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Micromorphological response towards altered environmental conditions in subsequent stages of *in vitro* propagation of *Morinda coreia*

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Abstract

The foliar micromorphological alterations from *in vitro* to field environments of micropropagated plantlets of *Morinda coreia* Buch. and Ham. were compared as developmental changes across the culture conditions. There were gradual developmental responses in stomatal structures, vascular tissues (venation) and raphides towards the field environment. The leaves under *in vitro* environment were thin with poorly developed leaf characteristics; these were impaired through *ex vitro* rooting prior to field transplantation. *In vitro* leaves had lower vein islet density (8.6 ± 0.16) and vein terminations (0.0) and raphide density (8.0 ± 0.19) as compared to rooted and acclimatized leaves. The physiological and structural adaptations in terms of stomata development lead to effective regulation of transpiration. Improved vein density for proper translocation of nutrients was achieved during gradual acclimation. Development of crystals, raphides and trichomes in the field environment suggested improvement in defense mechanisms. The gradual changes in foliar micromorphological structures during subsequent stages were responsible for adaptability, leading to improved survival success of *in vitro* regenerated plantlets of *M. coreia* during their field trials.

Key words: acclimation, *in vitro* propagation, micromorphological studies, *Morinda coreia*.
Abbreviations: BAP, 6-benzylaminopurine; FAA, formaldehyde acetic acid ethyl alcohol; IBA, indole-3 butyric acid; Kin, kinetin; MS, Murashige and Skoog's medium.

Introduction

Micropropagation consists of some critical stages like establishment of explants, effective multiplication, proper rooting, and successful transfers of plantlets in the greenhouse and to the field. Variations in the temperature, light intensity, air humidity and CO_2 concentrations during regeneration affect development across the environments. *In vitro* grown plantlets with anatomical and physiological abnormalities are further impaired by the *ex vitro* environmental conditions (Pospisilova et al. 2007). The changes that occur from ambient *in vitro* environment to the field, such as development of cuticle, cuticular waxes, effective stomatal regulation, and photosynthetic efficiency ensure the autotrophic growth of micropropagated plantlets (Seelye et al. 2003; da Silva et al. 2011).

Morinda coreia Buch. and Ham. (synonyms *Morinda pubescens* J.E. Smith, *Morinda tinctoria* Roxb.) is a valuable medicinal and dye yielding plant of the family Rubiaceae. It originated in India, South-East Asia and Polynesia. It is commonly known as Nuna, Manjanuna and Manjanathi in India (Luberck, Hannes 2001; Mathivanan et al. 2006). It is an evergreen small tree with pubescent and glabrous leaves and an angular stem. The fruits are green and contain compound succulent berries (Nisha et al. 2011).

It is considered as an important ethno-medicinal plant as a potential source of secondary metabolites, such as anthraquinones, phenolics, aucubin, scopoletin, asperuloside, vitamin A and C, alkaloids, flavone glycosides, terpenoids, linoleic acid, saponins, tannins and phenols (Nisha et al. 2011). In India, the plant is used to make morindone dye from root bark (trade name "Suranji"), which is used for dyeing of cotton, silk and wool in shades of red, chocolate or purple with different mordants (Singh, Tiwari 1976).

The harvest of plant roots for the dye (anthraquinone derivatives) requires whole plant destruction which caused threat to the entire natural population of this plant (Sharma 2003; Sujit, Rahman 2011). Development of micropropagation protocol for *M. coreia* could alleviate the pressure of over-harvesting from the natural forests and help in sustainable utilization of this plant (Shekhawat et al. 2015a).

The morphology, physiology and internal anatomy of the plants are intrinsic to the environmental conditions where they survive. The widespread applications of *in vitro* regeneration technology are restricted by the difficulties during transfer of the plantlets to *in vivo* conditions

(Chandra et al. 2010). Therefore, the present study aimed to investigate the foliar micromorphological changes occurring at various levels of plant development in *M. coreia* from laboratory to the soil transfer.

Materials and methods

In vitro *propagation of* Morinda coreia

Morinda coreia plantlets were regenerated according to an earlier report on *in vitro* propagation of *M. coreia* (Shekhawat et al. 2015a). Briefly, nodal shoot segments from five years old healthy plants were cultured on Murashige and Skoog's medium (Murashige, Skoog 1962) supplemented with 4.0 mg L^{-1} 6-benzylaminopurine (BAP). The *in vitro* regenerated shoots with mother explants were subcultured on liquid MS medium augmented with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} 6-furfurylaminopurine (Kin). The cultures were incubated at 25 ± 2 °C temperature, 60 to 70% relative humidity and 40 to 50 µmol m^{-2} s^{-1} Spectral Flux Photon light intensity provided by cool white fluorescents tubes for 12 h per day. The microshoots were rooted in vitro on half strength agar-gelled MS medium containing 1.0 mg L^{-1} indole-3 butyric acid (IBA) under 15 to 20 µmol m^{-2} s^{-1} light intensity. *Ex vitro* rooting was achieved by treating the shoots with 200 mg L^{-1} IBA for 5 min and maintained then in the greenhouse for 5 weeks. The regenerated plantlets were transferred to ecofriendly paper cups/bottles containing Soilrite® and moistened with 1/4th MS macrosalt solution, and maintained in the greenhouse for 4 weeks, then shifted to nursery bags and finally established in the natural field conditions (Figs. 1A to 1G).

