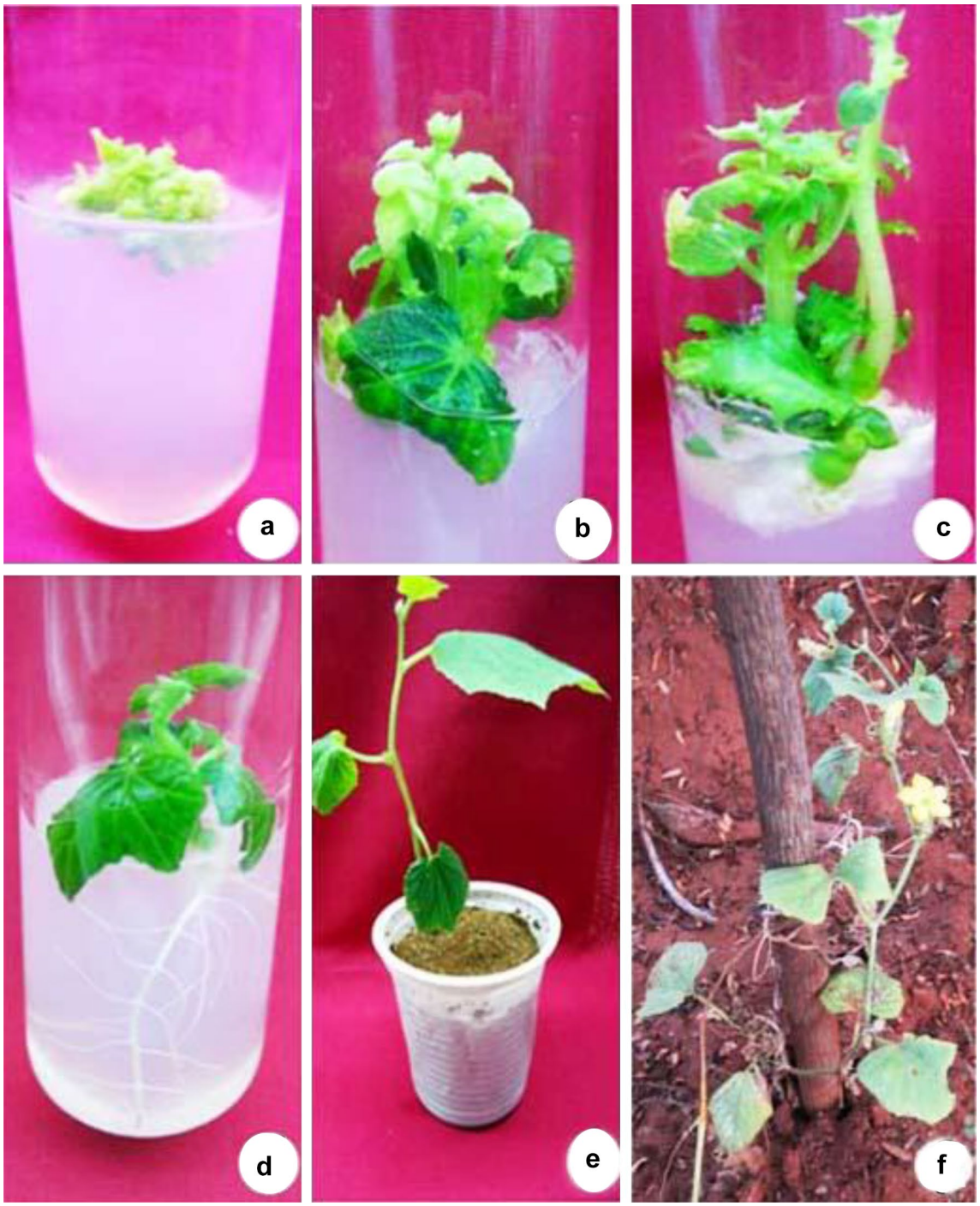

Fig. 1 Efficient plant regeneration from cotyledonary node explants of *Cucumis sativus* L., cv. 'Green Long'). Cotyledonary node explants cultured on MS medium for shoot bud initiation (**a**), adventitious shoot organogenesis from the initiated shoot buds (4 weeks old culture) (**b**, **c**), in vitro rooting (3-week-old plantlet) (**d**, **e**) and hardened plant growing in plastic cup with sand and soil (**f**)

Rooting and acclimatization

The results on percent of rooting and number of roots are depicted in Table 4. After 2 weeks of culture, the root initiation was noticed directly from the cut portion of the shoots. The percent of rooting was recorded after 4 weeks of culture (Fig. 1d). Interestingly, the percent of root initiation was increased with increasing the concentrations of auxin in the medium up to optimal level (IBA or NAA at 1.5 mg l^{-1}) and the rooting percentage was decreased

Table 2 Effect of different concentrations of BAP alone and/or in combination with two auxins on development of multiple shoots from cotyledonary node derived adventitious shoot cultures of cucumber

