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Factors Affecting *in vitro* Shoot Proliferation and Rooting of Mature *Tecomella undulata* (Sm.) Seem Tree

Hemshikha Tyagi, Uttar Kumar Tomar*

Forest Genetics and Tree Breeding Division, Arid Forest Research Institute, Jodhpur, India
*Corresponding author: uktomar@icfre.org

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Abstract *Tecomella undulata* (Marwar teak) is valuable timber yielding tree of Rajasthan. Micropropagation techniques are desirable in this species but commercially viable technique is still lacking. Thus *in vitro* propagation of *Tecomella undulata* using nodal segments of mature trees was refined. The *in vitro* shoot cultures can be established throughout the year but the most favourable months for bud break (75%) was January and February. Maximum 73% bud break with average 2.6 cm shoot length was observed on Murashige and Skoog (MS) medium supplemented with 0.54 µM NAA and 8.8 µM BA. Shoots derived from the apical part of the propagule resulted in highest increment in shoot length (33.3 mm) and shoot number (2.0) after four weeks when cultured on MS + 4.4 µM BA medium. *In vitro* regenerated shoots were rooted maximally (43.3%) by dip treatment for 15 minutes in NAA (537.06 µM) & Indole -3-butyric acid (IBA) solution (492.1 µM) followed by transfer on ½ strength Gamborg (B_5) basal medium in Jan-March months. Additives like Ascorbic Acid (567.8 µM) and Thiamine HCl (29.6 µM) were found best for root length and root number respectively. But, interaction of these additives was antagonistic for rooting. Correlation studies on different classes of shoot length and rooting revealed that the rooting percentage increases with the increase in shoot length. Shoots less than 2.5 cm long do not root. The rooted plantlets were successfully hardened. Flowering was also observed in tissue culture plants in first year as well as in second year.

Keywords: *clonal forestry, flowering, in vitro rooting, micropropagation, Rohida*

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1. Introduction

Tecomella undulata (Sm.) Seem is an economically important tree species of arid and semi arid region locally known as Rohida. It is mainly used as a source of timber for high prized furniture, carving and agricultural implements [1]. The species has been identified as an important source of environmental conservation in arid zone as a stabilizer of shifting sand dunes, providing shelter for wild life. It is also helpful for afforestation of the drier tracts due to its drought and fire resistant properties [2]. Over exploitation for timber and fuel, coupled with poor regeneration and sluggish growth has severely depleted the natural population of this valuable tree [3]. The United Nations Environment Programme's World Conservation Monitoring Centre at Nairobi, Kenya has included *T. undulata* under category I – Indeterminate [3,4]. Clonal reproduction of commercially important hardwood tree species is vital in a tree improvement program in order to provide improved planting stock for production forestry [5]. Clonal forestry techniques are operational world wide in many of the plant species *viz. Eucalyptus* in Brazil, Portugal, India and China [6,7] White spruce in Canada [8], *Pinus radiata, Cupressus macrocarpa* in Newzealand [9]. In many of the tree species clonal forestry is non-operational due to lack of proper and efficient clonal techniques. Efficient plant production clonal propagation methods for *T. undulata* tree species are still lacking. *In vitro* clonal propagation of *T. undulata* has been reported by various workers through seedling [10,11,12]. However, *in vitro* propagation through seedling has limitation in tree improvement programme. *In vitro* shoot cultures from nodal explants of mature trees were established and multiplied as reported by [13,14,15]. However, tissue culture protocol remains ineffective due to lack of reproducible rooting methods. Therefore, there is a need of improvement in shoot multiplication and long-term subculturing and better knowledge of root induction in this species. The present study was conducted to refine steps of tissue culture protocol and to improve rooting percentage by understanding factors affecting rhizogenesis.

2. Materials and Methods

Four 15 year old healthy trees of *T. undulata*, were selected in the year 2007 in AFRI campus, Arid Forest Research Institute, Jodhpur.

