



An improved micropropagation protocol for the *ex situ* conservation of *Mitragyna parvifolia* (Roxb.) Korth. (Rubiaceae): an endangered tree of pharmaceutical importance

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Abstract

Mitragyna parvifolia (Roxb.) Korth., commonly known as “Kadam,” is an endangered and pharmaceutically valued tree of the family Rubiaceae. The numerous medicinal properties are attributed to the various alkaloids of this plant. Poor seedling survival (due to very small size of seeds, approximately 10,000 per gm), overexploitation and habitat destruction are the major constraints in conserving the wild stocks of this species. This paper reports a significant, improved, and repeatable micropropagation protocol of *M. parvifolia* using nodal explants of a mature tree. Nodal explants harvested during spring season from the lopped tree differentiated the maximum number of axillary shoots (5.3 ± 0.82 per node) on full-strength Murashige and Skoog (MS) medium containing 3.0 mg L^{-1} 6-benzylaminopurine (BAP) and additives (25 mg L^{-1} each of adenine sulfate, L-arginine, and citric acid and 50 mg L^{-1} ascorbic acid). Shoots were amplified *in vitro* through (1) recurrent transfer of mother explants and (2) subculturing on fresh nutrient medium. The greatest number of shoots (13.4 ± 1.26) with an average length of $6.2 \pm 1.03 \text{ cm}$ was produced after 4 wk on MS medium containing 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} kinetin (Kin), 0.1 mg L^{-1} Indole-3-acetic acid (IAA), additives, 100 mg L^{-1} activated charcoal (AC), and 0.8% (w/v) agar. This is the first report of concurrent *ex vitro* rooting and acclimatization (CEVRA) in *M. parvifolia*. About 90% micropropagated shoots rooted *ex vitro* on pulse treatment of 500 mg L^{-1} Indole-3-butyric acid (IBA; for 5 min) and produced 8.5 ± 0.97 roots per shoot with an average length of $9.40 \pm 1.06 \text{ cm}$, after 5 wk. Over 80% of CEVRA plantlets were successfully transplanted to the soil in field. The defined protocol can be employed for conservation *ex situ* and restoration/rehabilitation/reintroduction *in situ* of *M. parvifolia*.

Keywords Endangered tree · *Ex situ* conservation · *Ex vitro* rooting · Micropropagation · *Mitragyna parvifolia*

Introduction

The genus *Mitragyna* (Rubiaceae, the coffee family) consists of ten species which are mainly distributed in tropical and arid/semi-arid parts of Africa, India, China, Bangladesh, Myanmar, Sri Lanka, and south-east Asia (Govaerts *et al.* 2015). Most of

these species exhibit various therapeutic properties and have been used in local/traditional medicines for a number of ailments (Pandey *et al.* 2006). Leaves of *Mitragyna speciosa* (Korth.) Havil. are ethno-medicinally used as a substitute for opium (from a non-opium source) or in treatment for opium addiction in many countries; and its demand continues to increase in the western world (Brown *et al.* 2017). A significant contribution towards the alkaloidal pattern/chemotaxonomic studies of *Mitragyna* genus was reported by Shellard and his co-workers (Shellard *et al.* 1969; Shellard and Houghton 1971). Recently, many researchers have extensively reviewed botanical, phytochemical, ethnomedicinal, pharmacological, and toxicological aspects of this genus (Hassan *et al.* 2013; Raffa 2015; Brown *et al.* 2017). However, the literature revealed few studies on *in vitro* regeneration/cultures of *Mitragyna* species (Roy *et al.* 1988) with most concentrated on *M. speciosa* (Phongprueksapattana *et al.* 2008; Zuldin *et al.* 2013).

This paper is dedicated to the frontline workers of COVID-19 Pandemic.

