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RESEARCH ARTICLE

MICROPROPAGATION OF CRITICALLY ENDANGERED MEDICINAL PLANT, *UTLERIA SALICIFOLIA* (BEDD. EX HOOK.F.) BRUYNS

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ABSTRACT

The present study aimed to develop *in vitro* micropropagation protocol for an endangered and highly valuable Ethnomedicinal plant, *Utleria salicifolia.* Micropropagation techniques applied for recovery of the selected plant species using leaf, cotyledon and node explants cultured on MS medium supplemented with different plant growth regulators. The maximum percentage of callus induction (81.1%) was observed in 1.0mg/L BA combination with 0.1mg/L Kn followed by 77.8% was recorded in 1.0mg/L 2,4-D combination with 0.1 mg/L Kn using leaf explants. The highest number of shoots (7.0shoot/explants) were observed in leaf callus explants was high in 1.0mg/L BA combination with 0.1 mg/L IAA. Indole butyric acid alone was more effective than other auxins for root development. The plantlets showed 97.5 % survival after acclimatization in soil.

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INTRODUCTION

Utleria salicifolia (Retz.) Wight. and Arn species is otherwise called as *Decalepis salicifolia* belongs to the family Apocynaceae and is commonly known as swallow root. Due to over exploitation by traditional healers, its population is gradually declining from wild and it came under endangered category and also endemic to Anaimali Hills, Tamilnadu (Radhakrishnan *et al.,* 1998; Sharma and Shahzad, 2014; Saradha and Samydurai, 2015). It is mainly located in rocky area with swallow tuberous root with vanilla like vanilla like aromatic fragrance (George *et al.,* 2011). The local tribes of Anaimalai hills are Malasar, Kadar, Muthuvam and they are called as Mahali kizhangu. Decoction of its tuberous roots used to treat debility due to tuberculosis, skin diseases and bleeding due to ulcer (Radhakrishnan *et al.,* 1998). It is known to have antiulcer (Rao *et al.,* 2004) hepatoprotective (Remya *et al.,* 2010), anti proliferative and inflammatory activities (Shailasree *et al.,* 2012; George *et al.,* 2016). The International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened species include a total of 560 species from India, out of which 247 species are in the Threatened category.

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On a global basis, the IUCN has estimated that about 12.5% of the world's vascular plants, total of about 34,000 species are under varying degree of threat (Phartyal *et al.,* 2002). The Foundation for Revitalization of Local Health Traditions (FRLHT), a non-governmental organization Southern India, has been monitoring populations of *D. hamiltonii* and established Medicinal Plant Conservation Areas (MPCA) focused on *in situ* conservation of *D. arayalpathra* and *D. salicifolia* (Sharma *et al.,* 2014). Micropropagation of threatened medicinal plants generally undertaken to multiply and conserve the germplasm especially when their population decline in wild. The population of medicinal plants has been decreased due to over exploitation by destructive harvesting (Sharma *et al.,* 2014; Samydurai *et al.,* 2016). The modern technology of micropropagation by tissue culture provides numerous advantages over conventional propagation methods like mass production of true-to-type and disease free plants of elite species in a highly speedy manner irrespective of the season within smaller space and tissue source (Patil, 1998; Karthik Prabu *et al.,* 2017; Samydurai and Rajendran, 2018). In the present study an efficient protocol developed for micropropagation of *U. salicifolia* using leaf, cotyledon and nodal segments.

MATERIALS AND METHODS

Plant material: The young and healthy leaf, fruit and nodal segments of *Utleria salicifolia* were collected from Anaimalai Hills, Western Ghats of Tamil Nadu, India.

Surface sterilization: The young fruit and nodal segments were thoroughly washed under running tap water for 10 minutes, followed by 1 mL/L tween 20 for 5 minutes, 5-7 times rinses with sterile double distilled water, finally using 0.1% (w/v) mercuric chloride for 3-5 minutes. After sterilization, the leaves were cut into small pieces with mid nerve, fruit decedents manually and used immature cotyledon and nodal region were used as explants.

