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Improved micropropagation and foliar micromorphological studies in *Turnera ulmifolia* L. – An important medicinal plant

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ABSTRACT

The present study reports an efficient *in vitro* propagation system for *Turnera ulmifolia* using nodal segments as explants. *Turnera ulmifolia* (Passifloraceae) is an important garden plant with multipotent medicinal values. Effective shoot proliferation was achieved on agar gelled MS medium (Murashige and Skoog, 1962). The maximum number of shoots (8.3 ± 0.57) per initial explant was obtained on MS medium supplemented with 8.88 mM of 6-benzylaminopurine (BAP) and 0.54 mM of α-naphthalene acetic acid (NAA). The highest number of shoots (59.5 ± 2.10) proliferated on semi-solid MS medium (with agar) augmented with 2.22 mM of BAP and 2.32 mM of kinetin (Kin) along with 0.54 mM of NAA. Longer (4-5 cm) and healthy shoots were rooted (12.0 ± 0.10 roots per shoot) on half-strength MS medium fortified with 9.84 mM of indole-3 butyric acid (IBA). The *in vitro* regenerated plantlets were hardened in the greenhouse and transferred to the field. Significant developmental changes were observed in the foliar micromorphology of *in vitro* raised plantlets when these were transferred to the field. The stomatal index was gradually reduced (26.72 to 21.25) in the leaves from *in vitro* to field environments. But, vein-islets and veinlet terminations (13.4 and 7.6) were increased (39.7 and 18.4) respectively from *in vitro* to *in vivo* grown plants. Simple, unicellular, less frequent and underdeveloped trichomes were observed with the leaves of *in vitro* plants but fully developed trichomes recorded in the field transferred plants. The study could help in understanding the response and adaptation of tissue culture raised plantlets towards changed environmental conditions.

Keywords: acclimation, agar-gelled medium, *in vitro*, micromorphological response, Passifloraceae

INTRODUCTION

Turnera ulmifolia L. belongs to the family Passifloraceae (formerly Turneraceae) is one of the important flowering plant with medicinal properties. This polymorphic polyploid species is a dense compact shrub with dark green foliage and bright yellow flowers. The plant is commonly known as yellow alder, chanana, damiana, buttercups, sage rose, twelve o'clock flower etc. It is native to the tropical America, Africa, India and the Southeast Asia with widespread geographic distribution from Guyana to the North Eastern regions of Brazil (Schultz, 1987). In India, this species is distributed in west Bengal, Orissa and the coastal areas (Pio Correa, 1984).

Yellow alder contains number of valuable secondary metabolites such as flavonoids, alkaloids, tannins and phenolic compounds (Antonio and Brito, 1998; Gracioso et al., 2002; Nascimento et

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al., 2006), mixture of cyanohydrins, fatty acids, calamine etc. (Khare, 2007). Alpha and beta pinene, 1,8 cineole, p-cymene, thymol, alphacopene, gamma-cadinene and calamine were also reported from *T. ulmifolia* (Lilia et al., 2004; Smith et al., 2007). These phytochemicals contribute to its antiulcerogenic (Gracioso et al., 2002), anti-inflammatory, antioxidant, antibacterial, antifungal, emmenagogue and expectorant properties (Kumar and Sharma, 2007; Sethi and Ramasamy, 2012).

Traditionally, the herbal extracts of *T. ulmifolia* is used to treat asthma, rheumatism, indigestion, bronchitis, albuminuria, leucorrhea, melroxemia, sore throat, weakness, cold, fever, boils, vertigo, dysmenorrhea, hemorrhage, lumbago, dyspepsia, dysentery and furunculosis (Lilia et al., 2002; Kumar and Sharma, 2007). The herbal tea prepared from this plant is used for the treatment of gastric dysfunctions in Brazil (Antonio and Brito, 1998).

Conventionally, *T. ulmifolia* is propagated through the seeds only. The systematic cultivation of this plant is not reported and the plant parts are directly collected from the open forests by the herbal medicine practitioners. Therefore, the population of this plant is depleting day-by-day due to the over harvesting of the plants during flowering time before seed settings. The *in vitro* propagation techniques could be used as an alternative method for mass production for this plant. There are some reports on micropropagation of *T. ulmifolia* but an efficient protocol with good survival percentage during field transfer of plants is largely deficit (Shekhawat et al., 2014).

The comparative foliar micromorphological analysis of *in vitro* and field transferred plants could correlate physiological aspects with anatomical and developmental patterns of differentiation and photomorphogenesis. This study could help to understand the response of the plants towards altered environmental conditions and improve the survival chances of *in vitro* raised plantlets under field conditions (Sack et al., 2012).

The present investigation concerns an enhancement in micropropagation efficiency of *T. ulmifolia* using agar-gelled Murashige and Skoog's (MS) medium. An improved rate of shoots multiplication and roots induction was achieved in this study as compared to the earlier reports (Kalimuthu et al., 2014). Furthermore, micromorphological studies were conducted to observe the internal changes taking place in leaf surface morphology and venation pattern to understand the micro-structural and morphological differentiation of micropropagated plants when transferred from laboratory to the field environment.

MATERIAL AND METHODS

Plant material and initial explant preparation

The mature (two years old) donor plants of *T. ulmifolia* were selected from the south west coastal regions of India for the collection of healthy and disease free explants during March 2013 to February 2014. The plants were identified using the flora of Madras Presidency (Gamble, 1921). The nodal segments (2-3 cm in length) and shoot tips were used as explants. The explants were collected year round (every month) from the same donor plant to evaluate the effect of season on establishment of cultures. The explants were treated with broad spectrum antifungal agent Bavistin (0.1%; w/v) for 5-10 min. and thereafter washed with autoclaved double distilled water for 4-6 times. The surface sterilization of explants was rendered by 0.1% mercuric chloride (w/v) for 4-5 min. and rinsed 5-6 times with autoclaved double distilled water.

