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Research article

DIRECT SOMATIC EMBRYOGENESIS IN *SESAMUM INDICUM* (L.) CV-E8 FROM COTYLEDON AND HYPOCOTYL EXPLANTS

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ABSTRACT: In the present investigation, successful and reproducible protocol for somatic embryogenesis was developed for sesame (*Sesamum indicum* L.) cv. E8. Direct somatic embryo induction without an intervening callus phase is reported for the first time from 5 days old cotyledonary and hypocotyl explants. Embryogenic frequency as well as number of somatic embryos was dependent on concentration of 2, 4-D. The optimum concentration of 2,4-D required for induction high frequency and large number of somatic embryos was 3.0mg/l. Addition of cytokinins along with 2, 4-D, further enhanced the number of somatic embryos. Maximum number of somatic embryos per explant was noted on MS medium supplemented with 3.0 mg/l 2, 4-D + 1.0 mg/l BAP. Cotyledonary explants proved superior over hypocotyl explants and BAP over Kinetin. Conversion of somatic embryos into complete plantlets was achieved on MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l ABA + 5.0 mg/l AgNO3. Proliferation of embryogenic cultures was confirmed by histological studies.

Key words: *Sesamum indicum*, Somatic embryogenesis, Seedling explants, $AgNO_3$, Histology.

INTRODUCTION

Sesamum indicum L. belongs to family Pedaliaceae and is the most important oil-seed crop of semi-arid tropics and a source of high quality cooking oil and protein (Jeyamary and Jayabalan, 1997). It is grown in India, China, Korea, Russia, Turkey, Mexico, South America and several countries of Africa. It is cultivated on a total area of over 7.7 million hectares with total production of 3.3 million tons (FAOSTAT, 2008). The whole seeds are edible and are used in baking of breads and hamburger buns and for extraction of oil for cooking. The seed contains 50-60% oil, which has excellent stability due to the presence of natural antioxidants such as sesamolin, sesamin and sesamol and has medicinal and pharmaceutical value (Brar and Ahuja, 1979). The first report of tissue culture in sesame was reported by (Lee et. al., 1985 and George et. al., 1987). Since then number of reports have appeared on micro propagation using different explants (Gangopadhyay et. al., 1998; Seo et. al., 2007 and Chattopadhyaya et. al., 2010). Among the explants used, cotyledon and /or hypocotyl explants gained utmost attention and have proved excellent source of explants for callus induction and subsequent regeneration (George et. al., 1987; Jeyamary and Jayabalan, 1997; Rajender Rao et. al., 2002; and Seo et. al., 2007), however to date; only two reports have focused on somatic embryogenesis in *S. indicum* initiated from callus derived from hypocotyl and Cotyledonary explant (Jeyamary and Jayabalan, 1997 and Xu et. al., 1997). However, only one report published on successful conversion of somatic embryos into complete plantlets (Xu et. al., 1997). To our knowledge there are no reports on direct somatic embryogenesis from seedling explants of *Sesamum indicum*. Somatic embryogenesis is a unique *in vitro* morphological appearance found only in plant system (Namasivayam, 2007) and somatic embryos have also been reported in many plant species (Ammirato, 1983 and Zimmerman, 1993). Somatic embryogenesis is shown to offer a number of applications; as a model system in embryological studies (Arnold et. al., 2002), as an alternative pathway for mass multiplication of elite cultivars within a short period (Ammirato, 1983 and Zimmerman 1993) and also to conserve germplasm and a tool for genetic transformation (Feher et. al., 2003 and Jimenez, 2005). Keeping in view the importance of somatic embryogenesis and sesame plant, the present investigation was taken up.

In this communication we report, for the first time direct (Without intervening callus phase), somatic embryo development from 5 days old *in vitro* raised cotyledon and hypocotyl explants and subsequent conversion into plantlets in *Sesamum indicum* L.

