Analysis of Extracts and Nutritional potential in plant species
Vol. 11 Spl 1 pp. 1-9, November, 2020
Available online https://www.interesjournals.org/plant-science.html
DOI: http:/dx.doi.org/10.14303/irjps.2020.0016

Full Length Research Paper

Development of an efficient protocol from *invitro* Micropropagation and Somatic embryogenesis for the Mass Multiplication of *Ventilago maderaspatana* Gaertn- A Medicinal Plant

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Abstract

An efficient *in vitro* method was developed for the micropropagation and mass multiplication of *Ventilago maderaspatana* Gaertn. Healthy and mature *V. maderaspatana*, plants stem segments (3-5 cm in height) are obtained from actively growing young shoots during the month of March from different locations at Western Ghats of Karnataka were used as explants. Woody Plant Media supplemented with different concentration and combinations for of plant hormones were used for shoot, callus and root induction. TDZ at 0.5 mg/L induced highest rate (86.1%) of regeneration with 6.83±1.71 shoots per explant. In callus formation, media supplemented with 0.5 mg/L NAA and 0.5 mg/L TDZ induced highest rate (87.9%) of regeneration with the formation 14.2±1.91 somatic embryos per callus culture. Highest rate (91.5%) of rooting was induced by 0.5 mg/L IBA with 4.08±1.23 roots per shoot. Regenerated plants were transferred into pots containing soil rite, vermiculate, coir peat, peat moss, sand and the mixture of sand and soil for hardening and acclimatization. Plants potted in Soil rite showed the highest rate (92.6%) of survival.Mature plants were reintroduced in to their natural habitat at Neerahalla (Hassan district, Karnataka) for conservation.

Keywords: *Ventilago maderaspatana* Gaertn, Micropropagation, Somatic Embryogenesis, Reintroduction, Medicinal plant and Thidiazuron.

INTRODUCTION

Biodiversity is the immense collection of all the species of plants, animals, insects and microorganisms residing the earth either in the aquatic or the terrestrial habitats. Global community has witnessed key changes as a result of the unfolding and globalization of revolution taking place since years (Mathur, 2013). In view of the shockingly growing world population, increasing anthropogenic actions, rapidly corroding natural ecosystem, etc the natural habitat for a prodigious number of herbs and trees are dwindling. Many of them are facing extinction (Sharma et al., 2010). An extremely conservative estimate states that this loss of plant species is between a hundred and thousand times above the expected natural extinction rate the planet is losing a minimum one potential major drug every 2 years (Phillips et al., 1994). According to the International Union for Conservation of Nature and also the World Wildlife Fund, there are between 50,000 and 80,000 flowering plant species used for medicinal purposes worldwide. Among these, about 15,000 species are threatened with extinction from over harvesting and environs destruction (Bonato et al., 2006) and 20 % of their wild resources have already been nearly exhausted with the swelling of human population and plant consumption (Chen et al., 2016).

To deal with this alarming situation, biotechnology has come as boon. Advances in plant biotechnology, particularly those associated to *in vitro* culture and molecular biology, have also provided influential tools to support and improve conservation and management of plant diversity. One of the techniques like plant tissue culture have been used to conserve endangered, rare, crop, ornamental, medicinal and forest species, allowing the conservation of pathogen-free material, elite plants and genetic diversity for short-medium- and long-term. *In vitro* conservation is especially

important for vegetatively propagated and for non-orthodox seed plant species (Kasagana and Karumuri,2011; Cruz-Cruz et al., 2013). It has also opened exciting frontier in the field of agriculture and offers opportunities for the increase in productivity, profitability, stability and sustainability(Kuras et al.,2004; Rameshkumaret al.,2017). Plant tissue culture techniques have also helped in large-scale production of plants through micropropagation or clonal propagation of plant species (Mathur, 2013).