Foliar micromorphological studies

Experiments were conducted to study the foliar micromorphological changes (leaf constants) during significant stages of plant developmental process from *in vitro* to field environments. Parameters examined were venation pattern, vein density (vein-islets and veinlet terminations), types of stomata, stomatal density, stomatal index, raphides and trichome density of the leaves of plants developed *in vitro* after 4th subculture in multiplication phase, after 5 weeks of rooting stages (*in vitro* and *ex vitro* rooting) and in field established plants (after 6th week). Randomly selected leaf specimens at different developmental stages under *in vitro* and in field conditions were used. Completely expanded entire leaf samples at third to seventh leaves from the base were used to investigate the surface microstructures on the adaxial and abaxial surfaces. The paradermal sections were prepared by standard method (Johansen 1940) for the observation of developmental changes.

To study the leaf architectural development (venation study), leaves procured from each stage were excised and fixed in advance with FAA solution (formaldehyde/glacial acetic acid/ethyl alcohol at a ratio of 1:1:3) for 24 to 72 h. The fixed leaves were stored in 70% ethanol (v/v) to remove the chlorophyll (12 to 24 h) and bleached with 5% (w/v) NaOH for 24 to 48 h. Thereafter the leaf materials were rinsed in distilled water and allowed to remain in saturated chloral hydrate solution for 12 h (Sass 1940). The cleared leaves were used to study the developmental changes in venation pattern, vein density (vein-islets and veinlet terminations) and raphide density in the tested environments. The terminology adopted for stomatal study

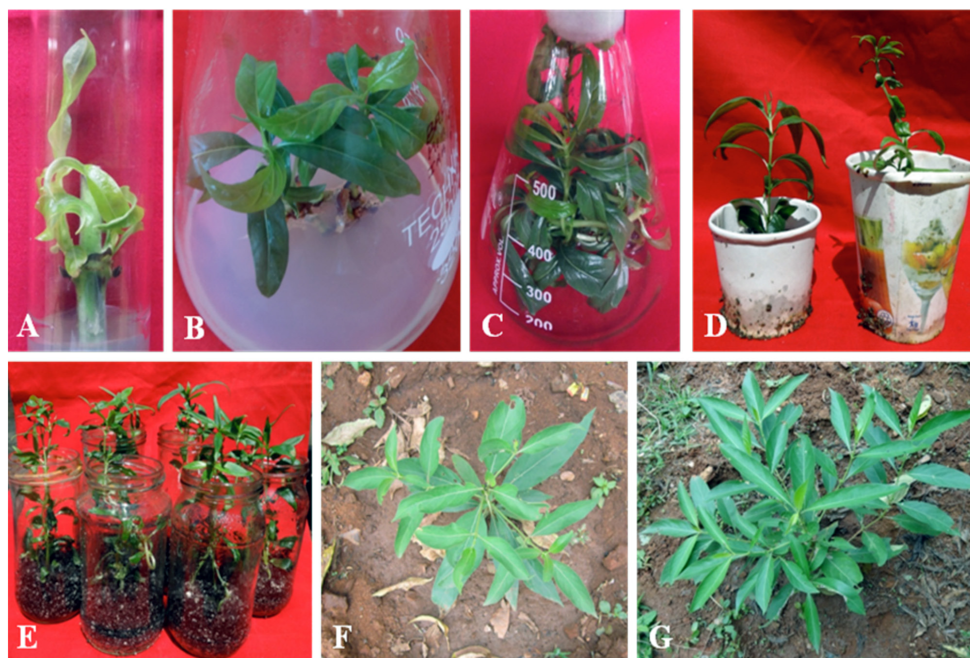

Fig. 1. Different stages in micropropagation of *Morinda coreia*.

and venation pattern was of Salisbury (1932) and Hickey and Wolfe (1975) respectively. Intermediate types of stomata have been described by following the classification and terminology as suggested by Croxdale (2000) and Prabhakar (2004). The materials were stained with 1 % (v/v) safranine (Loba Chemie, India) aqueous solution for 4 to 8 min, triple-rinsed in distilled water for 10 min to remove excess stain, mounted in water, examined under optical microscope (Olympus SZ61, USA) and analyzed by the software Moticam.

Observations and data analysis

Stomata, raphides, trichomes and vein densities were determined at the end of the culture period in all stages. The results were submitted to ANOVA using SPSS software (version.16). The experimental design consisted of four treatments (*in vitro* multiplication stage, *in vitro* rooting, *ex vitro* rooting and field transferred plants) with ten replicates and repeated thrice. Statistical differences between different stages were subjected to analysis of variance and the significance of differences among mean values was carried out using Duncan's Multiple Range Test and reported as mean ± standard error at $P < 0.05$ significance level.

Results

Developmental changes in foliar micromorphology

The leaves of *M. coreia* under *in vitro* multiplication stage were small, lanceolate, pale green in colour and less pubescent. Small, glabrous and pubescent leaves with increased green colour were observed at rooting stage of the shoots. The midrib and lateral veins were not prominent under *in vitro* conditions, but the leaves of *ex vitro* rooted plantlets in greenhouse were larger and pubescent.