MATERIALS AND METHODS

Preparation of explants

The seeds of *Sesamum indicum* L. genotype E8 were obtained from Agriculture Research Station, Gulbarga Karnataka State, India, and were used in the present investigation. The seeds were soaked in solution containing Bavistin (1.0% w/v, a fungicide) and dilute detergent (Tween 20) for 10-15 min then they were washed under running tap water and rinsed thrice with distilled water. The seeds were then surface sterilized with 0.1% (w/v) mercuric chloride for 45-50 sec and subsequently washed thoroughly in sterile distilled water to remove the traces of mercuric chloride. Further they were germinated on pre-sterilized culture tubes on Filter Paper Bridge and maintained under a 16 h/d photoperiod (36 µmol m^{-2} s^{-1}) at 26 ± 1 ^{0}C for 5 days. Healthy cotyledon and hypocotyls were excised exclusively from 5 days old seedlings, the explants were trimmed prior to inoculation. To achieve somatic embryogenesis in the present investigation, Murashige and Skoog (1962) medium with 3% sucrose as a carbon source, meso-inositol as organic compounds. To induce somatic embryos auxins like 2, 4-D (1.0, 2.0, 3.0 & 4.0 mg/l), 2, 4, 5-T (1.0, 2.0, 3.0 & 4.0 mg/l) and NAA (1.0, 2.0, 3.0 & 4.0 mg/l) were used. In order to enhance the frequency of somatic embryos, cytokinins like BAP (0.5, 1.0 & 1.5 mg/l) and Kn (0.5, 1.0 & 1.5 mg/l) were used as additives to the optimal concentration of auxin which induced, high frequency and maximum number of somatic embryos. For the conversion of somatic embryos into complete plantlets, torpedo stage embryos were placed on MS medium supplemented with BAP (1.0 mg/l), ABA (0.5, 1.0 & 1.5 mg/l) and AgNO3 (5.0, 6.0 & 7.0 mg/l). The MS medium was solidified with 0.8% agar and adjusted to pH 5.6-5.8 before autoclaving at 120^{0}C for 15 minutes. ABA was filter sterilized through 0.22 µm membrane filter and added to the medium after autoclaving. Culture conditions were as described above. About 40-50 explants were cultured per each treatment and the experiment was repeated three times. Different stages of somatic embryos *viz.,* globular, heart shaped and torpedo stages were noticed after 4-5 wks of cultures and the observations were recorded. Rooted plants were transferred on half strength MS medium for two weeks for primary hardening. The plantlets with stronger and thicker roots were transferred to pots containing sterile vermiculate for secondary hardening and later established in soil in the glass house where 60% of them survived and resumed growth.

Histological Studies

Specimens for histological studies were fixed in Formalin: Acetic acid: Alcohol (FAA 5:5:90) for 24h and were dehydrated in alcohol-butanol series before embedding in paraffin wax at 56^{0}C. Serial sections of 10 µm were taken using a microtome. After de-paraffinization, they were stained with 1% safranine and 0.1% fast green (Johansen, 1940).

Statistical Analysis

The experiments were conducted in a completely randomized design (RD). Data was analyzed by ANOVA and presented as mean ± SE/SD along with level of significance.

RESULTS AND DISCUSSION

Induction of somatic embryos

Direct development of somatic embryos was evident 8-10 days after culture initiation. Somatic embryos always developed from the adaxial surface of the cotyledonery explants irrespective of the auxin type or concentration. Among the various auxins tested 2, 4, 5-T and NAA elicited poor embryogenic response and the response of Picloram and Dicamba was negligible (Data not shown). Maximum embryogenic frequency of 95% and large number (35.66±4.38) of somatic embryos per responding Cotyledonary explant was obtained with 3.0 mg/l 2, 4-D followed by hypocotyl explants (Table 1). It is also been stated that, among auxins, the most frequently used was 2, 4-D (49%) followed by NAA (27%), IAA (6%), IBA (6%), Picloram (5%) and Dicamba (5%) (Raemakers et. al., 1995). The important role of 2, 4-D has been identified, by mentioning that in more than 65% of the recent protocols, this compound was applied alone or in combination with other PGRs (Gaj, 2004).

Auxins are the most likely candidates in the regulation of developmental switches (Nomura and Komamine, 1985 and Feher et. al., 2003). The influence of exogenously applied auxins particularly 2, 4-D on the induction of somatic embryo are well documented (Dudits et. al., 1991 and Yeung, 1995). It is suggested that 2, 4-D above certain concentration has a dual effect in the culture medium as an auxin directly or through metabolizing endogenous IAA (Michalczuk et. al., 1992a and 1992b) and as a stress inducing agent (Feher et. al., 2001, 2002 and 2003). However at higher concentrations of auxins the number and frequency of somatic embryo induction decreased (Table 1).