Hormone conc. (mg l^{-1})			Percent of shoot bud multiplication (mean ± SE)*	Number of shoots/culture (mean ± SE)*
BAP	IBA	NAA		
0.5	–	–	72.6 ± 1.60^{c}*	10.22 ± 0.76^{d}*
1.0	–	–	84.4 ± 1.80^{b}	12.87 ± 0.69^{c}
1.5	–	–	98.4 ± 1.28^{a}	19.85 ± 0.76^{a}
2.0	–	–	85.8 ± 1.24^{b}	13.00 ± 0.82^{b}
2.5	–	–	83.2 ± 1.36^{c}	10.14 ± 0.50^{d}
0.5	0.5	–	72.5 ± 1.15^{g}	6.85 ± 0.67^{g}
1.0	0.5	–	84.6 ± 1.20^{b}	10.66 ± 0.66^{d}
1.5	0.5	–	94.4 ± 1.80^{e}	11.40 ± 0.26^{e}
2.0	0.5	–	84.5 ± 1.24^{f}	7.16 ± 0.30^{f}
2.5	0.5	–	72.2 ± 1.18^{g}	5.30 ± 0.53^{h}
0.5	–	0.5	74.8 ± 1.18^{f}	5.40 ± 0.42^{c}
1.0	–	0.5	78.4 ± 1.40^{d}	6.20 ± 0.40^{e}
1.5	–	0.5	97.6 ± 1.62^{f}	10.60 ± 0.62^{i}
2.0	–	0.5	82.4 ± 1.36^{g}	2.20 ± 1.36^{j}
2.5	–	0.5	66.4 ± 1.20^{h}	1.85 ± 1.20^{k}

*Means followed by same letter within a column are not significantly different at ($p \leq 0.05$). Percent of shooting response and number of shoots per culture were recorded after 4 weeks of culture

Table 3 Effect of different concentrations of silver nitrate alone and/or in combination with BAP on shoot bud multiplication from cotyledonary node derived adventious shoot cultures of cucumber

BAP conc. (mg l^{-1})	$AgNO_3$ conc. (mg l^{-1})	Percent of shoot bud multiplication (mean ± SE)*	Number of shoots/culture (mean ± SE)*
–	1.5	67.5 ± 0.72^{h}*	12.2 ± 1.25^{g}*
–	3.0	72.3 ± 1.23^{g}	18.3 ± 0.87^{f}
–	4.5	83.4 ± 0.82^{e}	32.2 ± 1.23^{e}
–	6.0	78.7 ± 0.97^{f}	19.1 ± 0.75^{f}
1.5	1.5	92.6 ± 0.60^{d}	30.4 ± 0.77^{d}
1.5	3.0	97.8 ± 0.44^{b}	41.5 ± 1.13^{b}
1.5	4.5	100.0 ± 0.00^{a}	54.6 ± 2.20^{a}
1.5	6.0	94.3 ± 1.20^{c}	35.4 ± 0.75^{c}

*Means followed by same letter within a column are not significantly different at ($p \leq 0.05$). Percent of shooting response and number of shoots per culture were recorded after 4 weeks of culture

at higher doses. Among the IBA concentrations tested, the maximum percent of rooting noticed was 91.4% on half-strength MS medium supplemented with 1.5 mg l^{-1} IBA followed by 2.0 mg l^{-1}. In the case of IBA and KIN combinations used, the highest percent of root induction (96.2%) with maximum number of roots (8.4 roots/shoot) was observed on half-strength MS medium containing

Table 4 Effect of different concentrations of two auxins (IBA and NAA) alone and/or in combination with 0.5 mg l^{-1} KIN on initiation of roots from elongated shoots of cucumber

Hormone conc. (mg l^{-1})			Percent of root induction (mean ± SE)*	Number of roots/shoot (mean ± SE)*
IBA	NAA	KIN		
0.5	–	–	68.2 ± 0.10^{g}*	5.1 ± 1.02^{c}*
1.0	–	–	77.2 ± 0.24^{e}	6.2 ± 0.22^{b}
1.5	–	–	91.4 ± 0.30^{e}	6.4 ± 0.52^{b}
2.0	–	–	83.2 ± 0.70^{c}	7.0 ± 0.30^{a}
0.5	–	0.5	72.1 ± 0.40^{e}	5.2 ± 0.20^{c}
1.0	–	0.5	84.2 ± 0.80^{d}	6.8 ± 0.90^{b}
1.5	–	0.5	96.2 ± 0.83^{a}	8.4 ± 0.10^{a}
2.0	–	0.5	90.6 ± 1.12^{f}	9.8 ± 0.34^{e}
–	0.5	–	64.8 ± 0.40^{i}	2.5 ± 0.10^{g}
–	1.0	–	75.2 ± 0.62^{j}	3.55 ± 0.44^{f}
–	1.5	–	89.7 ± 0.90^{g}	4.24 ± 0.30^{e}
–	2.0	–	81.6 ± 0.35^{k}	5.30 ± 0.90^{g}
–	0.5	0.5	70.5 ± 0.80^{h}	2.8 ± 0.58^{g}
–	1.0	0.5	88.0 ± 0.40^{c}	6.8 ± 0.62^{b}
–	1.5	0.5	94.4 ± 0.10^{b}	7.2 ± 0.52^{a}
–	2.0	0.5	88.62 ± 0.64^{g}	8.2 ± 0.32^{f}

*Means followed by same letter within a column are not significantly different at ($p \leq 0.05$). Percent of rooting response and number of roots per shoot were recorded after 3 weeks of culture

