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Synergism of *m*-topolin with auxin and cytokinin enhanced micropropagation of *Maytenus emarginata*

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Abstract

A synergistic effect of *meta*-topolin (*m*T) with auxin and cytokinin has been applied to stimulate large-scale shoot regeneration from *Maytenus emarginata* mature tree nodal explants. The efficacy of *m*T and BAP was compared during induction and multiplication stages of regeneration. At the shoot bud induction phase, BAP showed a greater response than *m*T. Shoots were multiplied when shoot clumps of four to five shoots were harvested from mother explants and subcultured on medium containing low concentrations of cytokinin-cytokinin or auxin-cytokinin combinations. Among all the plant growth regulator (PGR) types and concentrations evaluated, a combination of 0.5 mg L^{-1} *m*T, 0.25 mg L^{-1} Kin and 0.1 mg L^{-1} IAA was found most effective for shoot proliferation with 80.20 ± 0.89 shoots. Synergistic effect of BAP, Kin and IAA was compared in combination with *m*T, Kin and IAA, and it was observed that the number of shoots (34.58 ± 3.68) regenerated on medium containing 0.5 mg L^{-1} BAP; 0.25 mg L^{-1} Kin was significantly lower than the combination of 0.5 mg L^{-1} *m*T, 0.25 mg L^{-1} Kin and 0.1 mg L^{-1} IAA. The result of this study clearly indicates that *m*T alone is not effective but synergism of *m*T with Kin and IAA has a great impact on shoot proliferation. Shoots were able to induce root only when initially treated with higher concentration (200 mg L^{-1}) of IBA for 5 min and then transferred to PGR-free ¼ strength MS medium containing 100 mg L^{-1} activated charcoal. About 74% of micropropagated shoots were rooted with an average number of 10.27 roots per shoot. About 80% rooted plantlets were acclimatised successfully under greenhouse condition. An improved plant propagation system developed by replacement of cytokinin BAP by *m*T in multiplication medium may be applicable for large-scale multiplication of the medicinal tree *M. emarginata*.

Keywords Acclimatisation · Auxin-cytokinin synergism · *In vitro* propagation · *Maytenus emarginata* · *meta*-topolin

Introduction

Maytenus emarginata (Willd.) Ding Hou, belonging to the Staff vine family Celastraceae, is a spiny tree or shrub of Indian Thar Desert and proximate Aravallies (Bhandari 1990). This tree is popularly known as "Kankero" in Hindi and "Thorny Staff tree" in English (Purohit and Shekhawat 2012). It is also distributed in Arabia, Pakistan, Afghanistan, Sri Lanka, Burma and Malaya. *M. emarginata* has immense medicinal properties to treat fever, asthma, rheumatism and gastrointestinal disorders (Sagwan *et al*. 2011). Its roots are used to cure dysentery, tender shoots used in the treatment of mouth ulcer (Spivey *et al*. 2002) and paste of bark mixed with mustard oil used to kill lice in the hair. Decocted leafy twigs are used as mouthwash to relieve toothache, pulverised leaves given to children in milk as a vermifuge and fruits used to purify blood (Bhandari 1990). *M. emarginata* is reported to contain a number of phytochemicals such as alkaloids, flavonoids, isoflavonoid glycosides, tannins and steroids (Moteriya *et al*. 2014). Sesquiterpene pyridine alkaloids have been isolated from *M. emarginata*, namely, emarginatine B and emarginatine F, and showed cytotoxic effect against human KB cells, ileocecal adenocarcinoma, melanoma and medulloblastoma tumour cells (Kuo *et al*. 1990, 1994; Shekhawat et al. 2018). It is a drought- and heat-resistant tree which produces biomass as a good source of fuel. The timber of *M. emarginata* is strong and durable and is used for making

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agricultural appliances. This plant holds a sacred value for the "Bishnoi" community residing in Thar Desert of North West India (Purohit and Shekhawat 2012).

