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RESEARCH ARTICLE

Optimized protocol for *in vitro* callus induction and micropropagation of *Urena lobata* L.: A fast-vanishing important medicinal plant

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Abstract *Urena lobata* (L.) is a valuable medicinal plant used widely in Ayurveda and Siddha to treat several ailments. Due to rapid urbanization, weeding, and excessive use of nitrogenous fertilizers, *U. lobata* population is dwindling, and it needs to be conserved to save the valuable medicinal resource. Thus, an optimized protocol has been developed for callus induction and micropropagation from the stem/leaf explants of *U. lobata*. Various growth regulators have been tested to achieve the best callus formation, such as 0 to 4 mg/l of 2,4-D, Kn, IAA, IBA, NAA, and BAP supplemented to MS medium individually and in combination. MS medium supplemented with 2,4-D with Kn and NAA with BAP in specific doses led to maximum callus initiation specially from stem explants rather than leaf explants. Callus could be successfully induced using 2.0 mg/l 2,4-D, 1.0 and 2.0 mg/l NAA individually or using 1 mg/l 2,4-D with 2.0 mg/l Kn or 1.0 mg/l NAA with 1.5-2.0 mg/l BAP in combination. There was no callus formation in control sets (without PGRs). Supplementation of MS medium individually with 2.0 mg/l IAA or even kinetin in the same dose resulted in maximum roots production (30% frequency) and shoots (66% frequency) from stem explants at 27^{0}C. After primary hardening, healthy plants were transferred to the greenhouse. Interestingly 2^{0}C modulation of temperature led to morphogenetic transformation to callus formation at 25^{0}C. Thus, callus or plantlet could be obtained strategically as per demand. Callus can also be used for upscaled production of bioactive secondary metabolites, helping protect the parent plant from overexploitation.

Keywords: Callus, Micropropagation, Murashige and Skoog, Plant growth regulators, *Urena lobata.*

Introduction

India has a long history of understanding plant-based medicines and rich heritage of knowledge used in curative and preventive therapies (Rai and Nath 2005). About 6,000 plants have been used in herbal, ethnic, and traditional medicines (Dubey *et al.* 2004, Gao *et al.* 2015) in India and China. *U. lobata*, an annual herb of family Malvaceae is distributed worldwide, especially in tropical-subtropical regions of South America, Asia, and Africa (Babu *et al.* 2016). The extract of *U. lobata* has been reported to be used as an antibacterial agent Mshelia *et al.* (2013), as a diuretic, depurative, antitussive, emmenagogue, and expectorant (Mazumder *et al.* 2001). Lane (2007) has reported *U. sinuata* to be used for treating man's waist pain problems.

Leaves of plants have antidiarrhoeal activity (Yadav *et al.* 2008), antioxidant, cytotoxic (Ali *et al.* 2013) and antifertility activity (Dhanapal *et al.* 2012) and roots of the plant show hepatoprotection, antidiabetic (Omonkhua and Onagbe 2011) antioxidant potential (Lissy *et al.* 2006) and diuretic effects (Sajem and Gosai 2006, Jia *et al.* 2010). Traditional herbalists have used leaf and root extracts to treat various types of maladies, cold, fever, malaria, restlessness, gonorrhoea, leucorrhoea, hematoma, carbuncle, stroke, haemorrhage, arthritis-induced numbness, and wounds (Sajem and Gosai 2006, Jia *et al.* 2010). According to Parziale (2005) it is considered as expectorant, antitussive, depurative, and diuretic. Chinese medicine has also been used to treat angina, menorrhagia, colic, diarrhoea, burns, dysentery, calculus, dyspepsia, coughs, dog bite, bronchitis erysipelas, dysuria, boils, scalds, eruptions and filariasis.

