ORIGINAL ARTICLE

Induction of shoot regeneration in cotyledon explants of the oilseed crop *Sesamum indicum* L.

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**KEYWORDS**

*Sesamum; Recalcitrance; Cotyledon; Shoot regeneration; Sucrose*

**Abstract** *Sesamum indicum* is an ancient oilseed crop known for its high quality edible oil and its medicinally important lignans. The crop is said to be recalcitrant to plant tissue culture thus limiting the use of modern biotechnology for its genetic improvement. We present here a protocol describing plant regeneration through adventitious shoot formation from cotyledons dissected from sesame seeds soaked for four hours in water. Subculturing of the cotyledons after two weeks of culture on a fresh Murashige and Skoog medium leads to differentiation of adventitious shoots from the proximal cut end of the explant. Culture of cotyledons on a medium containing 9% sucrose for a couple of weeks prior to transfer to MS medium supplemented with 3% sucrose induced a higher frequency of shoot regeneration. The highest frequency of 25% adventitious shoot regeneration was observed for *S. indicum* variety UMA. This variety also turned out to be the best among the ten genotypes tested for shoot regeneration. While addition of IAA marginally improved regeneration, silver nitrate was found essential for enhancing the frequency of shoot regeneration. The regenerated shoots formed roots on full strength MS medium supplemented with 1 mg/l IBA and the rooted plants were established in soil.

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1. Introduction

*Sesamum indicum* L. (syn *S. indicum* spp. *Orientale*) is an ancient oilseed crop known for its high quality edible oil and its varied uses in Ayurveda, Chinese and Tibetan traditional systems of medicines [4,5,19]. According to Index Kewensis *Sesamum*, of the family Pedaliaceae, is represented by 36 species distributed in East Africa and India [2,18]. Among these *S. indicum* is the only species that is cultivated. This species has a number of varieties cultivated in the USA, India, Russia, Kenya, China, and South Korea and to a lesser extent in Japan constituting a valuable sesame gene pool [14]. While 70% of the sesame seeds produced is used for oil extraction, the remaining is used in the manufacture of bakery and confectionery products [23]. Sesame seed yields 46–50% oil which is rich in mono and poly unsaturated fatty acids, 20–25% protein and various minerals [9]. Recently this crop has been receiving prominence due to its unique lignans such as sesamin, sesamolin, sesameol and tocopherols that have potential to cure hypertension, obesity and cancer. Even though sesame is in cultivation for a long time, the crop remains unattended

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due to low yields caused by biotic and abiotic stresses [19]. Lack of efficient tissue culture shoot regeneration system further limits genetic engineering of this crop for developing improved lines. Attempts have been made earlier to regenerate sesame in tissue cultures using explants such as shoot tips [11,15], nodes [10], cotyledons [26,28] and hypocotyls [3,31]. Significant success has been achieved for plant regeneration through somatic embryogenesis using hypocotyl or cotyledon as explants [13,16,31]. Excepting in rice, somatic embryogenesis has hardly met with success in genetic engineering of crop plants as compared to direct de novo shoot regeneration from explants [20]. The shoot tips and nodes are unsuitable for Agrobacterium mediated transformation as they give multiple shoots from pre-existing primordial shoot and/or shoot buds [24]. Due to lack of a proper regeneration system, genetic engineering of the crop has yielded no tangible result so far [1,7,27,30]. Rather shoot regeneration reported in sesame so far is found to be unsuitable for transformation of any of the Indian varieties of sesame. It is known that the genotype of a crop strongly influences shoot regeneration efficiencies [16]. Therefore in this study we made an attempt to develop a protocol for shoot regeneration by evaluating the effect of genotypes on shoots regeneration from cotyledons thereby identifying the best variety of S. indicum for genetic engineering. To the best of our knowledge this is the first report on effect of genotype on shoot regeneration in Indian genotypes of sesame. The role of BAP, IAA and Silver Nitrate on shoot regeneration from the excised cotyledons of sesame is also reported here.

2. Materials and methods

2.1. Plant Materials

Sesamum indicum varieties used for screening in this study were TMV3, TMV4, TMV5, TMV6, VR1, AKT64, TC25, RT127, Rajeshwari, and UMA. Seed samples of these varieties were procured from National Bureau of Plant Genetic Resources (NBPRG), New Delhi and maintained routinely in the experimental garden of our Department. These varieties differed in their seed color and the province for which they have been recommended for cultivation.

