European Journal of Biotechnology and Bioscience
ISSN: 2321-9122; Impact Factor: RJIF 5.44
Received: 22-03-2020; Accepted: 10-04-2020; Published: 21-04-2020
www.biosciencejournals.com
Volume 8; Issue 1; 2020; Page No. 15-20

In vitro culture establishment and seed studies of *Diospyros buxifolia* (Blume) hiern: A slow growing timber species of Western Ghats

Tresa Hamalton[1*], Rekha Jaganathan[2], Almas Khanam[3]
[1-3] Silviculture and Forest Management Division, Institute of Wood Science and Technology, Bangalore, Karnataka, India

Abstract
Diospyros buxifolia is a timber species, conventionally propagated through seeds, which have very short viability. A protocol for *in vitro* culture establishment of *D. buxifolia* using leaves, nodes and seeds as explants is reported. In this study, morphological characters of the fruits and seeds were also examined. Surface sterilization of explant is the first and most important step in establishing *in vitro* culture. Under the regime employed in this study, the most critical factor was the use of autoclaved antioxidant solution. For *in vitro* culture establishment, explants (leaf and node) collected in autoclaved antioxidant solution, followed by pretreatment with high concentration of M-45, Streptomycin and $HgCl_2$ significantly increased number of aseptic cultures (>95%). The method reported herein for *in vitro* establishment of *D. buxifolia* cultures can be successfully used for conservation strategies of *D. buxifolia* which is otherwise a slow growing tree.

Keywords: Diospyros buxifolia, *seed studies, surface sterilization, micropropagation*

1. Introduction
Diospyros is the largest genus of the pantropical family *Ebenaceae.* It is one of the largest angiosperm genera and consists of over 500 species distributed in the tropics and subtropics [1, 2]. Majority of the species (200-300) occur in Asia and the Pacific area [3]. In India, more than 40 species of *Diospyros* are distributed mostly in Deccan, Assam and Bengal, with few species in north India [4].
Diospyros buxifolia (Blume) Hiern, syn. *D. microphylla* Bedd., is a large ornamental, lofty, buttressed tree with 30m height and 1m diameter. In India, *D. buxifolia* is widely distributed in evergreen and semi evergreen forest of Western Ghats, from North Kanara to Anamalai hills and Travancore [4]. The stem is straight, cylindrical, buttressed, black, scaly, flecked with white and green. Young branches and flowers are covered with long, pale ferruginous hairs. The leaves are small, ovate-oblong, distichous and subsessile. The flowers are minute, white, 4-5 merous and dioecious. The fruit of *D. buxifolia* is oblong, single-seeded and purple in color. [4, 5, 6, 7, 20] The wood is reddish grey, streaky, hard and close grained and used in making excellent walking stick, poles, match boxes and splints [4, 5, 6]. So far micropropagation has been reported in persimmon species of *Diospyros* such as *D. kaki* [8], *D. virginiana* [9] and *D. lotus* [10], but not in commercially valuable timber species of this genus.
D. buxifolia trees growing naturally are indiscriminately logged for its wood, which is valued as a type of ebony. Conventionally, *D. buxifolia* is propagated through seeds produced once a year, but they have very short viability. [18] Also, the seedling growth is decidedly slow in all *Diospyros* species [11]. Hence micropropagation is imperative for large scale production and quick rejuvenation of diminishing natural populations. Appropriate choice of growth regulators and their correct concentration can facilitate *in vitro* regeneration. However, lack of knowledge of genetic background is the main disadvantage associated with seedling material, whereas, excessive leaching of phenolics, endogenous contamination and poor multiplication rate are the problems associated with explants from mature trees [12]. In view of the above, the present study was undertaken to standardize the most effective technique for *in vitro* culture establishment of *D. buxifolia* for micropropagation using leaves, nodes and seeds as explants.

2. Materials and Methods
2.1 Seed studies
2.1.1 Source and collection of seeds
Fruits of *D. buxifolia* were collected from forest area of Seethanadi, Karnataka during the month of June. Fruits were washed with water and brought to the laboratory, where they were dusted with Bavistin and stored in room temperature for further analysis.

