An efficient protocol for in vitro direct shoot organogenesis of *Sesamum indicum* L. using cotyledon as explant

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Abstract

Establishment of a suitable regeneration protocol is a pre-requisite to carry out transformation study in *Sesamum indicum* L. (sesame). In this paper, different parameters of regeneration were standardised to develop an efficient protocol for in vitro plant regeneration via direct adventitious shoot organogenesis using de-embryonated cotyledons of sesame as explants. Among the various treatments of MS medium supplemented with 6-benzylaminopurine, thidiazuron and indole-3-acetic acid, maximum regeneration frequency ($25.93 \pm 2.21\%$) was obtained in BTI 4 medium (MS supplemented with $33.33 \mu\text{M}$ BAP with $2.85 \mu\text{M}$ IAA) within 6 weeks of culture. Regeneration frequency increased further ($50.37 \pm 2.49\%$) by fortifying BTI 4 with $29.43 \mu\text{M}$ silver nitrate (AG 3 medium). Pre-culture of cotyledon explants in AB 3 medium (AG 3 supplemented with $3.78 \mu\text{M}$ abscisic acid) for 14 days followed by sub-culture in AG 3 medium further improved the regeneration frequency ($68.15 \pm 2.68\%$). The highest rate of shoot regeneration ($94.82 \pm 1.34\%$) was obtained by pre-culturing 4-day-old cotyledon in a vertical position in AB 3 medium for 14 days and sub-culturing in AG 3 medium for 4 weeks. Regenerated shoots proliferated in MS medium supplemented with $4.44 \mu\text{M}$ BAP and $1.44 \mu\text{M}$ gibberelic acid (GA_3). The highest frequency ($65.33 \pm 3.78\%$) of root induction was achieved by culturing the elongated shoots in MS medium supplemented with $2.69 \mu\text{M}$ α -naphthalene acetic acid (NAA) for 6 weeks. Rooted plants were acclimatised in soilrite and transferred to soil after 6–8 weeks. The rate of acclimatisation of plants was 76%.

Keywords Adventitious shoot · Direct organogenesis · Explant orientation · Regeneration · Scanning electron microscopy · Sesame

Abbreviations

ABA	Absciscic acid
AgNO_3	Silver nitrate
BAP	6-Benzylaminopurine
DMSO	Dimethyl sulfoxide
GA_3	Gibberelic acid

GUS	β -glucuronidase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and skoog
NAA	1-Naphthalene acetic acid
PGR	Plant growth regulator
SAAT	Sonication-assisted <i>Agrobacterium</i> -mediated transformation
SAM	Shoot apical meristem
SE	Standard error
SEM	Scanning electron microscopy
TDZ	1-Phenyl-3-(1, 2, 3-Thidiazol-5-yl)-urea (Thidiazuron)

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Introduction

Establishment of an efficient regeneration protocol is a pre-requisite for crop improvement by genetic transformation. *Sesamum indicum* L. (sesame) is difficult to regenerate

(Were et al. 2006). Various efforts have been made over the past two decades for in vitro regeneration in sesame. Regeneration of *S. indicum* has been achieved from hypocotyl and cotyledon (Taskin and Turgut 1997; Were et al. 2006; Seo et al. 2007; Yadav et al. 2010; Al-Shafeay et al. 2011) by direct organogenesis and from hypocotyl (Kwon et al. 1993; Wadeyar and Lokesh 2011) and leaf-derived callus (Raja and Jayabalan 2010) by indirect organogenesis. Somatic embryogenesis has also been reported from cotyledon, and hypocotyls-derived callus (Ram et al. 1990; Xu et al. 1997; Baskaran and Jayabalan 2006). Micropropagation has been achieved from shoot tip and nodal culture (Geroge et al. 1987; Gangopadhyay et al. 1998; Abdellatif et al. 2010). The result of plant regeneration in the available literature varies from 4.5 to 88%, and this is because in vitro regeneration of sesame is highly genotype dependent (Taskin and Turgut 1997; Yadav et al. 2010).

Previously, we have standardised an efficient protocol of gene transfer in sesame using a relatively less known technique named “sonication-assisted *Agrobacterium*-mediated transformation” (SAAT) (Debnath et al. 2014). The optimised protocol facilitated $85.56 \pm 0.35\%$ transient GUS activity in sesame cotyledon. We failed to obtain any stable transformed plantlet due to the lack of efficient regeneration protocol for the cultivar used by us. Therefore, it was necessary to design a suitable regeneration protocol of sesame which will help us to obtain a reasonable frequency of stable transformed plantlet shortly.

In this report, in vitro regeneration from cotyledon explants of sesame was studied. Different combinations of plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP), 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea (thidiazuron) (TDZ), abscisic acid (ABA), gibberellic acid (GA_3), 1-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) were used to optimize in vitro regeneration via adventitious shoot organogenesis, shoot and root elongation protocol. Regeneration frequency was checked in different genotypes of sesame to prove the efficiency of this protocol. Scanning electron microscopic study was used to show the organogenic nature of sesame regeneration in this protocol.

Materials and methods

Preparation of culture media and stock solutions of PGRs and silver nitrate

All the experiments were carried out using full-strength basal MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose (w/v) and 0.8% agar (w/v) except for seed germination, where $\frac{1}{2}$ MS medium (half-strength MS salts, 1.5% w/v sucrose and 0.6% w/v agar) was used.