Despite its varied uses, callous harvesting led to the loss of this valuable germplasm from arid and semiarid regions. Traditionally, *M. emarginata* is propagated through seeds. However, being a cross-pollinated species, seed derived from *M. emarginata* exhibited high genetic variability and it takes 12 to 15 y for showing superior characteristics at its reproductive maturity (Shekhawat *et al.* 2014). In addition, most of the trees from fragile ecosystems of arid and semiarid regions are difficult to propagate vegetatively (Shekhawat *et al.* 2012, 2014). Therefore, alternative means for large-scale propagation of this important plant is necessary. Rapid clonal propagation of woody plants through tissue culture technique can be used for afforestation, production of woody biomass and elite germplasm conservation (Shekhawat *et al.* 2014). Earlier, an attempt has been made to multiply this tree *via* tissue culture technique using 6-benzylaminopurine (BAP) (Rathore *et al.* 1992). To overcome the problem associated with *in vitro* rooting in *M. emarginata*, Purohit and Shekhawat (2012) tried *ex vitro* rooting in micropropagated shoots by deploying different chemical and physical factors. However, there was a need to refine the existing protocol particularly in the rate of shoot multiplication and *in vitro* rooting and acclimatisation of plants. Other than *M. emarginata*, little progress has been made on tissue culture of other *Maytenus* species, *i.e. M. aquifolium* (Pereira *et al.* 1995), *M. senegalensis* (Matu *et al.* 2006), *M. canariensis* (Gutiérrez-Nicolás *et al.* 2008) and *M. ilicifolia* (Coppede *et al.* 2014; Paz *et al.* 2017).

We used *meta*-topolin (*m*T) for the first time for *in vitro* regeneration of *M. emarginata*. *Meta*-topolin has been widely used as an alternative cytokinin in tissue culture studies for shoot multiplication (Werbrouck *et al.* 1996; Bairu *et al.* 2007; Amoo *et al.* 2011; Aremu *et al.* 2012). *Meta*-topolin has the potential to not only induce good-quality shoots with high rate of shoot multiplication but also help in rooting and acclimatisation (Valero-Aracama *et al.* 2010; Amoo *et al.* 2011; Gentile *et al.* 2014, 2017; Kucharska *et al.* 2020). In comparison to BAP, *m*T is metabolised much faster and transported in all parts of plant during acclimatisation (Werbrouck *et al.* 1996; Aremu *et al.* 2012). It is well known that the synergistic or antagonistic interaction between auxin and cytokinin is the basis of plant growth in plant tissue culture which highly depends on the plant species and tissue type (Coenen and Lomax 1997; Gaspar *et al.* 2003; Aremu *et al.* 2012). Although synergistic/antagonistic interactions of BAP with other auxins or cytokinins on morphogenesis *in vitro* have been extensively studied, very little published work is available on the role of *m*T and its interaction with other auxins or cytokinins. Therefore, the present investigation was carried out with an objective of establishing an improved micropropagation protocol for *M. emarginata* from mature tree nodal explants. We compared the effect of *m*T and BAP on *in vitro* shoot induction and multiplication and also evaluated the synergism among *m*T and auxin and/or cytokinin and their effects on shoot multiplication.

Materials and Methods

Explant selection and surface sterilisation Fresh and healthy shoot sprouts were collected from an 18- to 20-y-old tree of *Maytenus emarginata* (Willd.) Ding Hou growing in the new campus of Jai Narain Vyas University, Jodhpur, Rajasthan, during March to April and July to August. Leaves and spines were removed from shoots and cut into shoot segments with one to two nodes. These shoot segments were selected as explant for culture establishment of *M. emarginata*. Sterilisation of explants was performed under aseptic condition in laminar air flow cabinet. Explants were treated with 0.1% (*w/v*) Bavistin (BASF India Ltd., Mumbai, India) for 12 to 15 min followed by surface sterilisation with 0.1% $HgCl_2$ (*w/v*) (Hi-Media®, Mumbai, India) for four to five min and rinsed with autoclaved distilled water for three to four times after both treatments. To overcome the problem of phenolic exudation, nodal shoot segment explants were treated with autoclaved and chilled solution of antioxidant (0.1% ascorbic acid and 0.05% citric acid; Hi-Media®) for 15 min as described by Rathore *et al.* (1992).

