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IN VITRO REGENERATION OF UNDER EXPLOITED ETHNO-MEDICINAL PLANT LIMONIA ACIDISSIMA L.

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Abstract

In the present study, a protocol was optimized for establishment of callus and in vitro regeneration of medicinally important fruit tree Limonia acidissima L family Rutaceae for production of useful pharmaceuticals & conservation of the plant. Various explants viz. Epicotyl, hypocotyl, cotyledon and intrenodal segment were obtained by in vitro seed germination and established on MS medium supplemented with various concentrations and combinations of kinetin, BAP and 2,4-D. Best callusing response was observed on MS medium supplemented with 2,4-D (1.0mg/L) in combination with kinetin (0.5mg/L). Internodal segments showed best shooting response on MS medium supplemented with BAP (0.5mg/L) and Kinetin (0.5mg/L). In vitro rooting was done on different concentrations of IAA. 1.5 mg/l IAA was found to be best for rooting. The in vitro cultures developed could be exploited for production of pharmaceuticals and large scale production of this medicinally important tree.

Keyword: Cotyledons internodal segments, , epicotyls, hypocotyls, in vitro culture, Limonia acidissima L

1.INTRODUCTION

Limonia acidissima L. (LA) (synonym: Feronia limonia L.), commonly known as wood apple is the only species of its genus, belongs to family Rutaceae. It is a rare and an endangered tree species and in Rajasthan it has vulnerable status. It is one of the under valued and under exploited potential tree whose fruit are edible and other parts also have potent traditional application but it has not been much studied. The plant have great potential for exploitation in view of the value of their pharmaceutical products, use as food, fodder, medicine, energy and industrial purposes. Taking to agro-climatic condition of Rajasthan there is tremendous scope of cultivation of this undervalued unexploited fruit crop.

In spite of possessing high nutritive and medicinal value [1] in the fruit, the crop has neither been given due attention for commercial cultivation nor exploitation of the genotypes available in the state (Rajasthan). It is therefore, necessary to develop genetically superior planting material for assured uniformity and desired quality. Conventional propagation of Limonia acidissima can be achieved from seed, which result in a high degree of genetic and phenotypic variation. Micropropagation can provide an opportunity to obtain large number of homogenous plant [2].Many rare and endangered plant species are propagated in vitro because they do not respsond well to conventional methods of propagation. Despite great advancements of plant tissue culture techniques the success with woody plants has continued to be a challenging task [3]. The media composition and qualitative and quantitative aspects of plant growth regulators play a vital role in micropropagation. Therefore optimization of these conditions is a prerequisite for in vitro related work [4]. In the present study attempt has been made to optimize a simple protocol for callus culture and recurrent propagation of Limonia acidissima through the shoot regeneration from hypocotyls, epicotyls cotyledons and internodal segments derived from in vitro grown seedlings.

2.MATERIAL AND METHODS

Fruits of Limonia acidissima were procured from wild region of Bikaner (Rajasthan) and the plant was botanically authenticated from Herbarium (Voucher specimen D.C.M.1850: DCB NO.-1219; BSI-3355) , Department of Botany, Govt. Dunger college, Bikaner,

Rajasthan. Seeds were removed from the fruit, washed with running water for 10-15 min, immersed in 5% solution of liquid detergent (teepol) for 5min. and washed with 0.1% mercury chloride (2-3 times) then rinsed with sterile distilled water 3-4 times. After surface sterilization seeds were inoculated on MS [5] Medium for germination and development. Various explants viz. Epicotyl, hypocotyl, cotyledon and internodal segments were excised from 6-8 wk old seedlings. These explants were then established and maintained by frequent subculturing after 4 wk on MS [5] Medium supplemented with various concentrations and combinations of kinetin and 2,4-D for callus induction and kinetin and BAP for induction of multiple shoots. Cultures were maintained in growth chamber with regulated temperature (26±2oC), relative humidity (55±5%), 3000 light lux intensity. Data was recorded after 2, 4,6,8 and 10 wk and growth indices were calculated. For rooting, the in vitro shoots were transferred on MS5 Medium supplemented with different concentrations of IAA.