Under field conditions, the occurrence of seed dormancy in *U. lobata* due to an impermeable seed coat (testa) was detected by Horn and Natal

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(1942), leading to the delayed or low percentage of field emergence from such seeds (Harris 1981, Kirby 1963, Crane and Acuna 1945). A similar result showed by Horn and Natal 1942, Juillet 1952, Gerrard 1955. Urbanization and high weed density are other factors that reduce the germination percentage and plant population in natural growing areas, making them more valuable and unavailable. The present investigation aims to conserve *U. lobata* against environmental pressures.

Many valuable plant medicinal resources are endangered due to over-exploitation and misuse. To maintain biodiversity, various research groups worldwide have focused on plant conservation (Malik *et al.* 2005, Nadeem *et al.* 2000, Ray and Bhattacharya 2008). Over the past several years, plant tissue culture has rapidly evolved as one of the primary research tools and is being used for *ex-situ* conservation besides exploring the value without exploitation (Sharma and Patni 2006, Meena *et al.* 2010), including raising uniformly vigorous plants and the production of therapeutic compounds (Hirai *et al.* 1997).

This work aims to develop an optimal callus induction and micropropagation strategy through *in vitro* culture of *U. lobata* using leaf and stem explants. The callus formation protocol can be utilized to upscale secondary metabolites under stress.

Materials and Methods

Plant materials

Plant sample of *U. lobata* was collected from different undisturbed patches in Meerut city, Uttar Pradesh. About two months old, four-leafed saplings are transplanted in the experimental area of the Department of Botany. Plants were obtained from the seeds of the transplanted plant and used for further studies (Figures 1A, B, and C). Young leaves and stem explants were collected from the apical >3 cm length of the main twig for callus initiation and micropropagation (Figures 2A, B, and 3A).

PGRs treatment concentrations

In this experimental work, plant growth regulator concentration and combinations were standardized initially for optimal callus induction and growth in MS medium. Thus, six set of 2,4-D (1.0 to 4.0 mg/l), NAA (1.0 to 4.0 mg/l), IBA (1.0 to 3.0 mg/l), IAA (0.5 to 2.0 mg/l), Kn (0.5 to 2.0 mg/l) and BAP (0.5 to 2.5 mg/l) were prepared. The explants (leaf and stem) were (Table 1) surface sterilized and cultured on Murashige and Skoog (MS) media, supplemented as above following Hasan *et al.* (2016, 2019) and Saini *et al.* (2014) method.

For surface sterilization standard method of washing under running tap water for 30 minutes to remove dust particles on an exterior surface, followed by washing with diluted Tween-20 solution, rinsing 3 to 4 times with sterile water, and immersing in sterilant 0.1% mercuric chloride solution for 3 minutes, soaking in fungicide 1% Bavistin solution, and washing with double-distilled sterile water. Finally, aseptic explants were cut into pieces (1.25 - 2.0 cm) and inoculated in a PGRs supplemented medium. 15 replications per experiment were taken, and each experiment was performed twice.

Preparation of culture media and incubation conditions

MS basal medium (Murashige and Skoog 1962) procured from Hi-Media Laboratory Pvt Ltd; Nashik, India, was used for all the experiments. The pH of the media after supplementation with required PGRs was adjusted between 5.6-5.8, followed by autoclaving, cooling, and pouring approx. 33 ml of MS media into each conical flask (100 ml) and sealed with cotton plugs, paper/foil. These flasks were sterilized in autoclave for 20–25 min at 15 psi and 121°C. All culture flasks were incubated at 8hr light (PAR) with 16hr dark exposure at 25±2℃, and 27±2℃ temperature with 15 replicates each. The degree of response was noted on 5^{th} to 7^{th} week after incubation (Table 3).

Data analysis

Due to inherent fungal infection, some flasks were destroyed. However, the remaining culture flasks noted 100% response for callus induction or micropropagation. The data were statistically analyzed.

Figure 1: Morphology of *Urena lobata.* L; **A**- *In vivo* grown aerial parts of *Urena lobata* (2-year-old), **B**- Flower of plant, and **C**- Capsule.