Nutrient medium, culture establishment and culture conditions Murashige and Skoog (1962) medium with additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} each of citric acid, adenine sulphate and L-arginine; Hi-Media®) and 3% sucrose (Qualigens Fine Chemicals, Mumbai, India) were used for culture initiation, multiplication and subculturing as reported by Rathore *et al.* (1992). Agar-agar at 0.8% (*w/v*; Qualigens Fine Chemicals, India) was used as the medium gelling agent. The pH of the culture media was adjusted to 5.8 ± 0.02 using 0.5 N NaOH or HCl (Qualigens Fine Chemicals) prior to autoclaving at 1.05 kg cm^{-2} pressure and 121°C temperature for 15 min. Explants were inoculated vertically in culture tubes and kept in diffused light conditions (20 to 25 μmol m^{-2} s^{-1} photon flux density (PFD)) for two to three d and thereafter shifted to culture room having light intensity of 40 to 50 μmol m^{-2} s^{-1} PFD provided by cool white fluorescent tubes (Philips, Kolkata, India), at 26 ± 2°C temperature and 55 to 60% relative humidity.

Shoot bud induction and multiplication For shoot bud induction, nodal shoot segments were cultured on MS medium with additives and different concentrations (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 mg L^{-1}) of BAP, kinetin (Kin) or *m*T (Hi-Media®). For further shoot multiplication, mother/original explants (after harvesting the axillary shoots) were transferred from medium

containing optimised concentrations of cytokinins to lower concentrations (0.25, 0.50, 0.75 and 1.0 mg L^{-1}) of respective cytokinin.

To evaluate the interactive effect of auxin and cytokinin on shoot multiplication, clumps of four to five shoots produced *in vitro* were harvested from mother explant and subcultured on MS medium supplemented with additives and various concentrations of cytokinin (BAP, Kin or *m*T) in combination with or without indole-3-acetic acid (IAA; Hi-Media®). Subcultures were done regularly after an interval of 30 to 32 d.

***In Vitro* rooting of micropropagated shoots** For rooting under *in vitro* conditions, microshoots were individually harvested from shoot clumps. Continuous treatments with lower concentrations (0.0 to 5.0 mg L^{-1}) of auxins were unable to form roots from micropropagated shoots. Therefore, these shoots were initially pulse treated with different concentrations (0.0 to 500 mg L^{-1}) of indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) (Hi-Media®, India) for 5 min, then inoculated on auxin-free MS medium of different strengths (full MS, ½ MS, ¼ MS) with 100 mg L^{-1} of activated charcoal (Hi-Media®) and 0.7% agar. Additives used for shoot proliferation were not added in rooting medium. The cultures were initially kept in the dark for two to three d and then transferred to light conditions.

Acclimatisation of plantlets For acclimatisation, the *in vitro* rooted plantlets were removed from culture vessels, washed with distilled water and transferred to glass bottles containing autoclaved soilrite (procured from Keltech Energies Limited, Bengaluru, India) moistened with quarter strength of MS salts, capped with polycarbonate caps and placed near pad section (regions with 80 to 90% relative humidity and 28 ± 2°C) of greenhouse. After partial acclimatisation of plantlets, the bottles were moved from the pad section to fan section (regions with 60 to 70% relative humidity and 32 ± 2°C). The polycarbonate caps were gradually loosened and finally removed. The acclimatised plantlets were then transferred to black polybags containing mixture of field soil:soilrite in 1:1 ratio and kept in greenhouse for four to six wk and finally moved to the nursery.

Experimental design and statistical analysis All the experiments were set up in complete randomised block design, with a minimum of 15 replicates taken in each treatment and each experiment was repeated three times. The results were expressed as mean ± SD. The significance in differences among mean values was carried out using Duncan's multiple range test (DMRT) at $P < 0.05$. The data was analysed using one-way analysis of variance (ANOVA) by SPSS v.17 (SPSS, Chicago, IL).

Results and Discussion

Cut ends of cultured nodal explants of *M. emarginata* showed browning during culture initiation due to phenolic exudation. Woody plant species of desert habitats produce phenolics as they thrive in harsh environmental conditions (Rathore *et al.* 1992; Shekhawat *et al.* 1993). Browning/darkening of explants and medium is the result of oxidation of polyphenols which is the major constraint in cloning of woody species (Rai *et al.* 2010). The problem of phenolic exudation from cut end and browning of explants observed in this study was overcome by pre-treating explants with chilled antioxidant solution of 0.1% ascorbic acid and 0.05% citric acid prior to culture. The antioxidant treatment of explants to reduce phenolic exudation has been reported in many woody plant species including *M. emarginata* (Rathore *et al.* 1992; Shekhawat *et al.* 1993; Phulwaria *et al.* 2012; Rathore *et al.* 2014; Sharma *et al.* 2017).