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Mitragyna parvifolia (Roxb.) Korth. (Fig. 1a), known as “Kadam” in Hindi (Bhandari 1995), is distributed from Indian subcontinent to Myanmar (Catalogue of Life 2020). Kadam is well recognized for its innumerable medicinal properties and is widely used by the ayurvedic practitioners. The numerous medicinal properties are probably attributed to the presence of indole and oxindole alkaloids (Brown *et al.* 2017). The indole alkaloids recognized are akuammigine, ciliaphylline, hirsutine, rhynchociline, and tetrahydroalstonine (Shellard and Houghton 1971; Shellard and Lala 1977). The oxindole alkaloids are 16, 17-dihydro-17b-hydroxy isomitraphylline, 16, 17-dihydro-17b-hydroxy mitraphylline, isomitraphylline, mitraphylline, pteropodine, isopteropodine, speciophylline, and uncarine F (Shellard *et al.* 1969; Pandey *et al.* 2006). *M. parvifolia* is commercially important in timber and paper industry (Chatterjee *et al.* 1982). Wood quality of Kadam is equal to that of teak and therefore used in making furniture,

agricultural appliances, and construction materials; bark yields cordage fibers (The wealth of India 1998). This plant flowers (Fig. 1b) and produces fruits (Fig. 1c) during the months of June to August (Bhandari 1995).

In nature, *M. parvifolia* propagates through its very small seeds (approximately 10,000 per gm), and germinated seedlings are delicate and often wash away with heavy rain or excess water (Roy *et al.* 1988). Middleton (2009) reported downfall in the population of *M. parvifolia* at Keoladeo National Park (KNP), Bharatpur, India (a United Nations Educational, Scientific, and Cultural Organization (UNESCO) World Heritage Site and a Ramsar site under the Convention on Wetlands of International Importance, and a managed wetland of ecological significance). UNESCO – IUCN Enhancing Our Heritage Project (2003) in a report stated that KNP is possibly the only natural wetland site in the Yamuna River’s floodplains where naturally growing

Fig. 1. *Mitragyna parvifolia* (Roxb.) Korth. (a) Habit. (b) An inflorescence. (c) Fruiting stage.



M. parvifolia trees flourished. Moreover, other constraints such as frequent droughts, herbivory, and inter- and intraspecific competition between species are causes of dwindling population of *M. parvifolia* (Bidalia *et al.* 2017). Therefore, both *in situ* and *ex situ* studies are essential to understand the deterioration of Kadam populations in its natural habitats.

The innumerable pharmaceutical/commercial importance, overexploitation, and habitat destruction has resulted this plant to be categorized as an endangered tree species of Rajasthan (Panwar and Tarafdar 2006; Rai and Lalramnghinglova 2011); though, this species is also facing threat of extinction in its natural habitat (Bidalia *et al.* 2017). Furthermore, conventional methods of propagation such as rooting of stem cutting, grafting, and layering were unsuccessful for this slow growing species (Roy *et al.* 1988). Consequently, there is an immediate need to conserve the wild stocks as well as to multiply/propagate this plant species through alternative approaches. Therefore, micropropagation technology can be efficiently employed for the large-scale multiplication/propagation of *M. parvifolia* in a short span of time. Also, micropropagation as an “integrated plant conservation” strategy can combine *in situ* as well as *ex situ* approaches to ensure conservation of threatened plant taxa (Werden *et al.* 2020). Earlier, an attempt has been made to propagate this plant through tissue culture (Roy *et al.* 1988), but there was a need to refine/improve upon the existing protocol. Therefore, the present work was done to develop a significantly improved micropropagation protocol using nodal explants obtained from a mature tree for sustainable propagation and *ex situ* conservation of *M. parvifolia*. In order to reduce the cost, time, labor, and resources for the protocol, conditions for concurrent *ex vitro* rooting and acclimatization (CEVRA) have been optimized for the first time in *M. parvifolia*. The preliminary data of this work has been published as an abstract in 2016 World Congress on *In Vitro* Biology (Patel *et al.* 2016a), and the complete experimental data are presented here in this manuscript.

Materials and Methods

Explant preparation and surface sterilization A *Mitragyna parvifolia* (Roxb.) Korth. plant, more than 40-yr-old, growing in the garden of Department of Botany, Jai Narain Vyas University, Jodhpur, was used as the source plant for micropropagation. The old branches of the mother plant were removed (lopped) during the preceding winter (Dec to Jan) season. Fresh sprouts of following spring (Feb to Mar), summer (May to Jun), autumn (Sept to Oct), and winter (Dec to Jan) seasons were used as explants to study the influence of different seasons on culture establishment. Nodal segments (4 to 5 cm long) with one node each were washed thoroughly and surface sterilized using mercuric chloride (0.1% HgCl_2 (w/v))

for 4 to 5 min, followed by 5 to 6 washing with autoclaved tap water under a laminar air flow hood. The surface sterilized explants were kept in a sterile pre-chilled antioxidant solution (ascorbic acid and citric acid 0.1% (w/v)) for 18 to 20 min prior to inoculation.