Ventilago maderaspatana Gaertn., (*V. maderaspatana*) is a woody liana belonging to family Rhamnaceae. It is distributed in the forest of low elevations in Southern Greece, India, Indonesia, Myanmar, and Sri Lanka. Commonly called as Red creeper (Ravidra et al., 2013; Sindhura and Eswaraiah, 2014). It shows the occurrence of vathraquinonesphyscion, isofuranonaphthaquinones, emodin, xanthrorin & xanthorin-5-methyl ether, islandiein, ventilone-c, Ventiloquinones E,G,J eleuthrin, enantiopure 1,3-dimethyl pyranonappthoquinones (Kwade et al., 2014; Sasikala and Sudhakarc, 2015). Due to its substantial amount of medicinally important bioactive compounds and various anthropogenic and natural activities, authors of this paper have witnessed gradual decrease in the population of *V. maderaspatana*in the region of Hassan and Mysore districts of Karnataka, India. As in such situation conservation of a plant's genetic diversity of a region becomes important. In general, conservation involves deeds such as collection, propagation, characterization, evaluation, disease indexing and elimination, storage and distribution. The conservation of plant genetic resources has long been realised as an integral part of biodiversity conservation. The goal of conservation is to support sustainable development by protecting and using biological resources in ways that do not diminish the world's variety of genes and species or destroy important habitats and ecosystems (Kasagana and Karumuri, 2011).

Hence, the present work is aimed to establish a micro-propagation protocol for the mass multiplication and *In vitro* conservation of *V. maderaspatana* from direct shoot multiplication, callus culture and somatic embryo culture.

MATERIALS AND METHODS

Seed germination test

Before the initiation of micropropagation procedures, the seeds *V. maderaspatana* were tested for their efficiency in germination by seed germination test. To test the seed viability, seeds of were germinated using methods such as Between paper method, Top of the paper method and Sand method prescribed by Ministry of Agriculture and Farmers welfare, available in Seed testing Manual (2017). Seeds sown as per the treatments were incubated in germinator maintained at required levels of temperature with 12-hour light and dark each, until completion of germination. Observation and Datarecorded at the end of 6^{th} week. For each type of treatment 12 replicates were maintained.

Seeds were also germinated on solidified Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with different concentrations and various combinations of BAP and KN. The cultures were kept for incubation under lab conditions at ± 22^0C for 6 weeks. Observation and Data recorded at the end of 6^{th} week. For each type of treatment 12 replicates were maintained.

Plant material and tissue cultureconditions

Healthy and mature *V. maderaspatana* plants explants, stem segments (3-5cm in height) are obtained from actively growing young shoots during the month of March from different locations at Western Ghats of Karnataka (Table 1). The samples were brought to tissue culture lab in Government Science College, Hassan, in sterile bags and processed immediately to reduce risk of contamination. Tender branches were kept in running tap water for 45mins. Few drops of Tween-20 were added followed by fungicide treatment with 0.3% (w/v) Bavistin (Carbendazim 50%, WP, BASF India) a systemic fungicide for 2hr. The branches were cut to give 1.5 – 2cm long shoot tip or nodal explants. These explants were disinfected with 0.6% (w/v) mercuric chloride for 7min for nodal explants and 5min for shoot tip explants, followed by thorough rinsing in sterile distilled water for at least four to five times. Explants were trimmed further before inoculation into the culture medium. The pH of the Woody Plant Medium (WPM) (Lloyd and McCown, 1981) was adjusted to 5.8. 50ml aliquots of molten medium were dispensed into screw capped glass bottles (10.5cm x 8.5cm) prior to autoclaving at 15psi and 121°C for 20mins.

Shoot initiation and multiplication

Multiple shoots were initiated from nodal explants on WPM supplemented with 2.0mg/l 6-benzylaminopurine (BAP), Kinetin (KN) and Thidiazuron (TDZ) along with 0.3% (w/v) Activated charcoal (LR, Ranbaxy, India) or soluble Polyvinylpyrrolidone – 40 (0.1% w/v; Sigma Chemical Co. St. Louis, MO USA). The pH was adjusted to 5.8 before autoclaving at 121^0C for 20 minutes. Cultures are incubated at ±22^0 C. After the 5^{th} week, *in vitro* developed shoots were subcultured along with the original nodal explant on to WPM with BAP (0.5mg) and Naphthalene Acetic Acid (NAA) (1.0mg/l) for shoot proliferation and elongation in nodal explants.

Micro shoots obtained from second subculture measuring between 1.5 – 4.0cm with at least one node were transferred to WPM with 0.3% activated charcoal or *ex-vitro* rooted by treatment with 10.0mg/l Indole-3-Butyric Acid (IBA) for 30 min. and immediately transferred into bottles and plastic pots containing sterile soil-rite. The pots were covered with polythene bags having punched holes. The *ex vitro* treated shoot were maintained in the growth chamber at 80% RH. After 5 weeks the number of roots per shoot and

Table 1: Survey and sample collection from different regions of Karnataka for Micropropagation of *V. maderaspatana.*