2.1. Explants Collection and Sterilization

Single nodal explants (2.0-2.5 cm length and 0.4-0.5 cm width) of *T. undulata* were collected and decontaminated using few drops of Tween-80 for 10 minutes followed by treatment with Bavistin (1 gm l^{-1}) and Streptomycin (0.5 gm l^{-1}). In the next step the nodal segments were surface sterilized with 2.5% Sodium hypoclorite solution (5% available chlorine) for 5-7 minutes. Subsequently, these explants were thoroughly washed with autoclaved distilled water to remove sterilizing agent.

2.2. Inoculation and Culture Conditions

The nodal segments were cultured on MS medium supplemented with 30 g l^{-1} sucrose, 0.54 µM NAA and 8.8 µM BA. In each case pH of the medium was adjusted to 5.8 with 1 N NaOH or 1 N HCl and solidified with 0.8% agar (Hi- Media) before autoclaving at 121°C for 15 minutes. The monthly pattern of temperature and relative humidity were recorded with data logger (Novus) programmed to record the data at 15 minutes interval. Annual pattern of temperature and relative humidity in culture room is given in Figure 1.

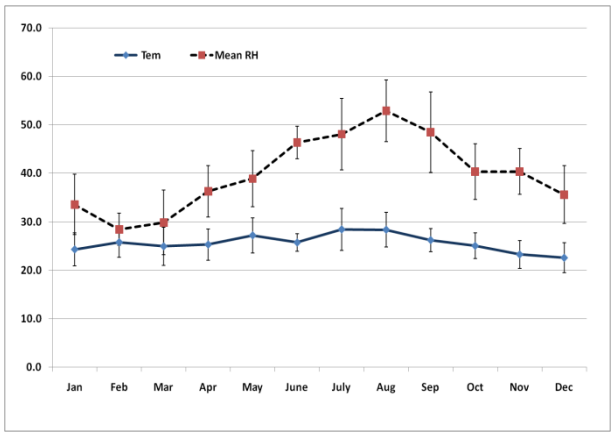

Figure 1. Monthly pattern of temperature (°C) and relative humidity (%) of tissue culture laboratory from June 2011 to May 2012

2.3. Maintenance of *In vitro* Shoot Cultures

The proliferated shoots were maintained through repeated subculturing on MS + 4.4 µM BA medium for five years as stock for shoot multiplication and rooting experiments. During the successive five years of study from 2007-2011, the cultures were incubated at 27 µmol m^{-2} s^{-1} light intensity for 16 hrs photoperiod. Average shoot multiplication of different years (2007-2011) is given in Figure 2.

2.4. *In vitro* Rooting and Hardening

In vitro multiplied shoots were initially subjected to MS medium lacking auxins and medium supplemented with auxins (IAA, IBA & NAA). Shoots inoculated on MS medium lacking auxins did not root, whereas, incorporation of IBA within media resulted in inconsistent (lack of repeatability) and low percentage of rooting with heavy callusing at the base of the shoots. Therefore, two step procedure [13] was followed for rooting which included pre-treatment of basal end of shoots (minimum 1.6 cm length) with the auxin solution of 492.1 µM IBA and 537.0 µM NAA for 15 minutes followed by transfer to medium devoid of auxin.

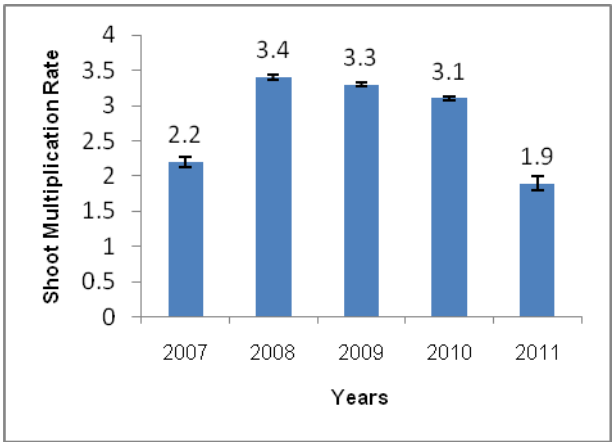

Figure 2. *T. undulata* average shoot multiplication rate of stock cultures on MS + 4.4 µM BA medium in five successive years (2007-2011).

