Optimization of *in vitro* and *ex vitro* regeneration and micromorphological studies of *Micrococca mercurialis* (L.) Benth.

Mani Manokari ¹, Mahipal S. Shekhawat ^{2*}

Mani Manokari ¹
e-mail: manokari01@gmail.com

Mahipal S. Shekhawat ^{2*}
e-mail: smahipal3@gmail.com

¹ Department of Botany, K.M. Center for Postgraduate Studies, Pondicherry, India

² Biotechnology Laboratory, Department of Plant Science, M.G.G.A.C., Mahe, Pondicherry, India

* corresponding author

Manuscript received: 09.03.2017
Review completed: 05.07.2017
Accepted for publication: 07.07.2017
Published online: 08.07.2017

ABSTRACT

A regeneration protocol has been successfully developed using nodal segments and leaf explants of *Micrococca mercurialis* (L.) Benth. The surface sterilized leaf explants were induced to proliferative green colored callus on Murashige and Skoog (MS) medium supplemented with 0.5 mg l⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D). Shoots were regenerated from the callus on MS medium supplemented with 0.5 mg l⁻¹ 6-benzylaminopurine (BAP). The nodal shoot segments responded on MS medium augmented with 1.0 mg l⁻¹ BAP. The shoots were multiplied on MS medium fortified with 0.5 mg l⁻¹ each of BAP, kinetin (Kin) and 0.1 mg l⁻¹ α -naphthalene acetic acid (NAA). The maximum 17.3 shoots with 8.58 cm average length were resulted on this media combination. The shoots were rooted *ex vitro* when pulse treated with 100 mg l⁻¹ indole-3 butyric acid (IBA). The plantlets were successfully established in the field with 96% success rate. Micromorphological studies were conducted for the successive stages of *in vitro*, *ex vitro* and *in vivo* grown plants to analyze the foliar developmental changes taken place across the environments. It has been concluded that the abnormal morphophysiology of *in vitro* plantlets were repaired while acclimatization under the greenhouse conditions before transferring to the field, which speed up the micropropagation process and increase the survival rate of the plantlets.

Keywords: *Micrococca mercurialis*, regeneration, *ex vitro* rooting, micromorphological studies, organogenesis

РЕЗЮМЕ

Манокари М., Шехават М.С. Оптимизация процесса регенерации *in vitro* и *ex vitro* и микроморфологические исследования *Micrococca mercurialis* (L.) Benth. Протокол регенерации был успешно разработан с использованием узловых сегментов и эксплантов листьев *Micrococca mercurialis* (L.) Benth. Из эксплантов листьев был получен пролиферативный зеленый каллус на среде Murashige и Skoog (MS) с добавлением 0,5 мг/л 2,4-дихлорфеноксиксусной кислоты (2,4-D). Побеги регенерировали из каллуса на среде MS с добавлением 0,5 мг/л 6-бензиламинопурина (BAP). Сегменты узловых побегов отреагировали на среду MS, дополненную 1 мг/л BAP. Побегли были размножены на среде MS, обогащенной 0,5 мг/л кинетин (Kin) и 0,1 мг/л α -нафталин уксусной кислотой (NAA). Максимальное число побегов – 17,3, со средней длиной 8.58 см было получено в этой комбинации сред. Побеги были укоренены *ex vitro* при периодической обработке 100 мг/л индол-3 масляной кислотой (IBA). Ростки были получены, коэффициент прорастания – 96 %. Микроморфологические исследования проводились для последовательных стадий развития растений *in vitro*, *ex vitro* и *in vivo* для анализа изменений развития листьев в разных средах. Сделан вывод о том, что аномальная морфофизиология проростков *in vitro* была компенсирована при акклиматизации в тепличных условиях до переноса в природную среду, что ускоряет процесс микроразмножения и увеличивает выживаемость проростков.

Ключевые слова: *Micrococca mercurialis*, регенерация, укоренение *ex vitro*, микроморфологические исследования, органогенез

Micrococca mercurialis (L.) Benth. belongs to the family Euphorbiaceae, is an annual erect herb and commonly known as badam or dese badam. The species is distributed throughout the India, Malaysia, Africa, Yemen, Sri Lanka and America. It prefers waterlogged alluvium clay and 365-1300 m altitude to grow (Choudhury et al. 2014). It is re-

ported as a rare and endangered plant species in India, and used as wild source of food with immense medicinal values (Shackleton et al. 1998, Sahoo 2001).

Traditionally, this plant is used as purgative, and to treat rheumatic pain, constipation, headache and filariasis of eyes (Tiware et al. 2011). The plant extract is reported to possess

anthocyanins, alkaloids, steroid, tannins, saponins, flavonoids, quinines, glycosides, terpenoids, phenols, phlobatanins, anthraquinones, coumarins, xanthoprotein and gums (Choudhury et al. 2014, Prasanna et al. 2015). Plant extracts exhibit potential against human pathogenic bacteria, and explored for its anticancer activity (Rao & Raju 1975, Jeyachandran & Bastin 2013).

The natural regeneration of *M. mercurialis* has been hampered due to the lack of cultivation practices. Continuous exploitation from wild for the commercial applications emphasizes the need to develop the in vitro propagation protocol for *M. mercurialis*. Literature survey ensures that, this plant is less explored in the field of biotechnology (Jeyachandran & Bastin 2013). Therefore, the development of an efficient in vitro regeneration protocol for *M. mercurialis* could be significant for the production of plantlets within short period of time.