Effect of cytokinins on shoot bud induction and multiplication The nodal explants cultured on MS medium with various concentrations of BAP, Kin or *m*T exhibited bud break within 1 to 2 wk. The percentage of bud-breaking varied from 59.25% (PGR-free MS medium) to 96.29% (MS containing 2.0 mg L^{-1} *m*T) on different concentrations of the three cytokinins tested (Table 1). Nodal explants cultured on MS medium without additive and PGRs (control) did not show any bud-breaking but few explants on PGR-free MS medium with additives showed bud-breaking with induction of a single shoot bud. However, medium containing Kin, BAP or *m*T was found to be more effective for shoot bud induction. Although frequency of bud-breaking was highest on MS medium containing 2.0 mg L^{-1} *m*T, the number of shoots induced on 2.0 mg L^{-1} BAP was slightly higher than that of medium containing 2.0 mg L^{-1} *m*T (Table 1). Two to three shoots were induced on MS medium supplemented with 2.0 mg L^{-1} BAP and *m*T (Fig. 1*a*, *b*). BAP is an active, affordable and effective cytokinin but it has many drawbacks including heterogeneity in growth and inhibition of rooting efficiency in many plant species (Werbrouck *et al.* 1996). Therefore, it is imperative to find a substitute for BAP. Owing to high shoot multiplication ability and efficacy in rooting and acclimatisation, *m*T is a cytokinin of choice in many plant tissue culture reports (Amoo *et al.* 2011; Aremu *et al.* 2012). In the present study, BAP is more effective than *m*T for shoot bud induction. In general, BAP has been preferred for shoot bud induction from mature plant explants because of its ability to be easily metabolised in cells and activate pre-existing meristems (Rai *et al.* 2010; Gupta *et al.* 2014).

For shoot multiplication, mother explants (after harvesting the axillary shoots) were transferred from optimised concentrations of BAP (2.0 mg L^{-1}), *m*T (2.0 mg L^{-1}) and Kin

Table 1 Effects of cytokinin (BAP, Kin, *m*T) on shoot bud induction from nodal explants of *Maytenus emarginata* (Willd.) Ding Hou

BAP (mg $L^{-1)}$	Kin (mg L^{-1})	*m*T (mg L^{-1})	Percent bud break (%) (mean ± SD)	Number of shoots per explant (mean ± SD)	Shoot length (cm) (mean ± SD)
0.00	0.00	0.00	59.25 ± 3.70^{h}	1.00 ± 0.00^{d}	0.70 ± 0.08^{i}
1.0	–	–	76.11 ± 2.00^{cd}	1.00 ± 0.00^{d}	1.69 ± 0.08^{d}
2.0	–	–	90.37 ± 3.05^{b}	2.45 ± 0.10^{a}	2.51 ± 0.32^{b}
3.0	–	–	74.81 ± 1.60^{de}	1.33 ± 0.20^{c}	3.13 ± 0.51^{a}
4.0	–	–	71.29 ± 2.31^{e}	1.027 ± 0.02^{d}	2.00 ± 0.07^{c}
5.0	–	–	63.33 ± 2.00^{fgh}	1.03 ± 0.03^{d}	1.49 ± 0.18^{de}
–	1.0	–	67.03 ± 3.34^{f}	1.00 ± 0.00^{d}	1.65 ± 0.13^{d}
–	2.0	–	86.11 ± 2.78^{b}	1.00 ± 0.00^{d}	1.17 ± 0.36^{f}
–	3.0	–	74.63 ± 2.24^{de}	1.04 ± 0.04^{d}	1.06 ± 0.03^{fg}
–	4.0	–	65.18 ± 1.39^{fg}	1.00 ± 0.00^{d}	0.98 ± 0.07^{fgh}
–	5.0	–	62.22 ± 1.46^{fgh}	1.00 ± 0.00^{d}	1.39 ± 0.08^{e}
–	–	1.0	81.29 ± 1.78^{c}	1.03 ± 0.03^{d}	1.16 ± 0.06^{f}
–	–	2.0	96.29 ± 1.60^{a}	2.18 ± 0.06^{b}	1.62 ± 0.13^{d}
–	–	3.0	86.66 ± 3.84^{b}	1.27 ± 0.12^{c}	0.87 ± 0.08^{ghi}
–	–	4.0	77.03 ± 2.24^{cd}	1.07 ± 0.02^{d}	0.83 ± 0.05^{ghi}
–	–	5.0	64.25 ± 3.34^{fg}	1.00 ± 0.00^{d}	0.76 ± 0.09^{hi}