The frontal view of epidermal peelings of the leaves possessed single layered epidermis on both surfaces. The *in vitro* multiplied leaves had unorganized, different shaped epidermal cells without any cuticular striations (Fig. 2A). Undulated epidermal cells were observed in leaves of *in vitro* rooted shoots (Fig. 2B) and highly undulated cells with thin sinuous anticlinal walls in *ex vitro* rooted plantlets (Fig. 2C). Polygonal, straight walled, organized and compact epidermal cells were recorded in field transferred plants of *M. coreia* (Fig. 2D). The midrib had also increased in size and was visibly prominent after transplantation of the plantlets in the field.

Stomatal analysis

The leaves were hypostomatic with paracytic type stomata and the stomata were restricted to the intercoastal area. Coastal cells were absent in leaves of *in vitro* multiplied shoots, but gradually developed in the period till field transplantation. The coastal cells were elongated with straight anticlinal walls and differed from epidermal undulations *in vitro* as well as *ex vitro* rooted and field transferred plantlets. The leaves of *in vitro* plantlets had higher stomatal density (52.0) and stomatal index (24.6) than in the other stages. The stomata differd in shape and size, and were oriented in all directions (Fig. 3A). The leaves of *in vitro* rooted plantlets developed unequal stomata with

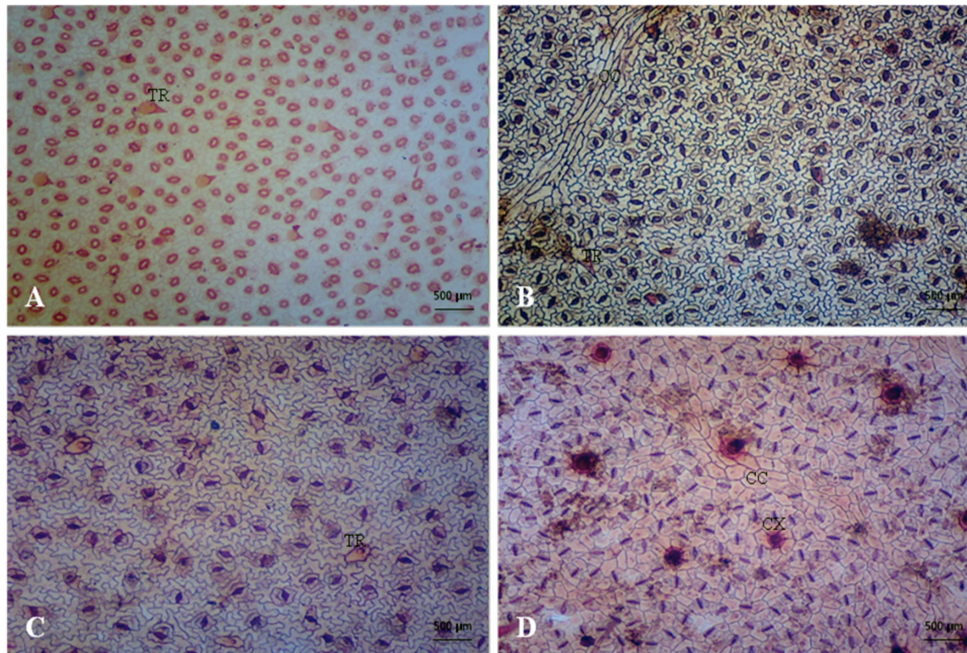

Fig. 2. Foliar epidermal peels of abaxial surfaces of *Morinda coreia* micropropagated plantlets. A, stomatal frequency in abaxial epidermis of *in vitro* emerged leaves at multiplication stage. B, stomatal frequency at *in vitro* rooting stage. C, stomatal frequency under *ex vitro* rooting (greenhouse) environment. D, stomatal frequency in leaves after field transplantation. TR, trichome; CC, coastal cells; CX, cicatrix. Scale bar = 500 µm.