1.5 mg l^{-1} IBA and 0.5 mg l^{-1} KIN combination (Table 4). When different concentrations of NAA were used in the medium, the maximum percentage of rooting (89.7%) with 4.2 roots/shoot was obtained on a medium containing 1.5 mg l^{-1} NAA. If NAA alone was used in the medium for rooting, callus formation was also noticed at the cut end of the shoot. In the case of NAA and KIN combinations tested, 1.5 mg l^{-1} NAA + 0.5 mg l^{-1} KIN combination produced the highest percent of rooting (94.4%) with more number of roots (7.2 roots/shoot). It is interesting to note that the number of roots per shoot was positively correlated with auxin concentrations. The rooted plantlets (> 5 cm length) were gently taken out from the culture tubes and washed initially to remove adhered agar traces to avoid contamination. Then, they were transferred to the plastic cups containing sterile sand and soil in the ratio 1:1 and covered with polythene bags to ensure high humidity and placed in the controlled environment (Fig. 1e). After 2 weeks, the polybags were removed and the plantlets were transferred to the greenhouse. Subsequently, the plantlets were established in the field conditions where about 72% of the plantlets were survived. The regenerated plantlets grew normally without showing any morphological variations similar to the control mother plants (Fig. 1f).

RAPD fingerprinting analysis

To assess the genetic fidelity, DNA from randomly selected 10 in vitro regenerated cucumber plantlets along with a control mother plant was used for RAPD fingerprinting analysis (Fig. 2). A total of 100 random oligonucleotide primers were tested for initial screening. Among these only 15 random primers produced clear and reproducible DNA bands. Each primer produced a unique set of amplification products ranging in size from 300 to 3000 base pairs. The number of bands for each primer varied from 4 to 8, with an average number of 5.9 bands per RAPD primer. The number and size range of amplified scorable bands for each RAPD primer are listed in Table 5. A total of 89 PCR amplicons were recorded and 87 DNA bands showed monomorphism and 2 were found to be polymorphic DNA bands in this study. It is important to mention that the frequency of monomorphism was 98.2% among the regenerated plants and control mother plants investigated. The RAPD fingerprints obtained after PCR amplification of genomic DNA from 10 randomly selected in vitro derived plantlets and the control mother plant of cucumber were scored for appearance as 1 (band present) and disappearance as 0 (band absent) for each plantlet. Results indicate that the RAPD fingerprinting approach