2.1.2 Fruit and seed morphology
About 20 fruits were used for measuring the length, diameter & weight of the fruits and de-pulped seeds using a Vernier Caliper. 10 seeds were used for determination of moisture content after drying in hot air oven for 7 days. The seed moisture content was expressed as percentage of the fresh weight. The remaining seeds were sown in sand for *ex vitro* germination.

2.1.3 Surface sterilization of seeds for *in vitro* germination
The fruits were rinsed with water before surface sterilization to remove Bavistin. Seeds were de-pulped and washed in Tween-80 for 15 minutes and then rinsed well with MilliQ water. In order to identify the most effective surface sterilization technique, different sterilizing agents were tested with varied durations of treatment. The seeds were pretreated with single concentration and combination of Bavistin and Streptomycin for different duration, and rinsed well with MilliQ water. In the laminar flow cabinet, the seeds were surface sterilized with 70% ethanol for 30 seconds. This was followed by shaking either with single

concentration of $HgCl_2$ for different durations, or with different concentrations of NaOCl for 30 minutes, and then rinsed well with autoclaved MilliQ water (Table 1). After the water was completely decanted, the dry seeds were inoculated into test tubes containing hormone free, half strength MS medium. The tubes were incubated in dark at 25°C and observed every alternative day for 5 weeks. The percentage of bacterial and fungal contamination was documented weekly.

Table 1: Treatments used for *D. buxifolia* seed surface sterilization

S. No.	Treatments	Duration (in minutes)
T1	Bavistin 0.5% + Streptomycin 0.1 % + $HgCl_2$ 0.1%	20 + 20 + 10
T2	Bavistin 0.5% + Streptomycin 0.1 % + $HgCl_2$ 0.1% + Antioxidant solution	20 + 20 + 10 + 15
T3	Bavistin 0.5% + Streptomycin 0.1 % + $HgCl_2$ 0.1%	30 + 30 + 30
T4	Bavistin 0.5% + Streptomycin 0.1 % + NaOCl 2.5%	30 + 30 + 30
T5	Bavistin 0.5% + Streptomycin 0.1 % + NaOCl 0.1%	30 + 30 + 30

2.2. *In vitro* culture establishment of *D. buxifolia* through leaf and node

For *in vitro* culture establishment, the procedure for surface sterilization of nodal and leaf explants of *D. buxifolia* was first standardized. Subsequently, the effect of different concentrations and combinations of plant growth hormones (PGRs) for axillary shoot induction and adventitious shoot induction, from nodal and leaf explants respectively, was tested.

2.2.1. Explant source

Seedlings of *D. buxifolia* were collected from Seethanadi, Karnataka and grown in the mist chamber till 4 to 5 years. The seedlings were then transferred under shade net and sprayed regularly with fertilizer (Micro elements of MS media) and fungicide (Bavistin) to maintain as healthy and disease-free plants. Mature leaves (3rd leaf onwards from the apex) and nodes (3rd node onwards from the shoot tip) were collected as explants from healthy lateral branches.

2.2.2. Surface sterilization of leaf and node

Leaves and nodes were freshly collected either in water or autoclaved antioxidant solution. The various treatments followed for standardizing surface sterilization are tabulated in Table 2. The explants were wiped with 70% ethanol and treated with Tween-80 for 2 minutes and then rinsed well with MilliQ water. The explants were surface sterilized with different concentrations and combinations of M-45 and Streptomycin for 5 minutes, followed by rinsing with MilliQ water. In the laminar flow cabinet, explants were surface sterilized with 70% ethanol for 30 seconds, followed by shaking with different combinations of $HgCl_2$ and PVP for 2 minutes, and then rinsed well with autoclaved MilliQ water. After surface sterilization, leaf explants were prepared for inoculation by removing the leaf margin, petiole and leaf tip. Nodal explants were prepared by trimming the cut ends and removing leaf, but retaining petiole, so that the final explant was 2 to 3cm in length. The leaf and node explants were inoculated into test tubes containing 10ml of MS medium.