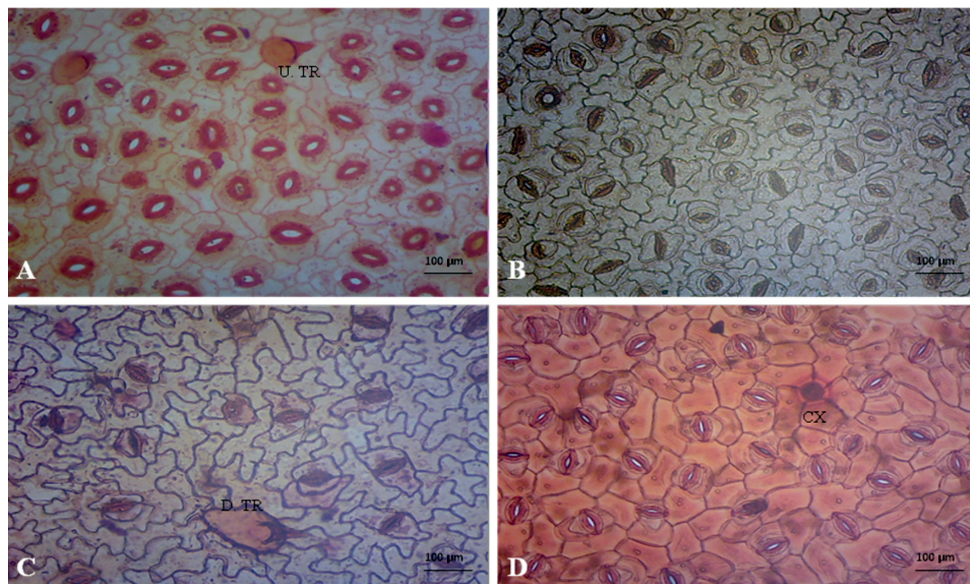

Fig. 3. Enlarged view of abaxial epidermis of *Morinda coreia*. Development in stomatal density at multiplication (A), *in vitro* rooting (B), *ex vitro* rooting (C) and field transferred stage (D) of plantlets. U.TR, underdeveloped trichome; D.TR, developed trichome; CX, cicatrix. Scale bar = 100 µm.

reduced stomatal density (46.0) and index (20.6) than the *in vitro* multiplied shoots (Fig. 3B). The stomata were always opened and non-functional in the multiplication phase, partially closed in the *in vitro* rooting stage, and functionally closed stomata were observed in *ex vitro* rooting and field transferred plants (Fig. 3C and 3D) in this study.

In vitro anomalies like arrested stomatal development and contiguous stomata were also observed in the plants (Figs. 4A and 4B). Plantlets in the *ex vitro* rooting stage had lower stomatal density (21.0) and index (17.0). The stomatal index increased from *ex vitro* to field transferred plants (18.8) during the successive stage of propagation (Table 1). Contiguous trichomes were also observed in the *in vitro* multiplied leaves, rarely in field plants and totally absent in the leaves of rooting stage (Fig. 4C and 4D).

Development in leaf architecture through various environments

Reticulate venation with organized aereoles were observed in leaves of *M. coreia*. Venation was obscure, non-prominent and unorganized in *in vitro* leaves. The vein-islets were

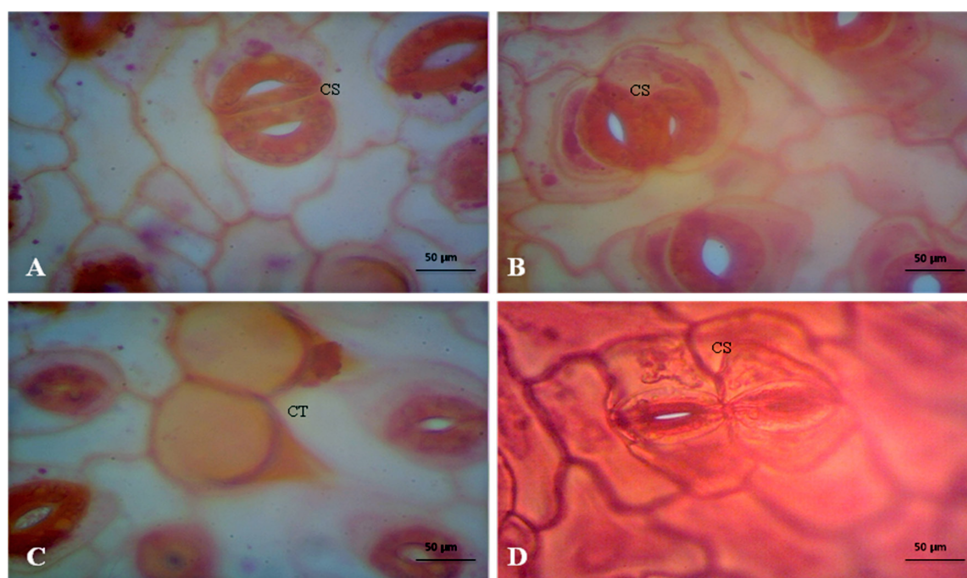

Fig. 4. In vitro induced foliar anomalies in *Morinda coreia* plantlets. A and B, different orientation of contiguous stomata in the *in vitro* multiplied leaves. C, contiguous and underdeveloped trichomes at multiplication stage. D, contiguous stomata in field transferred plants. CS, contiguous stomata; CT, contiguous trichome; U.TR, underdeveloped trichomes. Scale bar = 50 µm..

Table 1. Stomatal density and stomatal index in *Morinda coreia* plants in different stages of micropropagation. The values represented in corresponding column followed by same letters are not significantly different at $P < 0.05$