The differentiation of somatic embryos was asynchronous *viz*., embryos of all stages (globular, heart shaped, and torpedo shaped) were noticed in the same culture. Between the two explants cotyledons were more efficient than hypocotyls in terms of frequency and number of somatic embryo induction. (Table 1; Fig. 1a)**.** However, these findings are in contrast to the results obtained in *Sesamum indicum* (TMV 6) (Jeyamary and Jayabalan, 1997) where hypocotyl explants are reported to be more efficient than cotyledons. Cotyledons are reported to be excellent source of explant in responding to high frequency induction of somatic embryos in several oil seed crops like *Guizotia abyssinica* (Sarvesh et. al., 1994), *Carthamus tinctorius* (Tejavathi et. al., 2000; Mandal and Duttagupa, 2003), *Arachis* (Ozias et. al., 1992; Eapen and George, 1993) and Oil palm (Rajesh et. al., 2003).

Table 1. Effect of 2, 4-D, 2, 4, 5-T and NAA on induction of somatic embryos from cotyledon and hypocotyl explants of *Sesamum indicum* L.

Auxins	Conc. (mg/l)	Cotyledon		Hypocotyl	
		No. of somatic embryos per explant (SE)	Frequency (%)	No. of somatic embryos per explant (SE)	Frequency (%)
2, 4-D	1.0	19.00 ± 0.20^{c}	70	13.33 ± 0.15^{b}	45
	2.0	20.66 ± 2.30^{a}	80	16.00 ± 1.12^{b}	60
	3.0	35.66 ± 4.38^{a}	95	26.83 ± 1.89^{a}	70
	4.0	17.66 ± 0.11^{b}	60	17.00 ± 0.70^{c}	80
2, 4, 5-T	1.0	14.33 ± 1.27^{a}	65	12.33 ± 1.18^{a}	55
	2.0	19.33 ± 1.45^{a}	75	16.33 ± 1.23^{b}	65
	3.0	14.33 ± 0.50^{a}	60	20.66 ± 0.08^{b}	70
	4.0	09.00 ± 1.05^{b}	50	13.00 ± 0.85^{c}	60
NAA	1.0	14.60 ± 0.76^{b}	40	11.00 ± 1.18^{a}	40
	2.0	14.33 ± 0.89^{c}	52	09.00 ± 1.22^{a}	32
	3.0	11.33 ± 1.70^{c}	35	06.00 ± 1.75^{b}	35
	4.0	10.33 ± 1.52^{a}	30	04.00 ± 0.89^{c}	42

Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the different superscript in a column are not significantly different from each other. a = $P < 0.05$, b = $P < 0.01$ and c = $P < 0.001$ levels according to ANOVA.

Table 2. Effect of BAP and Kn with 2, 4-D on somatic embryo enhancement.

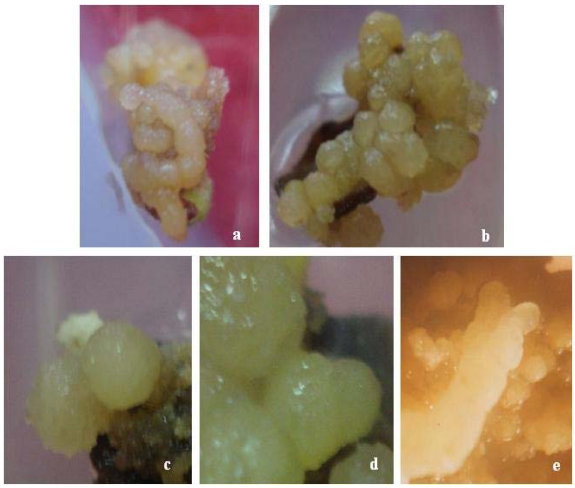
Auxin + Cytokinin	Conc. (mg/l)	Frequency (%)	Cotyledon
			No. of somatic embryos per explant (SE)
2,4-D + BAP	3.0+0.5	80	48.33 ± 6.93^{a}
	3.0+1.0	95	59.16 ± 4.30^{c}
	3.0+1.5	72	38.86 ± 0.14^{b}
2,4-D + Kn	3.0+0.5	60	20.66 ± 0.10^{c}
	3.0+1.0	55	20.00 ± 6.14^{a}
	3.0+1.5	48	19.00 ± 9.07^{c}

Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the different superscript in a column are not significantly different from each other. a = $P < 0.05$, b = $P < 0.01$ and c = $P < 0.001$ levels according to ANOVA.