It has been observed that the *ex vitro* rooting methods could address some of the problems in the large scale propagation of plantlets (Engelmann 2011) and reduce the cost of production (Lodha et al. 2014). The present communication reports the in vitro regeneration of shoots and *ex vitro* rooting in *M. mercurialis*. Foliar micromorphological studies were conducted at significant stages to study the plant developmental changes towards the changed environmental conditions. This study could help in understanding the response of plants towards changed environmental conditions.

MATERIAL AND METHODS

Selection of plant material and disinfection

Phenotypically healthy plants of *M. mercurialis* were collected from in and around Puducherry (a union territory in the southeast India) during monsoon months (November 2014 to January 2015). Two types of explants, namely fresh nodal segment (3–4 cm length), and freshly emerged leaves (4–5 cm) were selected. The explants were treated with 0.1 % (w/v) solution of Bavistin (systemic fungicide) for 5–7 min and washed with autoclaved distilled water. The explants were disinfected using 0.1 % (w/v) mercuric chloride (HgCl_2) for 4–5 min and thereafter washed with autoclaved distilled water for 5–6 times to make the explants free from sterility.

Culture medium and in vitro culture conditions

Murashige and Skoog medium (Murashige & Skoog 1962) was used to establish the cultures. The pH of nutrient medium was attuned to 5.8 ± 0.02 using 0.1 N NaOH or HCl prior to steam sterilization at 1.06 kg cm^{-2} pressure for 15 min at 121°C temperature. All the cultures were incubated at $16/8\text{-h d}^{-1}$ light/dark period in a growth room maintained with $40\text{--}50 \mu\text{mol m}^{-2}\text{s}^{-1}$ Spectral Flux Photon Density (SFPD) light intensity, provided by cool and white fluorescent tubes (Philips, India) at $28 \pm 2^\circ\text{C}$ temperature and 60–70 % relative humidity (RH).

Callus induction, proliferation and regeneration of shoots

The surface sterilized leaf explants dissected to an appropriate size ($2\text{--}3 \times 0.3\text{--}0.5 \text{ cm}$) were inoculated horizontally on MS medium enriched with additives (50 mg l^{-1} ascor-

bic acid and 25 mg l^{-1} each of adenine sulfate, citric acid and arginine), 3 % (w/v) sucrose, gelled with 0.8 % (w/v) agar and supplemented with various concentrations ($0.1\text{--}2.0 \text{ mg l}^{-1}$) of 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. The calli formed from the leaf explants were periodically subcultured after every four week on MS medium augmented with same concentration of 2,4-D for further proliferation. The calli were transferred to MS medium fortified with different concentrations ($0.25\text{--}2.0 \text{ mg l}^{-1}$) of 6-benzylaminopurine (BAP), kinetin (6-Furfurylaminopurine, Kin) or combination for regeneration of shoots. The MS medium devoid of PGRs served as control.

Establishment of cultures using nodal explants and multiplication of shoots

The surface sterilized nodal shoot segments were slightly trimmed from the cut ends and inoculated on MS medium enriched with sucrose and gelled with agar. Various concentrations ($0.1\text{--}2.0 \text{ mg l}^{-1}$) and combinations of cytokinins (BAP and Kin) were studied for shoots formation from nodal meristems. The initiated shoot clumps were divided into sub-clumps and transferred to fresh medium for further shoots proliferation. The multiplication medium was fortified with BAP and Kin (0.1 to 2.0 mg l^{-1}) alone or in combinations, and $0.1\text{--}0.5 \text{ mg l}^{-1}$ auxins (indole-3-acetic acid; IAA or α -naphthalene acetic acid; NAA). The cultures were regularly sub-cultured to fresh nutrient medium after an interval of 4–5 week.

Ex vitro rooting of regenerated shoots

Healthy and sturdy microshoots of 5–6 cm length were excised from mass cultures were pulse treated with various auxins [IAA, IBA (indole-3-butyric acid) or NAA] at different concentrations (50, 100, 150, 200, 300 mg l^{-1}) for 4 min for the induction of roots under *ex vitro* conditions. The pulse treated shoots were transplanted 1.5–2.0 cm deep in paper cups (size 150 ml) containing 50 gm autoclaved soilrite® (a mix of expanded perlite, Irish peat moss and exfoliated vermiculite in the ratio of 1:1:1 by volume). One-fourth strength MS macro salts solution was used to irrigate the shoots in *ex vitro* experiments. The plantlets were maintained in the greenhouse at 25°C to 28°C temperature and 80–85 % of relative humidity for five week. All the plantlets were covered with transparent polybags with minute punctures and water was sprayed continuously twice a day.

Acclimatization, hardening and field transfer of plantlets

The *ex vitro* rooted plantlets were transferred to the polybags containing soilrite®, garden soil and organic manure (1:1:1) in greenhouse for further acclimatization process. Upon growth, the plantlets were transferred to earthen pots ($25 \times 25 \text{ cm}$) containing sterile sand, soil and vermiculite (1:1:1), grown in greenhouse and irrigated regularly. After 5 week the plantlets were exposed to low humidity and high temperature area of greenhouse for 4–5 weeks and finally transferred to the field.

Foliar micromorphological studies

Experiments were conducted to study the micromorphological adaptation towards the environmental conditions during plant developmental processes at different

stages (*in vitro*, *ex vitro* and field environments). The foliar micromorphological changes such as stomatal index, venation pattern, vein-islets and veinlet terminations, density of trichomes and raphides of the plants were analyzed.