2.2. Surface sterilization of seeds and preparation of explant

About one hundred mature seeds of different accessions of sesame were washed thoroughly under running tap water. The seeds were then immersed in 50 ml of water containing 150 µl of Savlon, agitated for 10 min and then rinsed three times with distilled water. After removing the detergent, seeds were washed in water and immersed in 70% ethanol for 1 min followed by rinsing in sterile distilled water in a laminar airflow cabinet. Then the seeds were surface sterilized using sodium hypochlorite solution containing 2% chlorine for 5 min followed by a thorough wash in sterile distilled water for 3–4 times. The seeds were then left for soaking in sterile distilled water for 4 h. Aseptic seeds were then blotted on sterile Whatman No1 filter paper, and cotyledons devoid of embryonic axes were dissected from these sterilized seeds with a sterile blade under a biological microscope and transferred to the culture medium.

2.3. Culture medium and culture condition

In the present study MS basal medium is the one described in Murashige and Skoog [17], pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl before adding 0.8% agar powder for solidification, and then the medium was sterilized by autoclaving at 121°C, 15 psi for 15 min. The medium was cooled to 40 degree Celsius before fortifying with filter sterilized plant growth regulators. All the cultures were maintained at 25 ± 1 °C and 16/8 h (light/dark) photoperiod provided by a two 40 W cool white fluorescent tubes.

2.4. Effect of genotype and high sucrose concentration on shoot regeneration

To test the effect of genotype and high sucrose concentration on shoot regeneration, cotyledons isolated from the seeds of different varieties of S. indicum were cultured on two sets of media. In the first set, the culture medium (Medium A), consisting of MS + 6.5 mg/l BAP + 1 mg/l IAA + 5 mg/l AgNO3 supplemented with 3% sucrose was used. The hormone combination in the medium was in fact optimized and published in an earlier report for shoot regeneration in S. indicum variety VR1 [7]. In the second set (Medium B) the cotyledons were initially cultured for two weeks on the medium of same composition but supplemented with 9% sucrose instead of 3% sucrose. After 2 weeks of culture, the explants from both the sets were shifted to MS medium of same composition but with 3% of sucrose. Periodic observations were made until 4 weeks of culture. By the end of fourth week a final observation was made, and the data were recorded to calculate the frequency and intensity of adventitious shoot formation from the cultured cotyledons.

2.5. Effect of BAP and IAA on cotyledons

The MS basal medium supplemented with different concentration of BAP (0, 4.5, 6.5, 8.5 and 10.5 mg/l) with AgNO3 (5 mg/l) but with or without IAA (1 mg/l) was tested to find the effect of BAP alone or in combination with IAA on shoot regeneration in UMA variety of Sesamum indicum L. This experiment involved initial passage for two weeks on Medium B followed by transfer to Medium A as described in Section 2.4.

2.6. Effect of AgNO3 on in vitro shoot regeneration

This experiment consisted of five treatments. MS medium supplemented with 6.5 mg/l BAP and 6.5 mg/l BAP + 1 mg/l IAA described in Section 2.5, was used as basal media to test the role of AgNO3 (5 mg/l) on shoot regeneration from cotyledons. Medium devoid of hormones and supplements was used as control.

2.7. Rooting of shoots and hardening

The regenerated shoots formed in the above experiments were excised from surface of the explants and transferred to MS medium supplemented with 0, 0.1, 0.5, 1 mg/l IBA. The cultures were maintained as before and the data were recorded for obtaining the rooting efficiency. The rooted in vitro regen-
germinated plants after 4 weeks of culture on rooting medium were washed thoroughly under running tap water in order to completely remove the agar. These plantlets were then transferred to the soil: manure mixture (1:1) contained in the pots. The transplanted *in vitro* plants were kept covered using polythene bag to maintain humidity. The covers were removed gradually for acclimatization of the plant to field condition.

2.8. Data analysis

A minimum of 45 explants were used for each treatment, and each experiment was repeated at least thrice. The frequency and intensity of shoot regeneration were recorded, and the data were subjected to Duncan Multiple Range Test (DMRT).