Nutrient medium and culture conditions

The basal MS medium (Murashige and Skoog, 1962) was supplemented with additives (283.9 mM (Millimolar) of ascorbic acid, 67.9 mM of adenine sulphate, 143.5 mM of arginine and 130 mM of citric acid) and solidified with 0.8% (w/v) agar-agar (Hi-Media, India). The pH of the medium was adjusted to 5.8 ± 0.02 with 1N HCl or NaOH prior to autoclaving at 121°C for 15 min. 10 ml and 60 ml of media were poured per culture tube (volume 60 ml) and culture vessel (volume 350 ml), respectively. The cultures were maintained at 25 ± 2°C temperature for 12 h photoperiod with light intensity of 40-50 µmol m^{-2} s^{-1} Spectral Photon Flux Density (SPFD) provided by cool white fluorescent lamps (Philips India Ltd, New Delhi).

Bud breaking and multiplication of shoots

The surface sterilized explants were cultured on MS medium (one explant per culture tube) with different concentrations of 6-benzylaminopurine (BAP) (2.22, 4.44, 6.66, 8.88, 11.1 and 13.3 mM) and Kinetin (Kin) (2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 mM) either alone or in combination with α-naphthalene acetic acid (NAA) (0.54 mM) for shoot bud induction. The *in vitro* formed shoots were cut into segments (2.0-3.0 cm in length, each with one to two nodes) and subcultured on MS medium containing various concentrations of BAP

(0.44 to 8.88 mM) alone or optimized concentration of BAP (2.22 mM) with various concentrations of indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA) (0.57, 1.14 and 1.71 mM) for shoot multiplication. The cultures were further multiplied by repeated transfer of mother explants and subculture of *in vitro* produced shoots. The original explants were repeatedly transferred (after harvesting of long shoots) to fresh MS medium supplemented with optimized concentration of growth regulators (2.22 mM of BAP, 2.32 mM of Kin and 0.54 mM of NAA) to yield maximum number of shoots. The shoots were subcultured on agar gelled MS medium with each passage of 4-5 weeks interval for multiplication.

Rooting of shoots and hardening of plantlets

The healthier and sturdy *in vitro* multiplied shoots (4-5 cm long) were excised from shoot clumps and transferred to the rooting medium for the induction of roots. The nutrient medium (full, half and one-fourth strengths of MS media) contained activated charcoal (100 mg L^{-1}), gelled with 0.8% agar and supplemented with auxins at various concentrations [(IAA: 5.71, 11.42, 17.1 and 22.8 mM), (IBA: 4.92, 9.84, 14.8 and 19.7 mM) and (NAA: 5.37, 10.7, 16.1 and 21.5 mM)] was used. The shoots were initially incubated under diffused light for 2-3 days for *in vitro* root induction and thereafter shifted to the standard conditions with the light intensity of 35-40 µmol m^{-2} s^{-1} SPFD and 12 h photoperiod per day. After four weeks of rooting *in vitro*, the *in vitro* rooted plantlets were hardened in greenhouse. (throughout the year). The rooted shoots were transferred to the vessels (volume 350 ml) containing 50 g autoclaved soilrite® (a mixture of horticulture-grade perlite with Irish peat moss and exfoliated vermiculite supplied by Kel Perlite, Bangalore, India) and moistened with one fourth strength of MS basal salts. These were kept in greenhouse under high humidity (RH 80-90%) and temperature of 28 ± 2°C. The caps of bottles were gradually loosened over a period of two weeks and finally removed after four weeks. Thereafter, the plantlets were exposed to higher temperature (32 ± 2°C) and lower RH (60-70%). The hardened plantlets were transferred to the perforated nursery poly bags containing organic manure, sand and red soil (1:1:1; w/w) after 15-20 days and maintained in greenhouse. The hardened plantlets were shifted to the earthen pots filled with garden soil, red soil and vermi-compost (2:2:1; w/w) and finally reintroduced into the field.

Foliar micromorphological studies

The observations were performed to study the foliar micromorphological developmental differences in venation pattern and vein density, stomatal pattern, and trichomes of leaves of plants developed *in vitro* after 4th subculture in multiplication phase and after 6 weeks of field transferred plants (Rathore et al., 2013). Plants were randomly selected for the micromorphological experiments from both the environments. The entire leaves (foliar apparatus) at third to seventh leaves from the base were excised manually. The epidermal peels from the leaves were separated manually by standard method (Johansen, 1940) to observe the changes in structure of developing stomata. Leaves were fixed primarily in formalin, acetic acid and ethyl alcohol, FAA solution (1:1:3; v/v) and cleared in 70% ethanol (v/v) until the chlorophyll was removed (12-24 h), bleached with 5% (w/v) NaOH for 24-48 h and rinsed three times in distilled water for venation study. The leaves were then stained with 1% (v/v) safranine (Loba chemie, India) aqueous solution for 4-8 min. and rinsed carefully in distilled water to remove excess stain, mounted in water, examined under microscope (Labomed iVu 3100, USA) and analyzed using Pixelpro software.

Experimental design, data collection and statistical analysis

The experiments conducted with 20 explants for each treatment and every experiment was repeated thrice. The results were expressed as mean ± standard error (SE) of triplicates. The data were collected after every 4 weeks of incubation. The data were subjected to analysis of variance and the significance of differences among mean values was carried out using Duncan's Multiple Range Test (DMRT) at $p < 0.05$ using SPSS software, version 16.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Effect of the initial explant type and season of culture initiation on shoot development from the explants

Out of the two types of explants used for the establishment of cultures, the freshly sprouted nodal shoot segments were resulted better in induction of multiple shoots. Shoot tip explants responded quickly (within one week) but the number of shoots was less (2.0 ± 0.13) as compared to nodal shoot segments (8.3 ± 0.57), therefore, shoot tip as explants were avoided in further studies (Tabs 1 and 2).