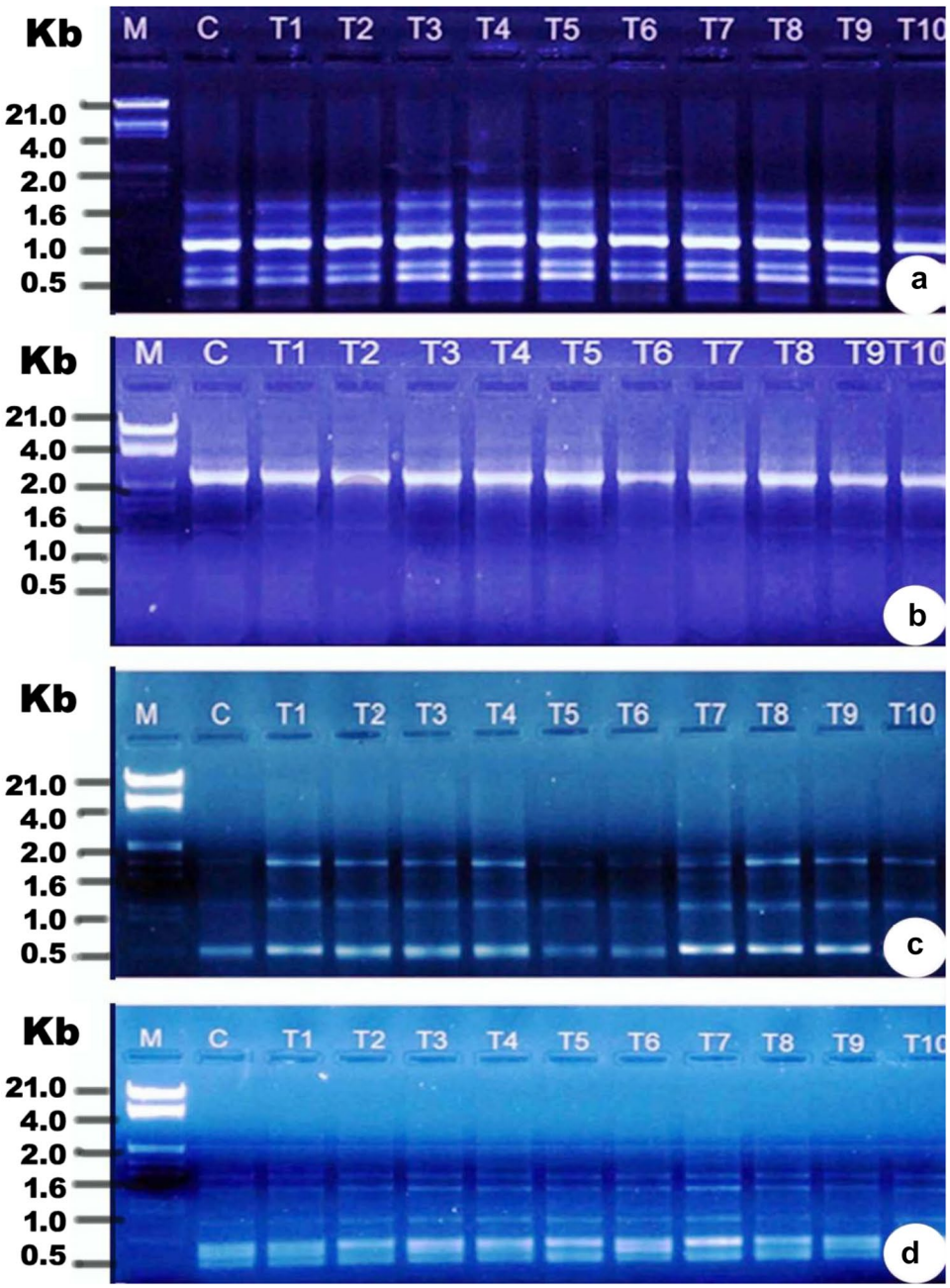

Fig. 2 RAPD banding patterns generated from the DNA samples of control mother plant and in vitro raised plantlets of *Cucumis sativus* L., cv. 'Green Long' with different primers OPA10 (**a**), OPB1 (**b**), OPC11 (**c**), and OPD06 (**d**); Lane M: molecular size marker (lambda DNA double digested by *Eco*RI/*Hin*dIII), lane C: control mother plant, lanes (T1–T10): in vitro regenerated plantlets from the cotyledonary node explants

Table 5 RAPD decamer primer sequences with G+C contents and number of the RAPD amplicons with monorphic and polymorphic DNA bands generated by PCR for genetic fidelity analysis in cucumber

Primer code	Primer sequence (5′–3′)	G+C content (%)	Size range of PCR amplicons in bp	Total number of amplified DNA bands	Total number of monomorphic DNA bands	Total number of polymorphic DNA bands	Percent of monomorphism
OPA02	TGCCGAGCTG	70	1800–550	7	7	–	100
OPA07	GAAACGGGTG	60	1100–650	5	5	–	100
OPA10	GTGATCGCAG	60	1600–300	8	7	1	87.5
OPA13	CAGCACCCAC	70	1200–550	7	7	–	100
OPA15	TTCCGAACCC	60	1800–300	6	6	–	100
OPB01	GTTTCGCTCC	60	3000–600	5	5	–	100
OPB04	GGACTGGAGT	60	1100–600	4	4	–	100
OPC05	GATGACCGCC	70	1800–550	6	6	–	100
OPC09	CTCACCGTCC	70	900–500	4	4	–	100
OPC11	AAAGCTGCGG	60	1800–300	5	5	–	100
OPC20	ACTTCGCCAC	60	1100–300	6	6	–	100
OPD06	ACCTGAACGG	60	1600–550	6	5	1	83.3
OPD11	AGCGCCATTG	60	1200–800	6	6	–	100
OPE06	AAGACCCCTC	60	1800–550	7	6	1	85.7
OPE14	TGCGGCTGAG	30	1200–550	7	7	–	100
Total				89	86	3	97.1

is an effective molecular tool for the detection of genetic variants among the in vitro raised plants in cucumber.

Discussion

The present study describes an efficient protocol for plant regeneration and confirmation of regenerants by genetic fidelity analysis in cucumber. The adventitious shoot bud initiation was achieved in all the concentrations of BAP tested but the number of shoot buds was enhanced up to 2.0 mg l^{-1} BAP. Further, no shoot bud growth was noticed on medium supplemented with KIN but later produced roots only. Therefore, the initiated adventitious shoot buds were further subcultured onto fresh medium containing the same concentrations of BAP alone and/or in combination with NAA/IBA for enhancement of multiple shoot bud regeneration. The results showed that the number of multiple shoots was increased significantly but the percentage of shoot bud regeneration was not altered when the shoot buds were subcultured onto MS medium containing BAP alone. Vasudevan et al. (2001) reported that BAP produced maximum number of shoots from shoot tip explants of cucumber. The superiority of BAP on shoot bud induction has also been well documented earlier in *Citrullus lanatus* (Pirinc et al. 2003; Ganesan and Huyop 2010). Ntui et al. (2009) stated that BAP alone has enhanced the percent of shoot bud induction and elongation in *Colocynthis citrullus* L. Among the BAP concentrations tested, BAP at 1.5 mg l^{-1} was found to be optimum dose for induction of maximum number of multiple shoot bud regeneration.