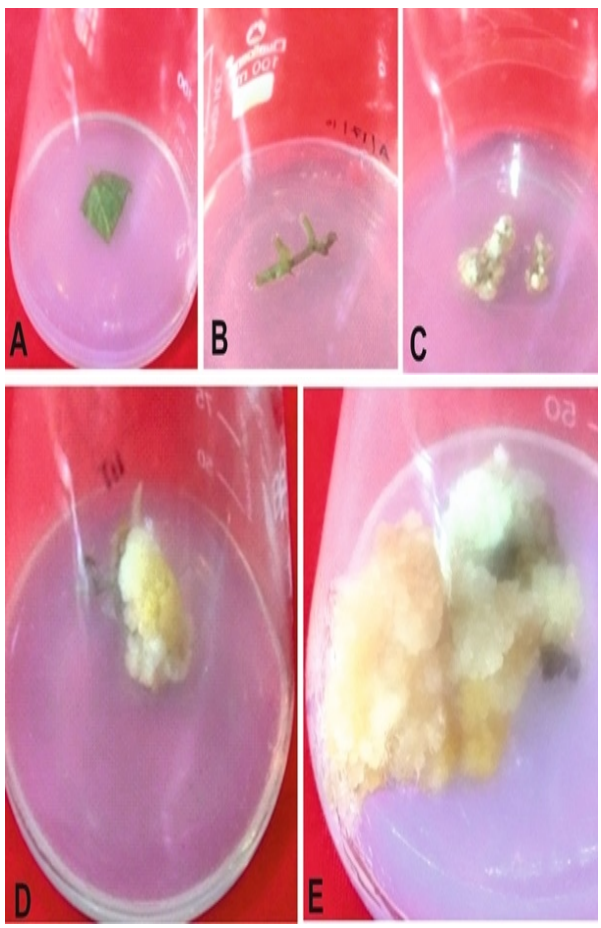

Figure 2: Effect of various Auxin and Cytokinin for callus induction in *Urena lobata;* **A**- Leaf explant, **B**- Stem explant, **C**- Initiation of callus formation in the nodal region from stem explants, **D** - Callus subculture, and **E** - Depicting proliferous growth on sub-cultured callus (1.0 mg/l 2,4-D with 2.0 mg/l Kn) from stem explant.

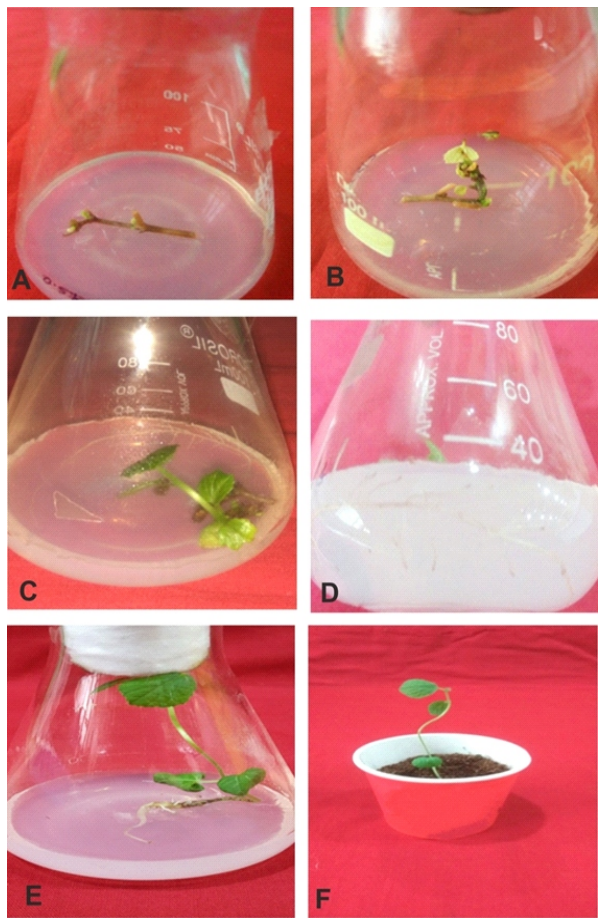

Figure 3: Micro propagation of *Urena lobata* from stem segments; **A**- Stem segment medium (Control/IAA/Kn) **B**- Shoot proliferation and elongation with Kinetin, **C** - Elongated shoots arising from the nodal region, **D**- Rooting in elongated shoots with IAA, and **E,F** - Plants undergoing hardening.