Table 2: Treatments for surface sterilization of *D. buxifolia* explants

S. No.	Medium for explant collection	M-45 + Streptomycin (5 min)	$HgCl_2$+ PVP (2 min)
T1	Water	0.2% + 0.05%	0.1% + 0
T2	Water	0.2% + 0.05%	0.1% + 0.01%
T3	Water	0.5% + 0.1%	0.1% + 0
T4	Water	0.5% + 0.1%	0.1% + 0.01%
T5	Antioxidant solution	0.2% + 0.05%	0.1% + 0
T6	Antioxidant solution	0.2% + 0.05%	0.1% + 0.01%
T7	Antioxidant solution	0.5% + 0.1%	0.1% + 0
T8	Antioxidant solution	0.5% + 0.1%	0.1% + 0.01%

2.2.3. Inoculation of nodal and leaf explants for multiple shoot induction

Two separate sets of experiments were performed for shoot initiation from nodal and leaf explants. The explants were surface sterilized following treatment T7 (Table 2), as it produced highest percentage of aseptic cultures, in the surface sterilization experiment (Table 5). Various concentrations and combinations of Zeatin and IAA (Table 3a) were tested for maximum bud break from nodal segments. The leaf explants were experimented for adventitious shoot induction using various concentrations and combinations of Kinetin and IAA (Table 3b). The culture medium used in both the experiments was MS (half nitrogen) supplemented with sucrose and additives (Ascorbic acid, Citric acid, Cysteine, Glutamine and 50mg/l PVP), and solidified with agar. The pH was adjusted to 6.0 after the addition of growth regulators before autoclaving. Observations were recorded weekly for up to 15 weeks, and the explants were subcultured to fresh medium after every 4 weeks.

2.3. Culture medium and growth conditions

Half strength MS basal medium [19] containing 3% (w/v) sucrose, additives (Ascorbic acid-50 mg/l, Citric acid-25mg/l, Cysteine-25mg/l, Glutamine-100mg/l and PVP-25mg/l), and agar 0.6% (w/v) was used. pH of the medium was adjusted to 6.0 and 10ml of the medium was dispensed into each 150x25mm Borosil culture tubes and plugged with non-absorbent cotton wrapped in cheese cloth. Dispensed media were autoclaved at 1.06kgcm^{-2} at temperature of 121ºC for 20 minutes. The antioxidant solution used in this study contained Ascorbic acid-50 mg/l, Citric acid-25mg/l, Cysteine-25mg/l and Glutamine-100mg/l. The inoculated cultures were kept under 25±2ºC at 37.5 µmol m^{-2} S^{-1} intensity light for 12h photoperiod. For seed germination, cultures were kept in dark till germination started, and was then transferred to 12h photoperiod. All the cultures were transferred to fresh medium at four weeks interval.

Table 3a: Treatments for shoot bud induction in *D. buxifolia*

PGR		IAA (mg/l)			
		0	0.01	0.1	0.2
Zeatin (mg/l)	0	T1	T6	T7	T8
	1	T2	T9	T10	T11
	2	T3	T12	T13	T14
	3	T4	T15	T16	T17
	5	T5	T18	T19	T20

Table 3b: Treatments for adventitious shoot induction in *D. buxifolia*

PGR		IAA (mg/l)			
		0	**0.01**	**0.1**	**0.2**
Kinetin (mg/l)	0	T1	T6	T7	T8
	0.5	T2	T9	T10	T11
	1.0	T3	T12	T13	T14
	1.5	T4	T15	T16	T17
	2.0	T5	T18	T19	T20

2.4. Experimental design, data collection and statistical analysis

The data for fruit and seed studies is expressed as Mean ± SD. For seed surface sterilization experiments, 10 replicates for each treatment were maintained. Seed germination was recorded till 60 days and then the percentage of seed germination was calculated. For surface sterilization of nodal and leaf segments, 5 and 15 replicates respectively were maintained, for each treatment. The cultures were observed weekly, and recorded the percentage of contamination and percentage of aseptic cultures up to 4 weeks. For shoot initiation from nodal segment and leaf segment 5 replicates were used for each treatment. Observation on number of days for bud break, percentage of shoot induction, number of shoots per explant and callus induction, was recorded weekly for 15 weeks. All the experiments were repeated thrice and were set up according to a completely randomized design, and the data was analyzed by one-way analysis of variance.

