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Meta-Topolin (*m*T) enhances the *in vitro* regeneration frequency of *Sesamum indicum* (L.)

Dhandapani Elayaraja [a], Kondeti Subramanyam [b], Venkatachalam Vasudevan [a], Selvam Sathish [a], Sampath Kasthurirengan [c], Andy Ganapathi [d], Markandan Manickavasagam [a,*]

[a] Department of Biotechnology, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India
[b] Department of Plant Sciences, University of Tennessee, Knoxville, TN, USA
[c] Breeding Department, J Oil (S) Pte. Ltd, I-Research Link, Singapore
[d] Bharathiar University, Coimbatore, Tamil Nadu, India

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ABSTRACT

An efficient micropropagation protocol developed for sesame using cotyledonary node explants derived from 7-day-old *in vitro* grown seedlings. The efficacy of *meta*-Topolin (*m*T) was evaluated during different stages of regeneration. Multiple shoots were initiated and proliferated on Murashige and Skoog (MS) medium supplemented with various concentrations of *m*T and N^6–Benzyladenine (BA), individually. Multiple shoots induced in the medium containing *m*T, responded well for shoot elongation, rooting and *in vitro* acclimatization. *m*T at a concentration of 6.21 μM in MS medium induced maximum number of shoots (23.36 shoots per explant) from 90.66% of cotyledonary node explants. About 73.33% of shoots induced on medium containing *m*T were elongated (5.93 cm per shoot) in liquid MS medium fortified with 5.77 μM GA_3, and 95.66% of them developed profuse roots (8.62 roots per shoot; 13.82 cm in length) in liquid MS medium containing 2.46 μM IBA. All the plantlets with roots were hardened and successfully acclimatized in the greenhouse with a survival rate of 94.33%. Multiple shoots obtained from the cotyledonary node in the medium containing BA showed significantly higher H_2O_2 content, enhanced antioxidant enzyme (SOD, APX, and CAT) activities, and lower chlorophyll than for the shoots derived from the medium containing *m*T. SCoT and RAPD markers revealed that all the regenerated plants from the medium amended with either BA or *m*T showed similar banding pattern to that of the *in vivo* grown plant, thus confirming the genetic stability of regenerated sesame plants.

1. Introduction

Sesame (*Sesamum indicum* L.) belonging to the family of Pedaliaceae is one of the economically important oilseed crops widely cultivated in tropical and subtropical regions. Sesame seeds contain 25% protein and 50% oil. Its, oil is rich in vitamin E and lignins such as sesamin, sesamolin, and sesaminol (Brar and Ahuja, 1979). These compounds play an important role as antioxidants, and hence, sesame oil is widely used in treating several human diseases (Anil Kumar et al., 2010). Natural antioxidants present in sesame oil and the nutritional benefits of sesame seeds promote the use and cultivation of sesame. Sesame cultivation is highly vulnerable to a wide range of abiotic and biotic stresses, which affects growth and production. Classical breeding play an important role in developing superior sesame cultivars. However, sexual incompatibilities and difficulty in obtaining fertile progenies hamper the success rates of breeding. In addition, breeding requires a lot of time to generate a variety/cultivar. Such difficulties can be overcome by developing successful genetic transformation techniques. Nevertheless, the success of genetic transformation of sesame is limited due to its recalcitrance nature towards *in vitro* regeneration. To date, few regeneration protocols have been established for sesame using various explants such as cotyledonary node (Yadav et al., 2010), cotyledon (Yadav et al., 2010; Seo et al., 2007; Rao and Vaidyanath, 1997a; Taskin and Turgut, 1997), hypocotyls (Karimi et al., 2013), and shoot tip (Rao and Vidyanath, 1997b; George et al., 1987). However, these regeneration protocols are time-consuming and not very efficient for a high frequency of shoot induction, multiplication and rooting, which in turn decrease the transformation efficiency. These problems could address by optimizing the *in vitro* regeneration medium with an efficient and optimum concentration of plant growth regulators (PGRs). To date, a wide range

* Corresponding author.Tel number:
E-mail address: manickbiotech@gmail.com (M. Manickavasagam).

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of cytokinins and auxins either individually or in combination at different concentrations were used for shoot induction and multiplication of sesame and among these, cytokinins played a significant role (Karimi et al., 2013; Yadav et al., 2010; Younghee, 2001). It has been reported that the shoots induced in the medium containing synthetic cytokinin such as BA have the likelihood of accumulating toxic BA metabolites (Aremu et al., 2012). Consequently, these metabolites interfere with shoot development, rooting and *in vitro* acclimatization of micropropagated plantlets (Aremu et al., 2012). However, naturally occurring cytokinins such as *meta*-Topolin (*m*T) is less stable and produce reversibly sequestrated metabolites, which play an important role in delaying senescence, increasing photosynthetic pigments, modulating the antioxidant enzyme activities, and thus improve the root and shoot development (Chauhan and Taylor, 2018; Aremu et al., 2012; Werbrouck et al., 1996). *m*T has been successfully used in various phases of regeneration process such as promoting shoot multiplication in *Ananas comosus* (Teklehaymanot et al., 2010) and *Aloe polyphylla* (Bairu et al., 2007), rooting in *Aloe polyphylla* (Bairu et al., 2007) and *Malus domestica* (Magyar-Tábori et al., 2001), and acclimatization in *Uniola paniculata* (Valero-Aracama et al., 2010) and *Aloe polyphylla* (Bairu et al., 2007). However, there has been no report describing the application of *m*T in *in vitro* shoot induction, proliferation, and rooting of sesame.

Micropropagation and development of plantlets from explant involve distinct stages and each stage requires several days to weeks. At each stage, the cultures are exposed to mechanical wounding, different chemicals, plant growth regulators (PGRs), temperature, and humidity. These conditions are very stressful to the cultures and result in the production of reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$) radicals, hydrogen peroxide (H_2O_2), and hydroxyl ($\cdot HO$) radicals. At low concentrations, ROS activate signal pathways and show adaptive responses against adverse conditions, while at high concentration, ROS induce oxidative stress and cause damage to macromolecules such as membrane lipids, nucleic acids and protein (Subramanyam et al., 2012). Over the decades, amino acids, phenolics, ascorbic acid, citric acid, and polyvinylpyrrolidone have been used as antioxidants in plant tissue culture medium to avoid the adverse effects of ROS. Cytokinins are generally considered as PGRs, as they have been found to be involved in cell division, cell growth, and differentiation. Recent reports indicate that inclusion of cytokinins such as BA and *m*T in the tissue culture medium enhanced the photosynthetic pigments and detoxified ROS by modulating the activities of antioxidant enzymes in several crops including, *Corylus colurna, Merwilla plumbea*, and *Nicotiana tabaccum* (Gentile et al., 2017; Amoo et al., 2015; Petit-Paly et al., 1999).