Culture medium and incubation conditions The surface sterilized and antioxidant-treated nodal segments were inoculated vertically on Murashige and Skoog (MS; 1962) medium supplemented with 1.0, 2.0, 3.0, or 4.0 mg L^{-1} BAP or Kin (HiMedia®, Mumbai, India) for axillary shoot bud induction. Influence of 25 mg L^{-1} each of adenine sulfate, L-arginine, and citric acid and 50 mg L^{-1} ascorbic acid (all additives were procured from HiMedia®, India) on shoot growth and development were studied. Also, the effect of MS, $\frac{1}{2}$ MS, WP (Lloyd and McCown 1981), or $\frac{1}{2}$ WP on bud-breaking/shoot bud induction were optimized. The WP and MS media contained 2.0 and 3.0% (w/v) sucrose, respectively, and solidified with 0.8% (w/v) agar (Qualigens Fine Chemicals, Mumbai, India). The pH of medium with PGRs was adjusted to 5.8 ± 0.02 prior to autoclaving at 1.1 kg cm^{-2} pressure and 121°C temperature for 15 to 16 min. The cultures were initially incubated in dark for 2 to 3 d and thereafter shifted to growth room maintained at temperature $26 \pm 2^\circ\text{C}$, photoperiod of 14 to 16-h with a light intensity of 40 to 50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ photon flux density (PFD) and relative humidity (RH) $60 \pm 2\%$.

Shoot multiplication and maintenance Shoots were amplified by two methods: (1) recurrent transfer of mother/original explants (after harvesting the first batch of *in vitro* formed shoots) on fresh nutrient medium containing BAP (0.5, 1.0, 1.5, or 2.0 mg L^{-1}) or IAA (0.05, 0.10, or 0.20 mg L^{-1}) for 4 wk and up to 4 subsequent 4-wk passages. The effects of successive transfers on shoot multiplication were recorded. To overcome the problem of oxidative browning during recurrent passages, different concentration of activated charcoal (AC; 50, 100, or 200 mg L^{-1}) were added to the medium. (2) Subculture of *in vitro* formed shoots (after decapitating apical shoot-tip and making a clump of 2 to 3 shoots, each 2 to 3 cm) on fresh MS medium supplemented with 0.25, 0.5, or 1.0 mg L^{-1} BAP or Kin alone or in combinations with 0.05, 0.10, or 0.20 mg L^{-1} IAA. After optimizing the best combination of PGRs, effect of gelling agents (0.8% (w/v) agar, 0.14% (w/v) gellan-gum (Sigma-Aldrich®, St. Louis, MO) or liquid) on shoot multiplication was evaluated. The shoot cultures were maintained for 2-y by regular subculturing every fourth/fifth week without any change in growth and vigor. During subculturing, sucrose was replaced in the medium with table sugar (Daurala Sugar Cubes, Noida, India).

Concurrent *ex vitro* rooting and acclimatization For rooting under *ex vitro*/greenhouse conditions, the shoots (3 to 6 cm) were individually excised from the multiplied cultures. The

base (2 to 3 mm) of shoots was dipped in 100, 300, 500, or 700 mg L⁻¹ of freshly prepared aqueous solutions of IBA or NAA for 1, 3, 5, or 7 min. These shoots were transferred to the bottles containing sterile soilrite® (a mixture of horticulture grade-expanded perlite, exfoliated vermiculite, and Irish Peat moss in equal ratio; procured from Keltech Energies Limited, Bengaluru, India) and enriched with quarter-strength MS macro-salts. The bottles were surmounted with polycarbonate-caps and placed near the pad section of greenhouse (fitted with cellulose pads that are continuously drenched with water, having high RH (85 to 90%) and moderate temperature (28 ± 2°C)). Once root formation was initiated/observed in treated shoots, the bottles were successively moved towards the fan section (fitted with heavy duty exhaust fans, having low RH (45 to 50%) and high temperature (36 ± 2°C)) of greenhouse. Meanwhile, polycarbonate-caps of the bottles were also unscrewed over a period of 2 to 3 wk and eventually removed thereafter.