Sl.No	Districts/ Region	Place of collection	Population	Climatic condition	longitude	latitude	Above the sea level (Feet)
1	Mysore	Chunchanahatte	Fragmented	Tropical wet and dry	76º16'50.63"E	12º30'32.56" N	2573
2	Madikeri	Makkuta	Fragmented	Tropical wet	76 º02'50.67"E	11 º58'02.13"N	3139
3	Hassan	Halebeedu	Dense	Tropical wet and dry	76 º04'25.20"E	13 º08'55.56"N	3094
4	Tumkur	Devarayandurga	Dense	Semi-arid	77 º12'35.11"E	13 º22'04.67"N	3355
5	Chitradurga	Jogimatti forest	Fragmented	Semi-arid	76 º23'49.48"E	14 º09'23.11"N	3475
6	Davengere	Rangaiahana durga	Fragmented	Semi-arid	76 º11'32.51"E	14 º38'21.24"N	2148
7	Uttrakannada	Yallapura taluk	Dense	Tropical wet	74 º43'24.55"E	14 º59'11.29"N	1808
8	Dakshina Kannada	Puttur	Dense	Tropical wet	74 º52'23.36"E	13º 02'25.11"N	144
9	Mandya	Adhichunchanagiri	Dense	Tropical wet and dry	76 º 44'51.10"E	13 º 01'27.39"N	2922
10	Chikmagalur	Charmudi ghat	Dense	Tropical wet	75 º 26'34.86"E	13 º 04'09.38"N	1131
11	Udupi	Kundapur	Fragmented	Tropical wet	74 º 47'30.25"E	13 º 39'44.43"N	71
12	Bellary	Hospet	Dense	Semi-arid	76 º 37'37.24"E	15 º 04'23.57"N	2404
13	Ramanagar	Thataguni estate	Fragmented	Tropical wet and dry	77 º 31'25.03"E	12 º 50'54.74"N	2740
14	Dharwad	Kalaghatagi	Dense	Tropical wet and dry	74 º 53'27.16"E	15 º 05'28.06"N	1796
15	Belagaum	Ramanagar	Dense	Tropical wet	74 º 31'2028"E	15 º 25'40.91"N	2287
16	Shimoga	Agumbe ghat	Fragmented	Tropical wet	75 º 06'13.90"E	13 º 30'36.85"N	2167
17	Haveri	Shigavvie	Dense	Tropical wet and dry	75 º 13'15.57"E	14 º 59'18.28"N	1993

their length was recorded and rooted plants were shifted to polytunnel.

Callus induction and Somatic Embryogenesis

Callus was initiated from the shoot tip explants on Petri dishes with clear lids containing WPM-solid medium supplemented with different concentrations and various combinations of BAP, 2, 4-D and TDZ plant hormone. The Petri dishes were then sealed along the rims with two layers of parafilm. 30 g of sucrose and 8% (w/v) agar was adjusted to pH 5.8prior to the autoclaving at 121°C for 20 min. The cultures were placed in dark for 3 weeks at 20 ± 2°C and then transferred to the diffused light (16 h photoperiod, PPFD at 30 µmol m−2 s−1) provided by cool-white fluorescent lamps under same culture conditions. Data for somatic embryo formation were taken after four weeks of culture. Selected calli were subcultured on same medium for one month. Highly embryogenic callus with small preglobular somatic embryos were maintained and multiplied in WPM medium with reduced TDZ.

Root initiation

To induce roots, elongated shoots were excised and transferred into woody plant rooting media containing different concentrations and various combination of Indole Acetic Acid (IAA), Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) plant hormone (Ayan et al., 2005). *In vitro* rooted plantlets were washed with distilled water to remove traces of medium followed by treatment with Bavistin solution (0.3% w/v) for 5 min. Plants were gently placed into screw capped bottles containing sterile soil-rite andweremaintainedingrowthchamberfor5 weeksat 80%RH.

Hardening and acclimatization

Well Regenerated plants were transferred into pots containing autoclaved sterile soil rite, Vermiculate, Coir peat, peat moss, Sand, Soil and a mixture of Sand and Soil for acclimatization. After 2-3 weeks they were potted into autoclaved soil mixture containing Soil rite, Vermiculate and Sand in the ratio of 1:1:1 under greenhouse condition to develop mature plants. Irrigation was done using normal tap water and were grown in greenhouse for a period of 3 – 4 months before moving to the originalhabitat.