MS medium, sucrose, and agar were purchased from HiMedia (Mumbai, India). Media were sterilised by autoclaving at 121 °C temperature with 15 psi pressure for 15 min. In all the media, pH was adjusted before autoclaving at 5.8 by the suitable addition of 1 N HCl and 1 N KOH solutions. To make stock solutions of BAP, NAA, and IBA, 1 N NaOH was used as a solvent. Stock solutions of IAA and GA_3 were prepared using absolute ethanol (Merck, Germany) as their solvent. DMSO (Sigma-Aldrich, USA) was used as a solvent to make the stock solution of TDZ. The concentration of all the stock solution of PGRs (Sigma-Aldrich, USA) was maintained at 1 mg/ml. Silver nitrate ($AgNO_3$) (HiMedia, India) was dissolved in double distilled water to make its stock solution having the concentration of 1 μ M. All the prepared stock solutions were sterilised by filtration and stored at 4 °C in an amber bottle. PGRs were added to media before autoclaving. After autoclaving, the media were cooled to around 45 °C to add $AgNO_3$ before plating.

Plant material and seed culture

The seeds of *Sesamum indicum* L. cv. *JK-1* were collected from National Bureau of Plant Genetic Resources, Pusa, New Delhi, India. Healthy and uniform seeds were washed with 50% commercial bleach (RIN ALA, Hindustan Unilever Limited, India) containing 4% chlorine (v/v) for 5 min. The cleaned seeds were surface-sterilised with 0.1% (w/v) aqueous solution of mercuric chloride for 2.5 min followed by 70% ethanol for 10 s. Surface-sterilized seeds were subsequently washed with sterile distilled water for four times, transferred to Petri dishes having 25 ml sterilised $\frac{1}{2}$ MS medium, and incubated at 28 ± 2 °C in the dark.

Preparation of explants, experiment designing, culture condition, and data-recording method

For all the regeneration experiments, 4–6-day-old de-embryonated cotyledons from germinated seedlings were used as explants (Fig. 1a). Fifteen explants were inoculated in 90×15 mm Petri dish containing 30 ml sterile medium. For each treatment, 45 explants were inoculated.

In the experiments for shoot elongation and root induction, inoculation of explants was carried out in Planton boxes (Tarson, India), each having 40 ml of sterile medium. A total of 25 clumps of regenerated shoot buds were excised from the regenerating explants with a sterile scalpel for each treatment of shoot elongation experiment. Five such clumps were inoculated in shoot elongation medium in each Planton box. For each treatment of root induction experiment, a total of 25 elongated shoot explants were cultured, five explants per Planton box in root induction medium.

All the cultures were incubated in the culture room having the temperature of 25 ± 2 °C and relative humidity of

Fig. 1 Different stages of plant regeneration of sesame via direct organogenesis of *S. indicum*. **a** Embryonated (left) and de-embryonated (right) cotyledons were used as explants; **b** vertical and **c** horizontal orientation of placement of cotyledon explants in medium; **d** appearance of callus-like growth from the proximal end of cotyledon after 9 days of culture; **e** initiation of adventitious shoot from callus-like growth after 12 days of culture; **f** fully regenerated shoot of

sesame after 35 days of culture; **g** a regeneration plate showing the regeneration from every cotyledon explant of sesame after 42 days of culture; **h** root induction from a regenerated shoot after 6 weeks of culture in rooting medium; **i** acclimatisation of well-rooted plantlet in soilrite. Picture was taken after 30 day post soilrite transfer; **j** regenerated plant in field condition. Picture was taken after 110 day post field transfer

60–65% under 16/8 photoperiod having an irradiance of $35 \mu\text{mol}/\text{m}^2/\text{s}$ photosynthetic photon flux density using daylight fluorescent tube lights (Philips Champion, India). Culture media were refreshed in every 2 weeks. Plant regeneration and root induction data were recorded after 6 weeks, and shoot elongation data were recorded after 2 weeks. In regeneration experiments, the frequency of regeneration was calculated by the percent of explants showing regeneration response among the total number of explants cultured. Besides that, the number of shoots per regenerating explants was also estimated. In shoot elongation experiment, the final

length of the elongated shoots was recorded. In root induction experiment, root induction frequency was calculated by the percentage of shoot having induced root among the total number of the cultured shoots. Besides that, the number of roots per explants and length of induced root was also calculated. For statistical analysis, data from three replicates were used for each treatment.

Basic in vitro adventitious shoot regeneration study by BAP, TDZ, and IAA

The media used for regeneration were MS supplemented with BAP of 11.11, 22.22, and 33.33 μM , TDZ of 10, 20, and 30 μM , and IAA of 2.85, 5.71, and 8.56 μM concentrations in all possible combinations of BAP or TDZ with IAA. MS without any PGR supplement was used as a control in this experiment. These media were designated as BTI 1–BTI 19.

Effect of AgNO_3 on in vitro adventitious shoot regeneration

In this experiment, cotyledon explants of sesame were cultured in the optimised BTA medium which consisted basically of the BTI medium showing highest regeneration supplemented with various AgNO_3 concentrations such as 0, 14.72, 29.43, 44.15, and 58.87 μM . Silver nitrate supplemented BTI media were designated as AG 1–AG 5.