3.RESULTS AND DISCUSSION

3.1. Influence of different explants on initiation of callus culture

Callus started appearing within 5-6 days of inoculation in the cultures.Varied response was shown by cultures on the same medium supplemented with different concentration and combination of PGRs. Callusing was observed along the epicotyls, hypocolyls and cotyledons, producing creamish green, fast growing and embryogenic callus. The best response for callus was observed on MS medium supplemented with Kinetin (0.5mg/L) and 2,4-D (1.0mg/L). Growth rate studies of these callus culture showed an increase in growth index up to 8 wk which declined in tenth wk. Cultures obtained from epicotyls showed maximum GI (10.4) whereas culture obtained from cotyledons showed minimum GI (5.2) in 8 wk. A plant growth regulator is a key factor responsible for callus initiation and development in plant cell cultures [6]. However, optimal concentration of these compounds may depend on many factors, such as genotype of the original plant, explants origin, peculiarities of the strain etc. Mathur et.al [7] and Ray et.al [8] reported maximum callusing (98 %) on MS medium supplemented with a combination of 2,4-dichlorophenoxy acetic acid (0.5 mg l-1) and kinetin (0.2 mg l−1). Zuraida et al. [9] reported that sub-culturing the callus on to medium with 0.2 mg/L 2,4-D showed enhanced callus proliferation rate up to 95% in Ruta graveolens.

3.2. Influence of different explants on micropropagation of shoots

Swelling and expansion in both hypocotyls and epicotyls were recorded with in a week of incubation and irrespective of type or concentration of cytokinin. This was followed by callus initiation at the cut ends of the explants which was more pronounced on MS medium containing Kinetin(0.5mg/L) and BAP(0.5 mg/L). Moderate callus was also produced on MS medium containing kinetin (0.5 mg/L) and BAP (0.1 mg/L) in both type of explants whereas very little or no callus formation was obtained in media containing Kinetin(0.5mg/L) and BAP (2.0mg/L). After 4 wk of culture greenish calli with small shoot bud were detected. After 8 wk of culture, shoots developed from the callus on medium supplemented with kinetin (0.5 mg/L) and BAP (0.5 mg/L). However direct regeneration was observed in case of internodal explants and no response was observed in case of cotyledons.

Shoot proliferation from intermodal segment was dependent on the interaction between concentration and combination of plant growth regulators (PGRs) in the medium. After 6 wk Creamish brown compact callus at the base of microshoots developed and new microshoots regenerated from the callus as well. Profused microshoots were developed after two cycles of sub culturing after 8 wk of inoculation.

In this study among the different concentrations and combinations of BAP and Kinetin tested the best formulation for multiple shoot proliferation (100%) and higher number of shoots (27.3+1.18) per explants was found in media supplemented with BAP (0.5 mg/l) and Kinetin (0.5mg/L). Whereas poor shooting response was observed on MS Medium supplemented with BAP and Kinetin alone however Hiregoudar et.al [2] recorded the optimum response and an average of 12 and 8 shoots from hypocotyl and internodal explants, respectively on the medium containing 2 µM BA in Limonia acidissima.

Parihar and Kumar [10] observed best multiple shoots from epicotyls of Aegle marmelos cultured on MS medium supplemented with BAP(1.5mg/L) and kinetin

(1.5mg/L) and reported that Kinetin was found better then BAP when explants were treated with individual phytohormones. Plant regeneration from epicotyl explants have also been reported by earlier researchers [11,12] in Citrus scientia. The superior effect of BAP on adventitious shoot regeneration from hypocotyl explants of wood apple has also been reported by Vyas et al. [13] and from cotyledon explants has been reported by Kouider et al. [14]

The role of cytokinins in shoot differentiation from nodal segments was reported in several woody species but only few reports were successful in inducing organogeneis from mature nodal explants[15]. Mahipal et al, [16] reported that MS medium augmented with 1.0 mgL−1 of each BAP and Kinetin (Kin) showed best multiplication of the shoots in vitro with maximum numbers of shoots (25.73 ± 0.06) in Passiflora edulis.

For root induction, in vitro regenerated, well developed and elongated shoots were excised and cultured on root induction media. Different concentrations of IAA (1.0, 1.5, 2.0, and 2.5mg/l) were used with MS Medium for root induction. Rhizogenesis occurred after 10 days of culture. Well developed roots were formed after 3-4 weeks of culture. Best rooting response (29.0+0.39) was observed on MS Medium with IAA (1.5mg/L). Increase in concentration of auxin didn't affected root induction. For successful micropropagation protocol for establishment of in vitro rooting from microshoots is crucial, and IAA is preferably used for the development of adventitious roots in vitro [17,18]. However Islam et al. [19] observed best rooting response on NAA (0.5 mg/L) and reported 80% shoots rooted within seven weeks in Feronia limonia L. whereas Tornero et.al. [20] reported, IBA as a potent auxin for root induction in Citrus limon. This might be due to genotypic variation of explants influenced by the cultural and environ-mental conditions [21] and indicates that either IAA or IBA or NAA may be used for rooting.

