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Efficient *invitro* plant regeneration from cotyledon-derived callus cultures of sesame (*Sesamum indicum* L.) and genetic analysis of True-to-Type regenerants using RAPD and SSR markers

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ABSTRACT

Sesame (*Sesamum indicum* L.) is an important oilseed crop which is rich in unsaturated fatty acids, proteins and natural antioxidants. Reports on tissue culture methods involving regeneration through callus phase are scanty, genotype dependent and have been reported with low regeneration frequency. This recalcitrant nature will impede the application of modern biotechnological tools for genetic advancement studies in sesame. An efficient protocol for *invitro* shoot organogenesis from callus cultures has been established using cotyledon explants excised from 1-week-old seedlings of sesame cv. TMV 3. Maximum frequency of greenish white friable compact (GWFC) callus (72.0%) was obtained on Murashige and Skoog (MS) medium supplemented with 4.54 μM of 2, 4-dichlorophenoxyacetic acid (2, 4-D). Organogenic GWFC callus cultured on MS medium containing 4.4 μM of 6-benzylaminopurine (BAP) and 10 μM of silver nitrate ($AgNO_3$) showed the optimum frequency of shoot regeneration (66%) and produced 4.6 shoots per 150 mg callus. Optimum rooting of 70.2%, an average of 6.02 ± 0.8 roots per shoot was achieved on MS medium fortified with 5.6 μM of indole-3-acetic acid (IAA). The rooted plantlets were acclimatized successfully in a greenhouse with a 65.8% survival rate. Non significant phenotypic aberrations were observed among the *ex vitro* transferred plantlets. The genetic stability of regenerants was assessed by using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers. PCR amplification using 10 RAPD and 10 SSR primers generated 71 distinct and scorable DNA bands. DNA banding patterns reveal the absence of somaclonal variation among the plantlets regenerated through indirect organogenesis compared to that of mother plant and confirms the genetic purity of the *invitro* raised plants. The developed protocol could be effectively employed for obtaining genetic traits *invitro* through genetic transformation studies.

1. Introduction

Sesame (*Sesamum indicum* L.) is a high value oil seed crop that is widely cultivated in Myanmar, India, China, Sudan and Tanzania. In India, it is cultivated in an area of 1.77 million hectares (next to groundnut and mustard), with an annual production of 0.81 million tons (FAOSTAT 2014). Sesame seed consists of 50 to 60% oil, 13.5% carbohydrates and 20% proteins (Arslan et al., 2007), and are commonly used as ingredients in buns, breads, biscuits and snacks (Pastorello et al., 2001). Sesame oil is a rich source of natural antioxidants like sesamin, sesamolin and sesamol and hence it is considered as the best oil for cooking purposes not just because of its antioxidant activity but also because of its free radical scavenging, antihypertensive, hypolipidemic, neuroprotective, anticarcinogenic and antimutagenic properties (Hou et al., 2003; Noguchi et al., 2004; Williamson et al., 2007).

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 (Rao et al., 2002; Daniel, 2008). 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. Hence, the solution to address these problems is to genetically improve sesame genotypes through genetic transformation techniques. Here again, the main prerequisite for genetic transformation is the availability of highly efficient and reproducible protocols of regeneration. Nevertheless, the main complication in genetic engineering of sesame is recalcitrant nature to regenerate *invitro*(Baskaran and Jayabalan, 2006).

In vitro regeneration system *via* direct shoot organogenesis from cotyledon and/or hypocotyl explants has been reported in *S.indicum*(Rao and Vaidyanath, 1997a; Sharma and Pareek, 1998; Were et al., 2006; Seo et al., 2007; Abdellatef et al., 2010; Chattopadhyaya et al., 2010; Zimik and Arumugam, 2017). Concomitantly, earlier reports regarding shoot regeneration from cotyledon/hypocotyls derived callus culture

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in sesame has been reported with very low regeneration frequencies (Taskin and Turgut, 1997; Rao and Vaidyanath, 1997b; Younghee, 2001; Baskaran and Jayabalan, 2006). Further, Plant regeneration through somatic embryogenesis pathway from zygotic embryo and hypocotyls-derived callus culture has been reported in sesame (Ram et al., 1990; Mary and Jayabalan, 1997; Xu etal. (1997), but conversion rate of somatic embryos into plantlets was not successfully accomplished. However, it has been concluded that the success rate of formerly published regeneration protocols in sesame were found to be low in most cases and were also genotype dependent.

Over the years, it has been reported by many researchers that tissue culture induced stresses like explants type, culture media, concentrations and combinations of plant growth hormones, subculture, light intensity, pH and temperature cause genetic variations among the regenerants (Amberger et al., 1992; Rani and Raina, 2000; Jain, 2001). The occurrence of somaclonal variation in regenerants will limit the commercial exploitation of crop species at larger scale. Therefore, it is crucial to retain the true-to-type nature of the *invitro* raised plantlets with respect to mother plant.

Several strategies were used to evaluate the genetic stability of *invitro* raised plantlets including phenotypic recognition (Kong et al., 2011), cytological analysis (Konieczny et al., 2010) and isoenzyme analysis (Northmore et al., 2011), but they were found to be time consuming and do not yield dependable results. Ahmad and Anis (2011) reported DNA-based marker technologies were used to assess the genomic stability of regenerated plants. These molecular markers are not influenced by environmental factors and hence generate reliable and reproducible results (Bhattacharyya et al., 2014). Among the molecular markers developed, Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) and Inter Simple Sequence Repeat (ISSR) are immensely useful in establishing the genetic stability of *invitro* regenerated plantlets in many crop species (Lakshmanan et al., 2007; Joshi and Dhawan, 2007). Out of several markers studied, SSR markers have gained considerable attention for genetic fidelity assessment because of reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and strong discriminatory power (Bairu et al., 2011). To our knowledge, no scientific reports are available concerning the detection of genetic variation in somatic clones of sesame using RAPD and SSR markers. The main objective of the study was to develop an efficient callus induction and subsequent plantlet regeneration system from cotyledon explants of sesame cv. TMV 3 and analysis of genomic stability of regenerants using RAPD and SSR markers.