Soil/field transfer of acclimatized plantlets The CEVRA plantlets were transplanted to the earthen pots containing a mixture of sand, garden soil, and farmyard manure (1:1:1) and irrigated with tap water three times a week for next 2 to 3 wk and then transferred to the field.

Experiment design, data collection, and statistics analysis All the experiments were set up in a completely randomized block design (RBD) with fifteen replicates per treatment and repeated three times. The observations, shoot number/length and root number/length, were recorded over first to fifth week depending on the experiment. Percentage response was calculated using the formula = (total number of responding explants/total number of cultured explants) × 100. The data were analyzed statistically using one-way analysis of variance (ANOVA) and differences amongst the mean values ($P < 0.05$) were calculated by Duncan's multiple range test (DMRT) using the SPSS (Statistical Package for the Social Sciences) software ver. 17 (IBM, Chicago, IL). All the results are presented in the form of mean ± SD of three independent experiments.

Results and Discussion

Effect of explanting seasons on culture establishment In the present study, culture establishment/response was influenced by the season of explant collection. The explants collected during the spring season (Feb to March) from the selected lopped tree were found the most responsive followed by the explants collected during autumn, summer, and winter seasons, respectively. Similar trends were reported in *Salvadora persica* (Phulwaria *et al.* 2011) and *S. oleoides* (Shekhawat *et al.* 2012). Explants collected during the spring season were

better in terms of percentage bud-breaking response, early bud-breaking, or days to respond and showed the least contamination in this study. Similarly, season-specific culture responses were earlier observed in *Tecomella undulata* (Chhajjer and Kalia 2017) and *Tinospora cordifolia* (Panwar *et al.* 2018). Funada *et al.* (2001) explained that during the spring season, more auxins are synthesized in young buds which trigger cellular division in the cambium and hence assist in enhancing active growth.

Phenolic leaching during culture establishment The problem of phenolic exudation/leaching was observed during culture initiation. To conquer this, sterilized nodal explants were treated with the sterile pre-chilled antioxidant solution (ascorbic acid and citric acid 0.1% (w/v)) for 18 to 20 min prior to inoculation. Similar superficial treatments of ascorbic acid and citric acid were adopted by many researchers in different plants (Phulwaria *et al.* 2012; Bhojwani and Dantu 2013). Initial incubation in dark for 2 to 3 d also assisted in reducing phenolic exudation to an extent. Additionally, such initial exposure of explants to dark/diffused light conditions for a certain period can promote morphogenesis by axillary meristem activation as reported by Panwar *et al.* (2018).

Effect of cytokinins on bud-breaking The nodal segments induced axillary shoot buds on MS medium containing BAP or Kin, within 1 to 2 wk. The frequency of bud-breaking varied from 20.7 to 89.4% on different concentrations of cytokinins tested (Table 1). Of the treatments evaluated, MS containing 3.0 mg L⁻¹ BAP was recorded as the best for bud-breaking on which the maximum number of shoots (approximately 4) was obtained (data not shown). On increasing the concentration of BAP beyond 3.0 mg L⁻¹, moderate callusing, fewer, and vitrified shoots with stunted growth were observed. Explants cultured on MS basal medium (control) did not show any bud-breaking. Similarly, the efficiency of BAP over other cytokinins in axillary shoot bud induction has been well reported in several tree/woody species (Shekhawat and Manokari 2016; Zarei *et al.* 2020; Machado *et al.* 2020).