Reintroduction of micropropagated plants to wild and Morphological examination

Nearly 100 micropropagated plants of *V. maderaspatana* were reintroduced in the area of Neerahalla (Hassan district, Karnataka), (Table 1) with the help of local people and our staff. The growth performance of reintroduced plants of *Ventilago maderaspatana* was recorded. For morphological examination, a set of 25 randomly tagged individuals among the 100 reintroduced plants that were established in the field and were selected of reintroduced plants. The morphological characters like height of the plant, inter-nodal length, number of leaves, leaf length and leaf breadth were recorded at the intervals of 6 months after transplanting. Different parameters like survival rate, height of plants, inter-nodal length, number of new shoots, leaf length and leaf breadth were recorded.

Statistical analysis

All results obtained were analysed for statistical significance using the one way The Analysis of Variance (ANOVA) and variations among means were compared using Post Hoc Duncan's Multiple Range test at P≤0.05 was considered significant. [SPSS version 11.5.0 (SPSS Inc., Chicago, IL)]. Data were expressed as mean±SEM.

RESULTS

Seed germination

Attempts were made to germinate seeds under laboratory condition. Techniques like Between Paper Method, Top of Paper Method and Sand Method were unsuccessful. Significantly lower germination of seeds was also found under *in vitro* condition. The seed explants were cultured on MS medium. As a control MS free medium showed a plant survival percentage of 66.6% while medium supplemented with BAP at 1.0 mg/l and 2.0 mg/l showed 58.3 % of plant survival and KN at 2.0 mg/l showed 75.0 % of plant survival. Media supplemented with both BAP and KN at 2.0 mg/l each showed highest plant survival rate (83.3%) in comparison with MS media supplemented with BAP and KN separately (Table 2).

Shoot initiation and multiplication

Nodal explants obtained from healthy mother plants of different locality were used for culture establishment. Shoot multiplications were obtained successfully from the nodal explants of *V. maderaspatanaon* WPM supplemented with different concentration and combination of BAP, KN and TDZ (Figure 1). The influence of cytokinins on multiple shoot formation from Nodal explants is summarized in (Table3). Multiple shoot initiation was observed in different hormonal treatment after 4 weeks of culture. Maximum shoots were obtained in the WPM medium supplemented with TDZ associated with the highest percentage (83.3%) of responding explants, number (6.83) of lateral buds, length (4.75 cm) of shoot buds and number (4.30) of nodes. Whereas the Kinetin showed lower number of shoot formation. TDZ was better when compared to BAP and combination of BAP+KN for shoot proliferation

Table 2: Effect of cytokinins on *in vitro* seed germination of *V. maderaspatana* after 6 weeks.

MS medium +growth regulators (mg/l)	Percent of survival plants
MS FREE	66.6
BAP (0.5)	50.0
BAP (1.0)	58.3
BAP (2.0)	58.8
KN (0.5)	68.2
KN (1.0)	66.6
KN (2.0)	75.0
BAP (0.5) +KN (1.0)	58.3
BAP (1.0) +KN (1.5)	66.6
BAP (2.0) +KN (2.0)	83.3

Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
Values followed by different are significantly different at P≤0.05
Treatments followed by the similar alphabet do not differ significantly from each other
Number of replicates for each treatment is 12
Data recorded at the end of 6 weeks
± S E: Standard Error of the mean

Figure 1. *In vitro* Shoot culture initiation, Multiple shoots formation and Shoot elongation of *V. Maderaspatana.*

Table 3: Effect of cytokinins on multiple shoot formation from nodal explant of *V. maderaspatana* after 6 weeks.

WPM medium + growth regulators (mg/l)	Explants producing Shoots (%)	Number of shoots/ Explants ± S.E	Mean shoot length (cm ± S.E)
BAP(0.5)	33.3	1.08±1.01	2.23±1.00
BAP(1.0)	33.3	1.83±0.77	3.56±0.85
BAP(2.0)	47.6	2.58±0.67	5.23±1.04
KN(0.5)	41.9	2.50±0.80	2.41±1.15
KN(1.0)	51.6	2.50±0.67	2.75±0.79
KN(2.0)	50.0	2.83±1.91	2.58±1.11
TDZ(0.5)	86.1	6.83±1.71	4.75±1.13
TDZ(1.0)	66.6	4.25±1.02	3.58±0.50
TDZ(2.0)	50.0	2.58±2.36	3.16±1.15
BAP(0.5)+KN(1.0)	58.3	2.50±1.02	3.00±0.81
BAP(1.0)+KN(1.5)	58.8	3.66±0.75	2.32±0.52
BAP(1.0)+KN(2.0)	66.6	3.91±1.91	4.33±0.52

Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
Values followed by different are significantly different at P≤0.05
Treatments followed by the similar alphabet do not differ significantly from each other
Number of replicates for each treatment is 12
Data recorded at the end of 6 weeks
± S E: Standard Error of the mean
Concentration of cytokinins are given in parentheses in mg/l

Callus induction and Somatic Embryogenesis

Callus was initiated from the shoot tip explants on WPM supplemented with different concentrations and various combinations of BAP, IBA, 2, 4-D, NAA and TDZ (Figure 2). Within 10 - 12 days the shoot-tip explants enlarged and developed calluses at the surface of shoot-tip explants in most of media tested, subsequently covered the whole surface of explants within 4 weeks. The response of explants varied with the different concentrations and the various combinations of plant hormones (Table 4). NAA showed the formation of nodular callus and 2, 4-D showed fragile callus formation. While IBA, showed the formation of root at different concentrations respectively. The combinations

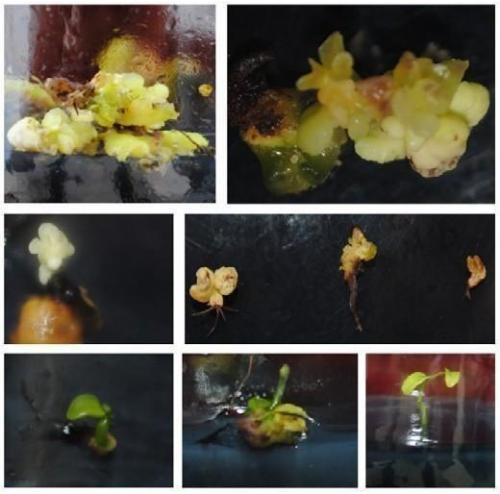

Figure 2. *In vitro* Callus initiation and Somatic embryogenesis of *V. maderaspatana.*

Table 4: Effect of different concentrations and various combination of BAP, 2,4-D and TDZ on the Somatic embryos and Callus formation form Shhot-tip cultured on WPM-solid medium for four weeks.

WPM medium + growth regulators (mg/l)	Explants response (%)	Explants Response	Number of Somatic Embryos ± S.E.M	Callus dry weight Mg/callus ± S.E
NAA(0.5)	33.3	Nodular Callus formation	---	62.2±1.00
NAA(1.0)	33.3	Nodular Callus formation	---	73.5±0.85
NAA(2.0)	45.1	Nodular Callus formation	---	55.2±1.04
2,4-D(0.5)	41.9	Fragile Callus formation	---	62.4±1.15
2,4-D(1.0)	41.6	Fragile Callus formation	---	72.5±0.79
2,4-D(2.0)	50.0	Fragile Callus formation	---	82.5±1.11
IBA(0.5)	83.3	Root formation	---	2.75±1.13
IBA(1.0)	66.6	Root formation	---	3.58±0.50
NAA(0.5)+BAP(0.5)	50.0	Nodular Callus formation	---	43.16±1.15
NAA(1.0) +BAP(1.0)	58.3	Nodular Callus formation	---	63.00±0.81
NAA(2.0) +BAP(2.0)	58.3	Nodular Callus formation	---	72.32±0.52
NAA(0.5)+TDZ(0.5)	87.9	Embryogenic callus formation	14.2±1.91	143.3±0.52
NAA(1.0) +TDZ(1.0)	66.6	Embryogenic callus formation	8.39±1.02	93.1±1.15
NAA(2.0) +TDZ(2.0)	33.3	Embryogenic callus formation	6.83±1.71	45.7±0.52

Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
Values followed by different are significantly different at P≤0.05
Treatments followed by the similar alphabet do not differ significantly from each other
Number of replicates for each treatment is 12
Data recorded at the end of 6 weeks
± S E: Standard Error of the mean
Concentration of auxins are given in parentheses in mg/l

of NAA and BAP at different concentration also showed the formation of nodular callus formation. Embryonic callus formation was occurred in WPM containing the combination of NAA and TDZ. Maximum explant response found with the concentration NAA (0.5mg/l) and TDZ (0.5mg/l).