2.5. Transfer of Rooted Plants in Pots

The rooted shoots were carefully washed off with water to remove agar and the rooted shoots were hardened in culture room conditions in autoclaved vermiculite along with ½ strength liquid MS medium devoid of sucrose. After one month the plants were transferred to polybags containing soil: compost (3:1) and kept in greenhouse and subsequently transferred to the field after proper hardening.

2.6. Data Analysis

In the present studies, completely randomized design (CRD) was used for statistical analysis. Data were evaluated by ANOVA test and means were compared with Duncan Multiple Range Test using SPSS (version 8.0) software to determine the significance of differences.

3. Results and Discussion

3.1. Establishment of Culture

The *in vitro* shoot cultures of *T. undulata* were easily established with stem nodal segment as described by previous workers [13,14,15] and there was no major problem of contamination and phenolic leaching in the medium. Bud break experiments were initiated according to the protocol developed as in [13].

3.1.1. Comparison of NAA and IAA on Bud Break

To study the requirement of auxin along with BA, MS + 8.8 µM BA medium was selected as control and different concentrations of IAA or NAA were added as treatments. Figure 3 shows the effect of different concentrations of NAA (0.27-2.7 µM) and IAA (0.3-2.9 µM) along with 8.8 µM BA incorporated in MS medium on shoot regeneration and shoot length.

Low to moderate shoot regeneration percentage (13 - 73%) was obtained in all the treatments, including the control. Concentrations of IAA decreased the shoot regeneration percentage when compared to control and NAA. There was an increase in the shoot regeneration

percentage (73%) as well as shoot length (2.6 cm) on 0.54 µM NAA but beyond the optimum concentration, there was a decrease in shoot production with an increased NAA concentration. ANOVA at 5% level shows significant difference on shoot length due to addition of NAA in MS + 8.8 µM BA medium. In *T. undulata*, incorporation of plant growth regulators (BA, NAA & IAA) are not showing significant effect on bud break but they are playing a positive role towards shoots growth [16].

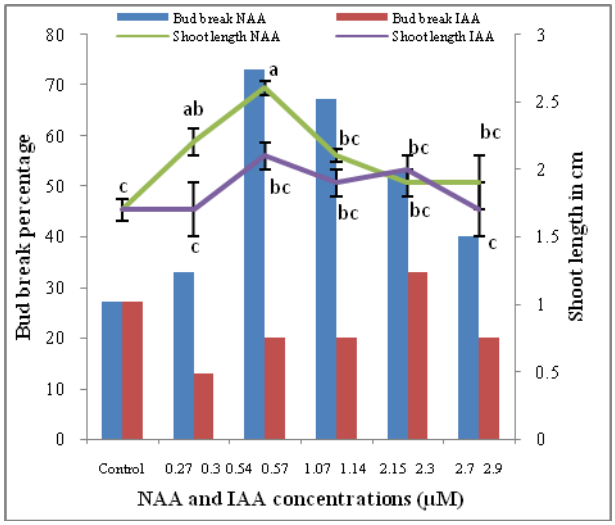

Figure 3. Effect of different concentrations of NAA (0.27 – 2.7 µM) and IAA (0.3 – 2.9 µM) along with 8.8 µM BA on bud break and shoot length of *T. undulata* after 40 days. Any two mean shoot length having different superscript are significantly different when separated by Duncan Multiple Range Test.

Shoot length response was found better in NAA supplemented medium than IAA. The effectiveness of BA in promoting *in vitro* axillary shoot production in woody plant is well documented [17,18]. Incorporation of NAA along with 8.8 µM BA was found better than IAA because NAA is more, light and heat stable [19] and IAA readily oxidized by light (photooxidation) as well as by enzyme (IAA oxidase). Similarly a combination of NAA (low concentration) and BA (higher concentration) in MS medium was effective in *Balanites aegyptiaca* [20], *Acacia auriculiformis* [21], *Aegle marmelos* [22] and *Ailanthus excelsa* [23].