Culture medium and growth chamber condition: Murashige and Skoog's (Murashige and Skoog's, 1962) medium was used for callus induction, shoot regeneration and *in vitro* rooting development were used half strength medium with appropriate plant growth regulators of auxins and cytokinin. The media were supplemented with sucrose (30 g/L), pH of the medium was adjusted to 5.8 before adding agar (8g/L) or phytagel (2.5 g/L) and autoclaving at 121ºC for 20 minutes. The cultures were incubated for 16h photoperiod at 24 ± 2ºC under a photon flux density about 37.037 µ mol m^{-2} s^{-2} emitted from cool white fluorescent tube lamps (Model Lifemax-A 73, Philips India Ltd., India).

Callus induction: Healthy and immature leaf, cotyledon and node of *U. salicifolia* were collected and used as explants for callus induction after proper sterilization. After sterilization the leaf explants were cultured on MS medium containing different concentrations (0.5-3.0 mg/L) of growth regulators, 6-benzyl amino purine (BA), 2,4-dichlorophenoxy acetic acid (2,4-D) and combined with (0.05-0.3 mg/L) kinetin (Kn) for callus induction.

Shoot formation: Leaf, cotyledon and node derived calli were inoculated on MS medium containing different concentrations of plant growth regulators like BAP (6-benzylaminopurine), 2,4-D (2,4-dichlorophenoxy acetic acid) and Kn (Kinetin 6-furfurylamino purine) for shoot formation. After 40-50 days intervals of culture, shoot induction percentage, total number of shoots/explant and length of the shoots were recorded.

Root development: Calli were inoculated on MS medium containing different concentrations of plant growth regulators of IBA (Indole butyric acid) and NAA (α-Naphthalene acetic acid) for root development. After two months of culture, rooting percentage, total number of roots/explant and root length were recorded.

Hardening: The well developed rooted plantlets were washed with sterile water to remove the medium adhering and plantlets were individually potted in sterilized potting media containing various hardening media (Table 4) and kept at mist chamber maintained at 70-80% relative humidity up to four weeks for hardening of plantlets. After hardening, the plantlets were transferred to polybags and kept again one month acclimatization at green house.
Finally the survivability percentage was calculated and shifted to the field for reestablishment.

RESULTS

Callus induction: The present investigation carried to develop micropropagation protocol for efficient regeneration and reintroduction of *Utleria salicifolia*. The highest percentage (81.1% and 77.8%) of callus induction was noted in leaf explants cultured on to the MS medium supplemented with growth regulators 1.0mg/L BA and 2,4-D combination with Kn 0.1mg/L (Table 1). The response of nodal explants for callus formation was lower and it was less than 58 % respectively. However, it was noticed that the response of leaf and cotyledonary explants for callus formation was satisfactory when cultured on to the MS medium supplemented with cytokinin and auxins. The results showed that the callus induction was varied according to the type of explants and concentration of various growth regulators. Among the three explants the leaf explant responded well for callus induction than the cotyledon and node explants.

Shoot formation: Well developed calli of leaf explants subculturing was made for effective shoot formation and observed that highest percentage (90%) of the callus responded effectively for shoot initiation in the basal medium containing the growth regulators of BA and IAA at 1.0 and 0.1 mg/L respectively and followed by 69% in the medium with BA and IAA at 0.5 and 0.05 mg/L respectively. The number of shoots (7shoots/callus) and shoot length (7.7 cm) were also higher in the MS basal medium containing BA and IAA at 1.0 and 0.1 mg/L respectively (Table 2 and Fig.1 B and C).

Root development: In Leaf callus derived shoots, it was noted that the IBA concentration at 0.6 mg/L effectively produced higher percentage of roots (90.5%). The same concentration of IBA present in the MS medium induced high number of roots (31.5 roots/shoot) with greater root length also (10.3 cm). The other individual supplementation of MS medium with NAA and IAA (88.1 and 81.9% respectively) also responded considerably to the rooting attributes of the leaf callus derived shoots of this species (Table 3).

Hardening: Hardening experiments were conducted for the study species by using various hardening media to determine the survivability rate of plantlets. For the leaf callus derived *in vitro* regenerated plantlets, the survivability rate was significantly higher (97%) in the hardening medium composed by garden soil, sand and vermicompost in the ratio of 1:1:1 by volume followed by the hardening medium consisting of red soil, sand and vermicompost 70% in the ratio of 1:1:1 by volume (Table 4).