Plants were randomly selected for the micromorphological experiments from the suitable environments. The plant materials (leaves) collected from *in vitro* at 4th subculture in multiplication stage, after 4th week of hardening in greenhouse environment and hardened plants transferred to the field (after 6th week). For anatomical comparison, the required leaf samples were collected at third to seventh leaves from the base. To observe the developmental changes in structures of the stomata, stomatal morphology and trichome distribution, the epidermal peelings/paradermal sections were obtained manually by standard methods (Johansen 1940). The stomatal density, frequency and stomatal index were calculated by the methods suggested by Salisbury (1932). Types of stomata have been described by following the classification and terminology as suggested latest by Prabhakar (2004).

Leaves from each stage were excised and fixed primarily in formalin acetic acid alcohol (FAA in the ratio of 1:1:3) solution for venation study. In addition, fixed as well as fresh leaves were cleared in 70 % ethanol (v/v) until chlorophyll was completely removed (12–24 h), bleached with 5 % (w/v) NaOH for 24–48 h, rinsed three times in distilled water and allowed to remain in saturated chloral hydrate solution for 24–48 h (Sass 1940). The cleared leaves were used for the study of venation pattern, vein-islets and veinlet terminations and trichomes. The terminology adopted for venation pattern was of Hickey and Wolfe (1975). The materials were stained with 1 % (v/v) safranin (Loba Chemie, India) aqueous solution for 3–5 min, examined under photomicroscope (Labomed iVu 3100, USA) and analyzed using software Pixelpro. Magnifications of the figures were indicated by the scale-bars.

Observations and data analysis

All the experiments concerning *in vitro* cultures and *ex vitro* rooting were carried out with 20 replicates per treatment and repeated thrice. The data of various experiments were recorded after 4 week of culture. The micromorphological experiments consisted of three treatments (*in vitro*, *ex vitro* and field transferred plants) subjected to statistical analysis by ANOVA using SPSS version 16 (SPSS Inc., Chicago, USA). The significance of differences among mean values was calculated by Duncan's multiple range tests at $P < 0.05$ and the results were presented as mean $\pm$ standard deviation (SD) of three experiments.

RESULTS AND DISCUSSION

Callus induction, proliferation and organogenesis

Out of various concentrations tested, 0.5 mg l⁻¹ of 2,4-D was reported most suitable for callus induction. About 96 % leaf explants responded within week and form callus on MS medium incorporated with additives and 0.5 mg l⁻¹ 2,4-D (Table 1). Callus induced on this medium was green, fast growing and organized (Fig. 1a). The entire explant converted into green, fast growing callus at the end of 3rd

Table 1. Effect of concentrations of 2, 4-D on induction, growth and morphology of callus.

Conc. of 2,4-D (mg l ⁻¹)	Callus induction response (%)	Growth and morphology of the callus
0.0	24.1 ^a	Creamy white, non-proliferative
0.1	79.0 ^d	Pale green, slow growing, unorganized
0.3	87.4 ^f	Green, slow growing, organized
0.5	96.0 ^g	Green, fast growing, organized
1.0	80.9 ^e	Yellowish green, less proliferative
1.5	72.2 ^c	Yellow, less proliferative, unorganized
2.0	64.4 ^b	Brown, hard, non-proliferative

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

week (Fig. 1b). Non-proliferative creamy-white colored calli (24% response) formed from the leaf explants inoculated on controlled medium (without 2,4-D). The higher concentrations of 2,4-D resulted in poor response and form less proliferative and unorganized callus.

Calli were further proliferated by sub-culturing of callus on fresh MS medium containing 0.5 mg l⁻¹ 2,4-D after 4 week interval. The proliferation capacity of callus was retained up to 3 months on this concentration (Fig. 1c). Continuous sub-culture of callus on 2,4-D alone is reported to inhibit the proliferation rate due to excessive accumulation of 2,4-D in the cells (Rai et al. 2010). Callus induction from leaf explants on 2,4-D was also reported in other Euphorbiaceae species such as *Manihot esculenta* and *Croton urucurana* (Raemakers et al. 2000, Lima et al. 2008). The shoots formation from the callus was achieved by transferring of calli onto MS medium supplemented with 0.5 mg l⁻¹ BAP (Fig. 1d) in this study. The maximum 15 shoots per culture vessel were regenerated on this medium combination (Table 2). Regeneration of shoots from callus could be directed by the growth regulators present in the medium (Kuppusamy et al. 2009).

Shoot induction from nodal segment explants

In current study the axillary buds were activated within 10 days from the nodal shoot segments placed vertically on the culture medium. The nodal segments showed initial axillary bud break response with all the concentrations and combinations of BAP and Kin tried, but no response was observed in control experiments. The maximum response in terms of percentage response, number and length of shoots was recorded on MS medium fortified with BAP alone (Table 3). Shoot segments with axillary buds are reported as excellent explants in many Euphorbiaceae members (Datta et al. 2007). The meristematic tissues in axillary buds play significant role in establishment of cultures with reduced rate of contamination (Lodha et al. 2014).