Similarly, Mohammadi and Sivritepe (2007) observed enhanced rate of shoot bud multiplication and proliferation on a medium fortified with BAP in *Cucumis sativus*. It has been well documented that BA has proved as potential cytokinin for induction of multiple shoot organogenesis in different plant species such as *Capsicum annuum* (Khan et al. 2011), *Moringa oleifera* (Saini et al. 2012) and *Stevia rebaudiana* (Thiyagarajan and Venkatachalam 2012). The number of multiple shoots was found to be decreased when a low concentration of auxins was included in the medium. Similar result was also reported earlier by Han et al. (2004) in bottle gourd. In contrast, Vasudevan et al. (2007) reported that the combination of BAP and NAA in MS medium triggered the initiation of adventitious shoot buds from embryonical axis explants of cucumber. In the present study, BAP was found to be the best cytokinin for multiple shoot bud regeneration. Similarly, it has been reported that BAP at lower concentration was found to be optimum for multiple shoot bud induction from nodal explants of *Cucumis sativus* (Ahmad and Anis 2005). However, the maximum number of multiple shoots in cucumber was obtained on medium containing the combination of BAP (3.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) by Ugandhar et al. (2011), Grozeva and Velkov (2014) and Jesmin and Mian (2016). It has been suggested that lower dose of auxin could trigger the cytokinin activity effectively for production of more number of multiple shoots. Also, plant regeneration from hypocotyl explants of cucumber cv Gergana was reported on medium fortified with

kinetin at 1.0 mg l^{-1} and it was inhibited at higher doses (Grozeva and Velkov 2014). This result further suggested that the cucumber genotype is considered as one of the major factors influencing shoot bud regeneration frequency and different genotypes are required various growth regulator combinations for plantlet development (Wang et al. 2015). Though the number of multiple shoots was enhanced with BAP alone and/or in combination with lower dose of auxins, no shoot bud regeneration was recorded in the cultures grown on medium containing KIN. Recently, Prem Kumar et al. (2016) proved that BAP showed superior effect on multiple shoot bud regeneration as well as shoot elongation in cotton compared to KIN. Similar observations on the superiority of BA over other cytokinins have been reported for *Psoralea corylifolia* and *Withania coagulans* shoot regeneration (Siva et al. 2015; Rathore et al. 2016).

To increase the number of multiple shoot buds, the regenerated shoots were further subcultured onto MS medium containing different doses of $AgNO_3$ alone and/or in combination with 1.5 mg l^{-1} BAP. It is interesting to note that the number of multiple shoot buds was significantly enhanced by addition of $AgNO_3$. The number of multiple shoots was positively correlated with $AgNO_3$ concentration. Ethylene is produced in in vitro plant culture systems and is known to inhibit plant growth and morphogenesis depending upon the species and culture stages. Consequently, inhibitors of ethylene actions such as $AgNO_3$ and polyamines can induce variable in vitro responses in plants (Giridhar et al. 2001). However, both the frequency of multiple shoot bud regeneration and number of shoots were slightly decreased at higher doses of $AgNO_3$. Similar results were also reported earlier by Park et al. (2012). Balkhande et al. (2013) demonstrated that the addition of $AgNO_3$ along with BAP enhanced the number of shoots in *Momordica cymbalaria*. Mohiuddin et al. (2005) reported that shoot regeneration was inhibited in *Cucumis sativus* when $AgNO_3$ (100 µM) was added in the shoot regeneration medium. In the present study, an enhanced rate of multiple shoot bud regeneration was noticed up to 4.5 mg l^{-1} $AgNO_3$ + 1.5 mg l^{-1} BAP combinations from cotyledonary node explants of cucumber. Similarly, Diao et al. (2009) also reported that addition of $AgNO_3$ in the medium enhanced the rate of somatic embryo formation in cucumber. One of the reasons for increased percent of multiple shoot bud development was due to the action of silver ions as competitive inhibitor of ethylene rather than inhibiting ethylene biosynthesis (Zhang et al. 2001). According to Kumar et al. (2009), the potent ethylene biosynthesis inhibitor, $AgNO_3$ could increase the arginine decarboxylase (ADC) activity that ultimately enhances the endogenous polyamines level in cultured cells. It has been reported that accumulation of polyamines level in cells exhibits improved rate of in vitro shoot bud multiplication in plants (Kumar et al. 2009). It is presumed that silver nitrate might have altered the ethylene action but triggered the polyamine production and thus it might have played a pivotal role in enhanced production of multiple shoots under in vitro. Similarly, Giridhar et al. (2003) also reported a positive role of $AgNO_3$ on enhanced production of in vitro shoot bud multiplication in *C. arabica* and *C. canephora*. Recently, Prem Kumar et al. (2016) also recorded the enhanced rate of shoot bud regeneration and multiplication in cotton by the addition of $AgNO_3$. Results altogether suggest that the addition of $AgNO_3$ along with BAP was greatly promoted the multiple shoot bud development as well as shoot bud elongation in cucumber.

Elongated shoots were cultured onto half-strength MS medium augmented with various concentrations of two auxins (IBA and NAA) alone and/or in combination with 0.5 mg l^{-1} KIN for rooting. Of the two auxins examined, IBA was found to be the superior auxin for induction of maximum percent of rooting over NAA. However, the IBA and KIN combination was found to be best for root initiation compared to the NAA and KIN combination. Both the frequency of rooting and number of roots were found to be enhanced by addition of lower dose of KIN in the medium. In general, IBA was proved as the best auxin for rooting response in a wide range of plant species including *Melothria maderaspatana* (Baskaran et al. 2009) and *Benincasa hispida* (Thomas and Sreejesh 2004). It is interesting to note that IBA and KIN combination produced the best rooting response when compared to IBA alone. Therefore, IBA was found to be a potent auxin for rooting in cucumber followed by NAA in the present study. Similar observation was also reported in cultivated *Cucumis* species (Compton et al. 2001; Selvaraj et al. 2002). Well developed plantlets were successfully transferred into plastic cups containing soil and sand initially and they were subsequently established in the field. The field grown plants appeared to be phenotypically similar to the mother plant and noticed normal flowering as well as seed set.