3. Results and discussion

3.1. Effect of genotype and high sucrose concentration on shoot regeneration

Within a week of culture, the white cotyledons started turning green and became swollen all through, with some of them showing more proliferative activities at the cut ends (Fig. 1A–C). By the end of third week, the explants showed appearance of leafy structures developing from the proximal cut end of the cotyledons (Fig. 1D). A closer view of the explants at an early stage of organogenesis revealed that the leafy structures develop from around the periphery of the cut end of the cotyledon and that they lacked a clear radical end indicating that the mode of shoot regeneration is *de novo* (Supplementary Fig. 1A).

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**Fig. 1** *In vitro* shoot regeneration in *Sesamum indicum* var. Uma. (A) A freshly excised cotyledon from sterilized seed; (B) and (C) cotyledon explants after 7 days and 14 days of culture on Medium B (MS + 9% sucrose + 6.5 mg/l BAP + 1 mg/l IAA + 5 mg/l AgNO₃) showing greening and swelling at cut end of cotyledons respectively; (D) and (E) shoots regenerating from the explant after one week and two weeks respectively of transfer to Medium A (same medium as in (B) except that the sucrose was 3%) from Medium B; (F) A shoot on MS + 1 mg/l IBA after three weeks of transfer showing rooting; (G) An *in vitro* plantlet showing well developed root system; (H) A regenerated plant after two weeks of transfer to soil.
Therefore in the present study only cotyledons directly excised from 5- to 7-day-old seedlings of sesame did not yield any tissue formation from the preexisting shoot tips or shoot buds. Juvenile explants denovo is the formation of shoots from the explant rather than a shoot regenerant from an existing shoot tip. The most preferred mode of regeneration was shoot tip has been the most popular one used for regeneration of several explants. The remaining genotypes showed a regeneration frequency ranging from 1 to 3 shoots per explant. The least regeneration frequency was observed for the sesame variety UMA followed by TC25 with 8.3%. The intensity of sucrose is necessary because the artificial condition prevailing in tissue culture environment limits the synthesis of carbohydrate necessary for growth and development of plants. In general starch grains in tissues accumulate during early shoot meristem formation which are later utilized during shoot differentiation process [25]. Higher concentration of sucrose might result in higher uptake of sucrose in explants which favors the formation of shoots [26].

Table 1 Effect of genotype and sucrose concentration on shoot regeneration in cotyledons of Sesamum indicum L. The data are mean of three replicates recorded after four weeks of culture on MS + BAP (6.5 mg/l) + IAA (1 mg/l) + AgNO₃ (5 mg/l).

| Genotype (variety) | % shoots regeneration (Mean ± SD) |
|--------------------|----------------------------------|
|                    | Medium A | Medium B |
| TMV3               | 12.80 ± 5.10b | 20.53 ± 10.40b |
| TMV4               | 1.30 ± 2.70b | 14.40 ± 5.30b  |
| TMV5               | 5.00 ± 6.50b | 15.50 ± 4.60b  |
| VR1                | 5.20 ± 3.20b | 12.20 ± 7.40b  |
| TMV6               | 3.40 ± 3.60b | 18.32 ± 7.40b  |
| RT127              | 2.70 ± 5.50a | 11.60 ± 9.70b  |
| RAJESHWARI         | 1.30 ± 2.80a | 15.50 ± 7.70b  |
| UMA                | 2.70 ± 5.50a | 25.55 ± 8.60a  |
| AKT64              | 0.60 ± 1.30a | 10.06 ± 5.04ab |
| TC25               | 13.20 ± 9.00a | 8.32 ± 3.40a   |

* Medium A & Medium B are explained in the text. Mean in the same column followed by different letters is significantly different at p < 0.05 according to DMRT.

and B). By fourth week these structure developed into leafy shoots (Fig. 1E). Well-developed shoots could be observed by 6th week (Fig. 1F). Effect of the genotypes on the two sets of sucrose treatment is presented in Table 1. It was observed that there is a clear cut difference in regeneration efficiency with respect to the treatment and genotypes of S. indicum tested. Among the two treatment tried a passage from 9% sucrose (Medium B) to 3% sucrose (Medium A) induced a better regeneration frequency than continued culture on 3% sucrose. Whereas continuous maintenance of culture on 9% sucrose resulted in browning of cotyledons, 3% sucrose yielded low frequency with an average shoot regeneration of 4.82% (Table 1). Similarly, among the genotypes tested maximum regeneration frequency was observed for the sesame variety UMA followed by TMV3 with 25% and 20%, respectively. The intensity of regeneration ranged from 1 to 3 shoots per explant. The least frequency of regeneration was exhibited by TC25 with 8.3% shoot regeneration. The remaining genotypes showed a regeneration frequency ranging from 10 to 15%.