Results and Discussion

Individual and combinational effects of different PGRs on callus initiation and micropropagation were observed on the stem (nodal) and leaf explants of *Urena lobata* L., demonstrating significant differences.

Callus induction

Stem and leaf explants of *U. lobata* were cultured on individual auxin (IBA, 2,4-D, IAA, and supplemented NAA) and cytokinins (Kn and BAP). Auxin is also studied for activation of expression of genes, which is controlled by cytokines, and its products are associated with biological activities such as cell division, callus formation, regeneration, chloroplast growth, and nutritional metabolism (Munazir *et al.* 2010, Tang *et al.* 2000, Taha 2017). Callus induction was observed between 35 to 45 days after incubation in most PGRs concentrations.

From stem explant, both 2,4-D and NAA with increased individual concentrations (1 to 3 mg/l) evolved from light brown to white with callus response after 45 days and later on to greenish-white at 4 mg/l concentration. White and light-brown callus colour, indicates the cells are in a state of stress. But the degree of response decreased with increasing individual concentration, at 1.0 mg/l 2,4-D with 0.5-3.0 mg/l Kn and 1.0 mg/l NAA with 0.5-2.5 mg/l IBA developed greenish-white callus colour, resulted in a better response. This was significantly better than the individual PGR concentrations. Further, the sub-culture of these calli was incubated on the same medium to multiply the callus (Table 1).

Maximum callus induction response after 45 days was observed from stem explants with 2.0 mg/l 2,4-D, 1.0-2.0 mg/l NAA, 1.0 mg/l 2,4-D with 1.0-3.0 mg/l Kn, 1.0 mg/l NAA with 1.5-2.0 mg/l BAP in supplemented with basal MS medium (Table 1 and Figure 2D, C, E). Greenish white callus induction was shown only at 2,4-D (1.0 mg/l) with KN (2.0 mg/l) concentrations compared to other combinations of auxin and cytokinin. In control (without PGRs Supplementation) sets, *U. lobata* failed to induce callus from stem and leaf explants. Various concentrations from 0.5 to 2.0 mg/l Kn and 0.5 to 2.0 mg/l IAA exhibited no callus induction from stem explant, indicating augmentation of both auxin and cytokinin for callus induction in place of individual IAA and kinetin to *U. lobata* explants (Table 1).

Maximum callus induction was observed at 45 days from leaf explant with 1.0–3.0 mg/l 2,4-D as white callus, whereas 1.0 mg/l 2,4-D with 1.0 mg/l Kn, 1.0 mg/l NAA with 1.5- 2.0 mg/l BAP induced greenish-white form of callus (Table 1). A lower frequency of callus induction was observed from the leaf than stem explants. However, the same treatment levels were followed on both explants (leaf and stem). Leaf explants of *Centella asiatica* showed high callus initiation reported by Rao *et al.* (1999). The present study demonstrates stem explants to be highly responding compared to leaf explants.

Shoot and root induction

With individual Kn or IAA supplementation to MS medium, *U. lobata* stems explants underwent only micropropagation, i.e., direct organogenesis without callus induction within 30 days. Interestingly, in *U. lobata*, shoot root frequency was up to 84% in control (Figure 3E). The frequency of root formation increased by 10 to 30% with 0.5 to 2.0 mg/l of IAA supplementation. Supplementation of 0.5-2.0 mg/l Kn only resulted in 30-66% shoot formation. The combination of IAA and Kn did not display any response for shoot and root formation. This indicates that the explant has the appropriate endogenous proportion of auxin and cytokinin for plantlet formation, which undergoes an additional imbalance of individual auxin and cytokinin, leading to only root or shoot formation (Table 2, Figures 3B, C, D, and E).

