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REGULAR ARTICLE

EFFICIENT *IN VITRO* ORGANOGENESIS AND PLANTLETS REGENERATION IN SESAME (*SESAMUM INDICUM* L.)-AN IMPORTANT OILSEED CROP

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ABSTRACT

The regeneration methods in Sesame (*Sesamum indicum* L.) already developed were genotype dependent and were unsuccessful for Indian cultivars. Hence, an efficient protocol for *in vitro* organogenesis and plant regeneration in sesame was attempted with TMV 3 cultivar. Immature cotyledons derived from 1-week old seedlings were cultured on MS (Murashige and Shoog) medium fortified with different concentrations of 6-benzylaminopurine (BAP), thidiazuran (TDZ) and kinetin for adventitious shoot induction. It was found that optimal medium for direct shoot formation was MS with BAP (2.0 mg/l) at a frequency of 74% with an average of 4.5 shoots per expalnt. The shoot regeneration frequency was significantly reduced with either TDZ or kinetin when compared with BAP alone. Elongated individual shoots were transferred on MS media supplemented with Indole Butyric Acid (IBA; 0.5 mg/l) showed rooting frequency of 70%. The rooted plantlets were acclimatized to potting mixture containing sand, soil and clay mixture and grown to maturity with survival rate of 65 %. No phenotypic aberrations were observed among the *ex vitro* transferred plantlets. The protocol described here assures a high frequency of shoot regeneration, root induction and also plant survival rate.

Keywords: Sesame, TMV 3, Cotyledon, Direct organogenesis

INTRODUCTION

Sesame (*Sesamum indicum* L., family: Pedaliaceae) is an important crop mainly cultivated in tropical and subtropical areas, and are rich in oil and protein. The seed contains about 50 to 60% of temperature stable edible oil. In addition, its oil is traditionally used for cooking and in industries [1]. It contains antioxidants such as sesamin, sesamolin, and sesamol, and it also exerts beneficial health effects including antioxidant activity, free radical scavenging, antihypertensive, hypolipidemic, neuroprotective, anticarcinogenic and antimutagenic properties [2-5]. India ranks first in area (1.86 million ha) and second in production (6, 36, 000 Mt) next only to Myanmar [6]. Sesame is the third most important oil seed crop in India after groundnut and mustard.

Even though the nutritive and medicinal properties of sesame seeds are well known, there was not much increase in area, production and productivity of this crop for many years. The reasons for such stagnant scenario can be grouped as crop productive and crop improvement. The crop productive reasons could be cultivation in marginal and nutrition-starved areas and inadequate water, nutrition and pest and disease management aspects, whereas lack of development of high yielding varieties and varieties resistant to pest and disease constitute crop improvement aspects. Insect pests like sphinx moth, gall fly, capsule borer and diseases such as Alternaria blight, Fusarium wilt, phyllody cause considerable damage and reduce the yield potential of this crop.

In crop improvement, backcross breeding approach for transferring pest and disease resistant genes from wild donors to cultivated species has not been successful due to post fertilization barriers [7]. Since not much progress could be made through classical breeding approaches, the development of pest and disease resistant sesame cultivar still remains a challenge. With the advent of novel molecular biology tools, plant transformation methods have become a routine progress. However, genetic transformation in this crop remains difficult due to the recalcitrant nature of sesame to *in vitro* regeneration [8]. There were reports of somatic embryogenesis from zygotic embryos [9], seedling-derived callus [10], and cotyledon and hypocotyl segments [11]. Previous studies showed the micropropagation of this plant, shoot-tip culture [12], nodal culture [13], and leaf disc culture [14], regernation from hypocotyls and de-embryonated cotyledon explants [12, 15-17] without much success rates.

