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An efficient *in vitro* regeneration protocol from leaf explant of *Lavatara cashmeriana* Cambess: an important endemic medicinal herb of Kashmir Himalaya

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ABSTRACT

An efficient *in vitro* micro propagation protocol from leaf explant of *Lavatera cashmeriana* Cambess, an endangered medicinal plant endemic to Kashmir Himalaya has been developed. The leaf explants were obtained from field grown mature plants. Indirect organogenesis (shoot and root regeneration) from leaf callus using cytokinins and auxins supplemented MS basal medium is being reported for the first time in this plant. Among the various strengths of growth regulators tested, best results were recorded when leaf explants from natural plants were cultured on MS basal medium supplemented with 2.0 mg/l 2,4-D for profuse callusing, 2.0

mg/ lBAP +1.0 mg/l NAA for shoot regeneration and 2.0 mg/l BAP+ 0.5 mg/l IBA for rapid shoot proliferation respectively. The root induction in *in virto* raise shoots was maximum in the medium containing half strength MS basal medium supplemented with 1.5 mg/l NAA. The rooted plantlets were hardened in small pots containing a mixture of sand: soil: vermiculite (1:1:1), in the greenhouse conditions and were successfully acclimatized and established in soil with 50-60% survival rate.

Keywords: *Lavatera cashmeriana*; endemic medicinal plant; cytokinin; auxin; shoot regeneration

1. INTRODUCTION

Lavatera cashmeriana Cambess commonly known as Saz Posh is a medicinally important plant belonging to family Malvaceae. It is an endemic species of Kashmir Himalaya growing in forests and meadows at an altitude of 2100-2500m (Malik, 2011). It is a principle source of Lavaterone, Lavaterepene, Lavateral, Lavaterosterol, Lavateronic acid (Hamid, 2002). Petroleum ether, chloroform and alcohol extracts of *L. cashmeriana* in various concentrations are found to be active against Gram-positive bacteria, while as only the alcohol extracts of this plant shows significant activity against Gram-negative bacteria. Chloroform extract of *L. cashmeriana* also shows a weak activity against Gram negative bacteria (Hamid, 2002). It finds its place in traditional as well as modern system of medicine. Plant extracts of this species are known for their anti-inflammatory, analgesic and antibacterial activity. The herb is given as a mild laxative. Current research has reported its seeds as protease inhibitors, which have anti proliferative activity against human lung cancer cell lines (Rakhshanda *et al*., 2012). The roots and leaves of *Lavatera cashmeriana* are used as mild laxative and for throat problems (Aijaz *et al.,* 2013). Its leaves and flowers have been reported to cure skin irritation in pregnant women (Mir, 2014).

The natural resurgence of *Lavatara cashmeriana* is through seeds, however their cultivation rate is very poor. The poor cultivation coupled with over exploitation for pharmaceutical use has depleted the species from natural habitat. Infact, the plant species is under severe pressure of overharvesting and has been categorized as endangered (Kaul 1997, IUCN, 1980). In view of the problems of conventional propagation, large scale multiplication of this species can only be met efficiently and economically in a sort span of time by *in vitro* propagation. Therefore, an efficient *in vitro* propagation system is required to further clarify its potential medicinal values and germplasm conservation. Since micro propagation plays an important role in rapid propagation, conservation and enhancement of the production of secondary metabolites to cater the demands of the pharmaceutical industry, the present study has been carried out

(i) To study the effect of different growth substances at varying concentrations on the successful regeneration.
(ii) To develop the most appropriate and rapid protocol for its efficient *in vitro* propagation.

2. MATERIALS AND METHODS

2.1. Washing and sterilization of explant

The explant for micropropagation purpose were collected from successfully established *Lavatara cashmeriana* plants growing in KUBG (Kashmir University Botanical Garden). Explant was cleaned by overnight washing under running tap water to remove all the adhering dust particles and microbes from the surface. The explant was then washed with liquid detergent (Laboline) for another 15-20 minutes and with tween twenty for 5 minutes respectively .The explant was again washed properly to remove the detergent and then transferred to the laminar air flow cabinet for surface sterilization. Sterilizing agents used for obtaining aseptic tissues were 4% sodium hypochlorite and 0.1% mercuric chloride .The most effective chemical sterilant for field grown leaf has been a solution containing $HgCl_2$ (0.1%) treated for 6 minutes resulting in100% sterilization.. Then explant was then rinsed 4-5 times with autoclaved double distilled water, each time leaving the explant in double distilled water for 5-8 minutes to remove the sterilant and to keep the explant hydrated. The explants was then inoculated on variously augmented MS media. All leaf cultures were maintained in an air conditioned culture room at a temperature of 22±2 ^{0}C.