In conclusion, in vitro growth and development of L. acidicssima was highly influenced by the concentration and combination of PGRs used. The double interaction of Kn with BAP influenced shoot regeneration ability. The results presented also demonstrate that internodal segments of L.acidissima offer great potential as a source explant for shoot induction. L. acidicssima which is rich in secondary metabolites can be cultured successfully. Callus cultures so obtained, can provide an efficient source for commercial exploitation. This confirms the increasing importance of in vitro technique for commercial exploitation of pharmaceutically important metabolites. The procedure reported in this study may facilitate improvement, conservation, and mass propagation of this important multipurpose tree.

Table-1 Effect of plant growth regulators on callus Initiation

S.No	Concentraion (mg/l)	Explants	No. of segments inoculated	Callus Initiation %	Nature of Response
1.	MS + KIN (0.5)+2,4-D (0.5)	Epicotyl	30	80	Cream Colour & Profuse Callusing
		Hypocotyl	30	80	Cream Colour & Profuse Callusing
		Cotyledon	30	75	Green & Profuse callusing
2.	MS + KIN (0.5)+2,4-D (1.0)	Epicotyl	30	100	Creamish Green & Embryogenic Callusing
		Hypocotyl	30	100	Creamish Green & Embryogenic Callusing

		Cotyledon	30	100	Creamish Green & Embryogenic Callusing
3.	MS + KIN (0.5)+2,4-D (1.5)	Epicotyl	30	60	Cream Colour & moderate Callusing
		Hypocotyl	30	70	Cream Colour & moderate Callusing
		Cotyledon	30	50	Green & feeble Callusing
4.	MS + KIN (0.5)+2,4-D (2.0)	Epicotyl	30	10	Poor Response
		Hypocotyl	30	8	Poor Response
		Cotyledon	30	5	Poor Response
5.	MS + KIN (0.5)+2,4-D (2.5)	Epicotyl	30	0	No Response
		Hypocotyl	30	0	No Response
		Cotyledon	30	0	No Response

Table-2 Effect of Cytokinins on shoot regeneration from internodal segments

MS basal medium cytokinins concentration (mg/l)		**% Explant producing Shoots**	**Mean No. Shoot per Explant ± SE**	**Shooting(%)**	**Nature of Response**
BAP	**KN**				
0.25	-	40	7.6 ±1.18	25.3	Feeble Shooting
0.5	-	40	8.3± 0.98	27.6	Feeble Shooting
1.0	-	50	12.6 ± 1.18	42.0	Moderate Shooting
2.0	-	60	10.6± 1.65	35.3	Moderate Shooting
2.5	-	10	3.0±0.47	10	Poor shooting
-	0.25	60	17.6 ± 1.78	58.6	Moderate Shooting

-	0.5	50	10.3± 1.65	34.0	Moderate Shooting
-	1.0	40	7.0 ± 1.41	23.3	Feeble Shooting
-	2.0	30	5.0 ± 1.20	16.6	Poor Shooting
-	2.5	10	3.0±0.47	10	Poor shooting
0.5	0.5	100	27.3 ±1.18	91.0	Embryogenic Shooting
	1.0	60	18.0±1.88	60.0	Moderate Shooting
	1.5	20	7.0±1.24	23.3	Poor Shooting
	2.0	0	0	0	No Response
1.0	0.5	80	24.6±1.44	82	Profuse Shooting
	1.0	50	15.0±1.41	50	Moderate Shooting
	1.5	20	6.3±0.72	21	Poor Shooting
	2.0	0	0	0	No Response
1.5	0.5	60	18.0±1.88	60	Moderate Shooting
	1.0	30	9.3±1.18	31	Poor Shooting
	1.5	10	3.0±0.47	10	Poor shooting
	2.0	0	0	0	No Response
2.0	0.5	30	8.3±1.44	27.6	Feeble Shooting
	1.0	15	4.16±0.48	13.8	Poor shooting
	1.5	0	0	0	No Response
	2.0	0	0	0	No Response