Medium: Murashige and Skoog containing additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} each of citric acid, adenine sulphate and L-arginine).

Data was recorded after 28 d of inoculation.

Mean ± SD followed by the same *letter* within each *column* are not significantly different according to DMRT at $P < 0.05$.

BAP 6-benzylaminopurine, *Kin* kinetin, *mT meta*-topolin.

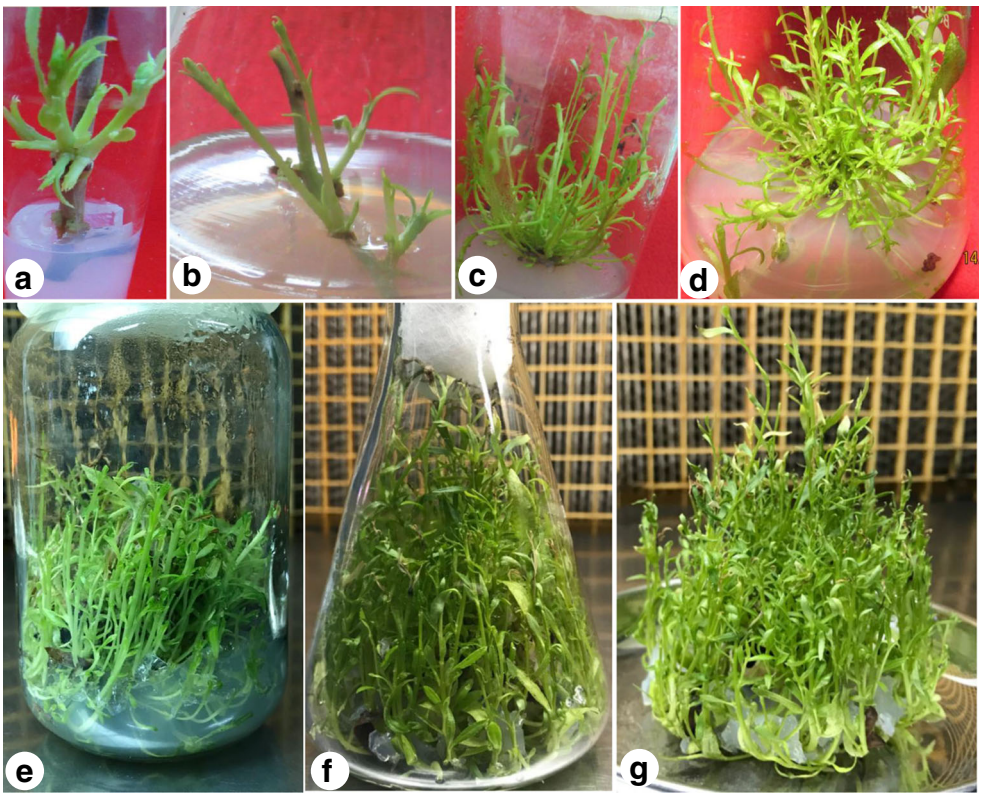

Figure 1. Shoot bud induction and multiplication from nodal explant of *Maytenus emarginata* (Willd.) Ding Hou. (*a*) Axillary shoot bud induction on Murashige and Skoog (MS) medium with 2.0 mg L^{-1} 6-benzylaminopurine (BAP). (*b*) Axillary shoot bud induction on MS medium with 2.0 mg L^{-1} *meta*-topolin (*m*T). (*c*) Shoot multiplication on MS medium with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} kinetin (Kin) + 0.1 mg L^{-1} indole-3-acetic acid (IAA) after four wk. (*d*) Shoot multiplication on MS medium with 0.5 mg L^{-1} *m*T + 0.25 mg L^{-1} Kin + 0.1 mg L^{-1} IAA after four wk. (*e*) Multiplication of shoots on MS medium with 0.5 mg L^{-1} *m*T + 0.25 mg L^{-1} Kin + 0.1 mg L^{-1} IAA after six wk. (*f*) Multiplication of shoots on MS medium with 0.25 mg L^{-1} *m*T + 0.25 mg L^{-1} Kin + 0.1 mg L^{-1} IAA after six wk. (*g*) Multiplied shoots ready for rooting treatment.