3. Results & Discussion

3.1. Seed studies of *D. buxifolia*

3.1.1. Morphology of Fruits and Seeds

Fruit and seed morphological characters were examined 3 days after collection. Fresh fruits were oblong berries and dark reddish purple in color (burgundy color) (Fig 1). As documented by Hiern, each fruit contained a single seed [20], which was russet colored (Fig 2). Mean length (mm) of the fruits and seeds were 17.88 ± 2.09 and 14.82 ± 1.51, respectively. Mean diameter (mm) of fruits and seeds were 9.00 ± 1.14 and 7.32 ± 1.21, respectively. Mean weight (g) of seeds was 0.56 ± 0.19 and of fruits was 0.98 ± 0.27. Moisture content percentage of the seeds was calculated using the fresh weight and dry weight. Mean moisture content (%) was found to be 50.55 ± 3.49.

Fig 1: Fresh fruit of D. *buxifolia*

Fig 2: De-pulped seed of *D. buxifolia*

Fig 3: Fruits and seeds of D. *buxifolia* 3 days after collection

3.1.2. Seed Surface Sterilization

The percentage of aseptic cultures, 5 weeks after the surface sterilization and inoculation of *D. buxifolia* seeds, is given in Table 4. Contamination rate is often high when the explants are collected from the forest, hence surface sterilization is essential for removing microbes like fungi and bacteria. Germinating seeds on sterile media can also be challenging due to systemic contamination, especially when the number of seeds available is limited. Therefore, to reduce the risk of fungal and bacterial contamination various sterilization treatments were used. From the results obtained, it is evident that production of aseptic cultures ranged from 0% to a maximum of 10% after 5 weeks.

Ramakrishna *et al*. reported NaOCl and $HgCl_2$ as effective against common fungus for surface sterilization of seeds [13]. However, in the present study, it was observed that surface sterilization with neither NaOCl nor $HgCl_2$ produced this effect for *D. buxifolia* seeds, as it resulted in 90 to 100% contamination (Fig 2), and this is attributed to the source of the seeds collected.

Out of various treatments tested, T3 (Bavistin 0.5% + Streptomycin 0.1 % + $HgCl_2$ 0.1%) recorded maximum (10%) aseptic cultures with 30% survival rate. This was followed by T2, which was same as T3 and also included rinsing in sterile antioxidant solution for 15 minutes, with 23% survival. It was observed that treatment with $HgCl_2$ was more significant than NaOCl, as all the seeds from T4 and T5 were completely contaminated within a week, with lesser fungal contamination (33 to 40%) than bacterial contamination (70 to 100%). On the contrary, treatment

with $HgCl_2$ resulted in only 33%, 27% and 10% contamination for T1, T2 and T3 respectively, after the same duration. At the end of 5 weeks, fungal contamination was more in all the treatments as compared to bacterial contamination, except in T3.

Table 4: Effect of different surface sterilization treatments on *D. buxifolia* seeds after 5 weeks

Treatment	Percentage of Bacterial contamination	Percentage of Fungal contamination	Percentage of Septic cultures	Percentage of Aseptic cultures
T1	8.33	91.67	91.67	8.33
T2	18.18	72.73	90.91	9.09
T3	60.00	40.00	90.00	10.00
T4	100.00	100.00	100.00	0.00
T5	100.00	100.00	100.00	0.00

Fig 4: Initiation of seed germination with bacterial and fungal contamination in D. *buxifolia* seed

3.1.3. Seed germination

The *in vitro* germination percentage of the surface sterilized seeds was calculated 60 days after inoculation, though germination had initiated within 7 days after inoculation (Fig 4). Out of 5 surface sterilization treatments (Table 1), seed germination percentage ranged from 8.33% (T1) to 33.33% (T4), and no germination was observed in T3. Systemic fungal contamination in the seeds was high in both *ex vitro* and *in vitro* germination studies. The seeds sowed *ex vitro* did not show any germination even after 60 days.

3.2. *In vitro* culture establishment of *D. buxifolia*

3.2.1. Surface sterilization of explants

Two types of explants (leaf and node) were used for experimenting explant surface sterilization. It was observed that, leaf explants collected in antioxidant solution and pre-treated with 0.5% M-45, 0.1% streptomycin combined with 0.1% $HgCl_2$ (T7) appeared to be the most effective pre-treatment as it yielded as high as 93.3% aseptic cultures. This was followed by T5 and T8 with 86.7% aseptic cultures, and T2 was the least effective (Table 5). In all the treatments, fungal contamination was more than bacterial contamination, except in T4. In nodal explants also, treatment T7 was most effective yielding 80% aseptic culture establishment. This was followed by T5 and T8 which were moderately effective (60% aseptic culture), and the remaining treatments were least effective resulting in 80% contamination.