According to George and Sherrington (1984), cytokinin has diverse effects on tissue culture depending on the chemical, culture method, and plant species. For shoot induction, young stem explants were incubated on medium with various compositions of 0.5 to 2.0 mg/l kinetin, leading to bud differentiation (Table 2, Figures 3B and C). Generally, cytokinin promotes the branching of lateral buds from the leaf axil and removes apical dominance of shoots (Murashige 1974). The nodal stem explant produced shoots, which increased the frequency of shoot initiation as the concentration of kinetin increased. After 4 weeks of incubation,

Table 1: The effect of auxin and cytokinin on *Urena lobata* explants

Tx	PGRs	(mg/l)	Callus colour	Callus nature	Response	Degree of response	Plantlet regeneration (%)
Stem explant							
T1	**2,4-D**	1	Light Brown	Friable	45 days	+	--
T2		2	White	Friable		++	
T3		3	White	Friable		+	
T4		4	Greenish White	Friable		+	
T5	**Kn**	0.5	--	--	30 days	--	Present (30%)
T6		1.0					Present (38%)
T7		1.5					Present (53%)
T8		2.0					Present (66%)
	2,4-D	**Kn**					
T9	1	0.5	Greenish White	Friable	45 days	++	--
T10		1.0		Friable		++	
T11		1.5		Friable		+++	
T12		2.0		Friable		++	
T13		3.0		Friable		++	
T14	**IAA**	0.5	--	--	30 days	--	Present (10%)
T15		1.0					Present (20%)
T16		1.5					Present (26%)
T17		2.0					Present (30%)
T18	**Control**						Present (84%)
T19	**IBA**	1.0	Green	Friable	45 days	+	--
T20		2.0	Green	Friable		+	
T21		3.0	-	-		-	
T22	**NAA**	1.0	Light Brown	Friable	45 days	++	
T23		2.0	White	Friable		++	
T24		3.0	White	Friable		+	
T25		4.0	Greenish White	Friable		+	
	NAA	**IBA**					
T26	1	0.5	Greenish White	Friable	45 days	-	--
T27		1.0		Friable		+	
T28		1.5		Friable		+++	
T29		2.0		Friable		+++	
T30		2.5	White	Friable		++	
Leaf explant							
T31	**2,4-D**	1	White	Friable	45 days	+	--
T32		2		Friable		+	
T33		3		Friable		+	
	2,4-D	**Kn**					
T34	1	1	Greenish White	Friable	45 days	+	
	NAA	**BAP**					
T35	1	1.5	Greenish White	Friable	45 days	+	
T36	1	2.0	Greenish White	Friable		+	

Table 2: Callus induction and shoot/root emergence in stem explant cultured on different concentrations of IAA, Kinetin, and combination of IAA and Kinetin (>15 replicates of each concentration).

Treatments	Stem explant	Plant Growth Regulator (mg/l)				
	Shoot-Root Emergence	0	0.5	1.0	1.5	2.0
Control	Shoot-Root (%)	84	-	-	-	-
IAA	Root (%)	-	10	20	26	30
Kinetin	Shoot (%)	-	30	38	53	66
IAA+ Kinetin	Shoot/Root (%)	-	-	-	-	-

well-developed shoots were sub-cultured on fresh MS medium containing an optimal cytokinin concentration for further shoot elongation. In the present work, stem explants on MS media (2.0 mg/l Kn) produced the highest shoot length and number, with 66% shoot regeneration response. The current results are in accordance with Grover and Patni (2011), in which *W. fruticosa* produced seedlings with high levels of Kn concentration. Shiny shoots were observed when the nodal segment was cultured on 1.0-3.0 mg/l kinetin media. However, fewer shoots were observed in medium supplemented with BAP (Table 2). Rahman et al. (2010) reported that the regenerated plantlet was feeble without NAA. Rueb *et al.* (1994) reported high amounts of BAP and kinetin to stimulate early cell division that plays an essential role in callus and plantlet regeneration. Similarly, kinetin and IAA contributed to the highest plantlet regeneration in *U. lobata*.