Micropropagation may trigger somaclonal variations. Hence, it is essential to check the genetic identity of micropropagated plants for their commercial utilization. Over the decades, several molecular markers are used to assess the genetic stability of several economically important species. Among the several molecular markers, random amplified polymorphic DNA (RAPD) and start codon targeted (SCoT) markers are widely used to assess the genetic fidelity of several micropropagated plant species. Each molecular marker has its own advantages and disadvantages in evaluating the genetic fidelity. Hence, it is advisable to use more than one molecular marker to assess the genetic stability of micropropagated plants (Vasudevan et al., 2017). RAPD is a simple, rapid, inexpensive and widely used method to identify the genetic fidelity of diverse plant species including, *Jatropha curcas* (Jaganath et al., 2014), *Ocimum basilicum* (Saha et al., 2014) and eggplant (Mallaya and Ravishankar, 2013). However, reproducibility of RAPD markers over other DNA based markers is low. Start codon targeted (SCoT) markers are novel, simple, reproducible and gene-targeted DNA markers based on the short conserved region flanking the ATG translation start codon in plant genes (Collard and Mackill, 2009). SCoT markers were successfully used to analyze the genetic fidelity of micropropagated plants of *Abutilon indicum* (Seth et al., 2017), *Albizia julibrissin* (Rahmani et al., 2015) and *Cleome gynandra* (Rathore et al., 2014).

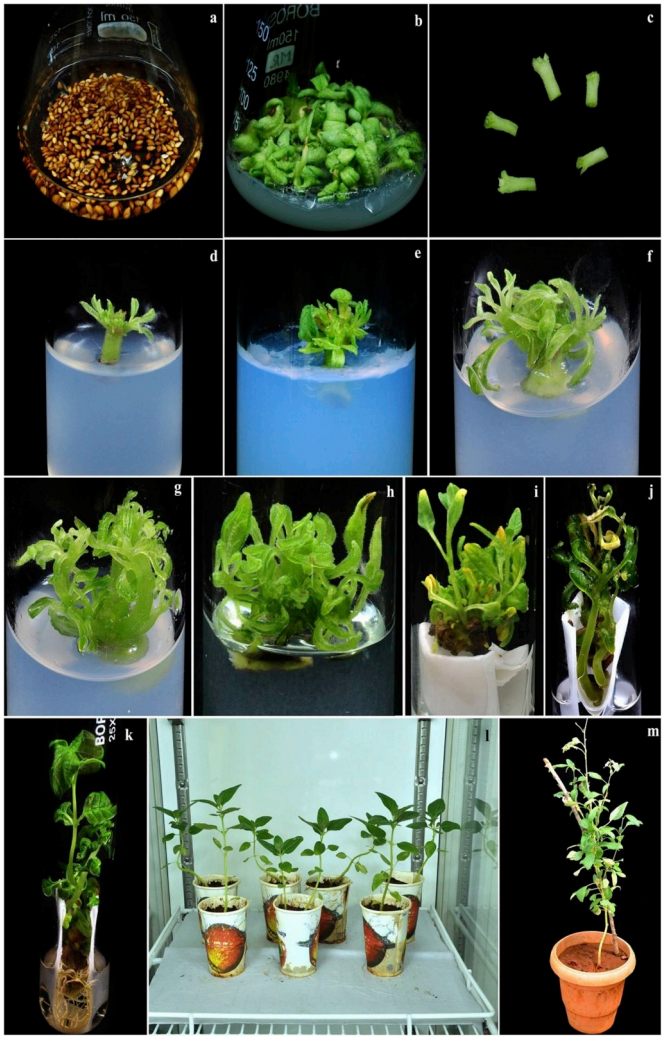

Fig. 1. Influence of *meta*-Topolin (*m*T) treatment on direct regeneration using cotyledonary node explants of sesame cv. TMV–3. **a** surface sterilized seeds; **b** seven-day-old *in vitro* grown seedlings; **c** cotyledonary node explants prepared from *in vitro* grown seedlings; **d-f** initiation of shoots from cotyledonary node on SIPM containing 6.21 μM *m*T after 1, 2, and 3 weeks of initial culture, respectively; **g** proliferation of shoots on SIPM containing 13.32 μM BA after 4 weeks of initial culture; **h** proliferation of shoots on SIPM containing 6.21 μM *m*T after 4 weeks of initial culture; **i** elongated shoots in SEM containing 5.77 μM GA_3 after 3 weeks of culture (shoots were obtained from the SIPM containing 6.21 μM *m*T); **j** elongated shoots in SEM containing 5.77 μM GA_3 after 3 weeks of culture (shoots were obtained from the SIPM containing 13.32 μM BA); **k** rooted shoot in RM containing 2.46 μM IBA after 3 weeks of culture; **l** hardened rooted plantlet in the growth chamber; **m** acclimatized plant in the greenhouse.

Based on the above-said facts, the present investigation has been carried out with an objective of evaluating the efficacy of *m*T along with other PGRs in high-frequency shoot induction, multiplication, and rooting using cotyledonary node as an explant. In addition, we also evaluated the genetic fidelity and the accumulation of chlorophyll, H_2O_2, and antioxidant enzyme activities in the clonally propagated plants.

2. Materials and methods

2.1. Seed source and surface sterilization

Dried seeds of sesame (*Sesamum indicum* L. cv. TMV–3) were obtained from the Regional Research Station, Vridhachalam, Tamil Nadu, India. Healthy seeds were hand-picked and surface-sterilized with 70%

ethanol for 1 min, followed by 0.1% mercuric chloride for 10 min. Finally, the seeds were washed with sterile double-distilled water several times to remove the surfactants. All PGRs used in this study except for *m*T were obtained from Sigma, St. Louis, USA.

2.2. Seed germination and explant preparation

Surface-sterilized seeds (Fig. 1a) were inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing various concentrations (0.22, 0.45, 0.67, or 0.90 μM) of thidiazuron (TDZ) and incubated at 25 ± 2 °C under a 16 h photoperiod. Seven-day-old *in vitro* grown seedlings were used to prepare cotyledonary node explants measuring 5 mm in length (Fig. 1c) by eliminating primary shoot, hypocotyls, and cotyledons.

2.3. Optimization of shoot induction and proliferation

Cotyledonary node explants (prepared from seedlings germinated in the medium supplemented with 0.45 μM TDZ) were inoculated vertically with the shoot apical region facing up on shoot induction and proliferation medium (SIPM) made of MS agar medium supplemented with various concentrations of N^6–Benzyladenine [BA (2.2–22.2 μM)] or *meta*-Topolin [*m*T (2.07–12.42 μM)] (Himedia, Mumbai, India). After 2-weeks of initial culture, the cotyledonary node explants were subcultured onto fresh SIPM containing the same concentration of PGRs and incubated additional 2-weeks for shoot multiplication. Cotyledonary node explants cultured on hormone-free MS agar medium maintained as a control. All the cultures were incubated at 25 ± 2 °C under a 16 h photoperiod with 30 μmol m^{-2} s^{-1} light intensity.

2.4. Optimization of shoot elongation and rooting

Multiple shoots induced from the cotyledonary node on SIPM containing the optimum concentration of BA (13.32 μM) or *m*T (6.21 μM) were selected for shoot elongation and rooting. Cotyledonary node was separated from the base of multiple shoot clumps and discarded. Multiple shoot clump was dissected into several pieces measuring 4–6 mm in length and inoculated into liquid shoot elongation medium (SEM) made of MS liquid medium with various concentrations of gibberellic acid [GA_3 (1.44–11.54 μM)] or kinetin [KT (2.32–18.58 μM)] for shoot elongation. After 3-weeks, the individual elongated shoots (4–6 cm in length) were excised and inoculated into liquid rooting medium (RM) made of MS liquid medium supplemented with different concentrations of indole-3-acetic acid [IAA (0.57–5.13 μM)], indole-3-butyric acid [IBA (0.49–4.42 μM)] or naphthaleneacetic acid [NAA (0.53–4.83 μM)] for rooting. Multiple shoots and elongated shoots cultured in hormone-free liquid MS medium maintained as controls for shoot elongation and rooting, respectively. The liquid medium based cultures were incubated statically at 25 ± 2 °C under a 16 h photoperiod with 30 μmol m^{-2} s^{-1} light intensity.