Effect of ABA on in vitro adventitious shoot regeneration

For this experiment, the cotyledon explants of sesame were cultured in the optimised AG medium supplemented with ABA in the concentrations of 0, 1.89, 3.78, and 5.66 μM . ABA-supplemented AG media were designated as AB 1–AB 4 in this experiment. Culturing cotyledon explants in AB media are identified as “pre-culture” hereafter throughout this report. After 7 days of pre-culture, explants from every treatment medium were sub-cultured in fresh optimised AG medium for another 5 week.

Effect of sucrose concentration on in vitro adventitious shoot regeneration

In this experiment, cotyledon explants were cultured independently on optimised AG medium supplemented with 3–9% serially for 7 days. Subsequently, explants from every sucrose treatment were sub-cultured in fresh-optimised AG medium and cultured for another 5 weeks there.

Effect of duration of pre-culture on in vitro adventitious shoot regeneration

The effect of pre-culture duration was observed by pre-culturing the cotyledon explants in the optimised AB medium for 7, 14, or 21 days before sub-culturing in the optimised AG medium. The total culture duration in AB and AG media was maintained for 6 weeks.

Effect of explant orientation and age on in vitro adventitious shoot regeneration

The effect of explant orientation during initial explant inoculation was evaluated by culturing cotyledon explants either vertically dipping the cut proximal end in the medium (Fig. 1b) or horizontally on the surface of the optimised AB medium (Fig. 1c). The effect of explant age was assessed by culturing 4-, 5-, and 6-day-old cotyledon explants in the optimised AB medium. Every possible combinations of explant age with explant orientation were used to study the cumulative effect of these two parameters. Explants from each treatment were sub-cultured in optimised AG medium after pre-culturing in optimised AB medium for the optimal duration.

Shoot elongation

For elongation of regenerated shoot buds, MS medium was supplemented with the combination of BAP of either 2.22 or 4.44 μM and GA_3 of either 0.58 or 1.44 μM concentration.

Root induction

The suitable PGR and its optimum concentration for root induction was evaluated by culturing elongated shoots in basal MS medium fortified with one of the following PGRs independently—NAA of either 0.27 or 2.69 μM , IBA of either 0.25 or 2.46 μM , and IAA of either 0.285 or 2.85 μM .

Acclimatisation of rooted plantlet

Well-rooted plantlets were transferred to autoclaved soil-rite (a commercially available mixture of perlite, Irish peat moss, and vermiculite) (Keltech Energies Ltd., India) in small plastic glasses. Glasses were covered with transparent plastic to maintain high humidity (90–95%) for 2 weeks, and after that, covers were opened occasionally to acclimatise the plantlets in low humid condition. Plantlets were occasionally watered. Acclimatised plantlets were kept in the culture room for 6–8 weeks after that they were transferred to field.

Performance study of the optimised regeneration protocol in other cultivars of sesame

The performance of our optimised protocol was monitored in other cultivars (*JT-7*, *PADMA*, *RT-103*, *PHULE TIL-1*, and *TAPI-A*) of sesame to assess the genotype/cultivar dependency of sesame regeneration. Seeds of these cultivars were collected from National Bureau of Plant Genetic Resources, Pusa, New Delhi, India. De-embryonated cotyledon explants from the germinated seedlings of each cultivar were cultured

in the protocol optimised for regeneration in this study. The observed regeneration frequencies of each cultivar of sesame were compared.

Scanning electron microscopy

To assess the nature of regeneration of sesame in the optimised protocol, samples were intended for scanning electron microscopy (SEM). Collected samples were fixed in FAA (10 ml formalin, 5 ml glacial acetic acid, 50 ml 95% ethanol, and 35 ml double distilled water) and dehydrated in series of alcohol gradient. Dehydrated samples were then subjected to critical point drying in CO₂ (E3100 Critical Point Dryer, Quorum Technologies, UK), fixed to metallic stub using double-sided adhesive tape, and sputter coated (S150 Sputter coater, Edwards, UK) with colloidal gold for 120 s at 10⁻¹ torr vacuum and 20 mA current. Processed samples were observed at FEI Quanta 200 (USA) scanning electron microscope at 10 kV.

Statistical analysis

Experiments were performed three times for all the treatments. Data were analysed by one-way ANOVA (Analysis Of Variance) to calculate statistical significance. The significant difference of treatment data (mean $\pm$ standard error) was assessed through Duncan's multiple range test

at $p < 0.05$ level (Duncan 1955). Both of the analyses were done in SPSS version 19 (IBM Corporation, USA). Graphs were prepared in GraphPad Prism version 6.01 (GraphPad Software, Inc., USA).

Results

Effect of BAP, TDZ, and IAA on in vitro shoot regeneration

In this experiment, the appearance of dark green coloured swollen growth was observed at the cut site of the proximal end of the cultured cotyledon within 7 days irrespective of PGR combination used in the culture media. Visible appearance of shoot bud could be observed after 5–12 days of culture in all media. After 6 weeks of culture, maximum regeneration frequency of $25.93 \pm 2.21\%$ was found in the BTI 4 medium (MS supplemented with $33.33 \mu\text{M}$ BAP and $2.85 \mu\text{M}$ IAA) followed by $20.74 \pm 1.88\%$ of regeneration in BTI 19 medium (MS supplemented with $30 \mu\text{M}$ TDZ and $8.56 \mu\text{M}$ IAA). The number of shoots per explant was also the highest in BTI 4 medium (2.96 ± 0.16) (Table 1).