Field No.	Multiplication stage	*In vitro* rooting stage	*Ex vitro* rooting stage	Field transferred plants
1	51.6 ± 0.18b	45.7 ± 0.24b	20.8 ± 0.13a	32.8 ± 0.14a
2	52.1 ± 0.10c	45.5 ± 0.17a	21.5 ± 0.24b	33.1 ± 0.17b
3	53.0 ± 0.22d	45.9 ± 0.12b	22.0 ± 0.16c	34.5 ± 0.20c
4	51.4 ± 0.14b	45.4 ± 0.18a	20.6 ± 0.26a	35.0 ± 0.00d
5	50.9 ± 0.20a	46.7 ± 0.12c	20.4 ± 0.18a	33.9 ± 0.11b
6	51.3 ± 0.11b	47.0 ± 0.29d	21.0 ± 0.11b	35.0 ± 0.21d
7	52.8 ± 0.26c	45.6 ± 0.21a	21.5 ± 0.29b	33.4 ± 0.19b
8	53.1 ± 0.19d	46.0 ± 0.19c	20.8 ± 0.30a	34.1 ± 0.24c
9	51.6 ± 0.10b	45.9 ± 0.33b	20.6 ± 0.12a	33.7 ± 0.21b
10	52.2 ± 0.13c	46.3 ± 0.16c	20.8 ± 0.19a	34.7 ± 0.17c
Mean	52.0 ± 0.27	46.0 ± 0.19	21.0 ± 0.33	34.0 ± 0.14
Stomatal index	24.6 ± 0.00	20.4 ± 0.21	17.0 ± 0.10	18.8 ± 0.16

lopsided with less vein-islets density (8.6) and the vein system was found open without veinlet terminations (Fig. 5A). The vein-islets were cubic and rhomboidal with increased density (10.2) in leaves of *in vitro* rooted plantlets. The veinlet terminations emerged to two per vein-islet and were mostly single, but branched veinlet terminations were also observed (Fig 5B). Vein-islets were clear (with 15.0 densities), organized, rhomboidal and comprised branched veinlet terminations (4.0) at this stage (Fig. 5C and Table 2). Vein density increased in terms of vein-islets (17.6) and veinlet terminations (6.0) in the field transferred plants (Fig. 5D). Vein-islets were rectangular shaped, and the veinlet terminations were comparatively more and highly branched at this stage.

Trichomes and cicatrix index

The *in vitro* multiplied shoots possessed underdeveloped, single celled trichomes with higher density (16.0) than the *ex vitro* and field transferred plantlets. The trichomes were gradually developed and attained maturity through *ex vitro* acclimatization and subsequent field transplantation (Fig. 6A). The density of trichomes in the *in vitro* rooting stage was higher (12.8) than in other stages of micropropagation of *M. coreia*. Single celled as well as multicellular trichomes were observed at this stage. There was a rapid reduction in trichome density (9.0) when the *in vitro* multiplied shoots rooted *ex vitro* (Table 3).

After transplantation to the field, the trichomes were fully developed in structure and functional aspect,

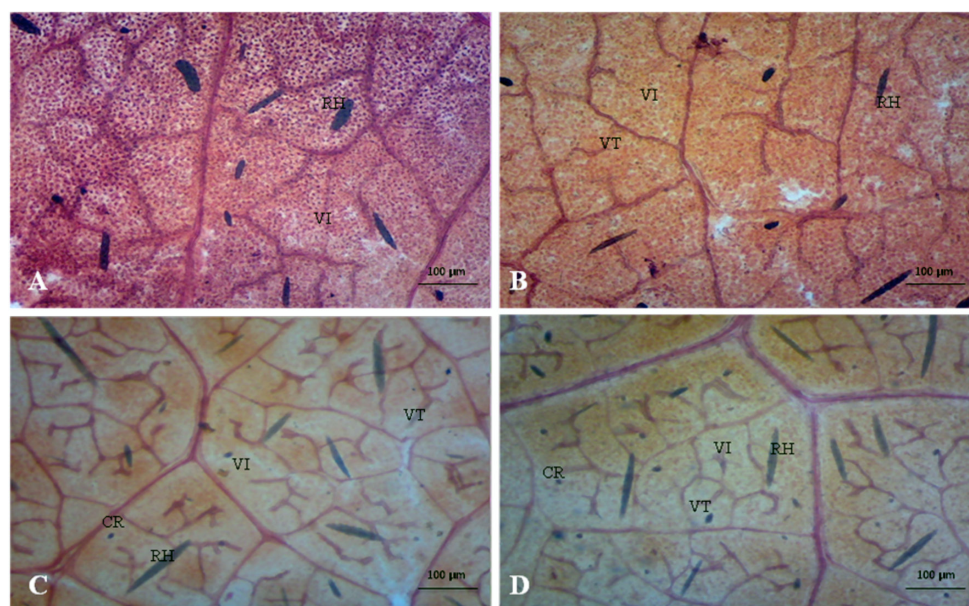

Fig. 5. Vein density and raphide density in micropropagated leaves from *in vitro* to field transferred plants of *Morinda coreia*. A, underdeveloped veination pattern and low raphide density in leaves at multiplication stage. B, development of vasculature and raphides during *in vitro* rooting stage. C, development of vein-islets, veinlet terminations and raphides at *ex vitro* rooting stage. D, increased vein density, raphide density and formation of crystals after field transplantation. VI, vein islet; VT, veinlet termination; RH, raphides; CR, crystals. Scale bar = 100 µm.