Table 1. Effect of different concentrations of cytokinins (BAP and Kin) with 0.54 mM NAA on the response in bud breaking and shoot induction from nodal explants of *T. ulmifolia*

Concentration of cytokinins (mM)		Response (%)	Number of shoots (Mean ± SE)	Shoot length (cm) (Mean ± SE)
Control	0.00	0 a*	0.0 ± 0.00 a	0.00 ± 0.00 a
BAP	2.22	67 d	3.3 ± 1.00 bc	2.12 ± 0.11 bc
	4.44	74 ef	5.2 ± 0.31 de	3.46 ± 0.19 de
	6.66	89 hi	6.7 ± 0.28 f	3.60 ± 0.14 ef
	8.88	100 j	8.3 ± 0.57 h	4.03 ± 0.22 g
	11.1	91 i	7.1 ± 0.20 g	3.23 ± 0.37 de
	13.3	83 g	6.0 ± 0.19 ef	2.00 ± 0.10 bc
Kin	2.32	55 b	2.7 ± 0.13 b	2.00 ± 0.21 bc
	4.65	65 d	3.1 ± 0.22 bc	2.29 ± 0.20 bc
	6.97	78 f	4.3 ± 0.40 d	2.78 ± 0.17 cd
	9.29	86 gh	4.8 ± 0.10 d	3.92 ± 0.12 ef
	11.6	73 e	3.6 ± 0.31 c	3.40 ± 0.33 de
	13.9	60 c	3.1 ± 0.00 bc	2.59 ± 0.19 bc

*Mean values represented in corresponding column followed by same letters are not significantly different ($p < 0.05$)

Table 2. Effect of different concentrations of cytokinins (BAP and Kin) with 0.54 mM NAA on the response in bud breaking from the shoot apex explants of *T. ulmifolia*

Concentration of cytokinins (mM)		Response (%)	Number of shoots (Mean ± SE)	Shoot length (cm) (Mean ± SE)
Control	0.00	0 a*	0.0 ± 0.00 a	0.00 ± 0.00 a
BAP	2.22	54 c	1.4 ± 0.73 f	1.15 ± 0.13 e
	4.44	67 g	1.9 ± 0.26 g	1.53 ± 0.27 j
	6.66	71 i	2.0 ± 0.20 h	1.60 ± 0.11 k
	8.88	84 l	2.0 ± 0.13 h	1.79 ± 0.10 l
	11.1	77 k	1.1 ± 0.58 d	1.46 ± 0.14 h
	13.3	64 f	1.0 ± 0.11 c	1.19 ± 0.24 g
Kin	2.32	48 b	0.6 ± 0.19 b	0.94 ± 0.17 b
	4.65	59 d	1.1 ± 0.56 d	1.17 ± 0.20 f
	6.97	64 f	1.4 ± 0.22 f	1.30 ± 0.31 g
	9.29	75 j	1.9 ± 0.16 g	1.51 ± 0.25 i
	11.6	68 h	1.3 ± 0.25 e	1.14 ± 0.00 d
	13.9	61 e	1.0 ± 0.30 c	1.05 ± 0.27 c

*Explanations: see Table 1

Earlier *in vitro* studies on *T. ulmifolia* also reported nodal shoots segments as better explants in terms of number of multiple shoots induction from the nodal meristems (Shekhawat et al., 2014). Lavia et al. (1994) used leaf explants for the establishment of cultures of *T. ulmifolia*. Cent percentage (100%) bud break was achieved within two to three weeks of inoculation on agar-gelled MS medium augmented with 8.88 mM of BAP and 0.54 mM of NAA in this study (Tab. 1).

The response of explants was significantly affected by the time of culture initiation. Maximum (100%) response was observed during the months of April-June (Fig. 1). The response of explants under *in vitro* conditions is mostly species specific. Phulwaria et al. (2011) reported maximum positive response during rainy season in *Salvadora persica* due to the dilution of growth-arresting factors. In this study, the highest number of shoots (8.3 ± 0.57) was obtained on medium containing 8.88 mM of BAP and 0.54 mM of NAA with additives (Fig. 2A, 2B, Tab. 1). Similarly, Shekhawat et al. (2014) reported 100% bud breaking on the medium with 8.88 mM of BAP with less number

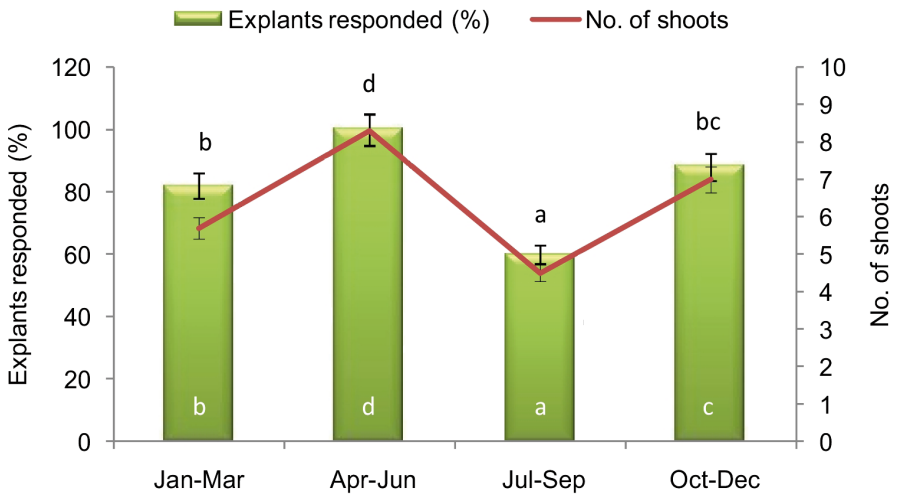

Figure 1. Seasonal effect on the response of explants and induction of shoots in cultures

of shoots (6.1 ± 0.42 per explant). The shoot induction frequency was comparatively low on MS medium supplemented with Kin (9.29 mM Kin + 0.54 mM NAA) and maximum 4.8 ± 0.10 shoots with 3.92 ± 0.12 cm length was achieved in this experiment. Kalimuthu et al. (2014) reported maximum 2.33 ± 1.36 shoots per explants on MS medium supplemented with BAP and TDZ with 86.5% response. Lavia et al. (1994) induced callus from the leaf explants on MS medium augmented with NAA and Kin. The explants cultured on MS medium without growth regulators (control) were failed to respond in this study (Tab. 1).