Table 1 Effect of BAP, TDZ and IAA on in vitro shoot regeneration of *S. indicum*

Name of medium	Amount of PGRs (μM)			Regeneration frequency $\pm$ SE (%)	Number of shoots per explant $\pm$ SE
	BAP	TDZ	IAA		
BTI 1	0	0	0	0 ± 0 d	0 ± 0 d
BTI 2	11.11	0	2.85	14.81 ± 0.83 bc	2.34 ± 0.06 a
BTI 3	22.22	0	2.85	22.96 ± 1.99 abc	1.45 ± 0.22 ab
BTI 4	33.33	0	2.85	25.93 ± 2.21 a	2.96 ± 0.16 a
BTI 5	11.11	0	5.71	21.48 ± 1.49 bc	2.50 ± 0.14 a
BTI 6	22.22	0	5.71	14.07 ± 1.38 abc	2.85 ± 0.1 ab
BTI 7	33.33	0	5.71	19.26 ± 0.83 ab	1.87 ± 0.24 c
BTI 8	11.11	0	8.56	16.30 ± 1.49 c	2.16 ± 0.43 c
BTI 9	22.22	0	8.56	23.70 ± 3.01 bc	1.13 ± 0.04 cd
BTI 10	33.33	0	8.56	15.56 ± 1.44 abc	2.33 ± 0.22 bc
BTI 11	0	10	2.85	9.63 ± 0.38 cd	1.11 ± 0.09 b
BTI 12	0	20	2.85	11.85 ± 0.51 abc	1.13 ± 0.05 b
BTI 13	0	30	2.85	13.33 ± 2.02 ab	1.28 ± 0.1 a
BTI 14	0	10	5.71	17.78 ± 1.19 bc	1.06 ± 0.04 a
BTI 15	0	20	5.71	20.01 ± 1.19 abc	1.03 ± 0.01 b
BTI 16	0	30	5.71	18.52 ± 2.51 ab	1.22 ± 0.11 b
BTI 17	0	10	8.56	12.59 ± 0.69 abc	1.11 ± 0.04 b
BTI 18	0	20	8.56	17.04 ± 1.38 abc	1.03 ± 0.02 b
BTI 19	0	30	8.56	20.74 ± 1.88 a	1.25 ± 0.12 b

Data represent mean $\pm$ standard errors ($n = 45$). Different letters followed by the means represent statistical difference at $p < 0.05$

Effect of AgNO₃ on in vitro shoot regeneration

Regeneration frequency of sesame increased ($50.37 \pm 2.49\%$) in AG 3 medium, where BTI 4 medium was supplemented with AgNO₃ at the concentration of 29.43 μM (Fig. 2). The frequency of regeneration decreased when BTI 4 medium was supplemented with higher concentration of AgNO₃ beyond 29.43 μM as in AG 4 ($45.19 \pm 3.32\%$) and AG 5 ($27.41 \pm 2.53\%$).

Effect of ABA on in vitro shoot regeneration

Regeneration frequency increased when cotyledon explants were pre-cultured in AG 3 medium supplemented with various concentrations of ABA (AB medium) for 7 days and

Fig. 2 Effect of AgNO₃ on the rate of in vitro regeneration from cotyledon explants of *S. indicum* in the AgNO₃ supplemented BTA 4 medium. Data were recorded after 6 weeks of culture. Data represent mean $\pm$ standard errors ($n=45$). Different letters represent statistical difference at $p < 0.05$

Table 2 Effect of ABA on in vitro shoot organogenesis of *S. indicum*

Name of medium	Amount of PGRs (μM)				Regeneration frequency $\pm$ SE (%)	Number of shoots per explant $\pm$ SE
	BAP	IAA	AgNO ₃	ABA		
AB 1	33.33	2.85	29.43	0	48.89 ± 2.98 a	3.16 ± 0.21 b
AB 2	33.33	2.85	29.43	1.89	54.07 ± 1.99 a	3.43 ± 0.27 a
AB 3	33.33	2.85	29.43	3.78	68.15 ± 2.68 a	3.61 ± 0.26 a
AB 4	33.33	2.85	29.43	5.66	62.96 ± 2.76 a	3.20 ± 0.07 b

Data represent mean $\pm$ standard errors ($n=45$). Different letters followed by the means represent statistical difference at $p < 0.05$

subsequently sub-cultured in AG 3 medium devoid of ABA. Regeneration frequency was the highest ($68.15 \pm 2.68\%$) when cotyledon explants were pre-cultured in AB 3 (AG 3 medium supplemented with 3.78 μM ABA) (Table 2).

Effect of sucrose concentration on in vitro shoot regeneration

The cotyledon explants were initially cultured in AG 3 medium supplemented with various concentrations of sucrose for 7 days and were sub-cultured in AG 3 that was supplemented with 3% sucrose. The in vitro regeneration frequency of cotyledon explants was marginally better when they were initially cultured on AG 3 medium supplemented with of 4% sucrose ($54.81 \pm 2.82\%$) than 3% sucrose ($49.63 \pm 2.66\%$) (Fig. 3). An increase of sucrose concentration beyond 4% in the initial medium resulted in the decrease of regeneration frequency in the AG 3 medium. Majority of the explants showed necrosis in AG 3 medium when they were initially cultured in AG 3 medium supplemented with 7, 8, and 9% of sucrose concentration independently.