The highest response (97 %) and number of shoots (4.2 shoots/explant) was observed on medium augmented with 1.0 mg l⁻¹ BAP after 4 week of incubation (Fig. 1e). The shoots exhibited induction of *in vitro* flowering and fruits setting at this concentration (Fig. 1f). The combined effect of BAP and Kin was reported to be better (3.9 shoots per

Figure 1 *In vitro* propagation of *Micrococca mercurialis*: a to c – different stages in callus induction from leaf explants; d – organogenesis and development of shoots from callus; e – bud break from nodal explants; f – *in vitro* flowering and fruit setting from *in vitro* shoots; g and h – multiplication of shoots on MS medium; i. *ex vitro* rooted shoot in soilrite medium pretreated with 100 mg l⁻¹ indole-3 butyric acid.

explants) than the solitary effects of Kin (3.3 shoots). Jeyachandran & Bastin (2013) induced maximum 3.3 shoots from nodal explants of *M. mercurialis* using 2.0 mg l⁻¹ BAP and 3.0 mg l⁻¹ NAA. They also reported fruit setting in old cultures (after 170 days of incubation), but early development of flowers and fruits were observed in this study.

Multiplication of shoots *in vitro*

Shoots derived through callus regeneration and axillary bud induction were sub-divided into clumps and transferred to different hormonal concentrations of cytokinins and auxins. It was observed that incorporation of NAA at the place of IAA in the medium improved the growth of

the shoots (data not presented). Maximum 17.3 shoots with 8.58 cm length was recorded on MS medium fortified with 0.5 mg l⁻¹ each of BAP and Kin + 0.1 mg l⁻¹ NAA (Fig. 1g and 1h). Presence of BAP in the medium found appropriate for shoot multiplication. Naphthalene acetic acid (0.1 mg l⁻¹) in combination with BAP, and Kin induced maximum 12.5 and 10.2 shoots respectively (Table 4). The rate of shoots multiplication in our study was superior as compared to the reports of Jeyachandran & Bastin (2013) in this

Table 2. Effect of cytokinins (BAP and Kin) on regeneration of shoots from leaf derived callus.

Conc. of BAP (mg l ⁻¹)	Conc. of Kin (mg l ⁻¹)	Number of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
Control 0.0	0.0	00.0 ± 0.00 ^a	00.0 ± 0.00 ^a
0.25	0.0	13.7 ± 0.22 ^b	3.17 ± 0.19 ^{de}
0.5	0.0	15.0 ± 0.17 ⁱ	5.03 ± 0.13 ^g
1.0	0.0	10.4 ± 0.13 ^g	4.91 ± 0.20 ^g
1.5	0.0	07.5 ± 0.25 ^{cd}	4.12 ± 0.17 ^{fg}
2.0	0.0	06.1 ± 0.19 ^{ab}	3.76 ± 0.11 ^{ef}
0.0	0.25	07.4 ± 0.10 ^{cd}	2.98 ± 0.16 ^{cd}
0.0	0.5	08.9 ± 0.18 ^{ef}	4.50 ± 0.12 ^{fg}
0.0	1.0	07.0 ± 0.23 ^{cd}	3.88 ± 0.20 ^{ef}
0.0	1.5	06.7 ± 0.20 ^{bc}	3.62 ± 0.26 ^{ef}
0.0	2.0	05.3 ± 0.12 ^b	2.93 ± 0.19 ^{bc}
0.25	0.25	09.0 ± 0.15 ^{ef}	2.16 ± 0.21 ^b
0.5	0.5	10.5 ± 0.19 ^g	3.69 ± 0.11 ^{ef}
1.0	1.0	09.8 ± 0.10 ^{fg}	3.00 ± 0.17 ^{cd}
1.5	1.5	08.1 ± 0.16 ^{de}	2.79 ± 0.20 ^{bc}
2.0	2.0	06.3 ± 0.11 ^{bc}	2.52 ± 0.16 ^{ab}

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at P<0.05.

Table 3. Effect of cytokinins (BAP and Kin) on multiple shoot induction from nodal explants of *M. mercurialis*.

Cytokinins Conc. (mg l ⁻¹)	Response (%)	Number of Shoots (Mean ± SD)	Shoot Length (cm) (Mean ± SD)
Control 0.0	0	0.0 ± 0.00 ^a	0.0 ± 0.00 ^a
BAP			
0.1	62	2.2 ± 0.14 ^b	2.7 ± 0.22 ^{cd}
0.3	75	2.8 ± 0.19 ^c	3.8 ± 0.10 ^f
0.5	80	3.5 ± 0.00 ^e	4.3 ± 0.13 ^g
1.0	97	4.2 ± 0.16 ^f	5.0 ± 0.25 ^h
1.5	91	3.7 ± 0.20 ^{ef}	4.5 ± 0.12 ^g
2.0	84	3.0 ± 0.11 ^d	3.1 ± 0.26 ^{de}
Kin			
0.1	49	1.9 ± 0.00 ^b	2.4 ± 0.15 ^b
0.3	55	2.5 ± 0.13 ^c	3.6 ± 0.20 ^f
0.5	64	2.9 ± 0.10 ^{cd}	3.9 ± 0.29 ^f
1.0	79	3.3 ± 0.12 ^e	4.1 ± 0.18 ^g
1.5	70	3.0 ± 0.26 ^{cd}	3.5 ± 0.22 ^{ef}
2.0	63	2.6 ± 0.18 ^c	2.9 ± 0.00 ^d
BAP & Kin			
0.1	56	2.0 ± 0.11 ^b	2.0 ± 0.17 ^b
0.3	69	2.7 ± 0.00 ^c	2.5 ± 0.22 ^c
0.5	74	3.4 ± 0.23 ^c	3.2 ± 0.13 ^{de}
1.0	82	3.9 ± 0.11 ^f	4.4 ± 0.20 ^{ef}
1.5	79	3.1 ± 0.20 ^d	2.9 ± 0.12 ^d
2.0	71	2.8 ± 0.14 ^{cd}	2.0 ± 0.00 ^b

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at P<0.05.