Shoot multiplication and maintenance of the cultures

The shoots were further proliferated by subculturing of *in vitro* produced shoots on MS medium augmented with different concentrations and combinations of cytokinins and NAA. The shoot segments from the initiation stage were transferred to the fresh MS medium fortified with 2.22 mM of BAP, 2.32 mM of Kin and 0.54 mM of NAA for multiplication, and highest number of shoots (59.5 ± 2.10) with 7.5 ± 0.23 cm average length was achieved on this medium combination (Figs 2C-2E, Tab. 3). The shoots developed on the above medium

Table 3. Effect of different concentrations of cytokinins with 0.54 mM NAA on multiplication of shoots

Concentration of cytokinins (mM)		Number of shoots (Mean ± SE)	Shoot length (cm) (Mean ± SE)
Control	0.00	0.0 ± 0.00 a*	0.00 ± 0.00 a
BAP	0.44	15 ± 0.10 c	3.4 ± 0.14 c
	2.22	26 ± 0.32 fg	4.2 ± 0.35 ef
	4.44	40 ± 1.10 j	5.6 ± 0.21 h
	6.66	32 ± 1.37 h	3.9 ± 0.10 de
	8.88	24 ± 1.23 ef	3.4 ± 0.12 c
Kin	0.46	12 ± 1.91 b	3.0 ± 0.00 b
	2.32	22 ± 0.56 de	3.8 ± 0.27 d
	4.65	35 ± 1.22 i	4.4 ± 0.13 f
	6.97	28 ± 1.19 g	4.1 ± 1.10 def
	9.29	20 ± 1.43 d	3.0 ± 0.14 b
BAP + Kin	0.44 + 0.46	28.4 ± 1.36 g	5.2 ± 1.30 g
	2.22 + 2.32	59.5 ± 2.10 l	7.5 ± 0.23 j
	4.44 + 4.65	45.8 ± 0.13 k	6.3 ± 0.17 j
	6.66 + 6.97	32.1 ± 1.00 h	5.8 ± 0.21 i
	8.88 + 9.29	23.0 ± 0.22 e	5.1 ± 0.10 g

*Explanations: see Table 1

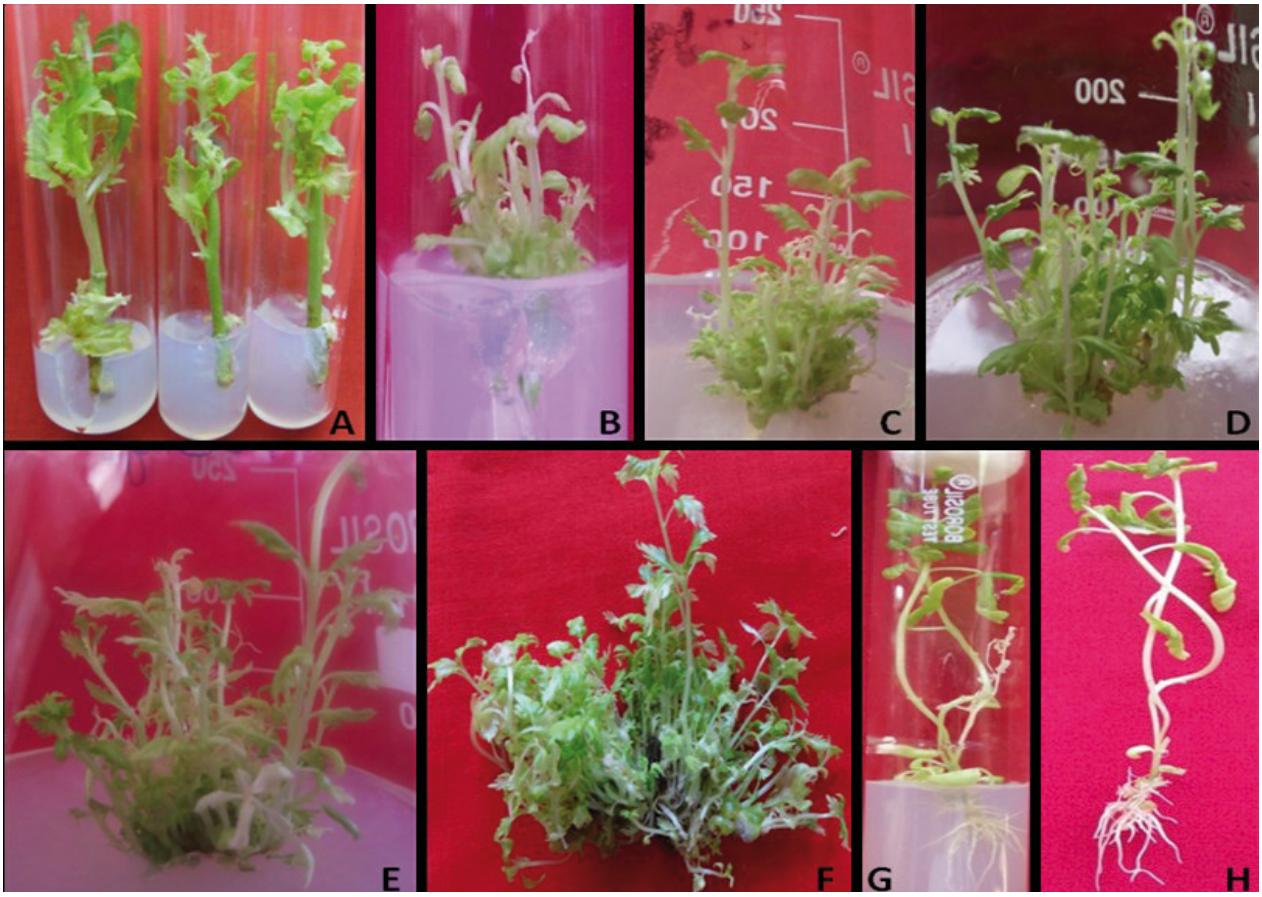

Figure 2. Micropropagation of *Turnera ulmifolia*: A) initiation of shoots from the nodal meristems; B-D) multiple shoots *in vitro*; E-F) large scale multiplication of shoots on agar gelled MS medium; G-H) induction of roots from the cut ends of the shoots