Effect of additives on bud-breaking Incorporation of additives in the BAP-3.0 mg L⁻¹ optimized MS medium, significantly improved shoot induction (5.3 ± 0.82 per node) and shoot health (Fig. 2a). In additives, adenine sulfate may have complemented the effect of cytokinins owing to its base structural similarity to cytokinins (Chhajjer and Kalia 2017). Likewise, addition of 15 mg L⁻¹ adenine sulfate improved multiple shoot formation in *Withania somnifera* (Sivanandhan *et al.* 2015). According to Greenwell and Ruter (2018), the molecular structure of arginine (C₆H₁₄N₄O₂) includes two extra nitrogen (imino) groups, and this higher percentage of reduced nitrogen (32.2%) may have been responsible for the improved shoot elongation

Table 1. Effect of Murashige and Skoog medium containing 6-benzylaminopurine (BAP) and kinetin (Kin) on bud-breaking from mature nodal explants of *Mitragyna parvifolia* (Roxb.) Korth. (after 4 wk)

Cytokinins (mg L ⁻¹)		% explants responded	Average shoot number ± SD	Average shoot length (cm) ± SD
BAP	Kin			
Control	-	0.0 ^g	0.0 ± 0.0 ^g	0.0 ± 0.0 ^f
1.0	-	57.1 ^d	1.9 ± 0.56 ^d	1.38 ± 0.29 ^d
2.0	-	72.3 ^b	3.1 ± 0.73 ^c	2.41 ± 0.40 ^c
3.0	-	89.4 ^a	5.3 ± 0.82 ^a	3.69 ± 0.59 ^a
4.0	-	75.2 ^b	3.9 ± 0.73 ^b	3.01 ± 0.41 ^b
-	1.0	20.7 ^f	0.7 ± 0.23 ^f	0.84 ± 0.26 ^e
-	2.0	48.2 ^c	1.3 ± 0.67 ^c	0.90 ± 0.31 ^e
-	3.0	63.0 ^c	2.1 ± 0.73 ^d	1.99 ± 0.41 ^c
-	4.0	72.4 ^b	3.2 ± 0.78 ^c	2.98 ± 0.47 ^b

Means in each column followed by same letters at superscript are not significantly different according to Duncan's Multiple Range Test at $P < 0.05$



Fig. 2. Micropropagation protocol of *Mitragyna parvifolia* (Roxb.) Korth. (a) Shoot bud induction on Murashige and Skoog medium + 6-benzylaminopurine (BAP; 3.0 mg L⁻¹) supplemented with additives (after 4 wk). (b) Shoot amplification through recurrent transfer of mother explant on MS medium containing BAP (1.0 mg L⁻¹), Indole-3-acetic acid (IAA; 0.1 mg L⁻¹), additives, and activated charcoal (AC; 100 mg L⁻¹) after second passage (after 4 wk). (c) and (d) Shoot

amplification through subculturing of *in vitro* formed shoots on MS medium containing BAP 0.5 mg L⁻¹, kinetin 0.25 mg L⁻¹, IAA 0.1 mg L⁻¹, additives, and AC 100 mg L⁻¹ on 0.8% (w/v) agar-gelled medium after 3 wk and 4 wk, respectively. (e) *Ex vitro* rooting in shoots treated with Indole-3-butyric acid (IBA; 500 mg L⁻¹) for 5 min (after 5 wk). (f) Hardened plants transferred to earthen pots (after 8 wk).

(3.69 ± 0.59 cm). Similarly, addition of 7.5 mg L^{-1} L-arginine facilitated the elongation of shoot buds in *Jatropha curcas* (Liu *et al.* 2016). Furthermore, ascorbic acid and citric acid work as antioxidants in the culture medium (Bhojwani and Dantu 2013). The advantageous effects of these additives in improving *in vitro* growth and development have been recognized in *Alhagi maurorum* (Agarwal *et al.* 2015) and *Blyttia spiralis* (Patel *et al.* 2016b). Therefore, these additives were regularly added to the forthcoming media.

Effect of media type on bud-breaking Of the type and strength of media evaluated, full-strength MS medium proved to be most suitable for bud-breaking. However, explants responded on all the media types and strengths but percent response, shoot number, and shoot length were less on WP, $\frac{1}{2}$ MS, and $\frac{1}{2}$ WP media, respectively. In comparison with MS medium, WP medium contains reduced amounts of nitrates and higher amount of calcium and thiamine, and lacks iodine or cobalt (Lloyd and McCown 1981). The superiority of MS medium over WP medium for tree species has been acknowledged in *Salvadora oleoides* (Shekhawat *et al.* 2012), *Terminalia bellirica* (Phulwaria *et al.* 2012), and *Neolamarkia cadamba* (Huang *et al.* 2020).