Table 2. Vein density (vein-islets and veinlet terminations) in *Morinda coreia* plants in different stages of micropropagation. The values represented in corresponding column followed by same letters are not significantly different at $P < 0.05$

Field No.	Multiplication stage	*In vitro* rooting stage	*Ex vitro* rooting stage	Field transferred plants
1	8.0 ± 0.17a	11.0 ± 0.33d	13.8 ± 0.19a	17.9 ± 0.13c
2	9.2 ± 0.20d	10.5 ± 0.00c	15.0 ± 0.29c	16.4 ± 0.10a
3	8.8 ± 0.38c	9.70 ± 0.27b	14.6 ± 0.14b	18.1 ± 0.19d
4	8.5 ± 0.12b	10.0 ± 0.21c	15.9 ± 0.00d	17.5 ± 0.25b
5	9.0 ± 0.00cd	9.80 ± 0.16b	15.0 ± 0.31c	16.8 ± 0.19a
6	8.2 ± 0.14a	10.6 ± 0.21c	15.1 ± 0.10c	18.0 ± 0.00d
7	8.6 ± 0.19b	11.0 ± 0.00d	15.9 ± 0.16	17.8 ± 0.15b
8	8.9 ± 0.11c	9.50 ± 0.16a	14.3 ± 0.12b	17.2 ± 0.29b
9	8.5 ± 0.27b	10.2 ± 0.12c	15.0 ± 0.19c	18.4 ± 0.24e
10	8.3 ± 0.20b	9.70 ± 0.25b	15.4 ± 0.00cd	17.9 ± 0.10c
Mean	8.6 ± 0.16	10.2 ± 0.17	15.0 ± 0.14	17.6 ± 0.11
Veinlet termination	0.0 ± 0.00	2.0 ± 0.12	4.0 ± 0.00	6.0 ± 0.18

observed on coastal as well as intercoastal regions and mingled with stomata (Figs. 6B and 6C). Such a spatial distribution was not clear in other stages evaluated. The density of trichomes increased (10.2) from the greenhouse (*ex vitro* rooting) environment. Underdeveloped trichomes were also reported in some leaves (Fig. 6D). The cicatrix was absent under *in vitro* environment, infrequent in the *ex vitro* rooting stage and frequent in the field transplanted plants (Table 3).

Crystal/raphide study

Greater number of rosette aggregate crystals was detected in field transferred plants; they were rare in *ex vitro* rooted plants, and completely absent in other stages. The *in vitro* multiplied leaves had underdeveloped raphide density (8.0) and they were comparably small in size with blunt tips (Fig 7A). The leaves of *in vitro* rooted plants possessed underdeveloped needle shaped raphides (8.2) with blunt ends. These were large, organized, needle shaped with increased density (10.0) in leaves of *ex vitro* rooted plantlets (Figs. 7B and 7C). The raphide density was greater (12.6) in field transferred plants as the leaf matured in the field environment (Fig. 7D and Table 4).

Discussion

Leaf characteristics like stomatal, veins, trichomes and raphides density are responsible for effective biochemical and physiological metabolism taking place during plant development (Carvalho et al. 2002; Durkovic et al. 2009; Blonder, Enquist 2014). Micromorphological studies of leaves from *in vitro* and field transferred plants of *M. coreia* indicated significant changes at all stages of micropropagation. The present investigation revealed that

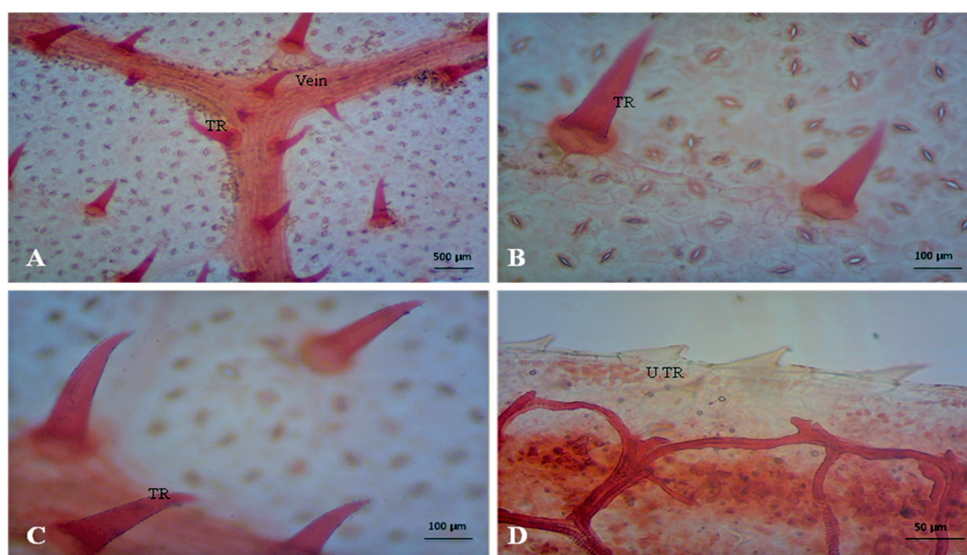

Fig. 6. Trichome development and contiguous stomata in field transferred plants of *Morinda coreia*. A, trichome density under field environment. B, trichomes on coastal cells. C, trichomes on intercoastal cells spread along with stomata. D, underdeveloped trichomes. TR, trichomes; CS, contiguous stomata. Scale bars = 500, 100 and 50 µm..