Embryogenic callus and Nodular calluswere slow-growing, compact, light brown or dark green. Some of these turned brown and died. Non-embryonic callus or fragile callus was fast growing, light yellow and loose. Subsequent subculture of these embryos from embryonic callusin the same medium for one month resulted in their multiplication and transferring them to a callus multiplication medium with lower concentration of TDZ for one month resulted in maturation of somatic embryos. Embryogenic callus with small pre-globular somatic embryos were maintained and multiplied in WPM medium with reduced TDZ. The regenerability of the somatic embryos was clearly visible as green tiny shoot primordia were observed within 3-4 weeks of transfer and after a week the green shoots emerged out (Figure 2). Some of the somatic embryos developed into shoot only, while most developed into shoots and root. Plantlets with 5-10 cm long from somatic embryos regeneration media were transferred to rooting medium.

Rooting of plantlets

Rooting was induced in shoots grown on WPM supplemented with auxins (Table 5). Roots emerged after 4 weeks of culture initiation. WPM medium supplied with NAA produced calluses and tufted roots at the bases of shoots. Combination of IBA and IAA exhibited lower effect

Table 5: Effect of auxins on rootingof microshoots of *V. maderaspatana,* Observations were made after 6 weeks ofculture.

WPM medium + growth regulators (mg/l)	Number of shoots rooted (%)	Mean root length (cm ± S.E)	Mean root number/ shoot ± S.E
IAA (0.5)	33.3	2.66±1.33	1.00±0.44
IAA (1.0)	33.3	3.33±2.60	2.33±0.84
IAA (2.0)	41.6	1.83±0.60	2.66±0.82
NAA (0.5)	41.6	2.02±0.82	1.91±0.82
NAA (1.0)	41.6	1.75±0,92	3.00±0.92
NAA (2.0)	33.3	3.67±1.66	2.02±1.23
IBA (0.5)	91.5	8.45±3.32	4.08±1.23
IBA (1.0)	66.6	6.86±2.60	5.41±1.66
IBA (2.0)	50.0	4.16±2.87	3.00±0.92
IAA (0.5) + IBA (1.0)	66.6	5.00±3.37	3.16±0.61
IAA (1.0) + IBA (1.5)	58.3	2.89±4.40	2.33±0.94
IAA (1.0) + IBA (2.0)	58.3	3.67±1.37	1.00±0.68

Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
Values followed by different are significantly different at P≤0.05
Treatments followed by the similar alphabet do not differ significantly from each other
Number of replicates for each treatment is 12
Data recorded at the end of 6 weeks
± S E: Standard Error of the mean
Concentration of auxins are given in parentheses in mg/l

Figure 3. *In vitro* Rooting, Hardening, Acclimatization and Reintroduction of *V. maderaspatana.*

in the root formation. MS media supplemented with IBA, root formation was better in the lower concentration. While media supplemented with 0.5 mg/l IAA showed least number of root formation. WPM medium supplemented with 0.50 mg/l IBA was the most effective one, associated with the highest percentage (91.5%) of shoots developed roots, the highest number (4.08) of roots per shoot and the tallest (8.45cm) roots (Figure 3).

Acclimatization and Transfer of plantlets to field

Regenerated plants were transferred into pots containing soil rite, vermiculate, coir peat, peat moss, sand and the mixture of sand and soil for acclimatization. Regenerated plants grown well under both growth chambers and mist chamber (Polyhouse) conditions. The survival rate during the hardening was high in Soil rite (92.65%) and in the mixture of Sand and soil (69.3%) in comparison with other substrates (Table 6). Survived and normally grown plants were, subsequently transferred to greenhouse conditions (Figure 3).

Morphological examination:

The morphological observation of 25 randomly tagged individual of reintroduced plants showed similar performance in morphological characters like height of the plant, inter-nodal length, number of leaves, leaf length and leaf breadth (Table 7).

Table 6: Effect of different substrate on hardening of plantlets of *V. maderaspatana,* observations were made after 6 weeks of transformation.

Substrate	Percent of survival plants
Vermiculate	33.3
Coir peat	33.3
Peat moss	41.6
Soil rite	92.6
Sand	41.6
Soil	33.3
Sand and Soil	69.3

Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
Values followed by different are significantly different at P≤0.05
Treatments followed by the similar alphabet do not differ significantly from each other
Number of replicates for each treatment is 12
Data recorded at the end of 6 weeks
± S E: Standard Error of the mean

Table 7: Growth performance of the introduced micro propagated plants of *V. maderaspatana.*

Growth parameter	After 6 months of reintroduction
Height (cm)	19.2±0.56[ab]
No. of leaves per plant	13.7±0.25[a]
Leaf length (cm)	5.6±0.32[ab]
Length of internodes (cm)	4.2±0.14[a]
No. of lateral Shoots	1.01±0.15[a]