Tissue culture of Sesame has been going on for almost three decades but no reliable protocol on successful shoot regeneration or transformation is reported so far. Of several explants, shoot tip has been the most popular one used for regeneration with a maximum of 70% response and with an intensity of 14 shoots/explant [11]. However, the most preferred mode of shoot regeneration suitable for genetic engineering of a crop is the formation of shoots denovo from the explant rather than from the preexisting shoot tips or shoot buds. Juvenile explants are the most totipotent for induction of de novo shoot regeneration. The culture of cotyledons and hypocotyls as explants from 5- to 7-day-old seedlings of sesame did not yield any tangible results so far. In sesame seed, cotyledons are the only visible juvenile portion that could be dissected and cultured. Therefore in the present study only cotyledons directly excised from the soaked seeds were used as explants. De novo shoot regeneration has been observed when de-embryonated cotyledon was used as explant in certain exotic and indigenous germplasm. But the regeneration frequency is so low that they fail to meet the requirement of genetic engineering of the crop for agronomic traits [7,26,28,30]. While VR1 was earlier reported to regenerate with a frequency of 57.33%, in the present study only 12.2% regeneration could be obtained [7]. The report described that the mode of plant regeneration observed was somatic embryogenesis rather than shoot regeneration. Since no data on shoot regeneration of uninfected control are given in the said report it is difficult to comment on the reasons for differences in the shoot regeneration frequencies observed. Frequency of shoot regeneration obtained in the present study was consistently higher than many of those reported earlier.

It is evident from our observation that the difference in the frequency of regeneration among different varieties can be attributed to the difference in the genetic constitution of germplasm. Genotype, among other factors, has resulted in different response to shoot induction medium, and studies shows that different cultivars elicit different regeneration efficiencies [26,28]. In this study a total of ten varieties of S. indicum were tested for induction of shoot regeneration. UMA, a light brown seeded variety, emerged as the most superior with highest regeneration frequency followed by TMV3, a black seeded variety. TC25 is a white seeded variety that regenerated with least frequency. In this context the present study demonstrates that the highest regeneration frequency that is possible among the ten genotypes tested is around 25%.

Our study further demonstrates that the composition of the culture media helps in enhancing the regeneration frequency. Sucrose added to the culture medium acts both as a carbon source and as an osmoticum in shoot regeneration. Supply of sucrose is necessary because the artificial condition prevailing in tissue culture environment limits the synthesis of carbohydrate necessary for growth and development of plants. In general starch grains in tissues accumulate during early shoot meristem formation which are later utilized during shoot differentiation process [25]. Higher concentration of sucrose might result in higher uptake of sucrose in explants which favors the formation of shoots [26].

As UMA variety of S. indicum showed the optimal shoot regeneration response under the prevailing culture condition, this variety served as source of explants for further experiments testing role of factors affecting shoot regeneration.

3.2. Effect of BAP and IAA on shoot regeneration in cotyledon explants

The data on regeneration frequency with respect to different hormones tested show that IAA alone was incapable of inducing shoot regeneration in cotyledon explants. However, BAP alone was able to induce shoot regeneration in all the concentrations tested and could be marginally improved in the presence of IAA (Table 2). Frequency of shoot regeneration declined when the BAP concentration was higher than 6.5 mg/l both in the presence and in the absence of IAA. It is well-known fact that cytokinins in general favor shoot formation. However, the response of an explant to externally supplied hormones depends on the endogenous level of hormone present in the explants. In this context we infer that the hormones used in the present study balance the hormone equilibrium to favor shoot regeneration.

3.3. Role of AgNO₃ on in vitro shoot regeneration

A separate experiment was conducted to study the role of silver nitrate (AgNO₃) on shoot regeneration. It was observed
that BAP alone was capable of inducing shoot regeneration in sesame (Table 3). Though the presence of IAA induced a marginal increase in shoot regeneration frequency, it went up to three times with the additional presence of AgNO₃ in the culture medium (Table 3). Thus it was concluded that the silver nitrate is essential for enhancing shoot regeneration frequency in sesame. Silver nitrate added to the culture medium acts as an ethylene inhibitor and prevents browning of the explants in vitro. Ethylene is said to be associated with poor regeneration or recalcitrant behavior of cultured tissues [6].