combination were healthy, sturdy and green, and the cultures were maintained for more than one year by regular subculturing on fresh media after every 3-4 weeks of intervals (Fig. 2F). Maximum 47.2 ± 0.08 shoots with 5.1 ± 0.23 cm length reported on MS liquid medium supplemented with 2.22 mM of BAP and 2.32 mM of Kin + 0.54 mM of IAA by Shekhawat et al. (2014) in this plant species. Kalimuthu et al. (2014) reported maximum 4.33 ± 1.03 shoots using MS medium combined with BAP and TDZ. Nine shoots were produced by Lavia et al. (1994) from the callus cultures of *T. ulmifolia* on MS medium fortified with NAA and BAP.

The efficacy of BAP over other cytokinins for the establishment of cultures and multiplication of shoots has been reported by several authors for many plant species (Rathore et al., 2013; Cheruvathur et al., 2015; Lodha et al., 2015; Manokari and Shekhawat, 2017b). Zhang et al. (2010) reported BAP as a better cytokinin for bud breaking and reinvigoration in *Pinus radiata* shoot cultures. The combined effect of cytokinin and auxin has also been recorded by Lavia et al. (1994) in proliferation of shoots of *T. ulmifolia*.

In vitro rooting and hardening of plantlets

Salt strength of MS medium is an important factor in influencing the rooting efficiency in several plant species (Kher et al., 2015; Shekhawats and Manokari, 2016; Patel et al., 2016). Half strength MS medium was reported superior (100% response) as compared to the full strength (74% response) and 1/4th strength (81% response) MS media for rhizogenesis in *T. ulmifolia* shoots (Tab. 4). The shoots were rooted on half strength MS medium fortified with 9.84 mM of IBA and activated charcoal (12.0 ± 0.10 roots per shoot) (Tab. 5, Figs 2G, 2H). The results were almost similar to the earlier reports with minor difference in the root

Table 4. Effect of different strengths of MS media on *in vitro* root induction

Strength of MS medium	Rooting response (%)
Full strength	74 a*
Half strength	100 c
One-fourth strength	81 b

*Mean values followed by same letter are not significantly different ($p < 0.05$)

Table 5. Effect of different concentrations of auxins on *in vitro* rooting of the shoots on half strength MS medium

Concentration of auxins (mM)		Rooting response (%)	Number of roots (Mean ± SE)	Length of root (cm) (Mean ± SE)
Control	0.00	0 a*	0.00 ± 0.00 a	0.0 ± 0.00 a
IAA	5.71	76 d	6.45 ± 0.51 d	3.19 ± 0.13 cd
	11.42	88 f	7.91 ± 1.70 g	3.40 ± 0.36 d
	17.1	84 e	7.49 ± 1.16 f	3.35 ± 0.13 d
	22.8	71 c	6.83 ± 0.20 e	3.03 ± 0.20 c
IBA	4.92	83 e	10.60 ± 1.27 i	2.58 ± 0.26 b
	9.84	100 g	12.00 ± 0.10 j	3.92 ± 0.48 ef
	14.8	91 f	9.13 ± 0.22 h	3.40 ± 0.29 d
	19.7	89 f	7.25 ± 1.10 f	3.00 ± 0.42 c
NAA	5.37	79 d	5.30 ± 0.40 b	3.78 ± 0.19 e
	10.7	83 e	5.79 ± 0.15 c	4.96 ± 0.20 g
	16.1	76 d	5.47 ± 0.31 b	4.73 ± 0.11 g
	21.5	62 b	4.51 ± 0.29 a	4.07 ± 0.26 f

*Explanations: see Table 1

numbers and length (Shekhawat et al., 2014). About 88% shoots were rooted on MS medium containing 11.42 mM IAA and 83% rooting was achieved with 10.7 mM NAA in this experiment (Tab. 5). The root initiation was visible on 15th day of inoculation from the cut ends of the shoots and these findings are slightly higher than the previous reports (Shekhawat et al., 2014). Maximum 7.91 ± 1.70 roots with 3.40 ± 0.36 cm average length were induced on 11.42 mM IAA and 5.79 ± 0.15 roots with 4.96 ± 0.20 cm length resulted with 10.7 mM NAA (Tab. 5).

The *in vitro* shoots exhibited less number of roots on full strength MS medium, but the shoots failed to survive on one-fourth strength of MS medium. There were no roots observed in the control experiments. These results are in contrast with the findings of Antonio et al. (1987), Lavia et al. (1994) and Lilia et al. (2002,) in *T. diffusa*. They observed *in vitro* rooting in *T. diffusa* on agar gelled MS medium without any growth regulators. Kalimuthu et al. (2014) reported maximum 87.34% rooting with 6.00 ± 0.63 roots per shoot using IBA in the medium but Lavia et al. (1994) recorded *in vitro* rooting on full strength MS medium containing NAA in *T. ulmifolia*.