Further, one recent report on sesame regeneration revealed that cotyledon explants derived from 1-2 w old seedlings were unsuccessful to facilitate in vitro adventitious shoot regeneration [17]. In addition, 1-week old cotyledon explants from seedlings were handy enough to execute tissue culture

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and also in vitro genetic manipulation studies as compared with cotyledons from mature and immature seeds. The aim of the study reported here is to establish a successful regeneration system using cotyledon explants derived from 1-week old seedlings of sesame.

MATERIALS AND METHODS

Plant material

Sesame seeds from the cultivar TMV 3 were collected from Oil Seeds Research Station, Tindivanam, Cuddalore district, Tami Nadu, India. Seeds were surface sterilized in 0.1% $HgCl_2$ for 2 min and rinsed five times with sterile water. Seeds were germinated for 1-week (7-8 d) for preparation of cotyledon explants.

Explants and media preparation

For isolation of cotyledon explants, surface sterilized seeds were aseptically transferred onto magenta box containing hormone free medium and cultured at 25±2 °C in the darkness for about 1-week. A 1-week old cotyledon was dissected and a cut was given at the proximal end before inoculation on media. All experiments were conducted using Murarhige and Skoog (MS) [18] basal medium. Unless otherwise stated, 30 g/l of sucrose was used as a carbon source and phytagel was used as the gelling agent at a rate of 4.0 g/l in all media; plant growth regulators (PGR) were added prior to autoclaving. The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl. The media were steam sterilized in an autoclave under 1.5 kg/cm² and 121 °C for 15 min. Around 25 ml of medium was dispensed into Petri dishes (90 x 10 mm) and cultures were maintained at 25±2 °C under 16 h cool-white fluorescent lights with 8 h dark period. Chemicals, medium and growth regulators were obtained from Hi-media, Mumbai, India.

Adventitious shoots regeneration

For direct shoot formation, cotyledons were excised from the in vitro grown seedlings and were cultured on different media consisting of MS (Murashige and Shoog) basal media supplemented with different concentrations of cytokinins viz., 6-Benzylaminopurine (BAP) (0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/l, Kinetin (0.5, 1.0, 1.5, 2.0, 4.0 and 5.0 mg/l and Thidiazuran (TDZ) (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l. Data were scored for percentage of explants forming shoots, number of shoots per explants and shoot length after 6 to 8 w of frequent sub-culturing. Nature of plantlets was evaluated qualitatively as normal shoots with leaves and abnormal stunted shoots. A total of twenty cotyledons were used for each treatment and the experiment was conducted with three replications. The data collected from the experiment was analyzed using IRRISTAT version 3/93 developed by Biometrics unit, International Rice Research Institute, Manila, The Philippines. The data were presented as mean±SE of three replications. Significant differences were assessed by using Duncan's Multiple Range test (DMRT) at the 5% probability level [19].

Rooting and hardening of plantlets

Plantlets with well-developed shoots (2 to 4 cm long) were excised from shoot clusters and placed on rooting media (half-or full-strength MS, MS+IBA (0.5 mg/l) and or MS+IBA (1.0 mg/l) for 4 to 6 w. The hardening was done as explained previously [26].

RESULTS AND DISCUSSION

An efficient and reproducible tissue culture protocol is an essential prerequisite for gene transformation as well as *in vitro* induction of mutation in crop plants. Several researchers reported that sesame is a recalcitrant species for *in vitro* regeneration and genetic transformation [7, 17]. An effective plant regeneration system that could be applicable to a wide group of genotypes for a given species is inconsistent among the cultivated varieties of sesame [7, 16, 17].

From the present study, it was found that around 90 % (data not showed) germination was observed on a full-strength MS basal medium (fig. 1 *a*). The cotyledons excised from such seedlings were given a cut at the proximal end to prevent apical shoot formation (Fig. 1 *b*). *In vitro* adventitious shoot regeneration from cotyledon explants derived from 1-week old seedling was achieved on MS medium supplemented with varying levels of cytokinins such as BAP, kinetin and or TDZ (table 1). After incubation over a period of 6 w, among the three cytokinins tested, BAP showed higher rate of shoot bud initiation as compared with TDZ or kinetin. With increase of BAP concentrations (more than 2.0 mg/l) in the culture medium, the rate of regeneration was reduced. In the present study, shoot organogenesis occurred directly from the proximal end of explants (fig. 1*c* and *d*).