In the present study, the genetic fidelity of the in vitro derived plantlets of cucumber was assessed through RAPD-PCR technology. DNA molecular markers are being considered to be the most effective tool to assess the variability found with the genomic sequence of the regenerated plants (Kumar et al. 2015). Earlier reports suggest that RAPD technology was effectively used to identify the somaclonal variations (Al-Zahin et al. 1999; DeVerno et al. 1999) and to identify the genetic fidelity of the tissue culture plants (Qin et al. 2006; Devarumath et al. 2007; Thiyagarajan and Venkatachalam 2012). Results show that a total of 100 random decamer primers were screened and only 3% polymorphism was recorded. The occurrence of polymorphism among the regenerated as well as mother plants of cucumber might manifest during cell division or differentiation under in vitro stress response imposed in the form of DNA methylation, chromosome rearrangements, point mutations (Phillips

et al. 1994). Similarly, low percent of DNA polymorphism (2%) was reported by analyzing the genetic stability of tissue culture plants of *Brassica oleracea* using RAPD-PCR (Qin et al. 2006). Recently, Kumar et al. (2015) reported plant regeneration in *Brassica oleracea* and noticed 10–12% polymorphism after genetic stability analysis of in vitro regenerated plantlets through RAPD analysis. It is reported that some percent of genetic variability might occur during in vitro cellular dedifferentiation and regeneration of plants (Kumar et al. 2015; Werner et al. 2015). The present results show that the plant regeneration from cotyledonary node explant is a highly reliable propagation procedure for multiplication of genetically identical plants (true-to-type). The low percent of genetic variability noticed could be due to the successive medium changes and the possibility of growth regulators causing variations or mutations (Werner et al. 2015). However, in the present study, about 97.1% of the DNA bands showed monomorphism for all the primers tested, describing genetic stability in the *Cucumis sativus* variety Green Long used for tissue culture. Earlier, RAPD-based DNA markers were successfully applied to prove the genetic fidelity of in vitro raised plants from different species including *Chlorophytum borivilianum* (Samantaray and Maiti 2010), *Drosera anglica* and *Drosera binata* (Kawiak and Lojkowska 2004), *Cuphea procumbens* (Fatima et al. 2012), and *Stevia rebaudiana* (Thiyagarajan and Venkatachalam 2012). In contrast, there was a genetic instability detected among the regenerated cucumber plants via somatic embryogenesis (Elmeer et al. 2009). In the present study, one of the reasons for the absence of genetic variability among the regenerated plants might be due to the development of adventitious shoot buds in which the callus phase was bypassed during plant regeneration.

Conclusion

In summary, an efficient plant regeneration protocol via direct adventitious shoot organogenesis from cotyledonary node explants of *Cucumis sativus* L. was established. Highest frequency of shoot bud development was noticed on MS medium containing 1.5 mg l^{-1} BAP. Maximum percent of shoot bud multiplication with highest number of shoots was obtained on MS medium supplemented with 1.5 mg l^{-1} BAP and 4.5 mg l^{-1} $AgNO_3$ combination. Results strongly suggest that silver nitrate showed positive effect on not only for enhancement of multiple shoot bud regeneration but also promoted rapid shoot bud elongation in cucumber. Further, the elongated shoots were rooted efficiently on half-strength MS medium containing IBA and KIN combination. The rooted plantlets were successfully established in the field conditions and the regenerated plantlets were found to be phenotypically similar to the control mother plant. DNA finger printing analysis indicated that the in vitro raised plants were found to be true to type. Therefore, the cotyledonary node explants could be successfully used for generation of transgenic cucumber plants in the future. To the best of our knowledge, this is the first report on high frequency of plant regeneration and assessment of clonal fidelity of in vitro regenerated plants from cotyledonary node explants of *Cucumis sativus* L.

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Authors contribution PV, PS Collected samples, conducted experiments; PV, UJ, PS, NG, SVS Analyzed the data and critically reviewed the manuscript, discussed the results and implications on the manuscript at all stages.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahmad A, Anis M (2005) *In vitro* mass propagation of *Cucumis sativus* L. from nodal segments. Turk J Bot 29:237–240

Al-Zahin MA, Ford-Llyod BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. Plant Cell Rep 18:473–477

Balkhande SV, Kure SR, Surwase BS (2013) Influence of silver nitrate on shoot regeneration from excised meristems of *Momordica cymbalaria* Hook.: a diminishing species. Res J Biotech 8:42–47

Baskaran P, Velayutham P, Jayabalan N (2009) *In vitro* regeneration of *Melothria maderaspatana* via indirect organogenesis. In Vitro Cell Dev Biol Plant 45:407–413

Compton ME, Pierson BL, Staub JK (2001) Micropropagation for recovery of *Cucumis hystrix*. Plant Cell Tissue Organ Cult 64:63–67