Effect of duration of pre-culture on in vitro shoot regeneration

In the previous experiment to observe the impact of ABA pre-culture, cotyledon explants were pre-cultured in AB 3 medium for 7 days and sub-cultured in ABA-minus AG 3 medium. In this analysis, the frequency of shoot regeneration from the cotyledon explants increased to $80.74 \pm 2.21\%$ when explants were pre-cultured for 14-days instead of 7 or 21 days in AB 3 medium. Mean shoot numbers in regenerated explants also increased (4.18 ± 0.17) (Table 3).

Effect of explant orientation and age on in vitro shoot regeneration

The cotyledon explants were pre-cultured in AB 3 medium for 14 days and were sub-cultured in AG 3 medium. Regeneration was favourable exerting higher frequency and number of shoots per explants for the 4- and 5-day-old cotyledons when cultured in the vertical orientation

Fig. 3 Effect of sucrose on the rate of in vitro regeneration from cotyledon explants of *S. indicum*. Cotyledons of sesame were pre-cultured in AG 3 medium supplemented with various concentrations of sucrose. Explants from all the treatments were sub-cultured in fresh AG 3 medium having 3% sucrose concentration. Data were recorded after 6 weeks of culture. Data represent mean $\pm$ standard errors ($n=45$). Different letters represent statistical difference at $p < 0.05$

Table 3 Effect of duration of pre-culture in AB 3 medium studied in rest of the optimised shoot regeneration protocol on in vitro shoot organogenesis of *S. indicum*

Sub-culture interval (days)	Regeneration frequency $\pm$ SE (%)	Number of shoots per explants $\pm$ SE
7	69.63 $\pm$ 1.67 b	3.58 $\pm$ 0.24 b
14	80.74 $\pm$ 2.21 a	4.18 $\pm$ 0.17 a
21	73.33 $\pm$ 2.50 b	2.25 $\pm$ 0.04 c

Data represent mean $\pm$ standard errors ($n=45$). Different letters followed by the means represent statistical difference at $p < 0.05$

instead of horizontal orientation. In the vertically cultured 4-day explants, $94.82 \pm 1.34\%$ of regeneration frequency was observed having 5.4 ± 0.04 shoots per explants; both were highest in this experiment among the other treatments (Table 4). However, in the 6-day-old cotyledon explants, the regeneration frequency ($71.85 \pm 2.76\%$) was lower and the number of regenerated shoots per explants ($4.44 \pm 0.20\%$) was higher when they were cultured vertically instead of horizontally.

Shoot elongation

Among the tested elongation media, the highest length of elongation (3.32 ± 0.37 cm) was obtained when the regenerated shoot buds were cultured in the basal MS medium supplemented with $4.44 \mu\text{M}$ of BAP and $1.44 \mu\text{M}$ of GA_3 (Table 5).

Table 4 Effect of explant orientation and explant age on in vitro shoot organogenesis of *S. indicum* studied in optimised shoot regeneration protocol

Orientation of explant	Explant age (days)	Regeneration frequency $\pm$ SE (%)	Number of shoots per explant $\pm$ SE
Vertical	4	94.81 $\pm$ 1.34 a	5.40 $\pm$ 0.04 a
	5	86.67 $\pm$ 1.84 a	4.86 $\pm$ 0.30 b
	6	71.85 $\pm$ 2.76 b	4.44 $\pm$ 0.20 c
Horizontal	4	81.48 $\pm$ 2.44 a	4.01 $\pm$ 0.17 a
	5	78.51 $\pm$ 3.15 a	3.30 $\pm$ 0.22 c
	6	76.30 $\pm$ 3.08 a	3.24 $\pm$ 0.27 b

Data represent mean $\pm$ standard errors ($n=45$). Different letters followed by the means represent statistical difference at $p < 0.05$

Table 5 Effect of different PGR combinations on the elongation of regenerated shoot buds of *S. indicum*

Amount of PGRs (μM)		Shoot length $\pm$ SE (cm)
BAP	GA_3	
0	0	0.49 $\pm$ 0.03 b
2.22	1.44	1.99 $\pm$ 0.29 ab
2.22	0.58	0.98 $\pm$ 0.05 ab
4.44	1.44	3.32 $\pm$ 0.37 a
4.44	0.58	2.47 $\pm$ 0.42 ab

Data represent mean $\pm$ standard errors ($n=25$). Different letters followed by the means represent statistical difference at $p < 0.05$

Root induction and plant acclimatisation

Root induction from the elongated explants of sesame was the best in MS medium supplemented with $2.69 \mu\text{M}$ of NAA (Fig. 1h). In this medium root induction frequency, the number of root per explant and root length was $65.33 \pm 3.78\%$, 5.97 ± 0.34 and 7.06 ± 0.13 cm, respectively, which was the highest among the tested media (Table 6). Application of different PGR other than NAA had no positive effect on root induction frequency, the number of root per explants, and the root length. Plants of 3–4 cm length and with well-developed root system were transferred to soilrite (Fig. 1i). The survival rate of plantlets in soilrite was 76%. Acclimatised plants of 15–20 cm length were transferred to soil (Fig. 1j).