The rooted plantlets were cautiously taken out from the culture vessels and the remnants of medium were removed with the help of a soft brush. All the rooted plantlets were exposed to the greenhouse conditions as mentioned in the materials and methods section. The specific temperature and RH in the greenhouse and gradual opening of the polycarbonate caps of vessels assisted in the adaptation of micropropagated plantlets to the *ex vitro* conditions (Figs 3A, 3B). About 94% of *in vitro* rooted plantlets were successfully hardened

Figure 3. Hardening and field transfer of *T. ulmifolia*: A) hardening of the plantlets in bottles; B) hardened plant transferred to the earthen pot; C) field transferred plants of *T. ulmifolia* after 6 weeks

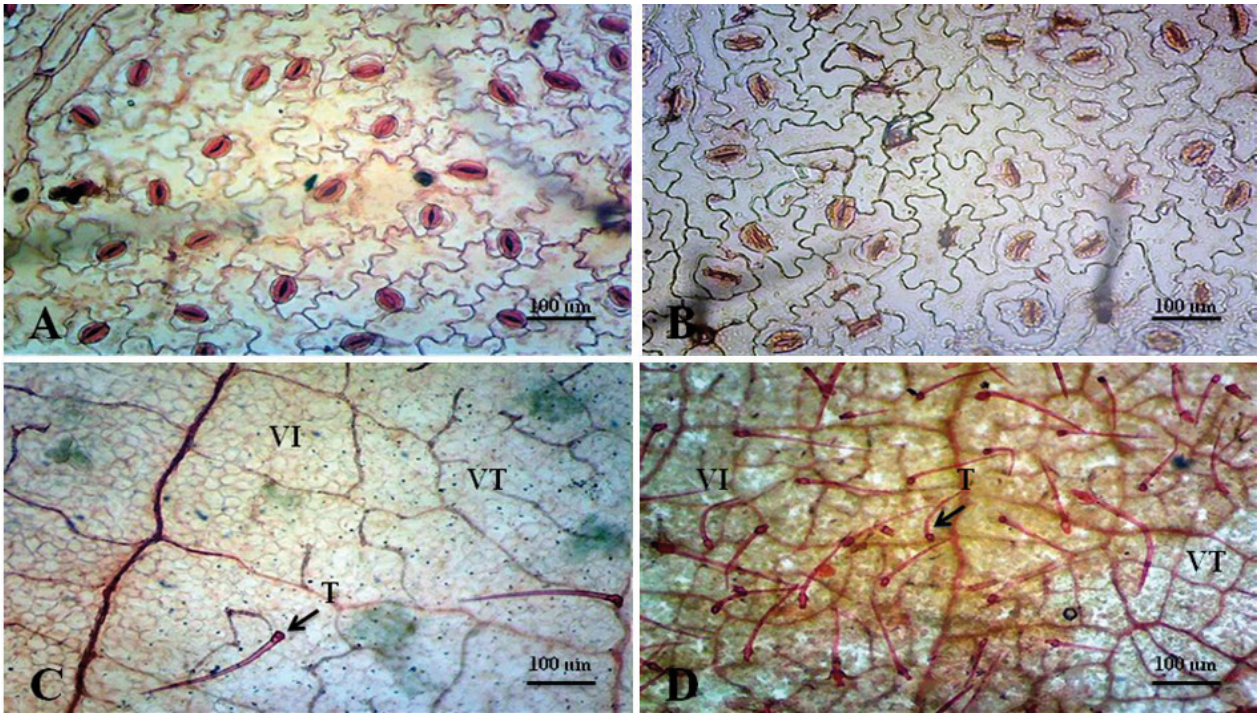

Figure 4. Micromorphological studies of *T. ulmifolia*: A) stomatal frequency of abaxial surface of *in vitro* raised shoots; B) stomatal frequency of abaxial surface of field transferred plants; C) venation pattern in leaves of *in vitro* raised shoots; D) venation pattern in leaves of the field transferred plants (VI – Vein-Islet, VT – Veinlet Termination, T – Trichome)

and established under the field conditions (Fig. 3C). The present survival rate is in connection with the success achieved by Shekhawat et al. (2014) and better than the findings of Lavia et al. (1994) and Kalimuthu et al. (2014) in *T. ulmifolia*.

Foliar micromorphological studies

The comparative foliar micromorphological studies of *T. ulmifolia* illuminated the gradual developmental pattern in plants from laboratory to the field habitat. The leaf architectural parameters such as stomatal index, vein islet density, veinlet termination numbers, crystals and trichome density revealed anatomical and micromorphological significance in plant developmental processes across the *in vitro* to *in vivo* environmental conditions. Leaves of *T. ulmifolia* were alternate, petiolate with serrate margins. The midribs were projected on abaxial surface of the leaves. The leaves were dorsiventral, amphistomatic but the frequency of stomata were more on the abaxial surface. The paradermal view revealed that both adaxial and abaxial epidermis consisted of single layered cells with highly undulated margins. Paracytic and anomocytic stomata were predominant, and the anisocytic and diacytic stomata were less frequent in *T. ulmifolia*. The epidermal cells overlaying the veins (coastal cells) were elongated, rectangular and polygonal in shape with straight anticlinal walls which differed from epidermal undulations. Rajagopal et al. (1972) reported paracytic, anomocytic, anisocytic and diacytic stomata in *T. ulmifolia* and *T. subulata*. Inamdar et al. (1973) observed anomocytic, cyclocytic, paracytic and anisocytic stomata with single subsidiary cell in some members of the family Passifloraceae.

The abaxial cells were small and undulated, and the stomata were always opened under *in vitro* conditions whereas, the cells were comparatively large and highly undulated in field transferred plants. The stomata were present on the inter-coastal regions, absent in the coastal regions and facing all the directions with irregular distribution. The number of stomata and stomatal index were observed more under *in vitro* environment as compared to the field grown leaves. The stomatal index was 26.72 in the *in vitro* grown plants (Fig. 4A) gradually reduced to 21.25 in the field transferred plants (Figs 4B and 6). Stomatal abnormalities such as contiguous stomata, stomata with single guard cell, and the guard cells with arrested stomatal development were occasionally observed in the field transferred plants. Plants systematically develop certain changes in both internal and external structures to repair the *in vitro* induced abnormalities and undergo adaptive