Shoot multiplication and maintenance

(i) Recurrent passage of original/mother explants

In the first approach for shoot amplification, the original/mother explants were recurrently cultured on fresh nutrient medium supplemented with cytokinin or cytokinin–auxin combinations, for four passages. Amongst these, MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA produced the greatest number of shoots (8.2 per explant) after the second recurrent passage (Fig. 2b). Although new shoots were formed up to the fourth passage, their multiplication rate decreased with successive passages. Shoot amplification through this approach is a faster, safer, and economic way to regenerate new flush of micro-shoots. This method of shoot amplification has been found successful in a number of tree/woody species such as *Salvadora persica* (Phulwaria *et al.* 2011), *S. oleoides* (Shekhawat *et al.* 2012), and *Couroupita guianensis* (Shekhawat and Manokari 2016).

Effect of activated charcoal Phenolic exudation has been a major concern for *in vitro* establishment and further growth especially in mature and woody tree species. To overcome this problem during recurrent transfers, 100 mg L^{-1} AC was found the best and produced sturdier shoots with well-developed foliage and without any phenolic leaching/oxidative browning. Similarly, beneficial results of AC were also observed in a few reports (Patel *et al.* 2016b; Zarei *et al.* 2020). According to Bhojwani and Dantu (2013), AC is added in culture medium to irreversibly adsorb toxic/brown exudates/

metabolites, phenolic inhibitors, and abscisic acid and to help in improving aeration. Additionally, AC takes part in various stimulatory activities by sustainably releasing its own natural substances, and the adsorbed PGRs and nutrients which ultimately become available to the growing cultures (Thomas 2008). Therefore, AC also became an important constituent of the subculturing media.

(ii) Subculturing of *in vitro* formed shoots

In second approach of amplification, *in vitro* shoots (after making a cluster of 2 to 3 shoots) was subcultured on MS medium containing cytokinin, cytokinin–cytokinin, or cytokinins–auxin combinations. In comparison with 0.25, 0.5, or 1.0 mg L^{-1} BAP alone, greater numbers of shoots were obtained from cultures grown on medium containing combinations of BAP (0.25, 0.5, or 1.0 mg L^{-1}) and Kin (0.25 or 0.5 mg L^{-1}) (Table 2). Similar is the case in *Tecomella undulata* where a combination of two cytokinins proved beneficial in comparison with a single cytokinin (Chhajer and Kalia 2017). Shoots subcultured on MS basal medium without any PGR failed to respond and turned black/brown within 2 to 3 wk. Amongst the concentration and combination of PGRs evaluated, MS medium containing 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} Kin, and 0.1 mg L^{-1} IAA gave the optimum multiplication response. On this medium, along with additives (25 mg L^{-1} each of adenine sulfate, L-arginine, and citric acid and 50 mg L^{-1} ascorbic acid) and 100 mg L^{-1} AC, the maximum numbers of shoots (13.4 ± 1.26) of mean shoot length 6.2 ± 1.03 cm was obtained after 4 wk incubation (Fig. 2c and d). In contrast to only BAP- or Kin-supplemented media, greater multiplication rate/response was observed on the medium supplemented with a combination of BAP, Kin, and IAA. In the present study, supplementation of IAA in subculturing medium significantly ($P < 0.05$) enhanced shoot multiplication by fostering axillary branching. A combination of two cytokinins (BAP and Kin) and an auxin (IAA) acted synergistically and positively regulated shoot amplification in this study. This auxin–cytokinin synergism is known to control various significant developmental activities, including the formation and maintenance of meristem that are essential for shoot branching/regeneration (Su *et al.* 2011). Similar synergism of auxin and cytokinin(s) for efficient shoot amplification has been reported in *Salvadora oleoides* (Phulwaria *et al.* 2014), *Tinospora cordifolia* (Panwar *et al.* 2018), and *Moringa oleifera* (Gupta *et al.* 2020). The replacement of sucrose with table sugar did not affect the multiplication rate/response in this study as evidenced by Chhajer and Kalia (2017).