2.5. Acclimatization

After 3-weeks, well-rooted plantlets were carefully separated from the RM, rinsed thoroughly with tap water to get rid of the adhered culture medium and then transplanted into paper cups filled with a mix of autoclaved soil, sand, and vermiculite (3:1:1v/v/v). The plantlets were hardened and acclimatized in the growth chamber and greenhouse (25 ± 2 °C, 80% relative humidity, and 16 h photoperiod), respectively. Percentage of survival of plantlets recorded after 4-weeks of transfer to the greenhouse.

2.6. Biochemical analysis

After 2-weeks of inoculation onto the SIPM medium containing 13.32 μM BA or 6.21 μM *m*T, cotyledonary node explants were subcultured onto the fresh SIPM medium containing respective PGRs. After 5 days of subculture, the cotyledonary node with multiple shoots were harvested for biochemical analysis. The shoots initiated from the cotyledonary node in SIPM lacking PGR were also subcultured into fresh SIPM medium (without PGR) and used as a control. The plant material was finely ground into powder using liquid nitrogen. Half a gram of plant powder was homogenized with 1 ml of ice-cold extraction buffer [50 mM potassium phosphate buffer (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β-mercaptoethanol, 2% polyvinylpyrrolidone (PVP), and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was centrifuged at 13,000 rpm at 4 °C for 20 min, following the retrieved supernatant was subjected to enzyme assays.

Superoxide dismutase [SOD (EC: 1.15.1.1)] activity was measured following the method of McCord and Fridovich, (1969). The final reaction contained 13 mM methionine, 50 mM sodium phosphate buffer (pH 7.8), 75 μM nitro blue tetrazolium (NBT), 33 μM riboflavin, 0.1 mM EDTA and 0.1 ml plant extract. Photoreduction of NBT at 560 nm, was monitored to calculate the SOD activity. Quantity of enzyme reducing 50% of the absorbance as compared to non-enzymatic control mixture considered as one unit and expressed the enzyme activity as Unit mg^{-1} protein min^{-1}.

Ascorbate peroxidase [APX (EC: 1.11.1.11)] activity was assayed following the method described by Chen and Asada (1989). The reaction mixture was made up of 0.5 mM ascorbic acid, 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 2 mM H_2O_2 and 0.1 ml plant extract. APX activity was calculated based on the reduction of absorbance for 1 min, at 290 nm using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed the enzyme activity as μmol ascorbate oxidized mg^{-1} protein min^{-1}.

Catalase [CAT (EC: 1.11.1.6)] activity was quantified according to the method described by Aebi et al. (1984) by monitoring the reduction in absorbance at 240 nm for 1 min. The reaction mixture consisted of 0.1 ml plant extract, 10 mM potassium phosphate buffer (pH 7.0) and 0.04% H_2O_2. Catalase activity was quantified using the extinction coefficient of 39.4 M^{-1} cm^{-1} and expressed the enzyme activity as μmol H_2O_2 reduced mg^{-1} protein min^{-1}.

H_2O_2 concentration was measured according to the method described by Velikova et al. (2000). Finely powdered leaf material (0.2 g) was homogenized in 800 μl of 0.1% (w/v) trichloroacetic acid (TCA) on ice. The reaction mixture consisted of 60 μl plant extract, 60 μl 1 M potassium iodide and 60 μl 10 mM potassium phosphate buffer (pH 7.0). The absorbance of the reaction measured at 390 nm, the H_2O_2 concentration was quantified using the standard curve and expressed the concentration as μmol g^{-1} of FW.

Total chlorophyll concentration was estimated spectrophotometrically according to the method described by Arnon (1949). Fresh leaves (0.2 g) were taken and ground to a fine powder in liquid nitrogen and then suspended in 10 ml of 80% acetone. Homogenate was then centrifuged for 10 min at 6000 rpm and then supernatant was collected. Measured the optical density of the filtrate at 645 nm and 663 nm using 80% acetone as a blank. Total chlorophyll content was quantified and expressed the concentration as mg g^{-1} of Fw.

2.7. Genetic fidelity analysis

Genetic fidelity of *in vitro* raised plants was evaluated using random amplified polymorphic DNA (RAPD) and start codon targeted (SCoT) markers. For this, genomic DNA was isolated from 8 randomly selected *in vitro* regenerated plants (obtained from the multiple shoots initiated on SIPM medium containing 13.32 μM BA or 6.21 μM *m*T) and *in vivo* plant raised from the seed, using GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). Ten RAPD and SCoT primers (Operon Technologies Inc., Germany) were used for analyzing the genetic fidelity of the micropropagated plants. The RAPD and SCoT-polymerase chain reactions (PCR) were carried out in a final reaction

volume of 25 µl containing 50 ng genomic DNA, 0.25 mM dNTP's, 0.4 µM of RAPD or SCoT primer, 1 U of Taq DNA polymerase (Sigma Genosys, Texas, USA) and 1X Taq buffer. PCR amplifications were performed in a PTC100™ thermal cycler (MJ Research Inc., Waltham, MA, USA), which was programmed for initial DNA denaturation at 95 °C for 10 min, and 30 cycles of 1 min denaturation at 95 °C, followed by 1 min annealing at 37 °C (55 °C for ScoT) and an extension at 72 °C for 2 min, with a final extension for 10 min at 72 °C. The amplicons were then analyzed by electrophoresis on a 1.0% agarose gel and photographs were taken by the gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA). Each PCR amplification was repeated three times, and the primers which showed consistent amplification at all three repetitions were selected to analyze the genetic fidelity. Consistent, well-resolved DNA fragments scored manually. The bands were scored on the basis of their presence ('1') or absence ('0') in the gel. Based on the obtained data, Jaccard's coefficient used to construct the similarity matrix, which then subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and the dendrogram was conjured with the help of NTSYS-pc version 2.02 software (Rohlf, 2000).

2.8. Statistical analysis

All the above-mentioned treatments were carried out in triplicates, with 50 explants per treatment. The data has been evaluated using one-way ANOVA, and the differences were contrasted using Duncan's multiple range test (DMRT). SPSS 20 software (SPSS Inc, USA) for Windows 7.0 was used to carry out the statistical analyses at the level of P value less than 0.05.

3. Results and discussion

3.1. Seed germination

There was no significant difference observed in the germination percentage (78.33%) between the seeds cultured on either hormone free or TDZ supplemented MS agar medium. Seedlings from the hormone-free medium had thin and long hypocotyls, profuse roots, and inconspicuous auxillary buds, while the seedlings from TDZ supplemented medium had thick and short hypocotyls with enlarged cotyledons and expanded auxillary buds. The auxillary buds from the seedlings that grew on 0.45 µM TDZ supplemented medium were bigger than those of the seedlings that grew on medium containing other concentrations of TDZ. It has been reported that the cotyledonary node with well-developed auxillary buds is essential for the development of multiple shoots. Shan et al. (2005) reported that the cotyledonary node explants prepared from 7-day-old soybean seedlings derived from 0.1 mg l^{-1} TDZ supplemented medium responded better than TDZ free medium for multiple bud tissue formation. Similarly, the explants prepared from chickpea, lentil, and pea seedlings grew on 1–50 µM TDZ supplemented medium responded better for multiple shoot induction and secondary shoot formation (Malik and Saxena, 1992). Further, Malik and Saxena (1992) reported that TDZ enhances the organogenesis, possibly by regulating the synthesis and release of endogenous growth regulators such as IAA or in combination with other metabolic changes, particularly in key regulatory enzymes and related proteins.