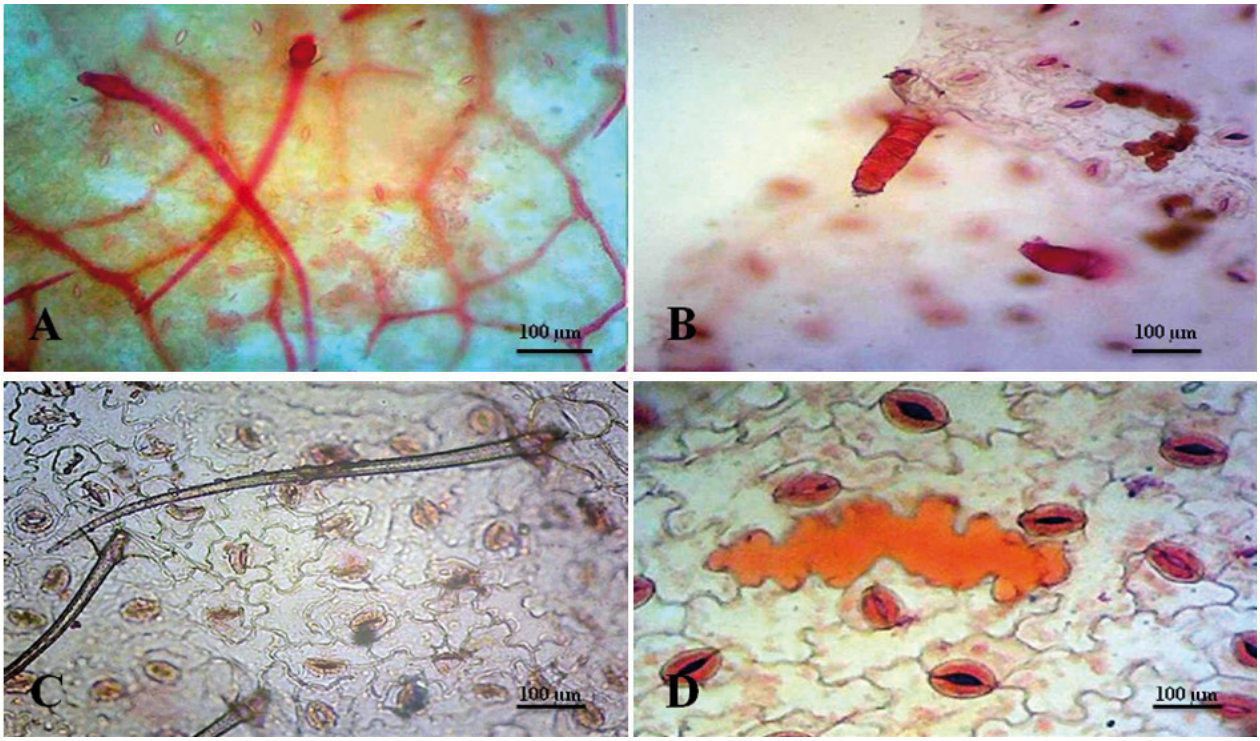

Figure 5. Peculiar foliar micromorphological features of *T. ulmifolia*: A) simple stellate hairs of *in vitro* raised shoots; B) uniseriate articulated hairs; C) simple stellate hairs of field transferred plants; D) mucilagenous cells present in field transferred plants

changes to respective environments (Yang and Miao, 2010).

The reticulate venation with rhomboidal vein-islets was observed in the leaves of *T. ulmifolia*. Veins and vein-islets were fewer with the *in vitro* leaves as compared to the field transferred plantlets. Number of vein-islets and veinlet terminations were increased and become distinct in shape during the hardening period (Fig. 4C, 4D). The vein-islets of *in vitro* plants were increased from 13.4 to 39.7 (Fig. 6) when transferred to the field. The veinlet terminations were simple, rarely branched and reported 7.6 in the *in vitro* leaves but simple as well as branched with increased numbers (18.4) observed in field transferred plants (Fig. 6). Similar findings were reported in *Cadaba fruticosa* (Lodha

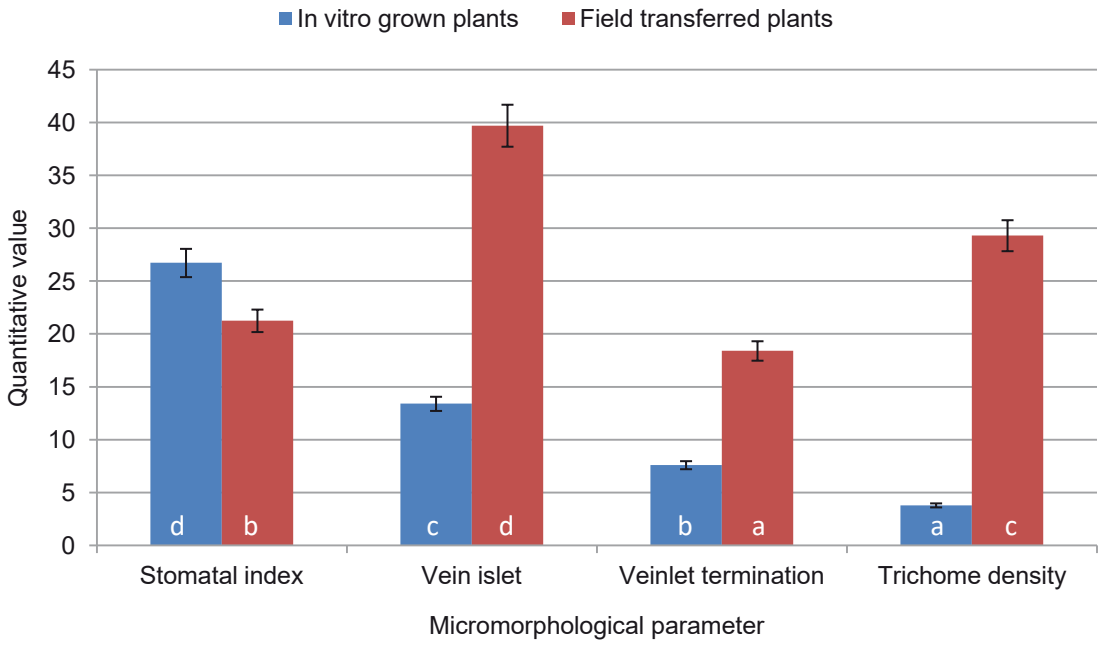

Figure 6. Various foliar morphological parameters of *in vitro* grown and field transferred plants