3.1.2. Seasonal Effect on Shoot Initiation

Stem nodal segments were raised every month on the best combination (MS + 0.54 µM NAA + 8.8 µM BA) to see the annual pattern for bud break and shoot length (Figure 4). The results indicate maximum bud break (75%) and shoot length (4.0 cm) in the month of January-February and minimum bud break (23%) in the month of July-August (rainy season). Increased production of shoots and shoot length was observed mainly in the months belonging to the winter season (Nov, Dec, Jan & Feb). The results on shoot length in different months were significantly different at 5% level as analysed by ANOVA and DMRT test. In *T. undulata* maximum bud break was observed in January-February, which differs from period reported by [13], where highest bud break was achieved in the month of August-September. This difference may be due to some change in climate as observed in last two decade in Jodhpur condition. Seasonal effect on bud break in mature tree's explants has been also reported in *M. esculenta* [24], *Acacia sinuta* [25] and *S. sebiferum* [26]. In all these species late winter *i.e.* December to March is ideal for high percentage of bud break. Luckily contamination was not a major problem in *T. undulata* and more than 75% of stem nodal explants were established by using normal sterilization procedures. Highest contamination (23%) was encountered with *T. undulata* during the rainy season. This may be because of ideal temperature and high moisture in rainy season which favours the growth of microbes. Similar results were obtained in *Pyrus pyrifolia* [27], *Arundinaria callosa* [28], *Casuarina equisetifolia* [29] and Banana [30]. However, the aseptic shoot cultures of *T. undulata* can be established throughout the year.

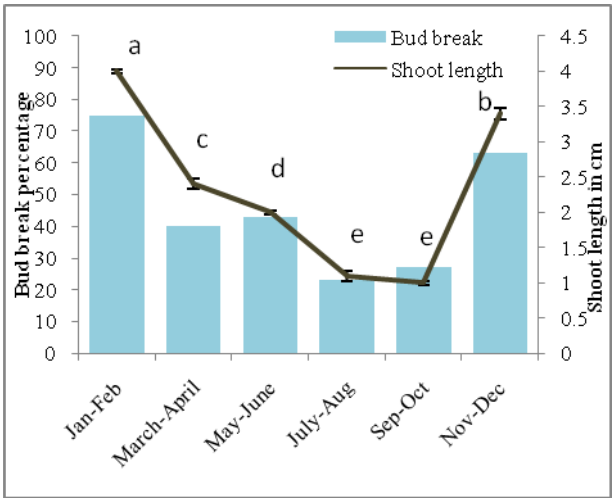

Figure 4. Effect of different months on bud break and shoot length of *T. undulata* on MS medium supplemented with 0.54 µM NAA + 8.8 µM BA after 40 days. Any two mean shoot length having different superscript are significantly different when separated by Duncan Multiple Range Test.

3.2. Shoot Multiplication

Shoot multiplication through successive subculturing provide major benefit of micropropagation over conventional methods. Earlier worker, reported maximum shoot multiplication in MS + 0.06 µM IAA + 4.4 µM BA [13]. In our experiments cytokinin was used alone due to callusing property of *T. undulata* during subculturing.

3.2.1. Effect of BA on Shoot Multiplication

Amongst cytokinins BA is most effective in shoot multiplication [13]. Therefore, different BA concentrations (4.4, 8.8 and 17.7 µM) supplemented in MS medium were used to observe its effect on shoot multiplication, shoot length and callus induction. The highest increment in shoot multiplication (2.5) was recorded in MS supplemented with 17.7 µM BA which was at par with 8.8 µM BA concentration according to DMRT at 5% level. However, maximum shoot length (20 mm) was observed on 4.4 µM BA and DMRT revealed that shoot length at 4.4 µM BA was significantly higher at 5% level than rest of the BA treatments and control. One-way analysis of variance also indicates significant difference on shoot length and shoots multiplication parameter at 5% level (Table 1).