In our experiment, elongated shoots were subjected to *in vitro* rooting by culturing them on MS medium with 0.5 to 2.0 mg/l IAA. Observations were recorded between the 4th and 5th week after incubation. The highest, 30% root formation was observed at 2 mg/l IAA (Figure 3D), and minimum roots at 10% were developed at 0.5 ml/l concentration. The formation of fibrous roots from *in vitro* conditions in *U. lobata* was not previously reported. The highest number of root formations per stem area was observed at 2 mg/l IAA. This is most likely to increase endogenous IAA concentration in the lower region of the shoot, which promotes root development (Martins *et al.* 2013). *In vitro* technique, reported by Krupa and Mglosik (2016), allows for a simpler correction, faster adaptation and helps in better plant survival.

Hardening and acclimatization

The plants were subjected to hardening, separating, and washing the plant roots, and transferred into pots containing sterilized Sand: Soil (1:1), covered with polybag for 10 days. The shoots of *in vitro* regenerated plantlets that reached up to a height of 6-7 cm with 5 to 8 healthy leaves and a good root system were considered hardened (Figure 3 F). Such plants were exposed to natural ambient conditions, and after 21 days, plants showed a phenomenal growth performance. Finally, saplings were planted in the field, where they grew as untreated, healthy saplings with no significant morphological change.

Table 3: The Effect of the temperature and light on plantlet regeneration and callus induction

PGR [Concentration (mg/l)]	Plantlet Regeneration				Callus Induction			
	Light		Temperature		Light		Temperature	
	8h	12h	25±2°C	27±2°C	8h	12h	25±2°C	27±2°C
Control	-	+++	-	+++	-	-	-	-
2,4-D [2]	-	-	-	-	+	-	+	-
2,4-D [1] + Kn [2]	-	-	-	-	++	-	++	-
NAA [1] + BAP [1.5]	-	-	-	-	+	-	+	-
NAA [1] + BAP [2]	-	-	-	-	+	-	+	-
Kn [0.5 – 2]	-	++	-	++	-	-	-	-
IAA [0.5 – 2]	-	+	-	+	-	-	-	-

Effects: +, ++, +++ and -, low, medium, high and no effects, respectively

Conclusions

Due to increasing inter and intraspecific competition and reducing land area, *U. lobata* plant population and distribution are declining proportionately. For medicinal purposes, optimized callus development and micropropagation protocol may help prospect various important bioactive secondary metabolites. Thus, in the present study, callus induction and micropropagation from stem explants of *U. lobata*. 1.0 mg/l 2,4-D with 2.0 mg/l Kn and 1.0 mg/l NAA with 1.5-2.0 mg/l BAP exhibited maximum callus initiation. The high-performing combination of PGRs for callus initiation/induction could not induce organogenesis. Plantlet regeneration was significant in controls without added PGRs, whereas increasing the supplemented concentration of IAA and Kn resulted in the only root or shoot formation. MS medium supplemented with 2.0 mg/l IAA produced maximum root proliferation. In the present investigation, the optimization strategy for *in vitro* callus development and micropropagation led to the understanding of slight modulation of exogenous auxin to cytokinin ratio, reiterating the inherent balance of auxin to cytokinin in the explants. Besides, 2℃ modulation (to 25^{0}C) of temperature transforms the morphogenetic pattern from an unorganized callus to an organized shoot/root formation. Interestingly, a combination of auxin and cytokinin could not support plantlet formation; instead, they supported unorganized callus formation. Individual PGR could display the defined morphogenetic role, though at a lower note, suggesting callus to be a better option for further research into upscaling of bioactive metabolites

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