Table 3. Effect of ABA and AgNO3 on adventitious shoot formation from embryos after 6 wks of cultures.

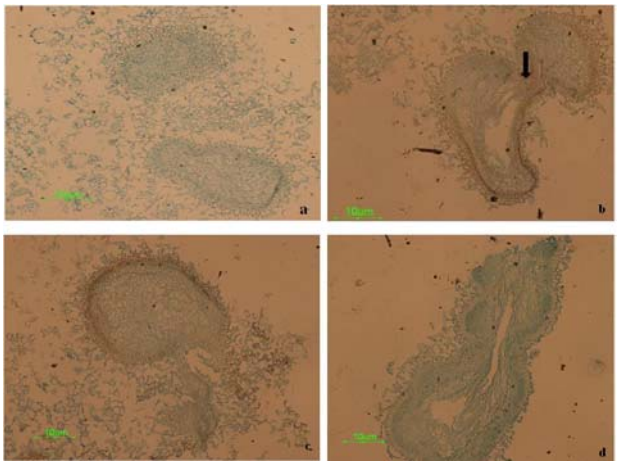
BAP (mg/l)	ABA (mg/l)	AgNO3 (mg/l)	Adventitious shoot conversion (%)	No. of adventitious Shoot/explants
1.0	0.5	5.0	43	44.30 ± 1.62^{b}
1.0	1.0	6.0	33	36.82 ± 1.40^{a}
1.0	1.5	7.0	26	25.46 ± 0.9^{c}

Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the different superscript in a column are not significantly different from each other. a = $P < 0.05$, b = $P < 0.01$ and c = $P < 0.001$ levels according to ANOVA.



(a) Initiation of Somatic embryos on 3.0 mg/l 2, 4-D.
(b) High frequency of Somatic embryos on 3.0 mg/l 2, 4-D + 1.0 mg/l BAP.
(c) Globular Somatic embryos.
(d) Heart shaped Somatic embryos.
(e) Torpedo stage Somatic embryos.

Figure 1. Different stages of somatic embryos of *Sesamum indicum* L.



(a) Initiation of Somatic embryos from Cotyledonary explant (bar =10 µm).
(b) Further growth of a somatic embryo (bar =10 µm).
(c) Heart shaped embryo (bar =10 µm).
(d) Cotyledonary embryo (bar =10 µm).

Figure 2. Histology of Somatic embryogenesis of *Sesamum indicum* L.

Interaction of 2, 4-D and cytokinins

It is reported that about 48% of the dicot species evaluated reacted to a combination of auxins and cytokinins for induction of somatic embryos (Raemakers et. al., 1995). Therefore, an experiment was conducted to investigate the interaction of BAP or Kn keeping the concentration of 2, 4-D constant (3.0 mg/l). Supplementing either of the two at low concentrations further enhanced the frequency and number of somatic embryos per explant, BAP at a concentration of 1.0 mg/l elicited best response (59.16±4.30/95%) than Kn (Table 2; Fig. 1b) suggesting need of BAP in this species. Requirement of BAP for somatic embryogenesis is reported in other plant species (Naik et. al., 2009 and Vengadesan et. al., 2005). In the majority of the species studied, in which addition of plant growth regulators is necessary to induce somatic embryogenesis, auxins and cytokinins are key factors in the determination of embryogenic response, probably because they strongly participate in cell division and cell differentiation respectively (Feher et. al., 2003; Gaj, 2004 and Suhasini et. al., 1996).