Cytokinins play significant role in shoot multiplication and shoot growth. Shoot multiplication experiment with cytokinin (BA), indicate that shoot multiplication rate is higher at 8.8 µM and 17.7 µM BA but at these levels BA enhance the callusing and reduces shoot length. Reference [31] also reported that explants of *Cajanus cajan* require 8.8 µM BA at the initial stage of shoot bud regeneration and multiplication but further growth and proliferation of the shoot was observed only after subculture to fresh medium with lower level of BA (4.4 µM). Therefore, MS + 4.4 µM BA was recommended for shoot multiplication in spite of low shoot multiplication than 8.8 and 17.7 µM in *T. undulata*. Similar results were reported in many of the other species *viz. Acacia senegal* [32], *Paulwonia kawakamii* [33], *Clitoria ternatea* [34], *Azadirachta indica* [35], *Lilium* species [36], *Mentha* spp. [37], *Withania somnifera* [38] and *Pogostemon cablin* [39].

Table 1. Effect of Different Concentration of BA on Shoot Multiplication and Shoot Length on MS Medium After 30 Days.

MS+BA (µM)	Mean shoot Number±SE	Mean shoot Length (mm)±SE	Associated callus
Control	1.4 ± 0.1^{b}	8.0 ± 0.5^{d}	++
4.4	1.8 ± 0.2^{b}	20 ± 0.8^{a}	+++
8.8	2.4 ± 0.1^{a}	15 ± 0.5^{b}	++++
17.7	2.5 ± 0.2^{a}	10 ± 0.4^{c}	++++

Shoot length: F cal (3, 60) (BA concentration) = 94.30*
Shoot multiplication: F cal (3, 60) (BA concentration) = 10.14*
Any two means having a common superscript are not significantly different when separated by Duncan Multiple Range Test.
('++' sign denotes low, '+++' moderate & '++++' heavy callusing)

3.2.2. Shoot Multiplication of Different Propagule Types

In many species, it has been reported that apical, middle and basal part of shoots have different potential of shoot multiplication. Therefore, to study the effect of apical dominance, these shoots were cut into three parts, apical, middle and basal and cultured on MS medium supplemented with 4.4 µM BA. Table 2 shows the effect of different part (apical, middle & basal) of the propagule on shoot multiplication. The results revealed that apical portion of shoots have highest potential for shoot length (mean shoot length increment 33.3 mm), followed by in basal part (22.9 mm) and lowest (17.8 mm) in the middle parts (Fig. 6c). Increment in mean shoot number was more or less same (2.0 fold) in the apical and basal parts followed by middle parts (1.3). ANOVA test indicate significant ($p \leq 0.05$) differences in shoot multiplication and shoot length. But apical and basal parts were at par for both growth parameters according to DMRT.

Preparation of propagules by cutting them in different way and removing unwanted callus, dead tissues and placing it on the fresh medium, play an important role in shoot multiplication and their health [40,41]. In *T. undulata* when shoot is cut into three parts *viz.* apical, middle and basal. Shoot multiplication was poor in cultures derived from middle parts. Apical and basal portion produced higher multiplication as compare to middle part. However, average shoot length was highest with apical portions. Similar results were observed in many species where an apical part of the propagule has shown higher length and low shoot multiplication as compared to the basal portion *viz. Ailanthus excelsa* [23] and *Paphiopedilum* orchid [41].

Table 2. Effect of different propagule position in shoot on shoot multiplication of *T. undulata* on MS + 4.4 µM BA medium after 30 days.

Propagule part	Mean shoot length (mm)±SE	Mean shoot no. ± SE
Apical	44.1 ± 4.0	3.0 ± 0.2
Middle	25.4 ± 1.2	2.3 ± 0.1
Basal	30.8 ± 4.6	3.0 ± 0.1

Shoot length: F cal (2, 74) (Propagule position) = 4.7*
Shoot multiplication: F cal (2, 74) (Propagule position) = 8.8*
*= Significant at 5 % level.