Optimised medium and cultural condition for in vitro shoot regeneration

The optimised regeneration protocol mentioned above can be summarised as follows. De-embryonated 4-day-old sesame cotyledon explants were cultured in the AB 3 medium (MS medium supplemented with $33.33 \mu\text{M}$ BAP, $2.85 \mu\text{M}$ IAA, $3.78 \mu\text{M}$ ABA, and $29.43 \mu\text{M}$ AgNO_3). During inoculation of the medium, proximal cut end of the cotyledon explants was dipped into the culture medium, and they were kept vertical to the surface of the medium. Proximal cut ends of cotyledons swelled, and callus-like growth was visible within 4–9 days of culture in AB 3 medium (Fig. 1d). Visible shoot buds appeared with 5–12 days in the same medium (Fig. 1e). Explants were cultured in AB 3 medium for 14 days and were sub-cultured in AG 3 medium subsequently (MS medium supplemented with $33.33 \mu\text{M}$ BAP, $2.85 \mu\text{M}$ IAA, and $29.43 \mu\text{M}$ AgNO_3). After a total of 6 weeks of culture in AB 3 and subsequent AG 3 medium, regenerated healthy shoots of 1–2 cm length were recovered (Fig. 1f, g) and were elongated in MS medium supplemented with $4.44 \mu\text{M}$ of BAP + and $1.44 \mu\text{M}$ of GA_3 . Roots were induced from the elongated shoots in MS medium supplemented with $2.69 \mu\text{M}$ of NAA (Fig. 1h). Rooted plantlets

were acclimatised in soilrite in culture room (Fig. 1i) and were subsequently transferred to the field (Fig. 1j). The whole process has been summarised in Fig. 4 in the form of a flowchart.

Fig. 4 Flow diagram representing the protocol of regeneration and plant acclimatisation of *S. indicum*

Table 6 Effect of different PGRs on root induction of the in vitro regenerated shoots of *S. indicum*

PGRs	Concentration (μM)	Root induction frequency $\pm$ SE (%)	Average number of roots per explant $\pm$ SE	Average root length $\pm$ SE (cm)
—	—	9.33 ± 1.85 b	0.80 ± 0.23 g	0.55 ± 0.35 f
NAA	2.69	66.67 ± 4.69 a	5.85 ± 0.16 a	7.35 ± 0.18 a
NAA	0.27	41.33 ± 2.31 ab	1.25 ± 0.05 f	2.47 ± 0.33 c
IBA	2.46	60.00 ± 3.20 ab	2.71 ± 0.12 c	1.08 ± 0.12 e
IBA	0.25	38.67 ± 3.61 ab	2.54 ± 0.18 d	0.67 ± 0.08 e
IAA	2.85	50.67 ± 1.85 ab	3.24 ± 0.17 b	2.22 ± 0.24 d
IAA	0.29	37.33 ± 3.95 b	1.56 ± 0.31 e	2.10 ± 0.38 b

Data represent mean $\pm$ standard errors ($n=25$). Different letters followed by the means represent statistical difference at $p<0.05$

Table 7 Effect of different genotype on in vitro shoot regeneration in the protocol optimised for *S. indicum*

Genotype	Regeneration frequency $\pm$ SE (%)	Number of shoots per explant $\pm$ SE
JK-1	94.07 $\pm$ 1.01 a	5.32 $\pm$ 0.05 a
JT-7	82.96 $\pm$ 1.49 ab	4.19 $\pm$ 0.10b
PADMA	57.78 $\pm$ 1.33 d	3.20 $\pm$ 0.05 f
RT-103	77.04 $\pm$ 1.25 abc	4.63 $\pm$ 0.08 c
PHULE TIL-1	68.89 $\pm$ 1.44 bcd	3.68 $\pm$ 0.04 d
TAPI-A	60.74 $\pm$ 1.38 cd	3.88 $\pm$ 0.05 e

Data represent mean $\pm$ standard errors ($n=45$). Different letters followed by the means represent statistical difference at $p < 0.05$

Effect of genotype on in vitro shoot regeneration

The efficacy of in vitro regeneration of cotyledon explants was found to be genotype-dependant. The frequency of regeneration was found to be lowest for the *PADMA* variety ($57.78 \pm 1.33\%$) and highest for the *JK-1* variety ($94.07 \pm 1.01\%$) of sesame (Table 7).

Scanning electron microscopy

Organised cell growth was initiated at the proximal end of cotyledons within the 5 days of culture (Fig. 5a, b). Callus-like organised cell mass lined up the proximal cut end within 7 days of culture (Fig 5c, d). After 10 days of culture, a newly formed small and iso-diametric group of cells developed one or several ‘meristemoid’ lump-like structure(s) near the organised cell growth at the proximal cut end of

Fig. 5 Scanning electron microscopic study of regeneration from cotyledon explants of *S. indicum*. **a** and **b** Initiation of organised cell growth (arrowheads in figure b) at the proximal end of cotyledon within 5 days of culture, figure b is the magnified view of the boxed region of figure a; **c** and **d** appearance of callus-like growth (arrowheads in figure d) at the proximal end of cotyledon within 7 days of culture, figure d is the magnified SEM view of the boxed region of figure c, explants of figure c and d are different but having similar physiologic condition; **e** appearance of one lump-like ‘meristemoid’ (arrowhead) within 10 days of culture; **f** appearance of the SAM after

15 days of culture; **g** magnified view of figure e showing SAM and leaf primordia; **h** SAM after 19 days of culture; **i** and **j** visual appearance of SAM in a cotyledon cultured for 15 days (arrowheads); **k** magnified view of the boxed region of figure j showing a cluster of SAM among which one dominated; **l** a view of cotyledon explant cultured for 29 days giving rise to a structure similar to figure k where a bunch of SAM appeared having a dominating SAM. *M* meristemoid, *PE* proximal end, *LP* leaf primordia, *SAM* shoot apical meristem, *T* trichome

cotyledon (Fig. 5e). Initially, one shoot apical meristem (SAM) appeared from one of the ‘meristemoids’ within 15 days of culture (Fig. 5f, g, h and i). Afterwards, this initial SAM was surrounded by the occurrence of several ‘meristemoids’ that would give rise to many SAM at the regeneration site (Fig. 5j, k). This structure gave rise to a cluster of regenerated plantlets having a larger shoot (Fig. 5l). The appearance of an enormous number of ‘meristemoids’, which gave rise to such regenerated plantlet cluster, also supported the presence of 5.4 average shoots per explant in this reported protocol.