Figure 2 Acclimatization of *M. mercurialis*: a – plantlet in greenhouse; b – field transferred plant.

plant species. Villaluz (2006) and Ricardo et al. (2007) reported that the addition of NAA in MS medium with BAP induced rate of shoot multiplication in many plant species.

Ex vitro rooting of shoots and field transfer of plantlets

Researchers estimated that the *ex vitro* rooting method is more economical which could reduce 70–75 % cost in the development of *in vitro* propagation protocols (Lodha et al. 2014). The information about *ex vitro* rooting is very scarce in the Euphorbiaceae family. The results of *ex vitro* rooting experiments clearly revealed that the concentration and treatment duration of auxins had significant role in *ex vitro* rooting. The shoots were rooted with all the concentrations of auxins tested for root initiation. About 94 % shoots were rooted under *ex vitro* conditions (in greenhouse) when pulse treated with 100 mg l⁻¹ of IBA for 4 min. Five roots with 3.92 cm length were differentiated from each shoot after 4 week. (Fig. 1i). Only 86 % of shoots were rooted (3.8 roots with 3.1 cm length) with the same concentration of NAA. Indole-3 acetic acid was reported to be less effective for the *ex vitro* rooting of the shoots of *M. mercurialis* and 83 % of shoots were rooted with 3.0 roots per shoot when pulsed with 100 mg l⁻¹ IAA (Table 5). The superiority of IBA in *ex vitro* rooting has also been reported in *Hemidesmus indicus* (Shekhawat & Manokari 2016b) and *Blyttia spiralis* (Patel et al. 2016) because the absorption of IBA was reported to be faster than other auxins (Van Der Krieken et al. 1993).

The plantlets were kept in the greenhouse for next 4 week for formation of fresh leaves (Fig. 2a). The hardened plantlets were successfully shifted to the natural soil conditions with 96 % rate of survivability (Fig. 2b). The *ex vitro* rooting mechanism in greenhouse provides positive gaseous exchange and CO₂ supply (Voráčková et al. 1998). The elevated concentration of CO₂ and higher irradiance in greenhouse also enhanced rate of photosynthesis (Kanechi et al. 1998).

Foliar micromorphological studies and developmental changes

Foliar micromorphological studies were conducted to analyze the micromorphological changes in *M. mercurialis* at the subsequent stages of *in vitro*, *ex vitro* (greenhouse) and field transfer (*in vivo*) of micropropagated plants. The leaves were lanceolate type and shown alternate arrangement in all the samples. The less prominent midrib was observed with the *in vitro* grown leaves, but prominent in *ex vitro* rooted and fairly prominent found in field transferred plants.

Table 4. Effect of concentrations of cytokinins (BAP and Kin) + 0.1 mg l⁻¹ NAA on multiplication of shoots.

Growth Regulators Conc. (mg l ⁻¹)	Number of Shoots (Mean ± SD)	Shoot Length (cm) (Mean ± SD)
0.0	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
BAP		
0.1	10.0 ± 0.26 ^g	6.44 ± 0.12 ^f
0.5	12.5 ± 0.42 ^h	8.90 ± 0.38 ^h
1.0	9.7 ± 0.19 ^{fg}	7.14 ± 0.21 ^{gh}
1.5	9.0 ± 0.33 ^f	6.72 ± 0.28 ^{fg}
2.0	8.2 ± 0.00 ^d	6.29 ± 0.20 ^f
Kin		
0.1	7.3 ± 0.18 ^{bc}	4.22 ± 0.33 ^c
0.5	10.2 ± 0.20 ^g	5.97 ± 0.12 ^e
1.0	9.0 ± 0.11 ^f	5.12 ± 0.40 ^d
1.5	7.5 ± 0.00 ^{bc}	4.16 ± 0.21 ^c
2.0	7.0 ± 0.17 ^b	3.91 ± 0.14 ^b
BAP + Kin		
0.1	12.9 ± 0.13 ^h	5.95 ± 0.22 ^e
0.5	17.3 ± 0.25 ^j	8.58 ± 0.17 ^c
1.0	15.0 ± 0.19 ^j	7.61 ± 0.00 ^e
1.5	10.2 ± 0.10 ^g	6.29 ± 0.16 ^f
2.0	8.7 ± 0.12 ^{de}	5.46 ± 0.20 ^d

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at P<0.05.

Table 5. Effect of different auxins and their concentrations on *ex vitro* root induction (pulsing for 4 min).

Auxins concent. (mg l ⁻¹)	Response (%)	Number of roots (Mean ± SD)	Root length (cm) (Mean ± SD)
0	0	0.0 ± 0.00 ^a	0.0 ± 0.00 ^a
IAA			
50	77	2.2 ± 0.18 ^c	1.88 ± 0.15 ^b
100	83	3.0 ± 0.25 ^c	2.46 ± 0.00 ^c
150	79	2.6 ± 0.12 ^d	2.06 ± 0.18 ^c
200	64	2.0 ± 0.20 ^c	1.84 ± 0.29 ^b
300	56	1.5 ± 0.15 ^b	1.66 ± 0.23 ^b
IBA			
50	78	2.6 ± 0.37 ^d	2.58 ± 0.10 ^{cd}
100	94	4.7 ± 0.15 ^g	3.92 ± 0.23 ^f
150	87	3.9 ± 0.12 ^f	3.20 ± 0.15 ^c
200	79	3.0 ± 0.28 ^c	2.91 ± 0.10 ^d
300	62	2.4 ± 0.20 ^{cd}	2.44 ± 0.22 ^c
NAA			
50	74	2.0 ± 0.13 ^c	1.95 ± 0.26 ^b
100	86	3.8 ± 0.11 ^f	3.10 ± 0.32 ^c
150	72	3.2 ± 0.26 ^{ef}	2.75 ± 0.29 ^d
200	69	2.4 ± 0.21 ^{cd}	2.17 ± 0.15 ^c
300	60	1.7 ± 0.00 ^b	1.73 ± 0.19 ^b

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at P<0.05.