The highest rate of adventitious shoot formation (74%) was achieved on MS medium fortified with BAP (2.0 mg/l) (fig. 1*c*). The shoot regeneration response of BAP was compared with other PGR (table 1). Of the various concentrations of TDZ or Kinetin tested, the cotyledon explants showed 65.4% and 56.5% regeneration with 0.5 mg/l and 1.0 mg/l of PGR respectively. The cultures treated with BAP (2 mg/l) were maintained another 2 to 3 w in same medium for multiple shoot initiation (fig. 1*e*). Kinetin and TDZ showed significantly less responsive than BAP for adventitious shoot formation and also multiple shoot induction.

The present study confirmed that BAP is considered as the most effective cytokinin for stimulating efficient shoot induction in sesame [7, 8, 16 and 20]. Substituting BAP with an equal concentration of kinetin or TDZ could not improve the shoot regeneration response from cotyledon explants of sesame [7, 16].

Several researchers reported that the age of explants (cotyledon explants from *in vitro* seedlings) is known to profoundly affect the shoot induction frequency and also regeneration efficiency. Few attempts were made to use cotyledon explants from different aged seedlings of sesame. To note, cotyledon explants from 3–4 d [16, 8–10] days [12, 21] and 1–2 w [17] seedlings were reported with varying regeneration frequencies. Furthermore, cotyledon explants derived from 1-2 w old seedlings showed either callusing or never initiated any adventitious shoots and or resulting in low regeneration frequencies [15, 17 and 20].

Our study reveals higher regeneration efficiency (74%) obtained from cotyledons of 1-week-old seedlings which is in contrast with findings of Seo et al. [17] and Yadav et al. [7] who reported lower regeneration efficiency from cotyledon explants obtained from the same aged seedlings. Variation in regeneration response may be due to genotype specific differences or different culture medium, as mentioned in previous regeneration studies on sesame [12, 13]. Simultaneously, the excised yellow colored cotyledon explants from seedlings cultured onto MS medium supplemented with either BAP or TDZ or kinetin at all concentrations were found dark greenish during entire culture period (from shoot initiation to shoot multiplication). Conversely to earlier findings, browning/necrosis and subsequent death of cotyledon explants was not noticed in the present study either on MS basal medium [8] or in the presence of a cytokinin alone [16].

The earlier researchers used cotyledon explants of mature, immature seed and also from 2-3 d old seedlings. The size of explants from above mentioned cases were tiny enough and very stiff to handle either for tissue culture and genetic engineering studies as well. The photographic documentations from the published articles of Were *et al. [*16] and Yadav *et al. [*7] remain unclear to sustain that adventitious shoot initials were originated from meristem-free organs and also multiple shoot initiation. Thus, to address these issues, we have used cotyledon explants from 7-d old or 1-week seedlings which are handy enough to work with it and also a precise transverse cut was made at the proximal end to cotyledon to entirely elude the meristematic region and also obtained multiple shoot induction (fig. 1*a*, *d* and *e*). Further, in the present investigation, exogenous addition of sucrose, abscisic acid and silver nitrate were not used which ultimately confirms that the protocol is simple and cost effective [22].