The shoot number achieved in this study (13.4 ± 1.26) was significantly greater than 8 to 10 reported by Roy *et al.* (1988) in the same taxon. The possible reasons behind this increment may be the various modifications that were made during the

Table 2. Effect of Murashige and Skoog medium containing additives, 100 mg L⁻¹ activated charcoal, and various concentration and combination of PGRs on shoot amplification through subculturing of *in vitro*-formed shoots of *Mitragyna parvifolia* (Roxb.) Korth. (after 4 wk)

Concentrations of PGRs (mg L ⁻¹)			Average shoot number ± SD	Average shoot length (cm) ± SD
BAP	Kin	IAA		
Control	-	-	0.0 ± 0.0 ⁱ	0.0 ± 0.0 ^g
0.25	-	-	2.1 ± 0.56 ^h	3.29 ± 0.39 ^f
0.5	-	-	4.2 ± 0.78 ^{ef}	3.79 ± 0.46 ^e
1.0	-	-	3.1 ± 0.73 ^g	4.69 ± 0.52 ^d
0.5	0.25	-	7.1 ± 0.87 ^d	5.19 ± 0.59 ^c
0.5	0.5	-	5.0 ± 0.81 ^e	5.8 ± 0.67 ^{ab}
0.5	1.0	-	3.9 ± 0.73 ^{fg}	4.19 ± 0.48 ^{de}
0.5	0.25	0.05	10.1 ± 0.99 ^b	5.4 ± 0.60 ^{bc}
0.5	0.25	0.10	13.4 ± 1.26 ^a	6.2 ± 1.03 ^a
0.5	0.25	0.20	8.7 ± 0.94 ^c	4.30 ± 0.52 ^d

Means in each column followed by same letters at superscript are not significantly different according to Duncan's Multiple Range Test at $P < 0.05$ 6-benzylaminopurine (BAP), kinetin (Kin), and Indole-3-acetic acid (IAA)

culture process over Roy *et al.* (1988) such as removing branches (lopping) of mother/source plant, season of explant collection, selection of an appropriate combination of PGRs, incorporation of additives, and AC in the medium.

Effect of gelling agents Of the two gelling agents evaluated, MS medium containing 0.8% agar (w/v) was found better in terms of both shoot multiplication and shoot quality. On 0.14% (w/v) gellan-gum solidified medium, shoot multiplication was significantly lower ($P < 0.05$) and shoots turned to yellow/light green color with excessive rooting at the base of shoot clump. These results are in consonance with Sharma *et al.* (2011) and Patel *et al.* (2016b). In this study, liquid medium proved the least effective for shoot multiplication. Agar is the most important and commonly used gelling agent in micropropagation owing to its stability, steadiness, high gel clarity and strength, and resistance to metabolism during culture. According to Ziv (1991), gelling agents may affect the water availability, humidity, headspace composition, and availability of other media components, especially cytokinins, in a sealed container. Therefore, MS medium containing 0.5 mg L⁻¹ BAP, 0.25 mg L⁻¹ Kin, 0.1 mg L⁻¹ IAA, 25 mg L⁻¹ adenine sulfate, 25 mg L⁻¹ L-arginine, 25 mg L⁻¹ citric acid, 50 mg L⁻¹ ascorbic acid, 100 mg L⁻¹ AC, 3% sucrose/table sugar, and 0.8% agar (w/v) was the optimal shoot multiplication medium.

Concurrent *ex vitro* rooting and acclimatization *In vitro* regenerated shoots were rooted *ex vitro* under greenhouse conditions when pulse-treated with IBA or NAA for different time durations. The frequency of rooting ranged from 35.7 to 90.1% across the four auxin concentrations tested (Table 3). The overall rooting performance increased with

increasing concentration of IBA up to the optimum level of 500 mg L⁻¹ IBA and decreased thereafter. The basal end of the shoots treated with 500 mg L⁻¹ IBA for 5 min proved the best in which 90.1% of shoots formed the highest number of roots (8.5 ± 0.97 per shoot) with an average length of 9.40 ± 1.06 cm, after 5 wk (Fig. 2e). This is the first report on *ex vitro* rooting in *M. parvifolia*. Micro-shoots failed to root *ex vitro* on the control treatment. Klerk *et al.* (1995) explained that the auxin pulse treatment improved rooting efficiency in apple cuttings by initiating the first cellular division within 24 to 96-h of the treatment. The efficiency of IBA over other root inducing phytohormones has been well recognized in many species including trees such as *Couroupita guianensis* (Shekhawat and Manokari 2016), *Campomanesia xanthocarpa* (Machado *et al.* 2020), and *Picea abies* (Zarei *et al.* 2020).