Surface sterilization of explant is the first and most important step in establishing *in vitro* cultures. The concentration of sterilants varies for different species and type of explants, and inappropriate concentrations can have adverse effect in cell metabolism, restricting the growth and development of explant. Therefore, appropriate concentration, combinations and duration of exposure of surface sterilant is essential to raise *in vitro* cultures successfully. Collecting explant in autoclaved antioxidant solution and pretreatment with high concentration of M-45, Streptomycin and $HgCl_2$ significantly increased number of aseptic cultures, as compared to collection of explants in MilliQ water. This is due to decreased microbial load in autoclaved antioxidant solution, and combined with high concentrations of antimicrobials, T7 sufficiently aided in reducing contamination during culture establishment.

Table 5: Effect of different surface sterilization treatments on leaf explants of *D. buxifolia* after 15 weeks

Treatments	Percentage of Bacterial contamination	Percentage of Fungal contamination	Percentage of Septic cultures	Percentage of Aseptic cultures
T1	27.33	53.33	80.0	20.0
T2	33.33	53.33	86.7	13.3
T3	26.67	46.67	73.3	26.7
T4	46.67	20.00	66.6	33.4
T5	6.67	6.67	13.3	86.7
T6	0.00	26.67	26.7	73.3
T7	0.00	6.67	6.7	93.3
T8	6.67	6.67	13.3	86.7

3.2.2. Shoot bud induction from nodal segments

In the preliminary response to growth hormones, bud break was observed within 40 days of inoculation (Fig 5). With increase in culture duration, callusing of explants in the base was also observed (Fig 6). The percentage of bud sprouting was significantly more in media containing 2mg/l Zeatin, as compared to all other combinations (Table 6).

Palla *et al*. produced shoots from nodal stem explants of *D. virginiana* by culturing them in MS medium containing only 10µM Zeatin, which was also routinely micropropagated in the same medium containing 5µM Zeatin alone [9]. Cooper & Cohen also reported that MS medium with 4.56µM or 13.69µM Zeatin alone produced the best shoot growth from nodal explants of *D. kaki* [14]. Fukui *et al*. have also used MS medium containing half strength nitrogen supplemented with only Zeatin at 10µM for production of multiple shoots [21]. Sarathchandra & Burch reported 60% shoot bud induction using 4.5 µM Zeatin in *D. kaki* [15]. In accordance with these studies, the percentage of bud sprouting in *D. buxifolia* was significantly more in media containing Zeatin alone (1 to 3 mg/l), compared to combinations of Zeatin and IAA. This was followed by 2mg/l Zeatin, either alone or with 0.1 mg/l IAA (Table 6).

Tao & Sugiura established a protocol for micropropagation of *D. kaki* using dormant buds cultured on MS medium containing half-strength nitrogen, with 20µM BA or 10µM Zeatin [8]. However, Kochanová *et al*. reported highest value of shoot regeneration from dormant buds of *D. kaki* on MS (1/2 N) medium, supplemented with 5 µM BAP and 1 µM IBA [16].

Table 6: Percentage of bud sprouting in *D. buxifolia* nodes after 8 weeks

PGR		IAA (mg/l)				Mean
		0	0.01	0.1	0.2	
Zeatin (mg/l)	0	0	0	0	20	5
	1	20	0	0	0	5
	2	40	0	20	0	15
	3	20	0	0	0	5
	5	0	0	0	0	0
Mean		16	0	4	4	SE=3

Fig 5: Bud break in *D. buxifolia* node in 2mg/l Zeatin+0.1 mg/l IAA after 40 days

Fig 6: Shoot growth from node of *D. buxifolia* in 2mg/l Zeatin+0.1 mg/l IAA after 75 days

3.2.3. Callus induction from leaf

In order to optimise the medium for adventitious shoot induction, the leaf explants were inoculated on medium containing different concentrations and combinations of Kinetin and IAA. Observation was recorded every 7 days and explants were subcultured to fresh medium after 28 days. The cut ends of all the explants turned brown/black initially, followed by leaf curling, and callus initiation at cut edges was observed after 35 days.