Devarumath RM, Doule RB, Kawar PG, Naikebawane SB, Nerkar YS (2007) Field performance and RAPD analysis to evaluate genetic fidelity of tissue culture raised plants of sugarcane. Sugar Tech 9:17–22

DeVerno LL, Park YS, Bonga JM, Barrett JD (1999) Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss]. Plant Cell Rep 18:948–953

Diao W, Jia Y, Hio S, Zhang X, Lou Q, Chen JF (2009) Efficient embryo induction in cucumber ovary culture and homozygous identification of the regenerants using SSR markers. Sci Hortic 119:246–251

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Elmeer KMS, Gallagher TF, Hennerty MJ (2009) RAPD-based detection of genomic instability in cucumber plants derived from somatic embryogenesis. Afr J Biotechnol 8:3219–3222

Fatima N, Ahmad N, Anis M (2012) *In vitro* propagation of *Cuphea procumbens* Orteg. and evaluation of genetic fidelity in plantlets using RAPD markers. J Plant Biochem Biotechnol 21:51–57

Gambley RL, Dodd WA (1990) An in vitro technique for the production de novo of multiple shoots in cotyledon explants of cucumber (*Cucumis sativus* L.). Plant Cell Tissue Organ Cult 20:177–183

Ganesan K, Huyop F (2010) *In vitro* regeneration of *Citrullus lanatus* cv. Round dragon. J Biol Sci 10:131–137
Giridhar P, Obul Reddy B, Ravishankar GA (2001) Silver nitrate influences in vitro shoot multiplication and root formation in *Vanilla planifolia* Andr. Curr Sci 81:1166–1170
Giridhar P, Indu E, Vijaya Ramu D, Ravishankar G (2003) Effect of silver nitrate on in vitro shoot growth of coffee. Trop Sci 43:144–146
Grozeva S, Velkov N (2014) *In vitro* plant regeneration of two cucumber (*Cucumis sativum* l.) genotypes: effects of explant types and culture medium. Genetika 46:485–493
Han JS, Oh DG, Mok IG, Park HG, Kim CK (2004) Efficient plant regeneration from cotyledon explants of bottle gourd (*Lagenaria siceraria* Standl.). Plant Cell Rep 23:291–296
Hussain Z, Tyagi RK, Sharma R, Agrawal A (2008) Genetic diversity in in vitro-conserved germplasm of *Curcuma* L. as revealed by RAPD markers. Biol Plant 52:627–633
Jesmin R, Mian MAK (2016) Callus induction and efficient plant regeneration in Cucumber (*Cucumis sativus* L.). J Biosci Agri Res 9:796–803
Kawiak A, Lojkowska E (2004) Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. In Vitro Cell Dev Biol Plant 40:592–595
Khan H, Siddique I, Anis M, Khan PR (2011) *In vitro* organogenesis from internode derived callus cultures of *Capsicum annuum* L. J Plant Biochem Biotech 20:84–89
Kontas J, Kintzios S (2003) Developing a scale up system for the micropropagation of cucumber (*Cucumis sativus* L.). The effect of growth retardants, liquid culture and vessel size. Plant Cell Rep 21:538–548
Kumar V, Parvatam G, Ravishankar GA (2009) $AgNO_3$—a potential regulator of ethylene activity and plant growth modulator. Electron J Biotechnol. https://doi.org/10.2225/vol12-issue2-fulltext-1
Kumar P, Gambhir G, Gaur A, Srivastava DK (2015) Molecular analysis of genetic stability in in vitro regenerated plants of broccoli (*Brassica oleracea* L. var. *italica*). Curr Sci 109:1470–1475
Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Rep 23:492–496
Mashayekhi K, Sharifani M, Shahsavand M, Kalati H (2008) Induction of somatic embryogenesis in absence of exogenous auxin in cucumber. Int J Plant Prod 2:163–166
Mohammadi J, Sivritepe N (2007) *In vitro* clonal propagation of *Cucumis sativus* L. by shoot tip culture. J Biol Sci 7:653–657
Mohiuddin AKM, Zaliha C, Abdullah M, Chowdhury KU, Napis S (2005) Enhancement of adventitious shoot regeneration in *Cucumis sativus* L. using $AgNO_3$. Plant Tissue Cult 15:15–23
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
Ntui VO, Thirukkumaran G, Lioka S, Mii M (2009) Efficient plant regeneration via organogenesis in Egusi Melon (*Colocynthis citrullus* L.). Sci Hort 119:397–402
Park EH, Bae H, Park WT, Kim YB, Chae SC, Park SU (2012) Improved shoot organogenesis of gloxinia (*Sinningia speciosa*) using silver nitrate and putrescine treatment. Plant Omics J 5:6–9
Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. Proc Natl Acad Sci USA 91:5222–5226
Pirinc V, Onay A, Yildirim H, Adiyaman F, Isikalan C, Basaran D (2003) Adventitious shoot organogenesis and plant regeneration from cotyledonasu of diploid diyarbakir watermelon (*Citrullus lanatus* cv. "Surme"). Turk J Biol 27:101–105