(2.0 mg L^{-1}) to lower concentrations of respective cytokinin. In comparison to BA, *m*T and Kin were found to be less efficient in shoot multiplication when mother explants were transferred to lower concentrations from higher concentrations of respective cytokinin (Table 2). Using this approach, the maximum number of shoots were regenerated on MS medium containing 0.5 mg L^{-1} BAP. It has been suggested that, after bud breaks on medium with higher levels of cytokinin concentrations, explants accumulate cytokinin which inhibits shoot growth and shoot regeneration (Sharma *et al.* 2017).

Interactive effect of cytokinin-cytokinin or auxin-cytokinin on Shoot Multiplication With the aim of multiplying shoots and subsequent plant regeneration, shoot clumps of four to five shoots were harvested from mother explants and subcultured on medium containing low concentrations of cytokinin-cytokinin or auxin-cytokinin combinations. The combination of two cytokinins with one auxin (BAP + Kin + IAA or *m*T + Kin + IAA) has a great impact on shoot multiplication than the combination of two cytokinins (BAP + Kin) or single cytokinin and auxin (BAP + IAA or *m*T + IAA). The synergistic effect of BAP, Kin and IAA was compared with the combination of *m*T, Kin and IAA and it was found that the number of shoots regenerated on the combination of *m*T, Kin and IAA was significantly higher than that on the combination of BAP, Kin and IAA (Table 3). The shoots regenerated on the combination of *m*T, Kin and IAA were vigorous and mostly were equal in length, while on the combination of BAP, Kin and IAA, few shoots were fully developed and others were weak and short in length (Fig. 1*c*, *d*). Among all the PGR types and concentrations tested, a combination of 0.5 mg L^{-1} *m*T, 0.25 mg L^{-1} Kin and 0.1 mg L^{-1} IAA was found most effective for shoot proliferation with 80.20 ± 0.89 shoots per explant (Table 3; Fig. 1*e*). Although the highest number of shoots proliferated on MS medium with 0.5 mg L^{-1} *m*T, 0.25 mg L^{-1} Kin and 0.1 mg L^{-1} IAA, length of shoots regenerated on this combination was lower in comparison to that on the combination of 0.25 mg L^{-1} *m*T, 0.25 mg L^{-1} Kin and 0.1 mg L^{-1} IAA (Table 3; Fig. 1*f*). It is well documented that auxin and cytokinin interact together and regulate many physiological processes related to plant growth (Coenen and Lomax 1997). The synergistic effect of cytokinin-cytokinin or auxin-cytokinin on shoot proliferation has been extensively studied. The effect of synergism of auxin-cytokinin or cytokinin-cytokinin can be attributed to the synthesis of endogenous PGRs in tissues and requirement of minimum threshold exogenous levels of PGRs (Gupta *et al.* 2020). In the present study, two cytokinins (*m*T and Kin) interacted synergistically with one auxin (IAA) and enhanced shoot proliferation significantly. Similarly, synergism of two cytokinins and one auxin for shoot multiplication has been reported in other tree species like *Terminalia bellirica* (Phulwaria *et al.* 2012) and *Mitragyna parvifolia* (Patel *et al.* 2020). At the shoot induction stage, *m*T alone did not respond satisfactorily. However, synergism of *m*T with Kin and IAA had a great influence on shoot multiplication. The potential effect of *m*T in mass propagation has been reported in several species including *Aloe polyphylla* (Bairu *et al.* 2007), *Musa* spp. (Bairu

Table 2. Effects of cytokinin (BAP, Kin, *m*T) on shoot multiplication of *Maytenus emarginata* (Willd.) Ding Hou