Kinetin is a potential cytokinin and is structurally similar to Zeatin. Hesar *et al*. reported greatest shoot length and highest shoot number in 2mg/l Kinetin during micropropagation of *Matthiola incana*, in which shooting and rooting were simultaneously obtained [17]. In this study, Kinetin at 2 mg/l, either alone or in combination with IAA up to 0.1 mg/l, resulted in increased callus induction. Maximum callusing was observed in cultures of *D. buxifolia* on medium supplemented with 2 mg/l Kinetin and 0.01 mg/l IAA. This was followed by medium containing 1 mg/l Kinetin with IAA, which was in par with 0.01 mg/l IAA with Kinetin (Table 7). Callus was initially creamy and friable, and later turned into black compact callus (Fig 7).

Sarathchandra & Burch reported regeneration of whole plants from leaf segments of *D. kaki* in medium containing 10 µM trans-Zeatin along with IAA, IBA or NAA [15]. Tao & Sugiura established a protocol for formation of adventitious buds from callus of *D. kaki* on MS medium (1/2N) containing 10 µM Zeatin, along with 0.03 to 0.1 µM IAA or 0.01 to 0.03 µM NAA [22]. Tamura *et al*. successfully initiated callus from leaf primordia of *D. kaki* winter buds in MS medium (1/2N) containing 10 µM Zeatin and 1 µM IAA, which, even after 210 weeks of culture, showed low organogenetic potential, when transferred to differentiation medium [23]. Likewise, the callus of *D. buxifolia* also failed to regenerate shoots even after repeated subcultures into fresh medium, for up to 15 weeks.

Table 7: Percentage of callus induction in *D. buxifolia* leaves after 5 weeks

PGR		IAA (mg/l)				Mean
		0	0.01	0.1	0.2	
Kinetin (mg/l)	0	0	0	40	0	10
	0.5	0	0	0	0	0
	1	20	40	0	40	25
	1.5	20	0	0	0	5
	2	40	80	40	0	40
Mean		16	24	16	8	SE=3

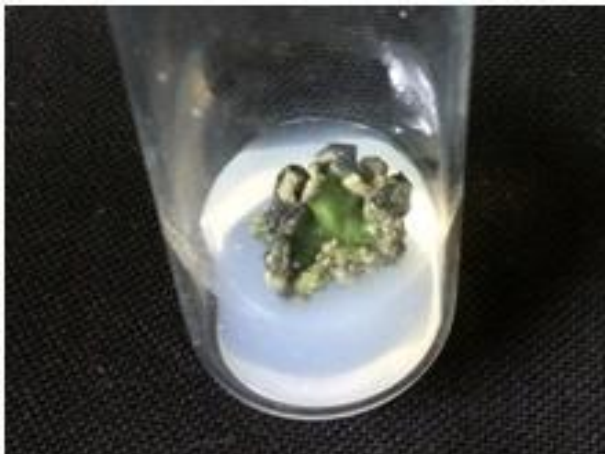

Fig 7: Callusing in D. *buxifolia* leaf in 2mg/l Kinetin+0.1 mg/l IAA after 40 days

Conclusion

Our results support the fact that, adequate surface sterilization prior to inoculation of explants is essential to completely inhibit contamination, which in turn helps in aseptic *in vitro* culture establishment. This is the first report on the seed characteristics of *D. buxifolia*, which can further the studies on seed storage behavior. From the present investigation it is evident that use of autoclaved solution for explant collection can greatly reduce contamination for *in vitro* culturing. Use of antioxidants and PVP in media, along with explant collection in antioxidant solution also reduced leaching from the explants during culture. Additional research needs to be focused on multiple shoot induction using a hormone cheaper than Zeatin, and subsequent rooting of shoots, followed by scale-up of the micropropagation technique in order to be successfully employed in quick rejuvenation of natural populations of *D. buxifolia*.

Acknowledgment

The authors are grateful to Indian Council of Forestry Research and Education, Dehradun for financial support.