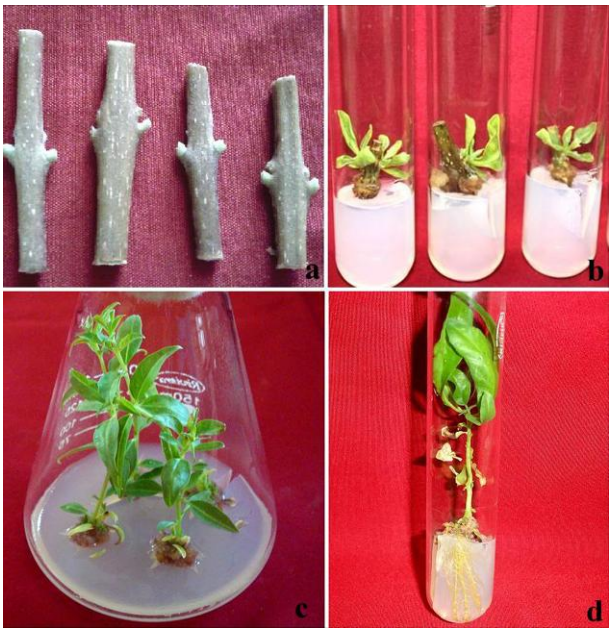

Figure 5. *In vitro* establishment and rooting of *Tecomella undulata.* (a) Stem nodal explants of *Tecomella undulata.* (b) Shoot induction from nodal segment on MS + 0.54 µM NAA + 8.8 µM BA after 4 wk. (c) Shoot multiplication of the apical part of propagule on MS + 4.4 µM BA medium after 4 wk. (d) *In vitro* rooting of regenerated shoots on ½ B_5 medium after 15 minutes treatment with IBA (492.1 µM) and NAA (537 µM) solution.

3.3. *In vitro* Rooting

Previous workers observed that auxin is required for rooting. Our results are also in agreement with previous workers. However, incorporation of auxins within media resulted in low percentage of rooting with heavy callusing at the base of the shoots. Therefore, two step procedure was followed where higher concentration of IBA and NAA were used alone and in combination to see their effect on rooting. Among these, combination of IBA (492.1 µM) and NAA (537.0 µM) proved best.

3.3.1. Effect of Different Medium on Rooting

Different medium viz. ½ MS, ½ B_5, ½ WPM & Hoagland were tried on induction of rooting of regenerated shoots collected from the shoots stocks maintained on 4.4 µM BA medium. All the treatments resulted in root production with frequencies ranging from 4.3 to 43.4%. Highest rooting percentage (43.4%) and roots per cultured shoot (3.1) was obtained on ½ B_5 medium with treatment of IBA (492.1 µM) + NAA (537 µM) solution for 15 minutes when compared with other media (Fig. 6d). ½ WPM and Hoagland media has shown lowest rooting percentage. ANOVA test indicate that there was significant difference in root length and root number of the shoots grown on different media (Table 3).

Table 3. Rooting Response of *T. Undulata* Shoots After 30 Days on Different Media After 15 Minutes Treatment of IBA (492.1 µm) and NAA (537 µm) Solution. Inoculation Date, 09 February 2010.

Treatment	Rooting%	Mean root length (cm) ± SE	Mean root number ± SE
½ MS	17.4	3.2 ± 0.8^{a}	2.8 ± 0.8^{b}
½ B_5	43.4	2.9 ± 0.4^{a}	3.1 ± 0.3^{a}
½ WPM	4.3	0.5 ± 0^{b}	1.0 ± 0^{c}
Hoagland	4.3	3.6 ± 0.1^{a}	1.5 ± 0.3^{bc}

Root length: F cal (3, 88) (Different medium) = 3.88*
Root number: F cal (3, 88) (Different medium) = 6.32*

Among different medium tried ½ B_5 medium was found best for rooting. The level of NH_4NO_3 and KNO_3 in half MS medium is higher as compared to half B5 medium. In apple cultivars the level of NH_4NO_3 in the medium from full strength to ¼ strength significantly increased the rooting percentage [42]. Similar to our observation B5 medium was found better for rooting in *P. orientale* [43] and *Argyrolobium roseum* [44]. It is well known that low salt concentrations favours rooting and B_5 medium has low salt levels than MS medium [45].

3.3.2. Effect of Different Additives

Thiamine HCl (vitamin B1) is required by all cells for growth. Ascorbic acid (vitamin C) acts as an antioxidant/anti browning agent. Thiamine HCl (THCl 29.6 µM) and Ascorbic acid (AA 567.8 µM) were used alone and in combination for rooting experiment. The shoots were given treatment of IBA (492.1 µM) and NAA (537 µM) solution for 15 minutes followed by transfer to the fresh ½ B_5 medium supplemented with the additives as given in table 4a.