BAP (mg L^{-1})	Kin (mg L^{-1})	*m*T (mg L^{-1})	Number of shoots per explant (mean ± SD)	Shoot length (cm) (mean ± SD)
0.25	–	–	6.08 ± 0.42^{bc}	1.44 ± 0.14^{cd}
0.50	–	–	10.38 ± 0.83^{a}	2.34 ± 0.28^{a}
0.75	–	–	6.68 ± 1.07^{b}	1.81 ± 0.29^{b}
1.0	–	–	5.31 ± 0.13^{cd}	1.20 ± 0.06^{cde}
–	0.25	–	3.37 ± 0.57^{fg}	1.11 ± 0.02^{de}
–	0.50	–	4.45 ± 0.30^{e}	1.30 ± 0.01^{cd}
–	0.75	–	3.34 ± 0.28^{fg}	1.47 ± 0.23^{d}
–	1.0	–	2.47 ± 0.17^{h}	1.16 ± 0.29^{de}
–	–	0.25	2.93 ± 0.13^{gh}	0.96 ± 0.05^{e}
–	–	0.50	5.90 ± 0.25^{bc}	1.98 ± 0.05^{bc}
–	–	0.75	4.56 ± 0.50^{de}	2.11 ± 0.13^{ab}
–	–	1.0	4.011 ± 0.21^{ef}	1.29 ± 0.03^{cde}

Medium: Murashige and Skoog containing additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} each of citric acid, adenine sulphate and L-arginine).

Data was recorded after 28 d of subculture.

Mean ± SD followed by the same *letter* within each *column* are not significantly different according to DMRT at $P < 0.05$.

BAP 6-benzylaminopurine, *Kin* kinetin, *mT meta*-topolin.

Table 3. Effects of combination of various plant growth regulators on shoot multiplication by subculturing shoot clumps of *Maytenus emarginata* (Willd.) Ding Hou

BAP (mg L^{-1})	*m*T (mg L^{-1})	Kin (mg L^{-1})	IAA (mg L^{-1})	Number of shoots per explant (mean ± SD)	Shoot length (cm) (mean ± SD)
0.50	–	0.10	–	8.77 ± 0.50^{hi}	1.69 ± 0.04^{i}
0.50	–	0.25	–	16.11 ± 0.96^{f}	2.45 ± 0.16^{g}
0.50	–	0.50	–	7.56 ± 0.51^{hij}	1.20 ± 0.04^{jk}
0.50	–	0.75	–	5.89 ± 0.38^{j}	1.07 ± 0.28^{k}
0.25	–	–	0.1	7.90 ± 0.26^{hij}	1.97 ± 0.13^{h}
0.50	–	–	0.1	13.56 ± 0.34^{g}	3.17 ± 0.33^{f}
0.75	–	–	0.1	6.99 ± 0.33^{ij}	1.90 ± 0.07^{hi}
–	0.25	–	0.1	3.89 ± 0.38^{k}	1.38 ± 0.06^{j}
–	0.50	–	0.1	8.00 ± 0.33^{hij}	2.37 ± 0.14^{g}
–	0.75	–	0.1	9.22 ± 0.51^{h}	3.25 ± 0.09^{f}
0.25	–	0.25	0.1	26.37 ± 1.30^{e}	5.66 ± 0.17^{a}
0.50	–	0.25	0.1	34.58 ± 3.68^{c}	5.05 ± 0.14^{b}
	0.25	0.25	0.1	51.22 ± 0.59^{b}	4.79 ± 0.14^{c}
–	0.50	0.25	0.1	80.20 ± 0.89^{a}	4.12 ± 0.05^{d}
–	0.75	0.25	0.1	30.00 ± 1.00^{d}	3.88 ± 0.07^{e}

Medium: Murashige and Skoog containing additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} each of citric acid, adenine sulphate and L-arginine).

Data was recorded after 45 d of subculture.

Mean ± SD followed by the same *letter* within each *column* are not significantly different according to DMRT at $P < 0.05$.

BAP 6-benzylaminopurine, *Kin* kinetin, *mT meta*-topolin, *IAA* indole-3-acetic acid.

et al. 2008), *Sorbus torminalis* (Malá *et al*. 2009), *Uniola paniculata* (Valero-Aracama *et al*. 2010), *Prunus* rootstock (Gentile *et al*. 2014), *Corylus colurna* (Gentile *et al*. 2017), *Manihot esculenta* (Chauhan and Taylor 2018), *Juglans nigra* (Stevens and Pijut 2018), *Pterocarpus marsupium* (Ahmad and Anis 2019), *Hedychium coronarium* (Behera *et al*. 2019) and *Ribes grossularia* (Kucharska *et al*. 2020).