3.2. Multiple shoot induction and proliferation

Cotyledonary node explants (Fig. 1c) cultured on hormone-free SIPM did not respond well for the shoot induction. However, the cotyledonary nodes cultured on SIPM containing BA or *m*T started producing shoots after a week of culture (Fig. 1d–f), and they proliferated within 4-weeks upon subculturing onto the fresh SIPM containing BA or *m*T (Fig. 1g and h). The percentage of response for shoot induction and shoot number gradually increased with increasing concentration of BA or *m*T up to 13.32 µM or 6.21 µM, respectively and thereafter, decreased with further increase in BA or *m*T concentration (Table 1). At an optimum concentration of BA (13.32 µM), 68.33% of cotyledonary node explants responded for shoot induction and produced 17.13 shoots per explant. Whereas, explants from 6.21 µM *m*T supplemented medium induced 23.36 shoots per explant with 90.66% response (Table 1). Till date, cytokinin (BA or TDZ) alone or in combination with auxin (NAA or IAA) used for the shoot induction from different explants of sesame (Karimi et al., 2013; Raja and Jayabalan, 2011; Yadav et al., 2010; Seo et al., 2007; Baskaran and Jayabalan, 2006; Were et al., 2006). However, in those reports, the percentage of response to shoot induction and number of shoots per explant was relatively less as compared to the *m*T used in the present study.

Bairu et al. (2008) reported the influence of BA and *m*T in the micropropagation of two important banana cultivars (Williams and Grand Naine). They observed a high frequency of shoot induction and a higher number of shoots from pseudostem explants cultured on *m*T (7.5, 15, and 30 µM) containing medium than for medium supplemented with BA. Further, they also noticed a higher number of abnormal shoots with BA treatment. Similarly, Ahmad and Anis (2019) and Nutan Singh and Suman Kumaria (2019) also observed the dominance of *m*T over BA in shoot bud induction and proliferation. Vijayakumar et al. (2017) evaluated the influence of different cytokinins (BA, Zeatin, kinetin, TDZ, and *m*T) individually on micropropagation and organogenesis of safflower and concluded that compared to other cytokinins tested, *m*T at a concentration of 3.5 mg l^{-1} induced higher number shoots from shoot-tip and nodal explants. The superiority of *m*T over BA was reported in *Malus domestica* (Dobrànszki et al., 2002), and *Beta vulgaris* (Kubalàkova and Strnad, 1992). In contrast, Gomez-Leyva et al. (2008) and Doil et al. (2008) reported that BA was more efficient than *m*T in shoot induction and multiplication of *Hibiscus sabdariffa* and *Hydrangea macrophylla*, respectively. Similar to these reports, we also observed a high frequency of shoot induction and a maximum number of shoots from cotyledonary node explants cultured on 6.21 µM *m*T compare to the other concentrations and PGRs tested.

In the current study, the shoots induced from the cotyledonary node cultured in SIPM containing BA showed hyperhydricity (Fig. 1g), while

Table 1
Effect of BA and *m*T on multiple shoot induction and proliferation from cotyledonary node explants derived from 7-day-old *in vitro* grown seedlings of *sesame* cv. TMV–3 on shoot induction and proliferation medium (SIPM) after 2 and 4-weeks of culture.

Plant growth regulators	Concentration (µM)	Percentage (%) of explants responding for shoot induction	Mean number of shoots per explant	
			After 2-weeks of culture	After 4-weeks of culture
Control	0.00	08.33 ± 0.43^{m}	2.62 ± 0.10^{j}	2.66 ± 0.10^{j}
BA	2.22	22.66 ± 0.52^{l}	4.32 ± 0.12^{i}	6.65 ± 0.08^{i}
	4.44	35.33 ± 0.34^{i}	6.16 ± 0.21^{g}	10.21 ± 0.12^{f}
	8.88	51.00 ± 0.41^{f}	9.42 ± 0.18^{e}	14.32 ± 0.06^{d}
	13.32	68.33 ± 0.32^{c}	12.32 ± 0.22^{c}	17.13 ± 0.10^{c}
	17.76	53.66 ± 0.66^{e}	7.00 ± 0.15^{f}	12.42 ± 0.21^{e}
	22.20	29.00 ± 0.51^{k}	5.24 ± 0.08^{h}	8.23 ± 0.06^{h}
*m*T	2.07	48.66 ± 0.32^{h}	6.21 ± 0.10^{g}	9.43 ± 0.04^{g}
	4.14	59.00 ± 0.21^{d}	12.00 ± 0.13^{c}	16.88 ± 0.09^{c}
	6.21	90.66 ± 0.65^{a}	16.42 ± 0.21^{a}	23.36 ± 0.11^{a}
	8.28	73.33 ± 0.27^{b}	13.23 ± 0.18^{b}	18.46 ± 0.04^{b}
	10.35	50.66 ± 0.31^{g}	10.43 ± 0.09^{d}	14.22 ± 0.10^{d}
	12.42	31.00 ± 0.18^{j}	4.38 ± 0.02^{i}	6.34 ± 0.08^{i}

For each treatment, 50 cotyledonary node explants were used and each treatment was repeated thrice. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level. Control: Treatment without PGRs.

Table 2
Effect of GA_3 on elongation of shoots obtained from the cotyledonary node explants cultured on SPIM containing 13.32 μM BA and 6.21 μM *m*T individually.

Plant growth regulator	Concentration (μM)	Percentage (%) of explants responding for shoot elongation		Mean number of elongated shoots per explant		Mean shoot length (cm)	
		Explant with shoots obtained from SIPM containing 13.32 μM BA	Explant with shoots obtained from SIPM containing 6.21 μM *m*T	Explant with shoots obtained from SIPM containing 13.32 μM BA	Explant with shoots obtained from SIPM containing 6.21 μM *m*T	Explant with shoots obtained from SIPM containing 13.32 μM BA	Explant with shoots obtained from SIPM containing 6.21 μM *m*T
Control	0.00	6.33 ± 0.06^{f}	9.33 ± 0.04^{f}	3.42 ± 0.03^{e}	5.32 ± 0.05^{f}	2.78 ± 0.04^{d}	3.03 ± 0.05^{d}
GA_3	1.44	28.66 ± 0.13^{e}	38.33 ± 0.10^{e}	5.34 ± 0.06^{d}	8.32 ± 0.02^{e}	3.00 ± 0.13^{c}	3.55 ± 0.11^{c}
	2.88	43.00 ± 0.18^{c}	52.66 ± 0.11^{c}	7.22 ± 0.11^{c}	11.45 ± 0.10^{c}	3.45 ± 0.13^{b}	4.29 ± 0.09^{b}
	5.77	60.66 ± 0.09^{a}	73.33 ± 0.08^{a}	9.42 ± 0.09^{a}	14.32 ± 0.12^{a}	4.22 ± 0.12^{a}	5.93 ± 0.10^{a}
	8.66	51.33 ± 0.06^{b}	64.00 ± 0.13^{b}	8.00 ± 0.12^{b}	12.11 ± 0.08^{b}	3.78 ± 0.14^{b}	4.68 ± 0.08^{b}
	11.54	35.00 ± 0.09^{d}	44.66 ± 0.08^{d}	5.65 ± 0.08^{d}	9.54 ± 0.06^{d}	3.22 ± 0.08^{c}	3.98 ± 0.07^{c}