Table 4a. Rooting Response of Shoots After 30 Days On ½ B_5 Medium Supplemented With Different Additives (Ascorbic Acid And Thiamine Hcl) Alone and in Combination After Treatment of IBA (492.1 µm) And NAA (537 µm) Solution For 15 Minutes. Inoculation Date, 12 March 2010.

Treatment	Rooting %	Mean root length (cm)±SE	Mean root number ± SE
Control	25	1.5 ± 0.3^{b}	4.5 ± 0.3^{ab}
567.8 µM AA	32	1.1 ± 0.1^{bc}	5.9 ± 1.0^{a}
29.6 µM THCl	33	3.3 ± 0.0^{a}	3.0 ± 0.0^{b}
567.8 µM AA + 29.6 µM THCl	5.4	0.6 ± 0.09^{c}	2.4 ± 0.4^{b}

Root length: F cal (3, 158) (Different additives) = 3.76*
Root number: F cal (3, 158) (Different additives) = 6.19*

The results indicate that there is significant difference among the different treatments for root length and root number. Interaction of Ascorbic acid and Thiamine HCl were antagonistic for rooting. In case of root length, ½ B_5 + 567.8 µM Ascorbic acid was best and for root number ½ B_5 + 29.6 µM Thiamine HCl proved best according to DMRT at 5% level of significance. The vitamin B complex is known to stimulate cell division [46] and Ascorbic acid used as an antioxidant agent can reduce browning of medium resulted due to exudation of phenolic compounds from mature explants and their oxidation. Therefore, it prevents necrosis also being an antioxidant in nature [47,48]. In the present study, medium incorporated with Thiamine HCl has improved rooting percentage and Ascorbic acid has favoured maximum number of roots/shoots. Our results are in agreement with the previous literature, which suggest that Thiamine is important for root growth [49]. Excellent root development due to Thiamine HCl was observed in *Matteucia struthiopteris* [50], Ostrich fern [51], *T. brevifolia* and *T. cuspidata* [49]. It has also been reported by [52], that application of Ascorbic acid in combination with an auxin (IBA) promotes rooting in terms of number of roots/cutting in various plant species. Ascorbic acid makes rooting earlier and improves the quality of roots as compared to those treated with auxin alone [53,54].

3.3.3. Effect of Shoot Length on Rooting

In past experiments, it was experienced that the long shoots respond better to the rooting treatments as compared to small shoots. Therefore, to know the effect of shoot length on rooting, the shoots were separated into 4 classes. Shoots with length 1.6-2.5 cm were regarded as class A, 2.6-5.0 cm as B, 5.1-7.5 cm as C and 7.6-9.0 cm as class D. The results clearly indicate that the class A with shoot length 1.6-2.5 cm do not root. The ANOVA test reveal that all the four classes are significantly different from each other in terms of rooting percentage and root number. To understand the relationship of shoot length and rooting, a set of rooting experiments and the data (n=162) of shoot length is divided in to 7 different classes. Shoot length from 1.6-2.4 cm. is regarded as class **1**, 2.5-2.9 as **2**, 3.0-4.0 as **3**, 4.1-5.0 as **4**, 5.1-6.0 as **5**, 6.1-7.0 as **6** and 7.1-9.0 cm as class **7** (Table 4b). The rooting percentage is increased with the increasing shoot length which shows the positive relationship of shoot length with rooting (Figure 5).

Pearson Correlation (2 tailed) analysis revealed significant (P = 0.05) positive correlation (r = 0.174) between these two variables (shoot and root length) in the data set (n = 162) given in Table 4c.

Annual pattern of relative humidity (RH) and temperature inside the culture room revealed variations in both parameters during different months of the year (Figure 1).

Table 4b. Effect of Shoot Length on Rooting of *T. Undulata* After 30 Days on ½ B_5 Medium With and Without Different Additives (Ascorbic Acid and Thiamine Hcl).