***In Vitro* rooting and acclimatisation of plantlets** MS medium without auxin or containing lower concentrations of auxins failed to induce roots from *in vitro* regenerated shoots. Individual shoots harvested from multiplied shoot clumps (Fig. 1*g*) were only able to induce roots when initially treated with higher concentrations of IBA for five min and then transferred to PGR-free ¼ strength MS medium. No rooting was

Table 4 Effects of pulse treatment of IBA on *in vitro* root induction in micropropagated shoots of *Maytenus emarginata* (Willd.) Ding Hou

IBA (mg L^{-1})	% of shoots showing rooting	Number of roots per shoot (mean ± SD)	Root length (cm) (mean ± SD)
0.00	0.00	0.00	0.00
25.0	0.00	0.00	0.00
50.0	0.00	0.00	0.00
100.0	66.67^{a}	8.78 ± 0.42^{b}	1.19 ± 0.17^{b}
200.0	74.07^{a}	10.27 ± 0.42^{a}	2.02 ± 0.18^{a}
300.0	25.92^{b}	2.9 ± 0.67^{cd}	0.62 ± 0.46^{d}
400.0	14.81^{c}	3.00 ± 0.5^{c}	0.86 ± 0.10^{c}
500.0	25.92^{b}	2.10 ± 0.59^{d}	0.93 ± 0.59^{c}

Medium: ¼ Murashige and Skoog containing medium containing 0.7% agar and 100 mg L^{-1} of activated charcoal.

Duration of IBA pulse treatment: 5 min.

Data was recorded after 28 d of inoculation.

Mean ± SD followed by the same *letter* within each *column* are not significantly different according to DMRT at $P < 0.05$.

IBA indole-3-butyric acid.

Figure 2. *In vitro* rooting and acclimatisation of *Maytenus emarginata* (Willd.) Ding Hou plantlets. (*a*) Root induction (*arrow*) in shoot pulse treated with 200 mg L^{-1} indole-3-butyric acid (IBA) for five min and cultured on ¼ strength Murashige and Skoog (MS) medium containing 0.7% agar. (*b*, *c*) A well-rooted shoot. (*d*) An acclimatised plant in glass bottle containing soilrite under greenhouse conditions. (*e*) Well-hardened plants of *M. emarginata* ready for transplantation in field conditions.

observed from shoots treated with NAA. Out of the various concentrations of IBA used for pulse treatment, 200 mg L^{-1} IBA induced maximum percent rooting (74%) with an average root number and root length of 10.27 and 2.02 cm, respectively (Table 4; Fig. 2*a*–*c*). Full and ½ strength MS media were not beneficial for *in vitro* rooting (data not shown). Auxins are required for the induction of roots, but in later stages of development inhibits the root primordia outgrowth and root formation (Quambusch *et al.* 2017). In accordance with our results, efficient rooting through an IBA pulse treatment rather than inclusion of PGRs in culture media has been reported in different plant species (Rathore *et al.* 1992; Shekhawat *et al.* 1993; Madhulatha *et al.* 2004; Quambusch *et al.* 2017; Khamushi *et al.* 2019). The *in vitro* raised plantlets were acclimatised successfully in greenhouse conditions with 80% survival rate and established in a nursery (Fig. 2*d*, *e*).

In conclusion, we report an improved micropropagation system for *M. emarginata* by incorporating *m*T in shoot multiplication medium. Although, *m*T alone is not as effective as BAP for shoot bud-breaking. However, the synergistic interactions of *m*T with Kin and IAA had a great influence on shoot multiplication. This is the first report describing the effect of *m*T on *in vitro* propagation of *M. emarginata*. Being a commercially important medicinal tree, the present regeneration protocol may be useful in large-scale propagation of *M. emarginata*.

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Authors' Contributions VK and JKS conceived and planned the work. JKS designed and performed the experiments and analysed the data. MKR gave input in experimental design. JKS and MKR wrote the manuscript. NSS and VK supervised the experiments and edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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