DISCUSSION

Micropropagation of *Utleria salicifolia* reported that the callus induction from the leaf explants was greater regeneration ability compared to other explants, as they have young and actively dividing cells in vascular cambium producing callus in many angiosperm plants (Hackett, 1985). It was proved in the present study were observed highest percentage of callus and shoots formation through leaf derived callus of *U. salicifolia*. Similarly, cotyledonary explants of *D. hamiltonii* produced highest percentage of callus (82%) on MS medium supplemented with 0.5 mg/L BA and 0.05 mg/L Kn (Samydurai *et al.,* 2016).

Table 1. Effect of different concentrations and combinations of BA, 2, 4-D with Kn in MS medium on callus induction from leaf segments, cotyledons and nodal segments

Plant growth regulators (Auxins: Cytokinin)			Percentage of callus induction (%)		
BA	2,4-D	Kn	Leaf	Cotyledon	Node
0.0	0.0	0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.5	0.0	0.0	67.3±0.61^{c}	64.4±0.52^{ab}	57.2±0.71^{bc}
1.0	0.0	0.0	68.5±0.69^{fg}	65.2±0.51^{ab}	58.5±0.68^{bc}
2.0	0.0	0.0	70.1±0.31^{d}	60.2±0.48^{cd}	56.3±0.70^{cd}
0.0	0.5	0.0	63.6±0.45^{ef}	58.1±0.42^{de}	51.7±0.39^{de}
0.0	1.0	0.0	67.7±1.10^{ef}	55.3±0.41^{ef}	54.6±0.91^{d}
0.0	2.0	0.0	69.1±0.79^{de}	53.6±0.40^{d}	50.2±0.49^{d}
0.5	0.0	0.05	72.8±0.83^{cd}	63.7±0.58^{e}	50.5±0.55^{e}
1.0	0.0	0.1	81.1 ±0.47^{a}	69.6±0.51^{bc}	55.4±0.53^{ab}
1.5	0.0	0.15	77.3±0.89^{c}	62.4±0.45^{bc}	54.3±0.52^{ef}
2.0	0.0	0.2	71.1±0.37^{bc}	61.2±0.41^{a}	50.2±0.48^{ef}
3.0	0.0	0.3	70.5±1.33^{c}	57.1±0.47^{ab}	46.0±0.45^{f}
6.0	0.0	0.6	51.6±0.62^{g}	49.3±1.13^{f}	39.4±1.20^{g}
0.0	0.5	0.05	74.1±0.82^{ab}	51.5±0.95^{b}	46.1±0.55^{a}
0.0	1.0	0.1	77.8±0.80^{b}	56.4±1.03^{ab}	47.2±0.62^{a}
0.0	1.5	0.15	76.7±0.71^{c}	50.2±0.35^{bc}	45.6±1.11^{ab}
0.0	2.0	0.2	70.4±0.92^{d}	48.4±0.26^{bc}	42.2±0.71^{b}
0.0	3.0	0.3	65.6±0.85^{e}	42.1±0.29^{e}	36.7±0.65^{c}
0.0	6.0	0.6	51.5±0.33^{gh}	40.1±0.26^{g}	33.3±0.61^{fg}

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT.

Table 2. Effect of different concentrations and combinations of auxins and cytokinins in MS medium on shoot initiation of *Utleria salicifolia* from Leaf derived callus

Plant growth regulators			Leaf callus		
BA	IAA	IBA	% of culture response	No. of shoots/Callus	Average shoot length (cm) (cm)
0.0	0.0	0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.5	0.0	0.0	69.5±0.62	4.0±0.63^{cd}	5.3±0.86^{e}
0.5	0.05	0.0	84.4±0.41	4.8±0.35^{c}	6.5±0.48^{c}
1.0	0.1	0.0	89.6±1.78	7.0±0.46^{a}	7.7±0.63^{a}
1.0	0.0	0.0	81.3±0.55	6.6±0.82^{ab}	6.9±0.48^{b}
2.0	0.0	0.0	79.2±0.71	5.3±0.45^{bc}	6.7±0.46^{c}
2.0	0.2	0.0	73.7±0.67	4.8±0.40^{c}	6.3±0.42^{cd}
0.0	0.5	0.0	71.3±0.75	3.5±1.07^{d}	5.3±0.80^{e}
0.0	0.5	0.05	80.9±0.92	6.1±0.63^{b}	5.5±0.53^{de}
0.0	1.0	0.1	74.5±1.46	6.8±0.66^{ab}	7.4±1.49^{ab}
0.0	1.0	0.0	75.5±1.17	5.3±0.61^{b}	6.2±0.41^{c}
0.0	2.0	0.0	70.7±0.41	4.9±0.57^{c}	5.8±1.53^{d}
0.0	2.0	0.2	68.5±0.60	4.6±0.70^{cd}	5.3±1.44^{e}
0.5	0.0	0.05	76.2±0.89	3.5±0.36^{de}	5.5±1.08^{de}
1.0	0.0	0.0	72.9±0.46	3.5±0.85^{de}	5.6±0.32^{de}
1.0	0.0	0.1	73.2±0.081	4.3±0.06^{cd}	6.9±1.84^{bc}
2.0	0.0	0.0	70.7±0.53	3.5±0.43^{de}	5.3±0.052^{e}
2.0	0.0	0.2	73.2±0.72	3.8±0.16^{d}	5.6±0.48^{de}