Class (cm.)	Rooting%	Root length (cm)	Root number
A(1.6-2.5 cm.)	0	0^{b}	0^{b}
B(2.6-5.0 cm.)	27	2.7^{ab}	1.0^{b}
C(5.1-7.5 cm.)	17	3.3^{ab}	0.9^{b}
D(7.6-9.0 cm.)	60	7.4^{a}	3.2^{a}

Root length: F cal (3, 158) (Different class of shoot length) = 1.755ns
Root number: F cal (3, 158) (Different class of shoot length) = 2.554*
*=Significant at 5% level, ns=non significant

Table 4c. Correlation Matrix Between Two Variables (Shoot Length & Root Length) for a Sample of Shoots Cultures (N=162).

Variable	Mean ± SD	1	2
1. Shoot length	4.16 ± 1.5	---	0.174*
2. Root length	0.28 ± 0.7	0.174*	---

* p< 0.05; **p< 0.01

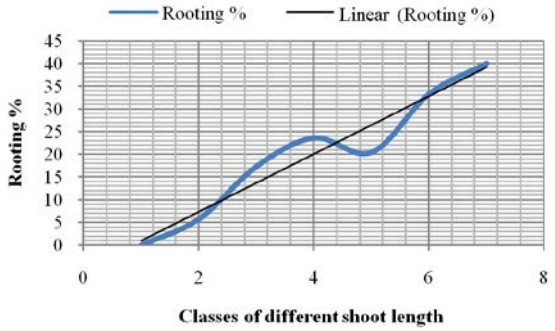

Figure 6. Represents the rooting percentage in shoots divided into 7 different classes according to their length.

When the results of all rooting experiments conducted in different months analysed (Data not shown) it was noticed that optimal rooting was recorded in winter period only *i.e.* December, January, February and March when the average temperature was 24 ± 1°C and RH 32%. It indicates that the culture conditions influence the rooting procedure. In this species similar annual pattern of bud break in nodal explants and rooting in stem cuttings was also observed [55]. These patterns under *in vitro* conditions may be due to carrying memory of physiological adoption of this species with ambient annual condition of Rajasthan.

3.4. Hardening

Maximum about 40% rooting was achieved in *Tecomella undulata* but due to poor health of the rooted shoots, about 50% of them were not found suitable for hardening. Shoot tip necrosis was the main cause for poor health of the shoots. The *in vitro* regenerated healthy plantlets were washed thoroughly with autoclaved distilled water. These plantlets were transferred to the Jam bottles containing vermiculite as potting mixture with ½ strength liquid MS media and kept in the culture room conditions for a period of four week. MS medium ½ strength was supplied initially for 15 days at five days interval and thereafter at 15 days interval. After one month these plantlets were transferred to thermocol cups containing vermiculite and kept in polyhouse for thirty days. Finally two months old plants were transferred in polythene bags containing soil mixture (sand and compost in 3:1). About 35% of micropropagated plants were survived in hardening. One tissue culture raised plant produced flower at two year of age after rooting, indicating the physiological maturity (Figure 7d).

Figure 7. *In vitro, ex vitro* hardening and flowering in *Tecomella undulata*. (a) *In vitro* hardening in autoclaved vermiculite. (b) *Ex vitro* hardening in mist polyhouse. (c) Hardened plants in shade house after one month. (d) Flowering in micropropagated plant after two years.

3.5. Conclusion

Present studies reveal some of the facts related to micropropagation of *T. undulata*. The shoot cultures from nodal explants initiated on MS basal medium in January-February months will give better results. Apical part of the shoot subcultured on MS + 4.4 µM BA medium will generate more rootable shoots. Rooting experiments done during January to March months with pretreatment of IBA (492.1 µM) and NAA (537 µM) solution for 15 minutes followed by transfer to ½ B_5 basal medium will give best rooting results. It appears *in vitro* rooting is following also an annual pattern and optimal rooting was observed in February and March only. This was similar to annual pattern of rooting of stem cuttings [55]. However, further researches are required to understand more about intrinsic factors of this species to further improve the rooting and hardening success. Flowering in tissue culture plants in first year and second year indicate that explants keeps memory alive even during long term sub culture on MS + 4.4 µM BA medium.

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