References

1. Duangjai S, Wallnöfer B, Samuel R, Munzinger J, Chase MW. Generic delimitation and relationships in Ebenaceae sensu lato: evidence from six plastid DNA regions. American Journal of Botany. 2006; 93(12):1808-1827.
2. Turner B, Munzinger J, Duangjai S, Temsch EM, Stockenhuber R, Barfuss MHJ, *et al*. Molecular phylogenetics of New Caledonian *Diospyros* (Ebenaceae) using plastid and nuclear markers. Molecular Phylogenetics and Evolution. 2013; 69:740-763.
3. Wallnöfer B. The biology and systematics of Ebenaceae: A review. Annalen des Naturhistorischen Museums in Wien. Serie B fürbotanik und Zoologie, 2001, 485-512.
4. The Wealth of India (Raw Materials) Vol. D-E, C.S.I.R. Publication, New Delhi, 1953, 76-77.
5. Gamble JS. Flora of the Presidency of Madras, 1921, 776-777.
6. Neginhal SG. Forest Trees of the Western Ghats, Bangalore, 2011, 213-214.
7. Saldanha CJ, Ramesh SR. Ebenaceae. In: Saldanha C J. Flora of Karnataka. Vol. 1. Oxford & IBH, 1984; 335.
8. Tao R, Sugiura A. Micropropagation of Japanese Persimmon (*Diospyros kaki* L.). In: Bajaj Y.P.S. (ed.) High-Tech and Micropropagation II. Biotechnology in Agriculture and Forestry, Springer, Berlin, Heidelberg, 1992, 18:424.
9. Palla KJ, Beasley RR, Pijut PM. *In vitro* culture and rooting of *Diospyros virginiana* L. HortScience. 2013; 48(6):747-749.
10. Xie Qi-xin, Mei-lianhuang, Xiao-ping wu, Dong-hongzhuang. Plant Regeneration from Leaves of Date Plum (*Diospyros lotus* L.) Scientia Agricultura Sinica. 2008; (2):607-612.
11. Troup RS. The Silviculture of Indian trees. Vol. 2. Nataraj publishers, Dehradun, 1921, 647.
12. Beena VB, Rathore TS. *In vitro* cloning of *Bambusa pallida* Munro through axillary shoot proliferation and evaluation of genetic fidelity by RAPD markers. International Journal of Plant Biology, 2012, 3:e6.
13. Ramakrishna N, Lacey J, Smith JE. Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. International Journal of Food Microbiology. 1991; 13(1):47-54.
14. Cooper PA, Cohen D. Micropropagation of Japanese persimmon (*Diospyros kaki*). Proc. Intl. Plant Prop. Soc. 1984; 34:118-124.
15. Sarathchandra SU, Burch G. Micropropagation of Japanese persimmon (*Diospyros kaki* Thun.) cv. 'Hiratanenashi'. New Zealand Journal of Crop and Horticultural Science. 1991; 19(2):113-120.
16. Kochanová Z, Onus N, Brindza J. Adventitious shoot regeneration from dormant buds of persimmon (*Diospyros kaki* Thunb.) cv. Hachiya. Journal of Agrobiology. 2011; 28(2):113-118.
17. Hesar AA, Livani BK, Tarang A, Zanjani SB. Effect of different concentrations of kinetin on regeneration of *Matthiola incana*. Plant Omics. 2011; 4(5):236-238.
18. http://tropical.theferns.info/viewtropical.php?id=Diospyros+buxifolia. 2 January, 2020.
19. Murashigae T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 1962; 15:473-497.
20. Hiern WP. A monograph of Ebenaceae. Transactions of the Cambridge Philosophical Society. 1873; 12(1):218.
21. Fukui H, Nishimoto K, Nakamura M. Varietal Differences in Rooting Ability of *In Vitro* Subcultured Japanese Persimmon Shoots. J Japan. Soc. Hort. Sci. 1992; 60(4):821- 825.
22. Tao R, Sugiura A. Adventitious Bud Formation from Callus Cultures of Japanese Persimmon. HortScience. 1992; 27(3):259-261.
23. Tamura M, Tao R, Sugiura A. Highly Stable Regeneration from Long-term Cultures of Japanese Persimmon Callus. HortScience. 1992; 27(9):1048.