Discussion

Sesame is often considered as “*The Queen of Oil-Seed*” due to its highly nutritious oil for human consumption. Since the yield of sesame is very low compared to the other oil-seed crops of India, a potential increase of productivity demands the prime importance to improve this ancient-most oil-seed crop (Debnath et al. 2014). The major constraint to achieve the goal is the inadequate information about sesame regeneration, which is a pre-requisite for this approach. Moreover, the regeneration efficacy of sesame is highly genotype dependent (Taskin and Turgut 1997; Yadav et al. 2010; Al-Shafeay et al. 2011). These factors usually result in failure of obtaining stable transgenic plants using “sonication assisted *Agrobacterium*-mediated transformation” (SAAT) method in sesame in our previous attempt (Debnath et al. 2014). Therefore, it was necessary to standardise a regeneration protocol for the sesame cultivar JK-1 used in this study, using which the improvement strategy of sesame can be designed using the transgenic approach in future research.

The previously reported potentiality of in vitro adventitious shoot regeneration made cotyledons of sesame to use as the choice of explant in this study (Were et al. 2006; Seo et al. 2007; Yadav et al. 2010; Al-Shafeay et al. 2011). In vitro regeneration of cotyledon explants was found to be marginally better when it was cultured in MS media supplemented with BAP and IAA rather than the supplementation of TDZ and IAA (Table 1). The optimum combination of PGRs for adventitious shoot regeneration was found to be 33.33 μM of BAP and 2.85 μM of IAA (BTI 4 medium) in this experiment. Previously, regeneration of sesame was reported in media supplemented with BAP + IAA (Were et al. 2006; Seo et al. 2007; Al-Shafeay et al. 2011) as well as with TDZ + IAA (Were et al. 2006; Wadegar and Lokeshia 2011). In this connection, fortification of MS medium with 29.43 μM AgNO_3 along with BAP and IAA (AG 3 medium) increased regeneration frequency (Fig. 2). The potency of AgNO_3 to improve in vitro regeneration is reported in various plants (Williams et al. 1990; Ashwani 2017; Panigrahi et al. 2017) including sesame (Seo et al. 2007; Abdellatef

et al. 2010; Al-Shafeay et al. 2011). Low concentration of silver ions reduced the effect of ethylene in plant cells by the probable effect of replacing copper co-factor of the ethylene receptor (ETR1) of the cell, thereby making it insensitive to ethylene, as described previously (Moshkov et al. 2008; Kumar et al. 2009). However, reduction of regeneration frequency at the higher concentration of AgNO_3 (AG 4 and AG 5 medium) in our experiment was probably due to the toxic effects of silver ion (Ratte 1999; De Jong et al. 2002).

The frequency of in vitro regeneration increased by the pre-culture of cotyledon explants in ABA-supplemented medium (AB 3 medium = AG 3 medium supplemented with 3.78 μM ABA) for 7 days and subsequent sub-culture in ABA-devoid medium (AG 3 medium) (Table 2). The presence of ABA in pre-culture medium might be associated with the in vitro sucrose uptake in the cultured explants as previously described in root tissue discs of beet (Saftner and Wyse 1984), fruit explants and cortex disc of strawberry (Archbold 1988), leaf veins of pea (Estruch et al. 1989), and tobacco leaf discs (Vreugdenhil and Kerckhoffs 1992). The increased cellular sucrose concentration by ABA-mediated sucrose uptake during pre-culture might facilitate the in vitro regeneration in our study. Previously, Seo et al. (2007) reported that pre-culture of cotyledon explants for 2 weeks on 9% sucrose and subsequent sub-culture to medium with 3% sucrose significantly enhanced adventitious shoot formation in sesame. In a recent study, Kim et al. (2016) reported that 4% sucrose concentration was necessary for shoot organogenesis in *Aloe saponaria*. For further investigation of the assumption of ABA-mediated sucrose uptake, cotyledons explants were cultured in varying concentrations of sucrose (3 to 9%) supplemented in the AG 3 medium independently for 7 days and explants from every treatment were sub-cultured in AG 3 medium that was supplemented with 3% sucrose only. This experimental condition mimicked the probable ABA-mediated sucrose uptake during the initial 7 days of culture duration. Increased frequency of regeneration ($54.81 \pm 2.82\%$) in AG 3 medium was observed when cotyledon explants were initially cultured in 4% sucrose supplemented medium (Fig. 3). However, compared to the initial culture in 4% sucrose supplemented medium, the frequency of regeneration was higher in AG 3 medium when the cotyledons were pre-cultured in AB 3 medium ($68.15 \pm 2.68\%$) (Table 2). It suggested the probable active uptake of sucrose in the presence of ABA during the pre-culture of cotyledon explants in AB 3 medium. Furthermore, it was observed that the in vitro regeneration frequency of cotyledon explants was increased by of pre-culturing them for 14 days in AB 3 medium instead of 7 days (Table 3). It indicated that the cellular concentration of sucrose by ABA-mediated uptake reached an optimal value by 14 days in AB 3 medium facilitating the increased regeneration frequency of cotyledon explants in AG 3 medium. However,