CEVRA has many advantages over the *in vitro* rooting: (1) the *ex vitro* formed roots have root hairs (lateral root system) which make them more effective during transplantation, (2) *ex vitro* rooting lacks callus formation at the root-shoot junction, (3) the vascular connection between *ex vitro* formed roots and the shoot is well developed (Bhojwani and Dantu 2013), (4) *ex vitro* rooting does not require additional acclimatization (an extra step in the *in vitro* rooting; Panwar *et al.* 2018), (5) it is economical as it requires less time, labor, and chemicals (Phulwaria *et al.* 2012). Acclimatization and hardening of regenerated plantlets is a bottleneck factor in transferring micropropagation technology from lab to field conditions. Thus, adoption of the CEVRA technique could be a boon, especially for tree species where rooting, acclimatization, and hardening are major constraints in transplantation. These steps are believed to be the most labor/resource

Table 3. Influence of Indole-3-butyric acid (IBA) and α -Naphthalene acetic acid (NAA) on *ex vitro* rooting of micropropagated shoots of *Mitragyna parvifolia* (Roxb.) Korth. (after 5 wk)

Auxins (mg L ⁻¹)		% shoots rooted	Average root number \pm SD	Average root length (cm) \pm SD
IBA	NAA			
Control	-	0.0 ^g	0.0 \pm 0.0 ^h	0.0 \pm 0.0 ^c
100	-	54.2 ^e	2.2 \pm 0.63 ^f	2.90 \pm 0.77 ^d
300	-	69.0 ^c	5.3 \pm 0.82 ^{bc}	5.39 \pm 0.84 ^b
500	-	90.1 ^a	8.5 \pm 0.97 ^a	9.40 \pm 1.06 ^a
700	-	75.3 ^b	6.1 \pm 0.87 ^b	5.89 \pm 0.90 ^b
-	100	35.7 ^f	1.1 \pm 0.73 ^g	2.30 \pm 0.68 ^d
-	300	54.1 ^e	3.2 \pm 0.78 ^e	3.10 \pm 0.75 ^d
-	500	66.1 ^c	5.0 \pm 0.81 ^{cd}	4.45 \pm 0.83 ^c
-	700	60.3 ^d	4.2 \pm 0.78 ^d	4.00 \pm 0.81 ^c

IBA and NAA pulse treatment duration: 5 min. Means in each column followed by same letters at superscript are not significantly different according to Duncan's Multiple Range Test at $P < 0.05$

intensive and determine the eventual success of a clonal propagation. It is estimated that CEVRA can reduce up to 30 to 70% cost of a micropropagation protocol and maximize the transplant survival rate in the field (Patel *et al.* 2016b; Panwar *et al.* 2018).

Soil/field transfer of acclimatized plantlets The CEVRA plantlets (10 to 12 cm) obtained from all the rooting experiments were transplanted to the earthen pots containing a mixture of sand, garden soil, and farmyard manure (1:1:1) and kept in the greenhouse near the fan section for the next 2 to 3 wk (Fig. 2f). CEVRA facilitated the plantlets to develop their phototrophic growth by enhancing leaf size, thickness, and cuticle development. Afterwards, successfully hardened plants with more than 80% transplant survival rate were transferred to the nursery and subsequently to the field. The micropropagated plants exhibited normal morphology and growth characteristics that are phenotypically analogous to the mother plant.

Conclusions

A simple, efficient, and repeatable micropropagation protocol using mature nodal explants of *Mitragyna parvifolia*, an endangered and pharmaceutically important tree of the Indian subcontinent, has been developed. This protocol is improvised over the earlier report (Roy *et al.* 1988) by standardizing the seasonal and micro-environmental factors influencing *in vitro* propagation such as removing branches (lopping) of mother plant, season of explant collection, media types and their strengths, recurrent transfer of mother explants, PGRs, additives, activated charcoal, and gelling agents. This is the first report on concurrent *ex vitro* rooting and acclimatization

(CEVRA) of *M. parvifolia* which made this protocol economically favorable for the *ex situ* conservation and for providing the raw material for extraction of numerous significant alkaloids.

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