Table 6. Stomatal index of plantlets under *in vitro*, *ex vitro* and field transferred plants.

Field No.	Stomatal Index (SI) (Mean ± SD)		
	<i>In vitro</i> plants	<i>Ex vitro</i> rooted (greenhouse)	Field transferred plants
1	5.3±0.10 ^d	19.4±0.11 ^c	23.0±0.29 ^c
2	4.8±0.00 ^{ab}	17.9±0.14 ^d	25.4±0.16 ^e
3	5.2±0.17 ^{cd}	18.6±0.19 ^a	22.6±0.31 ^b
4	5.0±0.11 ^c	19.3±0.13 ^b	24.1±0.17 ^d
5	5.1±0.25 ^c	18.6±0.10 ^{bc}	22.0±0.10 ^b
6	4.9±0.16 ^b	17.9±0.00 ^d	23.3±0.11 ^c
7	4.6±0.23 ^a	19.5±0.16 ^c	22.4±0.29 ^b
8	5.0±0.12 ^c	19.9±0.21 ^d	23.6±0.13 ^c
9	5.4±0.10 ^d	19.7±0.17 ^d	22.6±0.19 ^b
10	4.7±0.00 ^a	19.2±0.10 ^c	21.0±0.14 ^a
Mean	5.0±0.22	19.0±0.13	23.0±0.19

Note: Mean separation was analyzed using SPSS software (ver. 16.0), the values represented in corresponding column followed by same letters are not significantly different according to DMRT at P<0.05.

Figure 3 Frontal view sections of the abaxial epidermis of *M. mercurialis* under *in vitro*, acclimatization in greenhouse and field established plantlets (scale bar – 100 µm, 200 µm): a – stomata in abaxial surface of leaves under *in vitro* conditions (Pa – Paracytic, An – Anisocytic); b – stomata with the leaves grown under *ex vitro* conditions (Pa – Paracytic, An – Anisocytic, Dm – Desmocytic); c – stomata in leaves grown under field conditions (Pa – Paracytic, An – Anisocytic, Am – Anomocytic); d – enlarged view of stomata (*in vitro* leaves); e – enlarged view of stomata (*ex vitro* leaves); f – enlarged view of stomata with field grown leaves; g – venation pattern in leaves (*in vitro*); h – venation pattern in leaves (*ex vitro* conditions) (VI – vein islet, VT – veinlet termination); i – venation pattern in leaves (field conditions) (VI – vein islet, VT – veinlet termination, Rh – raphides); j – underdeveloped non-glandular trichomes of *in vitro* developed leaves; k – development of non-glandular trichomes of leaves under *ex vitro* conditions; l – increased trichome density in field transferred plants.

The abaxial epidermis was thin, single layered and consisted of irregular shaped cells in the *in vitro* grown leaves, but highly compact, thick walled, well organized with wavy outlined cells epidermis observed in the leaves *ex vitro* and field transferred plants. The overlaying epidermal cells on the veins were elongated differed from epidermal undulations in *ex vitro* and field transferred plants, but such types of cells were totally absent with the *in vitro* grown leaves.

Stomatal analysis

Although, the leaves of *M. mercurialis* are amphistomatic, but the stomatal frequency was less on adaxial surface, therefore the abaxial surface was used for the calculation of stomatal index. The leaves of *in vitro* plantlets presented less number of stomata than the *ex vitro* rooted and field transferred plants. Plantlets under *in vitro* culture conditions resulted in lower stomatal index (5.0). The stomatal index increased from *in vitro* to *ex vitro* (19.0) and field transferred plants (23.0) (Table 6). Similar increased stomatal density with reduced stomatal size in field transferred plants reported in *Prunus serotina* and *Rhododendron* sp. (Waldenmaier &

Schmidt 1990, Drew et al. 1992). Pospíšilová et al. (1998) reported that the number of stomata in *Nicotiana tabacum* was increased after *ex vitro* and field transfer due to enormous leaf area.

The stomata developed *in vitro* were sphere shaped but elliptical stomata were recorded in the leaves of *ex vitro* rooted and field transferred plants. The changes in stomatal shapes were also reported in *Vaccinium corymbosum* by Ticha et al. (1999). In present study the stomata were constantly opened under *in vitro* environment irrespective of time of collection of leaves (Fig. 3a). This condition is strictly due to the highly specific *in vitro* culture conditions, which inhibit the development of stomata and reduce their function. The culture vessels prevent the exchange of gaseous plant products lead the stomata to stay open to maintain the symmetry with the *in vitro* environment (Costa et al. 2009). The stomata of *ex vitro* rooted plants started to respond towards light and partially functional at 2nd week and fully functional till 4th week (Fig. 3b). The control of luminous intensity through partial shading, nutrient availability, 25°C to 28°C temperature and water holding capacity of soilrite® under

greenhouse conditions provide superior atmosphere to the developing plantlets.