the pre-culture for 21 days in AB 3 medium reduced the regeneration frequency in AG 3 medium. Besides that, culturing sesame cotyledon explants exclusively in AB 3 medium not only lowered the regeneration rate markedly, but also initiated blackening in some of the explants (data not shown). The blackening indicated the probable toxic effect of prolonged sucrose exposure (Tholakalabavi et al. 1994). However, much detailed investigation, which is beyond the scope of this study, is needed to conclude the phenomenon of ABA-mediated sucrose uptake during in vitro regeneration of sesame cotyledon.

The orientation of the explants during culture and the age of explants had significant role in sesame regeneration. In each experiment, regeneration was observed only from the cut proximal end of sesame cotyledon explants. Direct contact of the cut end of 4- and 5-day-old cotyledon explants with the culture medium during the vertical culture orientation in AB 3 and subsequent AG 3 media facilitated the regeneration more than the horizontal orientation of explants that had less contact surface of proximal end of cotyledon explants with the culture medium. Furthermore, by culturing the 6-day-old cotyledon in vertical orientation decreased the regeneration rate, but the number of regenerated shoot buds per explants was increased (Table 4). In plant systems, the level of endogenous and exogenous PGRs cumulatively affects regeneration (Maurya et al. 2013; Pulianmackal et al. 2014). The orientation of explants in culture medium affects the regeneration frequency probably by altering the total pool of endogenous and exogenous PGR levels and this phenomenon is well documented in previous reports in other plants (Sharma et al. 1990; Yadav et al. 1996; Garcia-Luis et al. 1999, 2006; Kumar and Reddy 2010). Our results indicated that the regeneration frequency and the number of regenerated shoots per explants were facilitated depending upon the age and orientation of explants, where the cumulative levels of endogenous and exogenous PGRs became optimum. Besides that, in our experiment, the observed highest regeneration frequency in the youngest explants (4-day-old cotyledon) indicated the increased capacity of regeneration of young explants in sesame. The crucial role of age of the explants on the onset of morphogenesis in in vitro cultures was well established in many other systems like *Solanum trilobatum* and *Brassica napus* (Jonoubi et al. 2004; Chakravarthi Dhavala et al. 2009).

In the optimised protocol of this study, the direct in vitro regeneration of adventitious shoots was observed from cotyledons explants of sesame with the highest frequency of $94.82 \pm 1.34\%$ having 5.4 ± 0.04 regenerated shoots per explants. Both these resultant values of our study are higher than the previous reports of in vitro direct shoot regeneration in sesame (Taskin and Turgut 1997; Were et al. 2006). However, it could not surpass the 100% efficacy of the report of indirect shoot organogenesis from hypocotyl-derived callus

explants (Wadeyar and Lokesh 2011). Similarly, our direct regeneration efficacy is not comparable to the reports of Gangopadhyay et al. (1998) and Abdellatef et al. (2010), wherein multiple shoot buds of high frequency were induced either from apical and axillary meristems for the efficacy of micro propagation.

The regenerated shoot buds were successfully elongated in MS medium supplemented with BAP and GA₃ in our study (Table 5). Combination of GA₃ and BAP had been found to improve shoot multiplication and elongation in sesame previously (Raja and Jayabalan 2010; Wadeyar and Lokesh 2011). Profuse root induction was achieved by the culturing of elongated shoot explants in NAA supplemented MS medium (Table 6). Use of NAA for root induction was reported earlier (Seo et al. 2007; Abdellatef et al. 2010).

The regeneration frequencies in the optimised protocol vary significantly among different cultivars of sesame due to the genetic variability among them (Table 7) (Grattapaglia and Machado 1998). Variability in the regeneration frequency indicated the cultivar dependent regeneration performance of this plant as reported earlier by many authors (Taskin and Turgut 1997; Yadav et al. 2010; Al-Shafeay et al. 2011).

Furthermore, through the scanning electron microscopic study, the appearance of shoot apical meristem (SAM) was observed at the proximal cut end of cotyledons, which was the particular region for regeneration for this explant in this plant (Fig. 5). This phenomenon suggested that the regeneration of sesame following the optimised protocol reported here was appeared to be an event of organogenesis.

Conclusion

In this paper, we report an efficient cotyledon-derived direct regeneration protocol of sesame. The regeneration frequency is the highest (94.82%) reported until date in sesame (*JK-1*). The protocol showed comparable high regeneration rate in other cultivars of sesame tested (*JT-7*, *PADMA*, *RT-103*, *PHULE TIL-1*, and *TAPI-A*). The physical parameters like the explants age and orientation in the medium played a major role in regeneration. Scanning electron microscopy conclusively identified the organogenic nature of the regeneration. A reproducible stepwise protocol in the form of flow diagram starting from explants to transplantation to soil is provided. It will pave the way for efficient *Agrobacterium*-mediated transformation of sesame for desirable traits.

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conducted experiments, analysed data, and wrote the manuscript. All of the authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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