All values are expressed in mean response in ±SEM (standard error of mean)
[a]Mean separation with in columns by Duncan's Multiple Range Test (DMRT)
[b]Values followed by different are significantly different at P≤0.05
Number of replicates for each treatment is 25
Data recorded at the end of 6[th] month

DISCUSSION

The independence of the next generation of plants begins withtheseed. Ithas theembryoasthenewplant in miniature, which plays an important role as a dispersal unit and is well provided with food reserves to sustain the growing seedling until it establishes itself as a self-sufficient, autotrophic organism (Derek, 1997). Attempts to germinate seeds under laboratory condition were unsuccessful. Seeds cultured on MS free medium showed a plant survival percentageof

66.6% while media supplemented with both BAP and KN at 2.0 mg/l each showed highest plant survival rate (83.3%) in comparison with MS media supplemented with BAP and KN separately. Seed germination failure and lowergermination rate in MS free medium clearly indicates the seed dormancy of *V. maderaspatana* in natural conditions. This may be due to the fact that seed may be sensitive to the basic requirements and environmental factors like water, light, temperature and minerals. There has been a large volume of published work and significant progress has been made in understanding seed dormancy. However, earlier reviews concluded that it is one of the least understood phenomena in the field of seed biology (Hilhorst, 1995; Bewley, 1997) and also there have been some potential sources of confusion that have been reported in the literature (Finch-Savage and Leubner-Metzger, 2006).

Institution of reliable procedures for regeneration of plants from tissue explants and callus cultures has been helpful of success in genetic engineering techniques of plants (Minocha R and Jain SM, 2000). Although great progress has been made in this regard with woody species, standard media formulations normally used for herbaceous plants (e.g. MS and B5) did not sustenance good growth of many woody plants (McCown and Sellmer, 1987). For woody plants many researchers found that Woody Plant media formulated by Lloyd and McCown (1981), Showed good result in comparison with MS media (Wolfe et al., 1983; Barney et al., 2007; McCown et al., 1987; Thakur and Kanwar 2008). Hence, in this study WPM with different combinations and concentration of plant hormones was used for the propagation of *V. maderaspatana.*

The nodal explants obtained from healthy mother plants showed a low rate of shoot germination in WPM supplemented with 0.5mg/L of BAP. Whereas, 0.5mg/L TDZ supplemented media showed high rate of regeneration. In a similar study conducted on *Musa spp.* MS media supplemented with 5.0 mg/L BAP and 0.5 mg/L of TDZ was considered to bethe best concentration. But media supplemented with 2.0mg/L TDZ showed a reduced shoot initiation. Lee (2001) reported that TDZ at 0.91 µM induced the largest number of shoots in Banana, but TDZ at a concentration 9.1 µM inhibited elongation of shoots was and clumps of small globular buds appeared at the base of shoots. A number of similar studies disclosed that high concentration of TDZ inhibit shoot formation or results in the formation of adventitious shoots (Mithila et al., 2003; Lee, 2005; Shirani et al.,2009; Murti et al., 2012)

In callus induction and somatic embryogenesis, NAA, 2,4-D, IBA and BAP at different concentrations formed nodular callus or fragile callus, whereas a combination of NAA and TDZ at various combination and concentration formed embryonic callus. A combination of 0.5mg/L NAA and 0.5mg/L TDZ obtained highest number of explant response. Earlier studies showed that high level of TDZ triggered rapid propagation and high stress levelresulted in direct somatic embryogenesis or by callus formation in strawberry (Biswaset al., 2009; Murti et al., 2012). Murthy et al., (1998) reported that high fraction TDZ induced Callus formation via axillary shoot proliferation and low concentration induces compact-green nodular callus. Either alone or in combination with other growth regulators TDZ also induces high-frequency somatic embryogenesis in many plant species. In *Ampelocissus latifolia* (belongs to Vitaceae a closely related family to Rhamnaceae)Murashige and Skoog medium supplemented with BAP (0.5 mg/l) and NAA (1.0 mg/l) showed the best result for callus induction (Anand et al., 2018). In *Cayratia pedata var. glabra* the maximum callusing frequency and a greater number of shoot formation was resulted in the lower concentration and combination of BAP (0.5 mg/l) and NAA (0.2 mg/l) (Sharmila et al., 2020)