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT.

Shoot regeneration from leaf callus was observed highest percentage (89.6%) among explants have taken for study and responsed in lower concentration of auxins and combination of cytokinin induced shoot formation in *D. salicifolia*. The higher concentration of auxin and cytokinin and combination of both plant growth regulators are inhibit the shoot regeneration and multiplication. In the present investigation, higher concentrations of BAP reduced shoot number and length, which is in agreement with the findings of Ganagaprasad *et al.,* (2005). In contrast to the synergistic effect of BAP in combination with an auxin has been reported in *Holostemma annulare* (Sudha *et al.,* 1998), *Hemidesmus indica* (Sreekumar *et al.,* 2000), *Holostemma ada-kodien* (Martin, 2002), *C.candelabrum* (Beena *et al.,* 2003), *Decalepis arayalpathra* (Gangaprasad *et al.,* 2005), *Ceropegia intermedia* (Karuppusamy *et al.,* 2009), *Decalepis hamiltonii* (Samydurai *et al.,* 2016). The endogenous levels of growth regulators in the members of Apocynaceae might be responsible for the observation of variation in the response and growth regulator requirement for in vitro shoot regeneration (Sri Rama Murthy *et al.,* 2010). Root development of Apocynaceae plant species of *Utleria salicifolia* have been achieved by *in vitro* derived shoot were cultured on various concentrations of IBA and NAA alone. The maximum root development was observed in the culture grown on the medium contained 0.6 mg/L IBA and NAA, when well

Table 3. Regeneration and sprouting ability of roots from the leaf derived callus shoots of *Utleria salicifolia* and their growth on different concentrations of auxins 60 days after inoculation

Plant growth regulators		Root sprouting %	No. of roots/shoot	Average root length (cm)
IBA	NAA			
0.0	0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.2	0.0	87.2±1.09	30.1±0.34^{a}	9.7±0.76^{ab}
0.4	0.0	72.3±0.20	26.3±0.65^{ab}	6.5±0.28^{de}
0.6	0.0	90.5±0.48	31.5±0.53^{a}	10.3±0.35^{a}
0.8	0.0	61.5±0.69	22.0±0.48^{bc}	6.4±0.65^{e}
1.0	0.0	53.9±0.26	20.2±1.04^{c}	6.2±0.94^{f}
2.0	0.0	45.4±0.47	18.2±0.65^{cd}	6.0±0.29^{f}
4.0	0.0	35.3±1.03	17.3±0.73^{d}	5.7±0.05^{fg}
0.0	0.2	81.2±0.45	27.4±0.36^{a}	9.3±0.39^{b}
0.0	0.4	83.2±0.47	28.6±0.65^{a}	9.5±1.43^{ab}
0.0	0.6	88.1±0.33	30.3±0.72^{a}	10.0±0.24^{a}
0.0	0.8	75.7±0.85	26.3±0.71^{ab}	9.2±0.64^{b}
0.0	1.0	70.5±0.54	23.3±0.45^{b}	8.4±0.82^{bc}
0.0	2.0	64.1±0.73	21.3±0.52^{bc}	7.8±0.54^{c}
0.0	4.0	45.8±0.27	18.2±0.34^{cd}	6.1±0.29^{f}
0.0	0.0	60.3±0.36	21.5±0.65^{bc}	7.2±0.84^{cd}
0.0	0.0	66.3±0.39	20.6±1.64^{c}	7.0±0.29^{d}
0.0	0.0	62.7±0.18	19.3±0.74^{cd}	6.7±0.45^{de}
0.0	0.0	60.0±0.89	17.2±0.35^{d}	6.5±0.63^{e}
0.0	0.0	81.9±0.78	26.5±0.63^{ab}	8.3±0.91^{bc}
0.0	0.0	77.8±1.23	23.8±0.11^{b}	6.7±0.28^{de}
0.0	0.0	68.5±0.42	21.2±0.43^{bc}	6.9±0.30^{de}