Table 3. Trichome density and number of cicatrix on the leaves of *Morinda coreia* plants in different stages of micropropagation. The values represented in corresponding column followed by same letters are not significantly different at $P < 0.05$

Field No.	Multiplication stage	*In vitro* rooting stage	*Ex vitro* rooting stage	Field transferred plants
1	15.6 ± 0.10b	12.0 ± 0.00a	8.6 ±0 .11ab	12.2 ± 0.16e
2	16.0 ± 0.21c	13.3 ± 0.22d	8.4 ± 0.18a	11.0 ± 0.00d
3	15.4 ± 0.00a	12.5 ± 0.19b	9.6 ± 0.10d	10.6 ± 0.11cd
4	15.9 ± 0.16b	13.0 ± 0.00d	8.9 ± 0.16b	9.4 ± 0.25a
5	16.0 ± 0.11c	12.8 ± 0.16c	9.1 ± 0.29c	10.0 ± 0.19c
6	16.4 ± 0.24cd	12.5 ± 0.12b	8.8 ± 0.00b	9.2 ± 0.00a
7	16.9 ± 0.18e	13.0 ± 0.19d	8.5 ± 0.25ab	9.8 ± 0.22b
8	15.6 ± 0.00b	13.2 ± 0.10d	9.3 ± 0.34c	9.5 ± 0.13b
9	16.2 ± 0.27cd	13.7 ± 0.13e	9.8 ± 0.22de	10.3 ± 0.27c
10	16.0 ± 0.20c	12.0 ± 0.17a	9.0 ± 0.16c	10.0 ± 0.11bc
Mean	16.0 ± 0.00	12.8 ± 0.21	9.0 ± 0.12	10.2 ± 0.18
Cicatrix	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.00	5.0 ± 0.10

how the micromorphological features of leaves of *M. coreia* changed from *in vitro* to field environments.

Morphologically the shoots raised under *in vitro* conditions resembled with the field established plants. Field transferred plants have normal bright green coloured leaves with increased pubescence. The increased colour intensity suggests increased photosynthetic pigment concentration and activity in the field transferred plants. Gaspar et al. (2002) reported that carbon (sucrose) addition to culture medium hindered chlorophyll synthesis, Calvin cycle and photosynthesis, and disturbed overall carbon metabolism in *in vitro* cultured plantlets.

M. coreia leaves were hypostomatic and contained paracytic stomata. The stomata were always open and non-functional stomata were observed in the multiplication phase, partially closed at *in vitro* rooting stage, and closed in *ex vitro* rooting and field transferred plants. Stomatal abnormalities like arrested and contiguous stomata were observed. Such types of stomatal anomalies under *in vitro* environments were also reported in *Nicotiana tabacum* (Pospisilova et al. 1999), *Castanea sativa* (Saez et al. 2012) and *Couroupita guianensis* (Shekhawat, Manokari 2016b). Stomatal development is considered to be of paramount importance in transpirational regulation from *in vitro* to the field environments. Contiguous stomata were reported to be formed from adjacently placed meristemoids or by readjustment during maturation (Patel, Inamdar 1971).

Most of the physiological disorders are not only limited to the period of *in vitro* growth but become more apparent upon acclimatization of regenerants (Hazarika 2006;

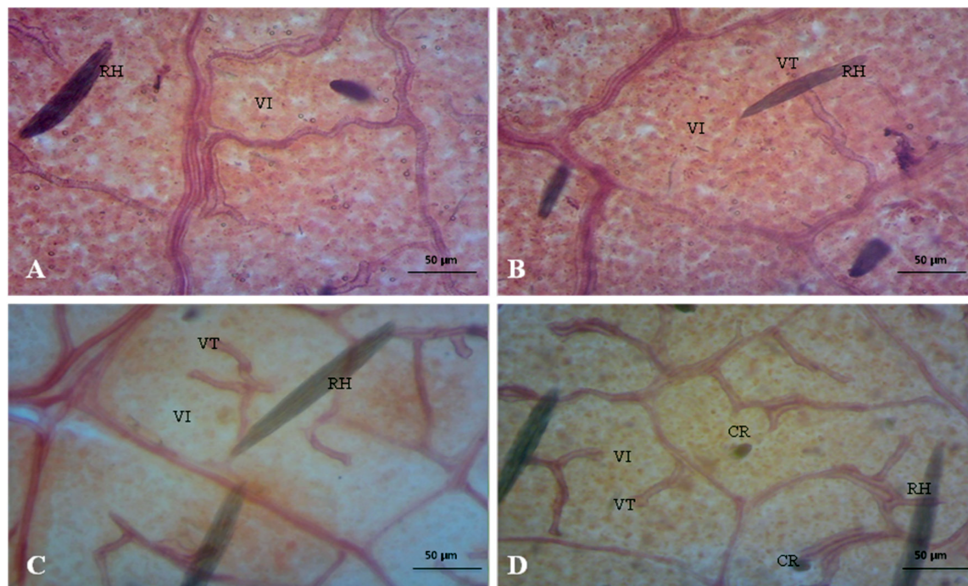

Fig. 7. Raphides and their density from *in vitro* to field transferred plants of *Morinda coreia*. A, underdeveloped raphides with blunt ends in multiplication stage. B, development of raphides during rooting stage. C, development of raphides and their structures during *ex vitro* rooting stage. D, mature raphides with organized structure in field transferred plants. VI, vein islet; VT, veinlet terminations; RH, raphides; CR, crystals. Scale bar = 50 µm. .