For each treatment, 50 cotyledonary node explants with multiple shoots were used and each treatment was repeated thrice. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level. Control: Treatment without PGRs.

the shoots developed in SIPM containing *m*T were healthy and green in color without hyperhydricity symptoms (Fig. 1h). Hyperhydricity is a morphological and physiological problem commonly occurring in the micropropagation due to either passive diffusion of water into tissues or metabolic disturbances (Pâques, 1991). Bairu et al. (2007) observed hyperhydricity in *Aloe polyphylla* shoots developed in a medium containing higher concentrations of cytokinins (BA, Zeatin or *m*T). However, at low concentrations (less than 7.5 μM), *m*T favored the development of good quality shoots with a high frequency of shoot multiplication, while at the same concentrations, BA and Zeatin induced hyperhydricity with reduced frequency of shoot regeneration. The importance of *m*T in controlling the hyperhydricity, necrosis, and delayed senescence was also reported by Mala et al. (2013) and Dobrànszki et al. (2002).

3.3. Shoot elongation

Shoot elongation is a crucial step in the regeneration process of a plant. In the present study, multiple shoots developed from the cotyledonary node inoculated into the SEM containing various concentrations of GA_3 and KT individually. The obtained results proved that the GA_3 is better than KT for shoot elongation. This is in agreement with the previous report where supplementation of the medium with low concentration of GA_3 favored the shoot elongation in sesame (Pratik et al., 2016). Multiple shoots derived from the cotyledonary node cultured on SIPM containing 6.21 μM *m*T responded better for shoot elongation than the multiple shoots derived from the explants cultured on medium containing 13.32 μM BA (Table 2). The percentage of response, the mean number of elongated shoots, and the shoot length gradually increased with increasing the concentration of GA_3 up to 5.77 μM and thereafter decreased with further increase in GA_3 concentration (Table 2). At an optimum concentration (5.77 μM) of GA_3, 73.33% of explants derived from the SIPM containing 6.21 μM *m*T responded for the shoot elongation and produced 14.32 elongated shoots per explant measuring an average shoot length of 5.93 cm (Table 2). Conversely, at 13.93 μM (optimum concentration) of KT, 61.33% of explants derived from the SIPM containing 6.21 μM *m*T responded for the shoot elongation and produced 13.32 elongated shoots per explant measuring an average

Table 3
Effect of IAA, IBA, and NAA on rooting of elongated shoots obtained from the cotyledonary node explants cultured on SPIM containing 13.32 μM BA and 6.21 μM *m*T individually.

Plant growth regulators	Concentration (μM)	Percentage (%) of shoots responding for rooting		Mean number of roots per shoot		Mean root length (cm)	
		Rooting of shoots obtained from SIPM containing 13.32 μM BA	Rooting of shoots obtained from SIPM containing 6.21 μM *m*T	Rooting of shoots obtained from SIPM containing 13.32 μM BA	Rooting of shoots obtained from SIPM containing 6.21 μM *m*T	Rooting of shoots obtained from SIPM containing 13.32 μM BA	Rooting of shoots obtained from SIPM containing 6.21 μM *m*T
Control	0.00	10.33 ± 0.39^{n}	$15.66 \pm 0.43^{\circ}$	1.23 ± 0.06^{p}	2.63 ± 0.04^{l}	2.82 ± 0.08^{n}	$0.54 \pm 0.06^{\circ}$
IAA	0.57	18.00 ± 0.66^{l}	25.33 ± 0.34^{m}	1.53 ± 0.09^{n}	3.32 ± 0.03^{i}	3.21 ± 0.03^{l}	4.54 ± 0.04^{m}
	1.71	28.66 ± 0.56^{j}	38.66 ± 0.28^{l}	2.04 ± 0.05^{l}	4.05 ± 0.06^{g}	3.98 ± 0.06^{j}	5.34 ± 0.08^{l}
	2.85	44.66 ± 0.43^{g}	49.33 ± 0.56^{i}	2.88 ± 0.05^{g}	5.07 ± 0.02^{e}	4.98 ± 0.03^{g}	7.88 ± 0.06^{g}
	3.99	67.33 ± 0.65^{b}	74.00 ± 0.48^{d}	3.89 ± 0.10^{b}	6.43 ± 0.04^{b}	6.34 ± 0.05^{b}	9.65 ± 0.04^{e}
	5.13	55.00 ± 036^{d}	62.33 ± 0.38^{g}	3.12 ± 0.08^{e}	5.88 ± 0.07^{c}	5.23 ± 0.02^{f}	8.21 ± 0.03^{f}
IBA	0.49	22.33 ± 0.12^{k}	48.00 ± 0.43^{j}	1.88 ± 0.03^{m}	3.22 ± 0.04^{j}	3.52 ± 0.08^{k}	7.62 ± 0.04^{h}
	1.47	32.66 ± 0.22^{i}	72.33 ± 0.28^{e}	2.89 ± 0.03^{g}	5.34 ± 0.03^{d}	5.64 ± 0.08^{d}	10.24 ± 0.08^{d}
	2.46	76.00 ± 0.34^{a}	95.66 ± 0.36^{a}	4.72 ± 0.05^{a}	8.62 ± 0.06^{a}	7.54 ± 0.12^{a}	13.82 ± 0.14^{a}
	3.44	59.33 ± 0.46^{c}	82.33 ± 0.54^{b}	3.69 ± 0.02^{c}	6.43 ± 0.02^{b}	6.32 ± 0.06^{b}	11.65 ± 0.05^{b}
	4.42	48.66 ± 0.54^{f}	75.33 ± 0.59^{c}	3.22 ± 0.02^{d}	6.00 ± 0.08^{c}	6.00 ± 0.05^{c}	10.89 ± 0.08^{c}
NAA	0.53	16.33 ± 0.47^{m}	20.33 ± 0.34^{n}	$1.38 \pm 0.01^{\circ}$	2.98 ± 0.03^{k}	3.02 ± 0.02^{m}	4.01 ± 0.03^{n}
	1.61	32.66 ± 0.34^{i}	38.33 ± 0.22^{l}	2.22 ± 0.05^{k}	3.88 ± 0.02^{h}	4.43 ± 0.05^{i}	5.68 ± 0.05^{k}
	2.68	52.33 ± 0.58^{e}	67.66 ± 0.54^{f}	3.02 ± 0.05^{f}	5.34 ± 0.05^{d}	5.43 ± 0.04^{e}	7.87 ± 0.03^{g}
	3.75	45.00 ± 0.43^{g}	52.00 ± 0.45^{h}	2.76 ± 0.02^{h}	4.41 ± 0.03^{f}	4.83 ± 0.02^{h}	6.62 ± 0.06^{i}
	4.83	38.33 ± 0.27^{h}	42.33 ± 0.32^{k}	2.43 ± 0.03^{j}	4.00 ± 0.02^{g}	4.02 ± 0.04^{j}	6.12 ± 0.04^{j}

For each treatment, 50 elongated shoots (4–6 cm in length) were used and each treatment was repeated thrice. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level. Control: Treatment without PGRs.