Induction of rooting is difficult process in sesame. Adventitious shoots at 2 to 3 cm in height (fig. 1*e* and *f*) were transferred to hormone-free half-strength or full-strength MS or MS+IBA (0.5 mg/l) and or MS+IBA (1.0 mg/l) for *in vitro* induction of roots (table 2). Rooting of shoots was induced at the base of shoots after 4 w and well developed roots appeared within 6weeks. The maximum percentage of root induction (70 %) was found with of MS+IBA (0.5 mg/l) (fig. 1 *g*) followed by 55 % with 1.0 mg/l IBA. This advantageous effect of IBA on *in vitro* induction of roots is well documented in previous tissue culture studies in sesame [7, 16]. The rooted plantlets were transferred to plastic pots containing sand, soil and clay mixture (fig. 1*h*) and then hardened in greenhouse using mud pots (fig. 1*i*) where 65 % of plants survived and grown to maturity.

Tissue culture induced genetic variations due to influence of several cultural conditions (culture media, type of explants, successive transfer of culture, temperature and pH) are a major and frequent event among the *in vitro* raised plantlets. There were no morphological or phenotypic variations were observed among the *in vitro* grown plantlets compared with seed derived plants of the same cultivar. This may be due to the use of optimal media condition and supplementation of exogenous hormones at very low concentrations. Further, the present protocol indicates direct regeneration of adventitious shoots without the intervention of callus phase which might have resulted in generation of genetically uniform plants [23, 24]. Direct regeneration of plants from explants is a faster and a time saving approach for obtaining whole plants without the callus interphase that can increase somaclonal variation [25].

Table 1: Effect of BAP, TDZ and kinetin on regenerative response of cotyledon explants of sesame cv. TMV 3

Phytohormones (mg/l)			% of explants forming shoots (mean±SE)[a]	Mean shoot length (cm) (mean±SE)	Mean no. of hoots/explants (mean±SE)	Nature of plantlets
BAP	TDZ	Kinetin				
0.0	0.0	0.0	0.0±0.00 j	0.00±0.00 d	0.00±0.00 d	-
0.2			30.2±1.21h	0.94±0.22 c	1.00±0.20 c	+
0.5	-	-	48.6±1.32 e	2.07±0.11 b	2.13±0.13 b	+
1.0	-	-	60.3±1.23 c	1.14±0.23 c	2.02±0.04 b	++
2.0	-	-	74.0±1.17 a	4.20±0.21a	4.50±0.08 a	++
3.0	-	-	50.7±1.22 e	0.78±0.09 c	1.10±0.14 c	+
5.0		-	41.5±1.31g	0.22±0.04 c	1.14±0.16 c	+
-	0.1	-	43.5±1.55 f	1.14±0.21 c	2.23±0.06 b	+
-	0.5	-	65.4±1.34 b	2.12±0.23 b	2.26±0.09 b	++
-	1.0	-	40.4±1.66 g	0.60±0.04 c	2.10±0.17 b	+
-	1.5	-	34.7±1.54 h	0.30±0.06 c	2.04±0.05 b	+
-	2.0	-	26.6±1.26 i	0.11±0.05 c	1.06±0.04 c	+
-	2.5	-	20.8±1.88 i	0.01±0.03 c	1.00±0.08 c	+
-	-	0.5	44.2±1.37 f	1.10±0.17 c	1.68±0.06 c	+
-	-	1.0	56.5±1.46 d	0.81±0.09 c	1.25±0.11 c	++
-	-	1.5	44.1±1.23 f	0.61±0.05 c	1.18±0.07 c	+
-	-	2.0	36.5±1.16 h	0.30±0.07 c	1.13±0.10 c	+
-	-	4.0	32.3±1.11h	0.10±0.06 c	1.09±0.02 c	+
-	-	5.0	21.9±1.05 i	0.01±0.06 c	1.02±0.04 c	+

Means having the same letter in columns are not significantly different by Duncan's multiple range test (P<0.05)., Each data represent mean±SE of three independent experiments, [a]Twenty cotyledons/treatment, cultured for shoot initiation, and experiment repeated thrice., Nature of plantlets was evaluated qualitatively as–: no shoots, +: abnormal stunted shoots++: normal shoots with leaves.