Table 4. Raphide density in the foliar cells of *Morinda coreia* plants in different stages of micropropagation. The values represented in corresponding column followed by same letters are not significantly different at $P < 0.05$

Field No.	Multiplication stage	*In vitro* rooting stage	*Ex vitro* rooting stage	Field transferred plants
1	7.7 ± 0.23a	8.0 ± 0.19ab	9.3 ± 0.22b	12.5 ± 0.22bc
2	8.2 ± 0.11b	8.6 ± 0.15c	10.0 ± 0.19c	12.2 ± 0.16b
3	8.1 ± 0.25b	7.8 ± 0.00a	11.1 ± 0.13d	13.0 ± 0.18d
4	7.9 ± 0.00a	8.5 ± 0.16c	10.0 ± 0.18c	12.8 ± 0.23c
5	8.4 ± 0.19bc	8.3 ± 0.22b	8.9 ± 0.21a	12.3 ± 0.17b
6	8.0 ± 0.21b	7.9 ± 0.00a	10.3 ± 0.33c	13.0 ± 0.24d
7	7.5 ± 0.13a	8.5 ± 0.12c	9.5 ± 0.17bc	12.9 ± 0.11c
8	8.1 ± 0.10b	8.1 ± 0.19ab	9.9 ± 0.10c	11.5 ± 0.16a
9	8.4 ± 0.27bc	8.0 ± 0.15ab	10.0 ± 0.26c	12.8 ± 0.25c
10	7.7 ± 0.15a	8.3 ± 0.20b	10.4 ± 0.00c	13.0 ± 0.18d
Mean	8.0 ± 0.19	8.2 ± 0.35	10.0 ± 0.14	12.6 ± 0.17

Pospisilova et al. 2007). The *in vitro* induced structural aberrations create serious practical problems and limit the profitability of the micropropagation technique. Pospisilova et al. (2009) reported that the incorporation of sugar in culture media increases sucrose and starch reserves in micropropagated plantlets, which may favour *ex vitro* acclimatization and accelerate physiological adjustments.

Unorganized reticulate type venation observed in *in vitro* leaves of *M. coreia*. The vein-islets were lopsided with less vein-islets density which increased from *in vitro* to the *ex vitro* rooted plantlets. This indicates development of vein density during the rooting stages. Auxin treatment at rooting stage promotes mitotic dedifferentiation and development of secondary vascular tissues, cambial cells and inter-fascicular supporting tissues (Vidal et al. 2003). Vein density was gradually increased via an *ex vitro* rooting mechanism. The exogenous application of auxins induces adventitious roots from microshoots, which gradually leads to the nutrient translocation to the aerial parts and promotes an autotrophic condition. According to Kull and Herbig (1995), leaves exposed to sun can show a higher ratio of veinlet termination than in shaded leaves. The marginal veins guarantee supply of sufficient water to leaf margins prone to high water stress (Nebelsick et al. 2001). The present findings revealed that the rooting stage could serve as a suitable marker of phase changes. Genes are expressed differently at the multiplication stage and at the end of rooting stage as reported in oak species using cDNA characterization (Gil et al. 2003).

Trichomes were observed on both the epidermal surfaces. Non-glandular trichomes were reported all over the epidermis in all treatments, with major differences in density and structure. The frontal evaluation of paradermal sections indicated development of trichomes in subsequent stages. The development of organized trichomes in the *in vitro* environments demonstrates morphological response to a highly sophisticated changed environment. Plantlets develop multicellular trichomes with organized structures characterizing pubescent nature under greenhouse conditions. These trichomes are reported to secrete active compounds responsible for the curative properties of the plant, anti-herbivore/defense mechanism towards environment and predators (Naidoo et al. 2009). The trichomes density increased from the *ex vitro* rooting to the *in vivo* environments. The increase inthis structural trait confers tolerance to wind, excessive heat and to reduce water loss in the field environment. Manetas (2003) reported that trichomes evolved as a physiological barrier and protect plant tissues against UV radiation.

The cicatrix index, scar left by the trichome on the leaf surface, increased from the *ex vitro* rooting stage to the field transplanted plants in *M. coreia*. The cicatrix index is also considered as a leaf constant in plant identification (Komsakorm et al. 1999).

Two types of crystals were observed through analysis of foliar micromorphological characteristics of *M. coreia*, i.e. calcium oxalate crystals (raphides) and rosette aggregate crystals. There was a gradual increase in raphide density from *in vitro* to the field transferred plants. The development of raphides in the field transferred plants reveals that photoautotrophic nutrition leads to potent metabolic, physiological activities and rigorous mechanical defense. The formation of crystals is linked with defensive activity against pathogens and herbivors under natural conditions (Konno et al. 2014).

In conclusion, the present study involved a comprehensive foliar analysis of *in vitro*, *ex vitro* and field transfer transition of tissue cultured plantlets of *M. coreia* using micromorphological techniques. The collective foliar micromorphological parameters highlighted the physiological and developmental alterations in tissue culture raised *M. coreia* plantlets and their adaptability to stressful field conditions by combating and alleviating the *in vitro* induced anomalies. The findings of micromorphological features could help in understanding the response of plants under changed environments.

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