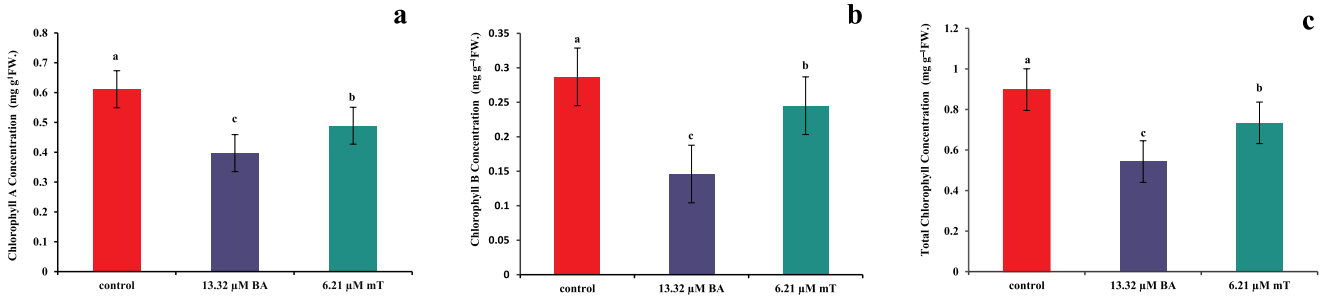

Fig. 2. Effects of 13.32 µM N^6–Benzyladenine (BA) and 6.21 µM *meta*-Topolin (*m*T) on the concentration of chlorophyll. **a** chlorophyll *a* concentration (mg g^{-1} FW.); **b** chlorophyll *b* concentration (mg g^{-1} FW.); c total chlorophyll concentration (mg g^{-1} FW.). Bars represent the mean values of three independent experiments with standard errors. Means followed by different letters are significantly different according Duncan's multiple range test (DMRT) at a 5% level.

shoot length of 4.56 cm. Similar to *Prunus* species, *Aloe polyphylla*, *Malus domesticam,* and *Beta vulgaris* (Gentile et al., 2014; Bairu et al., 2007; Dobrànszki et al., 2002; Kubalàkova and Strnad, 1992), in sesame, shoots induced in the *m*T containing medium and elongated in GA_3 containing medium were well developed, healthy, thick, and green in colour (Fig. 1i) than the shoots induced in the medium containing BA (Fig. 1j). There might be negative carryover effects on shoot elongation following shoot multiplication on BA supplemented medium. In contrary, shoots of *Prunus microcarpa* (Nas et al., 2010) and *Hibiscus sabdariffa* (Gomez-Leyva et al., 2008) initiated in the medium containing BA were able to regenerate better than the shoots initiated in the medium containing *m*T. This indicates that the regeneration response to PGRs depends on the plant species and explant used for the regeneration. Conversely, Mala et al. (2009) reported that none of the cytokinins (BA, *m*T, and MeOBAPR) used to initiate the shoots influenced the shoot regeneration process in *Sorbus torminalis*.

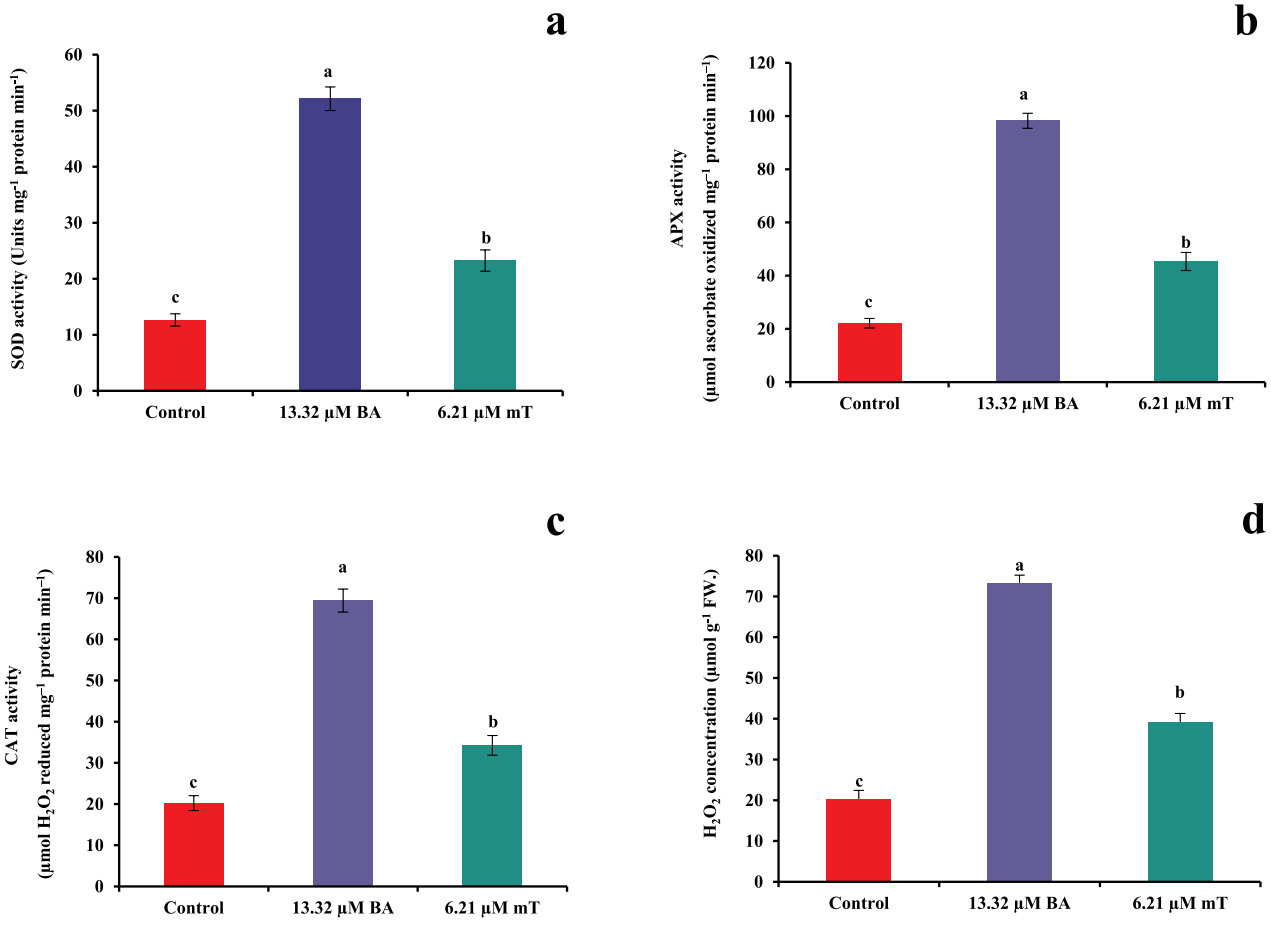

Fig. 3. Effects of 13.32 µM N^6–Benzyladenine (BA) and 6.21 µM *meta*-Topolin (*m*T) on antioxidant enzyme activities and the concentration of hydrogen peroxide (H_2O_2). **a** superoxide dismutase activity (Units mg^{-1} protein min^{-1}); **b** ascorbate peroxidase activity (nmol ascorbate oxidized mg^{-1} protein min^{-1}); **c** catalase activity (nmol H_2O_2 reduced mg^{-1} protein min^{-1}); **d** hydrogen peroxide concentration (µmol g^{-1} FW.). Bars represent the mean values of three independent experiments with standard errors. Means followed by different letters are significantly different according Duncan's multiple range test (DMRT) at a 5% level.