Maturation and germination of somatic embryos

In the present investigations it was noticed that the frequency of conversion of somatic embryos in to complete plantlets was very low (22%). Low efficiency of embryo maturation and germination and conversion to plantlets is a major problem in many species (Suhasini et. al., 1996 and Vahdati et. al., 2008). Exogenous application of ABA is known to have beneficial effect in maturation and germination of somatic embryos (Vahdati et. al., 2008; Tian et. al., 2000 and Mauri and Manzanera, 2003). We therefore, investigated the effect of ABA in maturation and germination of somatic embryos. In the present investigation attempts were made to convert bi-polar somatic embryos to adventitious shoot on MS medium fortified with various concentrations (0.5, 1.0 & 1.5 mg/l) of ABA. Addition of ABA at low concentrations (0.5 m/l) was found to be effective for the conversion of embryos into adventitious shoots. The frequency of embryo germination in to adventitious shoots was 22% on medium without ABA and AgNO3 (data not shown). Evident increase in frequency up to 43% was observed on medium with BAP (1.0 mg/l), ABA (0.5 mg/l) and AgNO3 (0.5 mg/l) after 6 wks (Table 3). The influence of ABA is well investigated in somatic embryogenesis, is known to be associated with embryo maturation both *in vivo* and *in vitro* (Ammirato, 1983) and also plays a role in nurturing seed maturation reducing secondary embryo formation (Zeevaart and Creelman, 1988) . Earlier reports showed that ABA is beneficial for embryo maturation in oil seed *Brassica campestris* (Finkelstein et. al., 1985 and Angoshtari et. al., 2009). There are several reports suggesting addition of AgNO3 to the culture medium to enhance regeneration efficiency in *Sesamum* (Younghee, 2001; Seo et. al., 2007 and Abdellatef et. al., 2010) and also in few other oil seed crops, *Brassica campestris* (Angoshtari et. al., 2009) and *Helianthus annuus* (Chraibi et. al., 1991). Silver nitrate is considered as an inhibitor of ethylene action in some species such as carrot, soybean and date palm (Roustan et. al., 1990; Al-Khayri and Al-Bahrany 2001).

Well developed shoots at 2-3cm in height were transferred on to hormone free half MS medium. Subsequently additional root induction (40-50%) was observed in 3-4 wks old cultures. Rooted plants were transferred on half strength MS medium 3-4 weeks to achieve hardening. The roots becomes strong and thick on this medium. After hardening the shoots were transferred to pots containing sterile vermiculate and later established in soil in the glass house, where 60% of them survived and resumed growth (Figs. 3 a, b and c).

(a) Adventitious shoots with roots.
(b) Individual seedling.
(c) Potted plantlet.

Figure 3. Regeneration through somatic embryogenesis of *Sesamum indicum* L.

The germinating embryoids possessing bipolar organization were considered for the histological studies. Histological preparations were according to (Johansen, 1940). Histological sections of bipolar embryoids by cotyledons confirmed the induction of the development process was embryogenic and not organogenic in nature. Development of somatic embryos appeared to progress through typical globular, heart, torpedo shaped and Cotyledonary stage embryo development. The first sign of embryogenesis was marked by the appearance of globular structures that were attached to the surface of the explant by a distinct stalk (Fig. 1c). The heart stage embryo (Fig. 1d), which was bilaterally symmetrical also showed a broad suspensor- like stalk. The Cotyledonary stage embryos showed the presence of two prominent cotyledons (Fig. 1e)**.** These histological and morphological observations enabled us to conclude that the stages are consistent with the general definition of somatic embryogenesis (Figs. 2a, b, c and d)**.**

CONCLUSION

In sesame, there is a paucity of information on SE compared to other oil seed crops. Overall the protocol established is better than previously reported in sesame through direct somatic embryogenesis. Induction of direct embryogenesis takes place from Cotyledonary and hypocotyl explants. 2, 4-D is efficient than other auxins tested, addition of BAP at low concentrations enhances the frequency and number of somatic embryos per explant, only torpedo shaped developed into complete plantlets on medium supplemented with ABA. Since the regeneration involves induction of somatic embryos and subsequent regeneration into the plantlets direct (without an intervening callus phase) from the cotyledon explant, this method can be implemented in developing transformation system for the crop which is recalcitrant and challenging.

Abbreviations: 2, 4-D- 2, 4-Dichlorophenoxy acetic acid; 2, 4, 5-T- 2, 4, 5- Trichlorophenoxy acetic acid; Kn- Kinetin; NAA - α-Naphthalene acetic acid; BAP- 6-Benzylaminopurine; ABA- Abscisic acid; AgNO3- Silver nitrate.

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