Table 2: Effect of different levels of auxins and MS medium strengths on rooting of adventitious shoots of sesame TMV 3

Culture medium	Rooting response (%) (mean±SE)[a]	Mean no. of roots/shoot (mean±SE)
Half MS	50±9.8 b	2.0±0.01 b
Full MS	58±8.4 b	2.4±0.08 b
MS+IBA (0.5 mg/l)	70±9.5 a	4.2±0.04 a
MS+IBA (1.0 mg/l)	55±10.2 b	2.0±0.05 b

Values followed by the same letter are not significantly different at P<0.05 according to Duncan's multiple range tests., [a]Ten adventitious shoots/treatment, cultured for root induction, and experiment repeated thrice, Each data represent mean±SE of three independent experiments

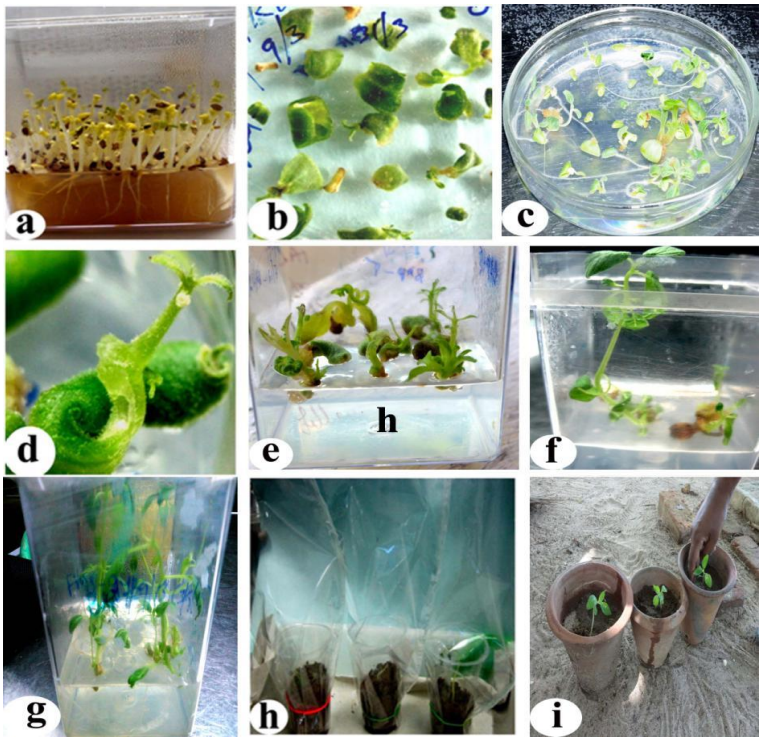

Fig. 1: *In vitro* plant regeneration form cotyledon explants of sesame cultivar TMV 3. (a) Yellow colored cotyledon on seedlings after 1-week on MS basal medium. (b) Cotyledon explants with a cut at proximal end. (c) Adventitious shoot regeneration from cotyledon explants on MS medium supplemented with 2.0 mg/l BAP. (d) Evidence showing shoots organogenesis at proximal cut end of cotyledon. (e) and (f) Multiple shoots on MS with 2.0 mg/l BAP after 6 w (e) and individual normal shoots with leaves (f). (g) Induction of roots from *in vitro* regenerated shoot cultured on MS basal medium supplemented with 0.5 mg/l IBA after 4 w of culture. (h) Plantlets acclimatized in pot mixture and covered with perforated polythene bag. (i) Mature plants grown in greenhouse. Scale bars: 2 mm (a, b, c, d, e, f, g, h and i)

CONCLUSION

In conclusion, the results obtained suggest that our regeneration protocol can be used for the *in vitro* propagation of sesame with a short duration of 8 to 10 w maintaining reproducibility and reliability. All the *ex vitro* transferred plants exhibited a homogeneity without morphological deviations. This protocol will be helpful for large scale propagation of sesame.

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