3.4. In vitro rooting and acclimatization

Inadequate rooting is one of several difficulties in micropropagation. In most of the micropropagated plants, there is a positive association between good rooting and their ability to acclimatize to the natural environment (Aremu et al., 2012). Hence, it is necessary to induce a good proportion of roots in micropropagated plants. In the present study, among the different PGRs (IAA, IBA, and NAA) analyzed for their potential in inducing roots from elongated shoots, the medium supplemented with IBA showed a better response (Table 3). The percentage of response for rooting, root number, and length of root increased gradually with increasing the concentration of IBA up to 2.46 μM and thereafter, decreased with further increase in IBA concentration (Table 3). The shoots initiated in the medium containing 6.21 μM *m*T responded better than the shoots originated in the medium containing 13.32 μM BA (Table 3) for *in vitro* rooting in RM containing IBA (Fig. 1k). At an optimum concentration (2.46 μM) of IBA, 95.66% and 76.00% of shoots initiated from the SIPM containing 6.21 μM *m*T and 13.32 μM BA responded for root induction, 8.62 and 4.72 roots per elongated shoot were obtained with an average root length of 13.82 cm and 7.54 cm, respectively (Table 3). The low number of roots from the shoots derived from BA containing medium might be due to harmful effects of N-glucosides or alanine conjugates formed during the BA metabolism in the shoots (Werbrouck et al., 1995). Moreover, the shoots derived from the medium containing BA has the tendency to accumulate toxic BA metabolites in the basal portion (rooting zone) of the micropropagated shoots and interfere with rooting (Werbrouck et al., 1995). The obtained results were consistent with the findings of Bairu et al. (2007), Gentile et al. (2014), Gentile et al. (2017), Naaz et al. (2019) and Saeiahagh et al. (2019) who observed that the shoots initiated in *m*T containing medium showed better rooting response than the shoots initiated in medium containing BA in *Aloe polyphylla*, *Prunus* species, *Corylus colurna, Syzygium cumini*, and *Actinidia chinensis,* respectively. In contrary to these reports, Bairu et al. (2008) reported that compared to BA, *m*T at 2.22 μM had an inhibitory effect on rooting of banana. Irrespective to the type of cytokinin used for the regeneration, higher concentrations negatively influences the rooting response. Valero-Aracama et al. (2010) observed that *m*T at 10 μM or higher concentrations had inhibitory effects on rooting of *Uniola paniculata*. Similarly, Escalona et al. (2003) also reported a progressive reduction in rooting as the concentrations of BA and *m*T increased in the range of 1.33–22.2 μM.

Well-rooted plantlets (Fig. 1k) were separated from the rooting medium and then acclimatized by transferring them into paper cups (Fig. 1l) filled with the mix of autoclaved soil, sand, and vermiculite (3:1:1v/v/v). After 2-weeks of acclimatization in the growth chamber, plants were transplanted to plastic pots containing autoclaved soil, sand, and vermiculite (3:1:1v/v/v) and grown in the greenhouse (Fig. 1m). The plants developed from the shoots initiated in *m*T containing medium showed a higher percentage (94.33%) of survival compare to the plants developed from the shoots initiated in BA containing medium. Generally, a well-developed root system is prerequisite for the successful establishment of plantlets in the *in vivo* condition. In the present study, the shoots originated from the cotyledonary node in *m*T containing medium produced a higher number and long roots compared to the shoots developed from the medium containing BA. *m*T metabolites are less stable and produce reversibly sequestrated metabolites (Werbrouck et al., 1995). The presence of a hydroxyl group in topolins gives them a structural advantage to undergo *O*-glucosylation and reduces the total cytokinin pool in the regenerated shoots and enhances the root formation and favours the acclimatization (Bairu et al., 2011). The results of this study are in agreement with Bairu et al. (2007) who reported that *m*T promoted the rooting, and more than 90% of the plantlets derived from the *m*T treatment successfully acclimatized compared to a 65% survival rate recorded with plantlets derived from BA treatment.

Table 4
List of RAPD primers, their sequences, number, and size of the amplified fragments from the sesame cv. TMV–3 plantlets generated from the shoots induced in *m*T containing medium.

S. No.	Primer Name	Primer sequence (5′ – 3′)	Number of scorable bands/ primer	Size range of bands (bp)
1	OPA3	AGTCAGCCAC	1	1000
2	OPA5	AGGGGTCTTG	2	1800–1200
3	OPA6	GGTCCCTGAC	4	1700–1000
4	OPA7	GAAACGGGTG	2	1150–650
5	OPA8	GTGACGTAGG	3	1050–660
6	OPA10	GTGATCGCAG	3	2200–450
7	OPA11	CAATCGCCGT	3	2500–800
8	OPA12	TCGGCGATAG	2	1000–680
9	OPA14	CTCGTGCTGG	1	1200
10	OPD15	TTCCGAACCC	1	1100
Total			22	2500–450

Table 5
List of SCoT primers, their sequences, number, and size of the amplified fragments from the sesame cv. TMV–3 plantlets generated from the shoots induced in *m*T containing medium.

S. No.	Primer Name	Primer sequence (5′ – 3′)	Number of scorable bands/primer	Size range of bands (bp)
1	S1	CAACAATGGCTACCACCA	2	1400–1100
2	S4	CAACAATGGCTACCACCT	3	2000–800
3	S6	CAACAATGGCTACCACGC	2	2200–1500
4	S11	AAGCAATGGCTACCACCA	3	2000–1000
5	S16	ACCATGGCTACCACCGAC	3	1900–1400
6	S17	ACCATGGCTACCACCGAG	3	2000–1300
7	S25	ACCATGGCTACCACCGGG	2	1400–1100
8	S26	ACCATGGCTACCACCGTC	3	1500–800
9	S32	CCATGGCTACCACCGCAC	3	1600–600
10	S34	ACCATGGCTACCACCGCA	4	2700–1000
Total			28	2700–600

3.5. Biochemical analysis

Early senescence is one amongst several problems encountered during the *in vitro* regeneration and severely affects the quality and productivity of the clonally propagated plantlets. Plant hormones such as abscisic acid and ethylene promote the senescence, while cytokinins act as senescence delaying hormones (Talla et al., 2016). The chloroplast is the first organelle affected by the senescence and results in the reduction of photosynthetic rate (Grover et al., 1987). Chlorophyll degradation rate is one of the main criteria for monitoring the leaf senescence (Nooden and Nooden, 1985). Supplementation of cytokinins in the medium reduces the chlorophyllase activity in the *in vitro* grown plant tissue and prevents chlorophyll degradation (Gentile et al., 2017). However, the type of cytokinin in the media influences the chlorophyllase activity and chlorophyll content (Dobránszki and Mendler-Drienyovszki, 2014). In the current investigation, the chlorophyll present in the shoots developed in the plant growth regulator-free medium was higher than the medium containing either BA or *m*T. However, multiple shoots developed from the explants (cotyledonary node) in the culture medium fortified with 6.21 μM *m*T appeared greener and accumulated higher chlorophyll *a*, chlorophyll *b*, and total chlorophyll (35.2%) than the shoots developed in the medium containing 13.32 μM BA (Fig. 2a–c). The results were in agreement with the reports where the *in vitro* cultures of *Actinidia chinensis, Corylus colurna* and *Pelargonium hortorum* accumulated higher chlorophyll when they regenerated on the medium containing *m*T in comparison with BA (Saeiahagh et al., 2019; Gentile et al., 2017; Wojtania and Skrzypek, 2014). Further, they reported that *m*T showed better effect than BA in reducing the chlorophyllase activity and chlorophyll degradation.