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 4. The survivability rate of the plantlets derived from the leaf callus of *Utleria salicifolia* on different hardening medium after 2 weeks of incubation period

Sl. No.	Hardening medium	Composition ratio (v/v)	Survivability %
1	Red soil + sand	1:1:1	92%
2	Garden soil + sand + vermicompost	1:1:1	97%
3	Decomposed coir waste + perlite + compost	1:1:1	90%
4	Vermicompost + soil	1:1	82%
5	Red soil + sand + vermicompost	1:1	92%

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT

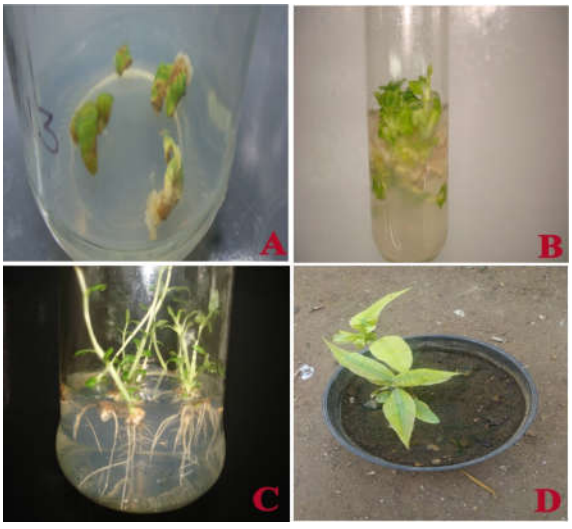

Fig.1. Showing the micropropagation of *U. salicifolia*; A-Callus induction from leaf explants; B-Shoot sprouting from leaf calli; C-Well developed rooted shoots; D-Hardened plantlet in plastic pot

developed *in vitro* shoots were cultured. Root development of *U. salicifolia* was observed in the present study is directly coincided with results of *Decalepis arayalpathra* (Gangaprasad *et al.,* 2005; Sudha *et al.,* 2005) and *D. hamiltonii* (Samydurai *et al.,* 2016). However, enhanced rooting was successfully brought on half strength MS basal medium supplemented with IBA by culture many species belong to Apocynaceae, *Brachystelma glabrum* (Revathi Lakshmi *et al.,* 2017), *Caralluma edulis* (Rathore *et al.,* 2008), *C. diffusa* (Karthick Prabu *et al.,* 2017), *Decalepis hamiltonii* (Anitha and Pullaiah, 2002; Samydurai *et al.,* 2016), three varieties of *Caralluma adscendens* (Aruna *et al.,* 2009), *Ceropegia intermedia* (Karuppusamy *et al.,* 2009), *Caralluma stalagmifera* (Sreelatha *et al.,* 2015) and macropropagation of *D. salicifolia* (Saradha and Samydurai, 2015). Acclimatization and survivability rate of *D. salicifolia* were significantly higher (97%) in the hardening medium composed by Garden soil:sand: vermicompost in the ration of 1:1:1. Similarly, the well developed *in vitro* derived plantlets of *Decalepis* species were hardened by different media composition and achieved survivability rate of 84% and 97.5% respectively (Gangaprasad *et al.,* 2005; Samydurai *et al.,* 2016).

Conclusion

D. salicifolia is an critically endangered woody medicinal plants from Anaimalai Hills, Southern Western Ghats of Tamil Nadu. This species being used as traditional medicine by local inhabitant tribes for various purposes and over exploitation and destructive collection it will become extinction from wild. Hence, an attempt has been taken to conserve it through micropropagation techniques for reintroduction into natural habitat and sustainable utilization.

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