The field transferred plants presented closed stomata, and fluctuations were observed in the function of the stomata towards the light and humid conditions of the field environment (Fig. 3c). Number of stomatal apparatus increased with the enlargement of leaf lamina is associated with the closure of stomata during harsh conditions are intrinsic to plant's internal stimuli to check transpiration and desiccation of plantlets in the field (Lamhamedi et al. 2003).

In the current study paracytic and anisocytic types of stomata were present with the *in vitro* leaves (Fig. 3a and 3d), but prominently paracytic and rarely desmocyctic and anisocytic stomata observed in *ex vitro* rooted plantlets (Fig. 3b and 3e). Paracytic, anisocytic and anomocytic stomata were observed under field grown leaves (Fig. 3c and 3f). Similar to our results, anomocytic, paracytic, anisocytic and diacytic types of stomata were also reported in this plant species by Rao & Raju (1975).

Venation and trichomes

Reticulate venation was observed in all the samples of the leaves of *M. mercurialis*. Veinlet terminations were absent but irregular, opened and unorganized vein-islets were recorded under *in vitro* conditions (Fig. 3g). The vein-islets were rhomboidal in shape in the leaves of *ex vitro* rooted and field transferred plants. Vein-islets and veinlet terminations were gradually increased from *in vitro* to the field transferred plantlets through acclimatization in greenhouse (data not shown) (Fig. 3h and 3i). The veinlet terminations were rarely branched in both the materials.

Non-glandular trichomes were observed all over the epidermis in all treatments with differences in density. The trichomes were underdeveloped in the *in vitro* leaves, which were gradually developed and attained maturity in acclimatized (*ex vitro*) and field transferred plants (Fig. 3j and 3k). The raphides, recorded in the field transferred plants were totally absent in the plants under *in vitro* and greenhouse conditions (Fig. 3l). Development of raphides is reported to be a defensive activity of plants under stressed environments (Konno et al. 2014). The *in vitro* induced abnormality in morphology, anatomy and physiology are recently reported in many plants (Lodha et al. 2015, Shekhawat & Manokari 2016a, b).

CONCLUSIONS

An efficient *in vitro* regeneration procedure has been developed for *M. mercurialis* using leaves and nodal segments as explants. The shoots were multiplied efficiently and 94 % shoots were rooted *ex vitro* under the greenhouse conditions in soilrite®. *Ex vitro* rooted plantlets were successfully transferred to the field conditions. Foliar micromorphological studies were conducted to analyze the developmental changes in the leaves in response to the altered environmental conditions during *in vitro* cultures, *ex vitro* rooting and field transfer of the hardened plantlets. The study could help in understanding the hardening process of the tissue culture raised plants which could ultimately help to enhance the survival percentage of the micropropagated plants in the field conditions.

ACKNOWLEDGEMENTS

Authors are grateful to the Department of Science, Technology and Environment, Government of Puducherry for providing financial support to our laboratory as Grant-In-Aid Scheme.

LITERATURE CITED

- Choudhury, S., C.H. Rahaman & S. Mandal 2014. *Micrococca mercurialis* benth-pharmacognostic analysis and antimicrobial activity of an important folk medicinal plant. *Journal of Biology, Agriculture and Healthcare* 4:122–128.
- Costa, F.H.S., M. Pasqual, J.E.S. Pereira & E.M. de Castro 2009. Anatomical and physiological modifications of micropropagated 'Caipira' Banana plants under natural light. *Scientia Agricola (Piracicaba Brazil)* 66:323–330.
- Datta, M.M., P. Mukherjee, B. Ghosh & T.B. Jha 2007. *In vitro* clonal propagation of biodiesel plant (*Jatropha curcas* L.). *Current Science* 93:1438–1442.
- Drew, A.P., K.L. Kavanagh & C.A. Maynard 1992. Acclimatizing micropropagated black cherry by comparison with half-sib seedlings. *Physiologia Plantarum* 86:459–464.
- Engelmann, F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In vitro cellular and developmental Biology – Plant* 47:5–16.
- Hickey, L.J. & J.A. Wolfe 1975. The bases of angiosperm phylogeny. Vegetative morphology. *Annals of Missouri Botanical Garden* 62:538–589.
- Jeyachandran, R. & M. Bastin 2013. An efficient protocol for *in vitro* flowering and fruiting in *Micrococca mercurialis* (L.) Benth. *International Journal of Natural and Applied Science* 2:18–22.
- Johansen, D.A. 1940. *Plant Microtechnique*. 1st edition, McGraw Hill Book Co., New York, London.
- Kanechi, M., M. Ochi, M. Abe, N. Inagaki & S. Maekawa 1998. The effects of carbon dioxide enrichment, natural ventilation, and light intensity on growth, photosynthesis, and transpiration of cauliflower plantlets cultured *in vitro* photoautotrophically and photomixotrophically. *Journal of the American Society for Horticultural Science* 123:176–181.
- Konno, K., T.A. Inoue & M. Nakamura 2014. Synergistic defensive function of raphides and protease through the needle effect. *PLOS ONE* 9: e91341. doi:10.1371/journal.pone.0091341.
- Kuppusamy, K.T., C.L. Walcher & Nernhauser 2009. Cross-regulatory mechanisms in hormone signaling. *Plant Molecular Biology* 69:375–381.
- Lamhamedi, M.S., H. Chamberland & F.M. Tremblay 2003. Epidermal transpiration, ultrastructural characteristics and net photosynthesis of white spruce somatic seedlings in response to *in vitro* acclimatization. *Physiologia Plantarum* 118:554–561.
- Lima, E.C., R. Paiva, R.C. Nogueira, F.P. Soares, E.B. Emrich & A.A.N. Silva 2008. Callus induction in leaf segments of *Croton urucurana* Baill. *Ciencia Agrotecnologia* 32:17–22.
- Lodha, D., A.K. Patel, M.K. Rai & N.S. Shekhawat 2014. *In vitro* plantlet regeneration and assessment of alkaloid contents from callus cultures of *Ephedra foliata* (Unth phog), a source of anti-asthmatic drugs. *Acta Physiologia Plantarum* 36:3071–3079.
- Lodha, D., A.K. Patel & N.S. Shekhawat 2015. A high-frequency *in vitro* multiplication, micromorphological studies and *ex vitro* rooting of *Cadaba fruticosa* (L.) Druce (Bahuguni): a multipurpose endangered medicinal shrub. *Physiology and Molecular Biology of Plants*. doi:10.1007/s12298-015-0310-6.