In vitro clonal propagation leads to major changes in the metabolism,

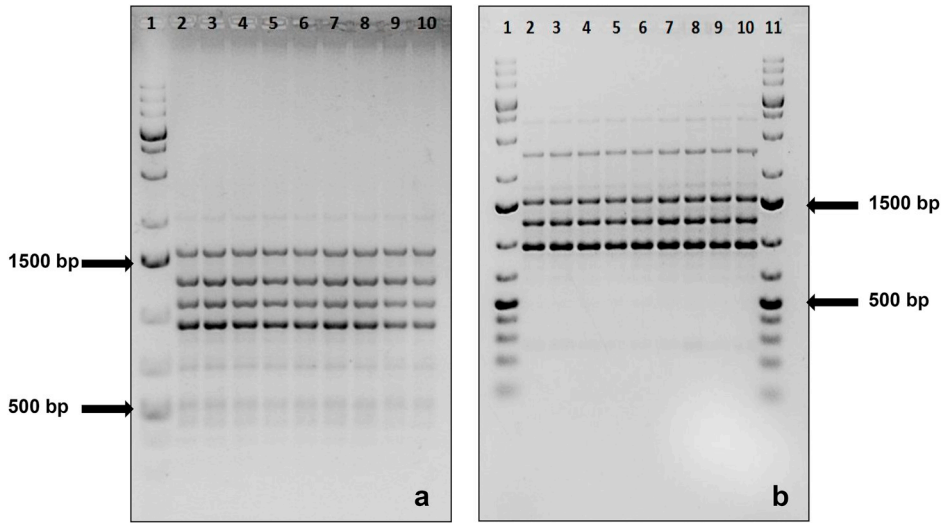

Fig. 4. Genetic fidelity analysis of the regenerated sesame plants (cv. TMV–3) obtained from multiple shoots initiated on SIPM containing 6.21 μM *m*T. **a** RAPD banding pattern obtained with primer OPA6. *lane 1* 1 kb DNA ladder; *lanes 2–9 in vitro* regenerated plants; *lane 10 in vivo* plant raised from the seed. **b** SCoT banding pattern obtained with primer S34. *lanes 1 and 11* 1 kb DNA ladder; *lanes 2–9 in vitro* regenerated plants; *lane 10 in vivo* plant raised from the seed.

physiology, and development of clonally propagated plants (Gentile et al., 2017). These changes favour the formation of ROS and hamper the growth and development of plants. Antioxidant enzymes such as SOD, APX, and CAT are the vital enzymes involved in ROS scavenging mechanism, and the maintenance of the constant level of free radicals and peroxides in the plant cell depends on the balance between the activities of SOD, APX, and CAT. The superoxide radicals formed during the photoreduction of oxygen at Photosystem I (PSI) are dismutated to H_2O_2 by SOD, and APX and CAT predominantly scavenge H_2O_2 into water and oxygen. Hence, in order to avoid the oxidative damage under stress conditions, plants should upregulate the antioxidant enzyme activities. In the present study, the multiple shoots developed on the medium fortified either with BA or *m*T exhibited higher SOD, APX, and CAT activity and H_2O_2 than the shoots developed in the medium lacking plant growth regulators. However, significantly higher SOD (124.3%), APX (116.7%), and CAT (102.8%) activity, and H_2O_2 (86.9%) were recorded in multiple shoots developed in the medium containing 13.32 μM BA when compared to the medium containing 6.21 μM *m*T (Fig. 3a–d). The highest antioxidant enzyme activities and H_2O_2 in the shoots derived from the medium containing BA indicate oxidative stress triggered by this hormone. Similarly, Gentile et al. (2017) and Amoo et al. (2015) reported the influence of *m*T and BA on antioxidant enzyme activities in the micropropagated *Corylus colurna* and *Merwilla plumbea*, respectively and concluded that plantlets regenerated on a medium containing BA showed higher antioxidant enzyme activity in comparison to the medium containing *m*T.

3.6. Genetic fidelity analysis

Obtaining the plants without any modifications or mutations in their genome is the main objective of the micropropagation of any plant species. Usage of synthetic hormones, modifications in the nutrients, and artificial climatic conditions may trigger somaclonal variations and mutations in the genome of clonally propagated plantlets (Lakshmanan et al., 2007). Hence, screening the *in vitro* clonally propagated plants for their genetic stability has primary importance in the commercial tissue culture plants production. Use of more than one marker has always been recommended for better analysis of genetic fidelity of micropropagated plants (Rohela et al., 2019; Vasudevan et al., 2017; Thakur et al., 2016). Hence, in the current study, RAPD and SCoT markers were employed to evaluate the genetic stability of the micropropagated plantlets developed from the medium containing BA or *m*T. From the genomic DNA of *m*T treated plantlets, 10 RAPD primers yielded 22 clear, reproducible and scorable amplicons with approximately 2.2 amplicons per primer (Table 4). The number of amplified fragments per primer varied from one (OPA3, OPA14, and OPA15) to four (OPA6), which varied in size from 450 to 2500 bp (Table 4). Ten SCoT primers produced 28 scorable bands with an average of 2.8 bands per primer and the size of the amplicon ranging from 600 to 2700 bp (Table 5). The amplicon number varies from 2 (S1, S6, and S25) to 4 (S34) in each primer (Table 5). The amplicons generated among the micropropagated and *in vivo* grew plantlets using RAPD and SCoT markers are identical and monomorphic in nature (Fig. 4a and b). Like *m*T treated plantlets, the plantlets developed from the medium containing BA also showed similar banding pattern with all RAPD and SCoT markers tested (Supplementary Fig. 1). The UPGMA cluster analysis of Jaccard's similarity coefficient, revealed that *in vitro* generated and *in vivo* grown plantlets has 100% similarity, which indicates that there were no genetic variations among the regenerated sesame plantlets. In the current study, BA or *m*T treatment during shoot induction and multiplication did not induce any somaclonal variations in the regenerated plantlets. The results were in agreement with the report of Bairu et al. (2008) who analyzed the somaclonal variations using RAPD markers in banana plantlets and concluded that there were no significant somaclonal variations among the plantlets developed from medium containing either BA or mT.

4. Conclusion

In conclusion, we herein report an efficient regeneration protocol for sesame by augmenting *m*T in the SIPM. *m*T positively influenced the shoot regeneration, rooting and ex vitro acclimatization of sesame plants. To the best of our knowledge, this is the first report describing the role of *m*T in the regeneration of sesame using cotyledonary node as an explant.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101320.

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