Rooting of micro shoots found out that IBA induced best percentage of roots. IBA at 0.5mg/L induced highest number of shoots (91.5%) and average root length was found to be 8.45 cms in length. Although average number of roots per shoot was highest in WPM supplemented with 1.0 mg/L of IBA. IAA and NAA individually at different concentration and with IBA did not show significant results. In the earliest studies of auxinic compounds in rooting and propagation assays, IBA was reported to be more effective than IAA (Preece, 2003). Larsen and Guse (1997) andKester et al., (1990) reported that Indolebutyric acid (IBA) is the most reliable rooting hormone. And is still probably the best hormone for general use because of being non-toxic to plants over a wide range of concentration levels.In many studies that the combination of cytokinins (BAP and kinetin) and auxins [NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA)] produced better results compared to the use of auxins or cytokinins alone (Yamini et al., 2003; Shirin et al., 2007). But in this study a combination of 0.5mg/L IAA and 1.0mg/L BAP induced 66.6% of root. While BAP alone induced 91.5% of roots. In a similar study, Peter et al., (2007) reported that Indole butyric acid at 10 and 100 µM gave 100% rooting and produced more roots per micro shoot and the quality of the rooted shoots was also superior than IAA (Peter et al., 2007).

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Direct transfer of *in vitro* raised plants to field/wild is not possible due to high rate of mortality. They are prone to rapid desiccation on transplanting, owing to poor development of epicuticular wax and ineffective control of stomatal function under the high humidity and low levels of illumination that obtain during culture (George and Sherrington, 1984; Sutter, 1985; Brainerd and Fuchigami, 1992). This is also because they regenerate in the culture condition that has been cosseted environment with a very high humidity, varied light and temperature condition and being protected from the attack of microbial and other agents. Direct transfer to sunlight also causes charring of leaves and wilting of the plants (Hiren et al., 2004; Lavanya et al., 2009). The ultimate success of *in vitro* propagation lies in the successful establishment of

plants in the soil (Saxena and Dhawan, 1999). In other words, the survival percentage is determined by the hardening of the plantlets. It is therefore, necessary to accustom the plants to a drier or natural atmosphere by a process called acclimatization or hardening (Deb and Imchen, 2010). The painstaking care required during this period (Murali and Ducan, 1995)

During the transfer of *in vitro* cultured *V. maderaspatana* plantlets for hardening great care has been observed. Plantlets were transferred into pots containing soil-rite, vermiculate, coir peat, peat moss, sand and the mixture of sand and soil for acclimatization. The survival rate during the hardening was high in Soil-rite (92.6%). In *ex vitro* culture of *Citrus aurantifolia* soil-rite ensured 95% to 100% survival of plantlets (Chaturvedi et al., 2002). Generally, soil-rite is the prime medium for primary hardening in tissue culture laboratories (Cronaur and Krikorian, 1984; Zimmerman and Debergh, 1991). Soil-rite is a combination of perlite, vermiculite and peatmoss. For secondary hardening of plantlets, generally a mixture of press mud cake and sand in a ratio 1:1 is employed (Zimmerman et al., 1991). On the contrary, a study found that vermicompost containing about 20-25 % carbon was found to be a good alternative to soil-rite, for primary hardening in *Musa spp* (Bhalsing et al., 2001).

The morphological observation of 25 randomly tagged reintroduced individual plants found to be normal as the characters like height of the plant, inter-nodal length, number of leaves, leaf length and leaf breadth were found similar with the plants growing in the natural condition (data not shown). And genetic similarity studies steered by the authors of this paper using RAPD and ISSR markers and dendrogram generated by cluster analysis using the UPGMA methods on Jaccard's coefficient conducted showed similarity in regenerated plantlets with mother plant (Chandra and Thoyajaksha, 2018).

CONCLUSION

The present work shows the complete success of protocols developed for *in-vitro* conservation and micropropagation of *V. maderaspatana*. Woody Plant Medium was used as prime medium for explant culture. A combination of KN and BAP was found to be effective in in-vitro seed germinations. It was observed that TDZ a cytokine used for the micropropogation is very effective in shoot initiation and callus induction. Among the auxins used IBA showed better root regeneration percentage. The protocols used for the present research were developed with a great efforts and care.The above procedure allows the micropropagation of *Ventilago maderaspatana* and the proliferation of cell mass, which could be used for studies on organic compounds of pharmaceutical interest and for research on woody climbers.

ACKNOWLEDGMENT

The authors of this paper are thankful to the UGC (University Grant Commission), Government of India, for sanctioning a major research project and financial support.

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