- Murashige, T. & F. Skoog 1962. A revised medium for rapid growth and bioassays with *Tobacco* tissue culture. *Physiologia Plantarum* 15:473–497.
- Patel, A.K., D. Lodha, K. Ram, S. Shekhawat & N.S. Shekhawat 2016. Evaluation of physiochemical factors affecting high-frequency plant regeneration of *Blyttia spiralis* (synonym: *Pentatropis spiralis*), a threatened climber of medicinal value. *In vitro Cellular and Developmental Biology – Plant*. doi:10.1007/s11627-015-9738-1.
- Pospišilová, J., N. Wilhelmová, H. Synková, J. Čatský, D. Krebs, I. Tichá, B. Hanáčková & J. Snopek 1998. Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. *Journal of Experimental Botany* 49:863–869.
- Prabhakar, M. 2004. Structure, delimitation, nomenclature and classification of stomata. *Acta Botanica Sinica* 46:242–252.
- Prasanna, S.C.G., M. Poongani & S. Karpagam 2015. Phytochemical content of the leaf, stem and root of *Micrococca mercurialis* (L.) Benth. A promising herb. *IOSR Journal of Pharmacy and Biological Sciences* 10:24–27.
- Raemakers, K., E. Jacobsen & R. Visser 2000. The use of somatic embryogenesis for plant propagation in Cassava. *Molecular Biotechnology* 14:215–221.
- Rai, M.K., P. Asthana, V.S. Jaiswal & U. Jaiswal 2010. Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. *Tree Structure and Function* 24:1–12.
- Rao, P.N. & V.S. Raju 1975. Development and diversity of stomata in *Micrococca mercurialis* Benth. *Current Science* 44:363–366.
- Ricardo, M.D., M. Faloci Mitra, M.A. Gonzalez, Mroginski & A. Luis 2007. *In vitro* cultured primary roots derived from stem segments of cassava (*Manihot esculenta*) can behave like a storage organs. *Annals of Botany* 99:409–423.
- Sahoo, S. 2001. *Conservation and Utilization of Medicinal and Aromatic Plants*. Allied Publishers, New Delhi, India.
- Salisbury, E.J. 1932. The interrelations of soil, climate and organisms and the use of stomatal frequency as an integrating index of relation of the plant. *Bech. Bot. Zbl.* 99:402–420.
- Sass, J.E. 1940. *Elements of Botanical Microtechnique*. McFraw-Hill Book Co., New York and London.
- Shackleton, S.E., C.M. Dzerefos, C.M. Shackleton & F.R. Mathabela 1998. Use and trading of wild edible herbs in the central Lowveld Savanna region, South Africa. *Economic Botany* 52:251–259.
- Shekhawat, M.S. & M. Manokari 2016a. *In vitro* propagation, micromorphological studies and *ex vitro* rooting of cannon ball tree (*Couroupita guianensis* aubl.): a multipurpose threatened species. *Physiology and Molecular Biology of Plants*. doi:10.1007/s12298-015-0335-x.
- Shekhawat, M.S. & M. Manokari 2016b. *In vitro* regeneration frequency, micromorphological studies and *ex vitro* rooting of *Hemidesmus indicus* (L.) R. Br.: a multi-potent endangered climber. *Indian Journal of Plant Physiology*. doi:10.1007/s40502-016-0216-5.
- Tichá, I., B. Radochová & P. Kadleček 1999. Stomatal morphology during acclimatization of tobacco plantlets to *ex vitro* conditions. *Biologia Plantarum* 42:469–474.
- Tiwari, P., B. Kumar, M. Kaur, G. Kaur, H. Kaur 2011. Phytochemical screening and extraction: a review. *Internationale Pharmaceutica Scientia* 1:99–106.
- Van Der Krieken, W.M., H. Breteler, M.H.M., Visser & D. Mavridou 1993. The role of conversion of IBA into IAA on root regeneration in apple: introduction of a test system. *Plant Cell Reports* 12:203–206.
- Villaluz, Z.A. 2006. Improvement of *in vitro* techniques for rapid meristem development and mass propagation of Philippine cassava (*Manihot esculenta* Crantz). *Journal of Food Agriculture and Environment* 4:220–224.
- Voráčková, Z., H. Lipavská & P. Konečný 1998. The efficiency of transfer of plants cultivated *in vitro* to *ex vitro* conditions as affected by sugar supply. *Biologia Plantarum* 41:507–513.
- Waldenmaier, S. & G. Schmidt 1990. Histologische Unterschiede zwischen in-vitro- und ex-vitro-Blättern bei der Abhärtung von *Rhododendron*. *Gartenbaumwissenschaft* 55:49–54.