Prem Kumar G, Sivakumar S, Siva G, Vigneswaran M, Senthil Kumar T, Jayabalan N (2016) Silver nitrate promotes high-frequency multiple shoot regeneration in cotton (*Gossypium hirsutum* L.) by inhibiting ethylene production and phenolic secretion. In Vitro Cell Dev Biol Plant 52:408–418
Qin Y, Li HL, Guo YD (2006) High frequency embryogenesis, regeneration of broccoli (*Brassica oleracea* var. *italica*) and analysis of genetic stability by RAPD. Sci Hort 111:203–208
Rathore MS, Mastan SG, Yadav P, Bhatt VD, Shekhawat NS, Chikara J (2016) Shoot regeneration from leaf explants of *Withania coagulans* (stocks) Dunal and genetic stability evaluation of regenerates with RAPD and ISSR markers. South Afr J Bot 102:12–17
Saini RK, Shetty NP, Giridhar P, Ravishankar GA (2012) Rapid in vitro regeneration method for *Moringa oleifera* and performance evaluation of field grown nutritionally enriched tissue cultured plants. 3 Biotech 2:187–192
Samantaray S, Maiti S (2010) An assessment of genetic fidelity of micropropagated plants of *Chlorophytum borivilianum* Santpau and Fernandes using random amplified polymorphic DNA (RAPD) markers. Biol Plant 54:334–338
Selvaraj N, Vasudevan A, Prem Anand R, Ramesh Anbazhgan V, Ganapathi A (2002) Micropropagation of *Cucumis sativus* L. from field grown plants. In: Proceedings of *Cucurbitaceae* conference. Naples, pp 149–156
Selvaraj N, Vasudevan A, Manickavasagam V, Ganapathi A (2006) *In vitro* organogenesis and plant formation in cucumber. Biol Plant 50:123–126
Selvaraj N, Vasudevan A, Manickavasagam M, Kasthurirengan S, Ganapathi A (2007) High frequency shoot regeneration from cotyledon explants of cucumber via organogenesis. Sci Hort 112:2–8
Singh BP, Dubey KK, Singh RP (2007) *In vitro* direct regeneration of multiple shoots from cotyledon explants of *Cucumis sativus* L. Plant Arch 7:103–107
Siva G, Sivakumar S, Prem Kumar G, Vigneswaran M, Vinoth S, Arunachalam S, Elango B, Senthil Kumar T, Jayabalan N (2015) Multiple shoot production from nodal explants and FTIR analysis of in vitro regenerated plants of *Psoralea corylifolia* L. Pharm Biol Eval 2:105–109
Sridevi V, Giridhar P (2014) Establishment of somaclonal variants of *Robusta coffee* with reduced levels of cafestol and kahweol. In Vitro Cell Dev Biol Plant 50:618–626
Thiyagarajan M, Venkatachalam P (2012) Evaluation of the genetic fidelity of in vitro propagated natural sweetener plant (*Stevia rebaudiana* Bert.) using DNA-based markers. Plant Cell Biotech Mol Biol 13:99–104
Thomas TD, Sreejesh KR (2004) Callus induction and plant regeneration from cotyledonary explants of ash gourd (*Benincasa hispida* L.). Sci Hort 100:359–367
Ugandhar T, Venkateshwarrlu M, Begum G, Srilatha T, Jaganmohanreddy K (2011) *In vitro* plantregeneration of Cucumber (*Cucumis sativum* L.) from cotyledon and hypocotyl explants. Sci Res Rep 1:164–169
Vasudevan A, Selvaraj N, Sureshkumar P, Ganapathi A (2001) Multiple shoot induction from the shoot tip explants of cucumber (*Cucumis sativus* L.). Cucurbit Genet Cooperative Rep 24:8–12
Vasudevan A, Selvaraj N, Ganapathi A, Kasthurirengan S, Ramesh Anbazhagan V, Manickavasagam M (2004) Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. Biol Plant 48:1215–1218
Vasudevan A, Selvaraj N, Ganapathi A, Choi CW, Manickavasagam M, Kasthurirengan S (2007) Direct plant regeneration from cucumber embryonal axis. Biol Plant 51:521–524
Vengadesan G, Selvaraj N, Prem Anand R, Gaba V, Ganapathi A (2005) Ontogeny of somatic embryos in Cucumber (*Cucumis sativus* L.). In Vitro Cell Dev Biol Plant 41:789–793
Venkatachalam P, Sangeetha P, Geetha N, Sahi SV (2015) Phytofabrication of bioactive molecules encapsulated metallic silver nanoparticles from *Cucumis sativus* L. and its enhanced wound healing potential in rat model. J Nanomater. https://doi.org/10.1155/2015/753193

Walden R, Wingender R (1995) Gene-transfer and plant-an easy-going general review. Regeneration techniques. Trends Biotechnol 13:324–331

Wang SL, Seong SK, Ye XG, He CF, Suk YK, Pil SC (2015) Current status of genetic transformation technology developed in cucumber (*Cucumis sativus* L.). J Integrat Agric 14:469–482

Werner ET, Soares TCB, Gontijo ABPL, Souza Neto JD, do Amaral JAT (2015) Genetic stability of micropropagated plants of *Crambe abyssinica* Hochst using ISSR markers. Genet Mol Res 14:16450–16460

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate in vitro. Plant Cell Tiss Org Cult 67:45–54