Values are mean of 30 explants

Table 3. Effect of IAA on in vitro root induction

MS basal medium with IAA Concentration (mg/l)	% of shoot forming root	Mean No. of roots devloped per Shoot ± SE	Rooting (%)	Nature of Response
0.5	NIL	NIL	NIL	NIL
1.5	100	29.0 ± 0.38	96.6	Normal rooting with lateral roots and optimum growth
2.0	70	18.0 ± 0.94	60.0	Normal rooting with lateral roots and optimum growth
2.5	50	12.6 ± 1.18	42.0	Limited rooting and growth

Values are mean of 30 explants

Table 4. Growth indices of callus culture of Limonia acidissima

Explants	Growth indices				
	2 week Callus	4 week Callus	6 week Callus	8 week Callus	10 week Callus
Epicotyl	5.1	6.8	9.4	10.4	7.3
Hypocotyl	4.9	6.9	8.2	9.1	8.5
Cotyledon	2.7	4.3	4.7	5.2	6.1

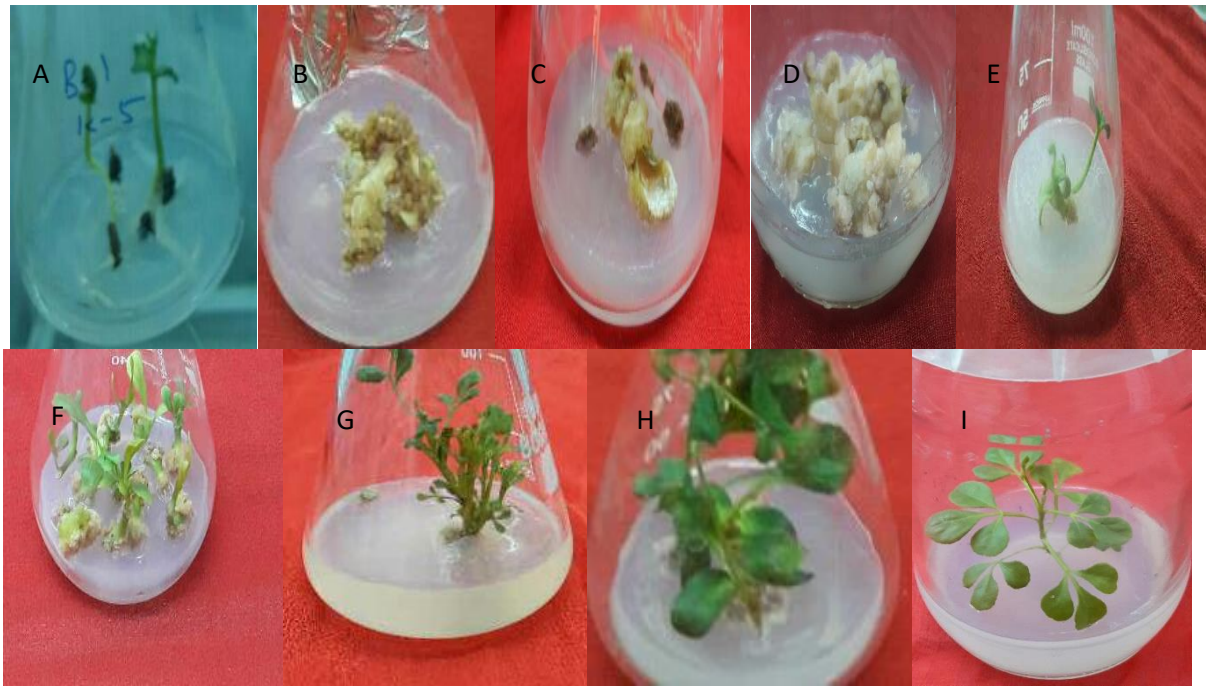

Fig. 1- In vitro propagation of Limonia acidissima : A. In vitro grown seedling; B. Initiation of callus from hypocotyls; C. Initiation of callus from cotyledons on MS medium supplemented with Kinetin (0.5mg/L) and 2,4-D (1.0mg/L); D. 8 weeks callus on MS medium supplemented with Kinetin (0.5mg/L) and 2,4-D (1.0mg/L); E. Initiation of Shoot development from hypocotyls on MS medium supplemented with Kinetin (0.5mg/L) and BAP (0.5mg/L); F Multiple shoots from callus on MS medium supplemented with Kinetin (0.5mg/L) and BAP (0.5mg/L); G. Multiple shoots from inter nodal segment along with basal callusing on MS medium supplemented with Kinetin (0.5mg/L) and BAP (0.5mg/L); H. Single shoot differentiation on MS medium supplemented with Kinetin (0.5mg/L) and BAP(0.5 mg/L); I. Rooting of micro shoot on MS media containing IAA .

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