The beneficial effect of AgNO₃ in stimulating organogenesis has been found in crops such as *Triticum aestivum* [21], *Helianthus annuus* [8] and *Brassica campestris* [6]. Addition of AgNO₃ was found to enhance conversion of somatic embryos into plants in Sesame as well [29]. It would be relevant to mention here that it took almost several years before a significant success could be achieved in shoot regeneration and subsequently in transformation of erstwhile recalcitrant crops such as *Oryza sativa* [12] and *Brassica campestris* [22]. It is our view that the regeneration frequency can be increased by choosing appropriate genotype from the vast germplasm by genotype screening. Alternately by trying increased number combination of hormones, explant source, age of explant and type of the explant it should be possible to achieve higher regeneration frequency in sesame.

### Table 2 Effect of BAP and IAA on *in vitro* shoot regeneration in cotyledons of *Sesamum indicum* L. var. Uma. Data recorded after 4 weeks of culture.

| Treatment No. | Supplement (mg/l) | % Shoot regeneration (Mean ± SD)² |
|---------------|-------------------|-----------------------------------|
|               | BAP   | IAA   | AgNO₃   |                           |
| T1            | 4.5   | 0.0   | 5.0     | 20.30 ± 4.70              |
| T2            | 6.5   | 0.0   | 5.0     | 20.40 ± 1.60              |
| T3            | 8.5   | 0.0   | 5.0     | 17.20 ± 6.70              |
| T4            | 10.5  | 0.0   | 5.0     | 13.30 ± 3.80              |
| T5            | 0.0   | 1.0   | 5.0     | 00.00 ± 0.00              |
| T6            | 4.5   | 1.0   | 5.0     | 20.80 ± 3.30              |
| T7            | 6.5   | 1.0   | 5.0     | 25.20 ± 5.10              |
| T8            | 8.5   | 1.0   | 5.0     | 18.70 ± 3.20              |
| T9            | 10.5  | 1.0   | 5.0     | 12.00 ± 10.60             |

* Mean values followed by different superscripts in the last column are significantly different according to DMRT (*P* < 0.05).

### Table 3 Effect of Silver Nitrate (AgNO₃) on *in vitro* shoot regeneration in cotyledons of *Sesamum indicum* var. Uma. Data recorded after 4-weeks of culture.

| S. No. | Supplement (mg/l) | Regeneration frequency (Mean ± SD)¹ |
|--------|-------------------|-----------------------------------|
|        | BAP   | IAA   | AgNO₃   |                           |
| 1      | 0.0   | 0.0   | 0.0     | 0.00 ± 0.00              |
| 2      | 6.5   | 0.0   | 0.0     | 12.00 ± 1.70              |
| 3      | 6.5   | 0.0   | 5.0     | 17.70 ± 1.50              |
| 4      | 6.5   | 1.0   | 0.0     | 8.70 ± 0.50              |
| 5      | 6.5   | 1.0   | 5.0     | 34.70 ± 4.60              |

* Mean values followed by different superscripts in the last column are significantly different according to DMRT (*P* < 0.05).

## 3.4. Rooting of shoots

Adventitious shoots formed as above were separated into individual shoots. These shoots on transfer to full-strength MS supplemented with IBA showed root induction within a week of culture (Fig. 1F). Of the three concentration of IBA tested for rooting maximal response was observed for 1 mg/l IBA (Table 4). Fully developed plantlets of about 6–8 cm were obtained by 3rd week of culture on this medium (Fig. 1G).

The rooted plants were successfully transferred to soil through a step of hardening (Fig. 1H).

In the present study, we established a regeneration system through adventitious shoot formation from excised cotyledons of *S. indicum* and were able to produce normal healthy plantlets. All the ten varieties responded and developed adventitious shoots on the medium tested. Even though the percentage of shoot formation were not very high the study shows that the varieties studied and the growth regulators used have the potential to induce shoot regeneration and therefore can be suitably used in several biotechnological studies. Further experiments to fine-tune the regeneration system by screening of the germplasm, testing of different growth regulators and additives can be pursued.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijgeb.2017.07.006.

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