2. Materials and methods

2.1. Seed materials and explants preparation

Genetically pure seeds of sesame cv. TMV 3 obtained from Oil seeds Research Station, Tindivanam (Tamil Nadu Agricultural University), Villupuram district, Tamil Nadu, India were used for this experiment. These seeds were multiplied by adopting the agronomic practices as recommended by research institute in the experimental field of Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Tamil Nadu, India. Disease free seeds of sesame cv. TMV 3 were soaked in distilled water for overnight, surface sterilized in 0.1% mercuric chloride for 2 min and rinsed five times with sterile double-distilled water to remove the contaminants. Disinfected seeds were germinated on MS(Murashige and Skoog, 1962) basal medium containing 3% (w/v) sucrose as a carbon source and 4% (w/v) phytagel as gelling agent without plant growth regulators. The seedlings were grown at 25 ± 2 °C under 16-h photoperiod with light provided by white fluorescent lamps at an intensity of 80 μmoL m^{-2} s^{-1}.

2.2. Callus initiation

In the present investigation, cotyledon explants excised from sterilized mature seeds, 1 and 2-weeks-old aseptically grown seedlings were used as explants for regeneration studies. These explants were cut at the proximal end to remove the embryonic axis (de-embryonated cotyledon) before culturing on media. All the de-embryonated cotyledons were cultured in MS basal medium supplemented with either 2, 4-dichlorophenoxyacetic acid (2, 4-D; 2.27, 4.54, 9.08 and 13.62 μM) or 2, 4, 5-trichlorophenoxyacetic acid (2,4,5-T; 2.17, 4.34, 8.68 and 13.02 μM) or indole-3-butyric acid (IBA; 2.5, 5.0, 7.5 and 12.50 μM) or α-naphthalene acetic acid (NAA; 2.63, 5.26, 7.89 and 13.15 μM) or indole-3-acetic acid (IAA; 2.8, 5.6, 8.4 and 14.0 μM) for callus induction. These cultures were subcultured on the same media once in every 15 days and maintained up to 8 weeks. For each treatment, data on frequency of callus initiation and its fresh weight were recorded after eighth week of culture. A total of 10 cotyledons were used for each replication, 3 replications per treatment and experiment was conducted 3 times. Nature of callus was evaluated qualitatively as no callus initiation (N), Greenish White Friable Compact (GWFC) and White Non Friable (WNF).

2.3. Adventitious shoot initiation

Well established GWFC calli (150 mg fresh weight) obtained from callus induction treatments were used for shoot regeneration studies. After 8 weeks, proliferated organogenic GWFC calli were transferred into MS basal medium supplemented with either 6-benzylaminopurine (BAP; 2.2, 4.4, 8.8, 13.24 and 22.0 μM) or Thidiazuron (TDZ; 2.27, 4.54, 9.08, 11.35 and 13.62 μM) or kinetin (2.4, 4.8, 9.6, 14.4 and 24.0 μM) for adventitious shoot initiation. Further, cytokinins such as BAP(4.4 μM), TDZ(2.27 μM) and kinetin (9.6 μM) in combination with NAA(2.63 μM), IBA(2.54 μM) and IAA(2.8 μM) were also studied for adventitious shoot regeneration. The efficiency of adventitious shoot regeneration was investigated by addition of silver nitrate ($AgNO_3$) as an additive to the MS medium containing 4.4 μM BAP with different concentrations of $AgNO_3$ (5, 15, 30, 40 and 50 μM). Cultures were transferred on fresh media after frequent subculturing once in every 2 weeks and maintained for 6 weeks. Observations on the percentage of shoot organogenesis per 150 mg callus piece, average number of shoots per callus and mean shoot length were recorded after 6 weeks. About 150 mg callus was used for each replication, 10 replications per treatment and experiment was conducted 3 times.

2.4. Rooting and hardening of plantlets

Shootlets about 2–3 cm long were excised from shoot clusters and placed on rooting media containing either hormone-freehalf-strengthMS or full strength MS basal medium or MS + IBA (2.5 μM) or MS + IBA (5.0 μM) or MS + IAA (2.8 μM) or MS + IAA (5.6 μM) for 3–4 weeks. The cultures showing root initiation were transferred to fresh media after 2 weeks and maintained up to 4 weeks. After 4 weeks, the rooted shoots were successfully uprooted and washed three times with sterile water to remove the adhering media and basal callus before transferring to a plastic pot containing sterilized sand, soil and clay mixture (1:1:1) for acclimatization. The plastic pots were then covered with polyethylene, and holes were made to facilitate exchange of gases. These potted plants were maintained in the plant growth chamber for 2 weeks and then moved to greenhouse. The plastic covers were removed once the plants get well established in greenhouse and they were maintained until flowering and seed set. The survival rate of plants was counted after one month in greenhouse. For each treatment, data on percentage of rooting response, average number of roots per shoot and mean root length were recorded after fourth week of culture. A total of five adventitious shoots were used for each