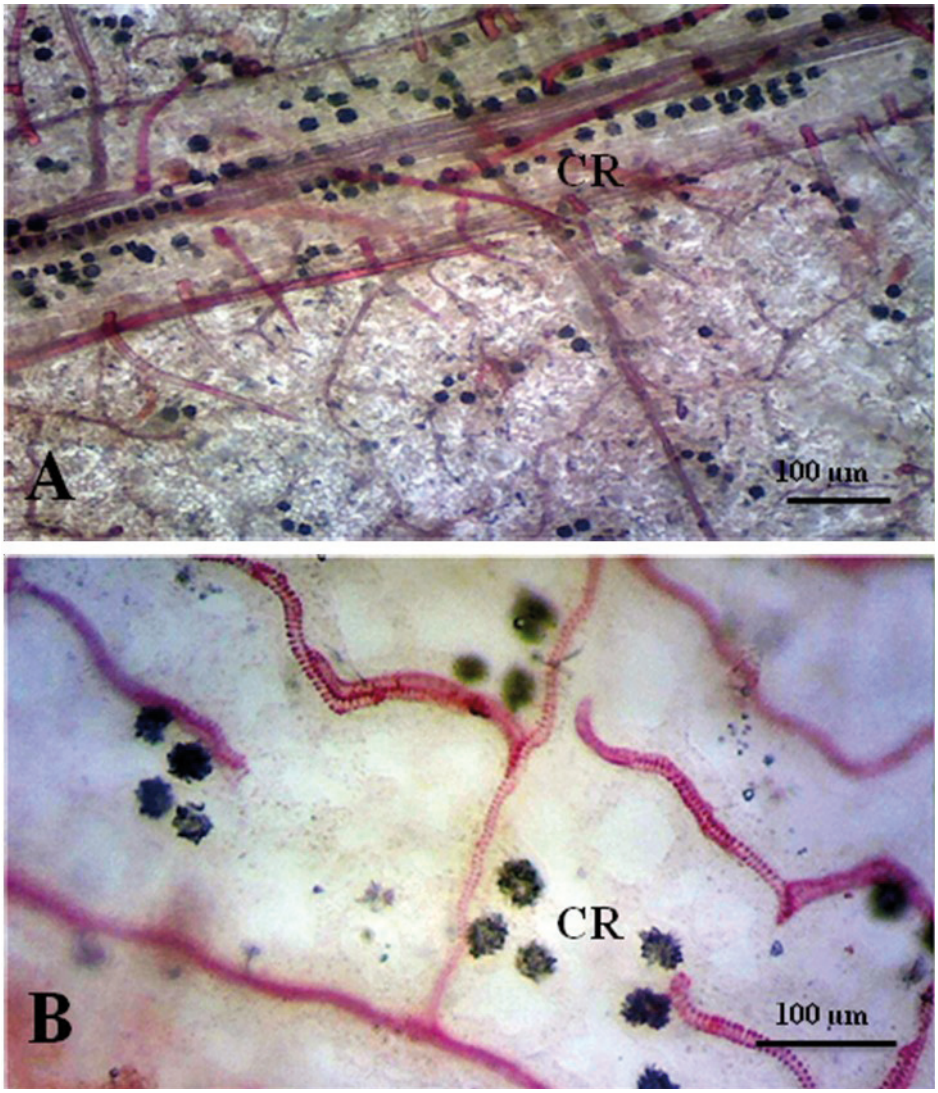

Figure 7. Calcium oxalate crystals in the leaves: A) distribution of calcium oxalate crystals on the mid rib and major veins of field transferred plants; B) magnified view of crystals arrangement in the mesophylls of field transferred plants (CR – Crystals, T – Trichomes)

et al., 2015), *Couroupita guianensis* (Shekhawat and Manokari, 2016) and *Hybanthus enneaspermus* (Shekhawat and Manokari, 2018). The development of increased vein densities from *in vitro* to the field transferred plants is to regulate soil water availability in the plants (Nebelsick et al., 2001).

Two types of trichomes were observed on abaxial surface of the leaves of this plant. These were less frequent, underdeveloped and simple unicellular in the *in vitro* grown plants (Fig. 5A) but fully developed trichomes detected in the field transferred plants. The trichomes were simple, unicellular, stellate and uniseriate articulated on the foliar surfaces of the leaves after field transplantation (Fig. 5B, 5C). Some mucilagenous cells were also observed in the leaves of field transferred plants (Fig. 5D) but these were completely absent in the *in vitro* grown leaves. The highly elongated unicellular hairs were also reported in *T. cearensis*, *T. blanchetiana* and *T. lamiifolia* by Solereder (1908). The trichome density was increased from *in vitro* (3.8) to field transferred plants (29.3) of *T. ulmifolia* (Fig. 6) which agreed with the reports of Rathore et al. (2013) in *Cleome gynandra*, Lodha et al. (2015) in *Cadaba fruiticosa* and Shekhawat and Manokari (2016) in *C. guianensis*.

Calcium oxalate crystals were reported in the leaves of *T. ulmifolia* (Kumar and Sharma, 2007) and *T. aphrodisiaca* (Kumar et al., 2006). Crystals were under-developed in the leaves of *in vitro* micro-shoots, which were well developed, distinct and arranged in orders along with the veins and mesophylls of the field transferred plants. The calcium oxalate crystals were uniformly grouped and randomly arranged with the mesophyll cells (Figs 7A, 7B). Similar findings of development

of crystals from *in vitro* to field adapted leaves were also reported in *P. edulis* by Manokari and Shekhawat (2017a).

CONCLUSIONS

An improved and efficient micropropagation protocol has been developed using agar-gelled MS medium in this study. The shoots were multiplied and rooted effectively using half strength MS medium. The protocol can be used for the propagation of this important medicinal plant. Comparative foliar micromorphological studies of *in vitro* grown and the field transferred plants could help to understand the adaptive response of the plants in the new environmental condition which can improve the hardening process and survival chances of the micropropagated plants.

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AUTHOR CONTRIBUTIONS

M.M. − designed experiments and performed statistical analysis; M.S. − equally contributed to manuscript writing.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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