2.2. Culture media, growth regulators and growth condition

During the present study MS medium supplemented with 3 % (w/v) sucrose and solidified with 0.8% agar was used prior to autoclaving at 121°C. The pH was adjusted at 5.8. Growth substances whose effects on callus production and subsequent organogenesis were studied, included auxins; 2,4-dichlorophenoxyacetic acid (2,4-D),indole butyric acid(IBA), indole acetic acid(IAA),naphthalene acetic acid (NAA)) and cytokinins; Benzyl amino purine (BAP) and Kinetin(Kn). These were used both individually and in combinations of different concentrations. Inoculations were performed under aseptic conditions in a laminar air flow cabinet, in cultures tubes (150 X 25 mm) containing 20-25 ml medium and conical flasks containing 35-40 ml medium and

plugged tightly with non-absorbent cotton.The media containing glassware were exposed to UV light for sterilization for 30 minutes before inoculation. All inoculated culture vials were incubated in an air conditioned culture room at 22 ± 2^0C on 16-18 hrs photoperiod at 3,000 lux light intensity (40W white fluorescent tubes, Philips, India.) and with 55-60 % relative humidity.

2.3. Callus induction

For callus induction, each of the auxins, was added in seven concentrations; 0,2, 0.5, 1.0,1.5, 2.0,2.5 and 3.0 mg/l . Thus seven individual treatments were obtained for auxin (2,4-D , NAA, IBA and IAA),with their total number as 28. For shoot regeneration auxins (2,4-D , NAA, IBA and IAA) were also combined in all possible combinations(0.5-2.0mg/l) with Kn and BAP(0.5-2.0mg/l). Thus BAP and Kn individually formed 16 types of treatment combinations with each of the auxins.

Callus masses from different leaf cultures were investigated for point of origin, colour, texture and signs of organogenesis. This was done across the various auxin- cytokinin combinations. The callus induction frequency on different callus induction media was calculated by using the following formula.

$$\text{Callus induction frequency} = \frac{\text{Number of cultures producing calli}}{\text{Total number of cultures}} \times 100$$

2.4. Sub culturing

After **callus induction**, calli were sub cultured within 3-4 weeks of interval on the fresh media for further proliferation. Since the callus formation was more on 2,4-D ,NAA, and BAP + NAA treatments combinations, all the further trials for callus proliferation were carried out in only these treatments. Watery, spongy brown and dead portion of calli were discarded during subcultures and only friable, nodular calli were maintained to develop organogenic nature.

2.5. Shoot regeneration

For shoot regeneration leaf explant as well as the healthy callus masses (originating from different leaf cultures) were inoculated on shoot regeneration medium, that include cytokinins, Benzyl amino purine (BAP) and kinetin (Kn) which were used both individually and in combination with different concentrations of auxins. Each of the cytokinin was added in seven concentrations; 0.2mg/l- 3.0 mg/l. The cultures were incubated in the plant culture room maintained at 16 h photoperiod. The data was recorded for the number of days taken for shoot primordial initiation, per cent callus forming shoot primordia, and number of shoots per leaf callus. The regeneration frequency was determined by counting the number of calli forming shoots.

2.6. Shoot elongation and Multiplication.

Various leaf cultures, with indirectly induced shoot buds, were used as propagules for *in vitro* shoot elongation and multiplication. Different concentrations of BAP+Kn and BAP or Kn with various concentrations and combinations of NAA and IBA were used for shoot elongation and multiplication. At the end of four weeks the number of shoots produced were counted.

2.7. Induction of rooting in shoots and hardening of plantlets

Regenerated shoots were excised from the parent cultures and transferred onto full strength MS basal medium and with half salt strength (MS1/2) MS medium supplemented with different concentrations (0.5-3.0mg/l) of auxins (IBA, IAA and NAA). The cultures were incubated under the same culture conditions as mentioned above. Data for days to root initiation, number of roots per shoot, root length and per cent rooting were recorded and analyzed for test of significance. The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1:1:1) transferred to the greenhouse conditions for acclimatization and hardening. Survival frequencies of *in vitro* regenerated plants were recorded.

3. RESULTS

3.1. Callus formation

During the course of present study, the inoculation of leaf explants of *L. cashmeriana* on MS medium supplemented with different concentrations and combinations of PGR's i,e cytokinins (BAP and Kn) and auxins (NAA/IBA/IAA /2,4-D) alone and in combination resulted in varied responses. Out of the various auxin (2,4-D/NAA/IAA/IBA) supplemented MS medium, no response was observed in explants inoculated on different concentrations of IAA. With rest of the auxins, callus was initiated on the cut surfaces of leaf, explant

and 2, 4-D was found to be most effective for callus induction followed by NAA and IBA. Calli were formed in all leaf cultures within 8-15 days in all the tested concentrations, ranging from 0.2-3.0mg/l of 2, 4-D (except in 0.2mg/l 2, 4-D), (Fig 1 A, B).

3.2. Shoot regeneration

The regeneration of adventitious shoot with varying shoot number and size, from leaf calli was evaluated within 25-60 days of culture on cytokinins(BAP and Kn) and also on various cytokinin + auxin combinations. Shoot buds also regenerated on various sub cultured leaf calli, when they were grown on the same shoot regeneration medium. The responses of the leaf explant and callus masses to various PGR's is given (Table 1).

3.3. Shoot regeneration from leaf explant using cytokinin(BAP) alone or in combination with Kn or auxins (2,4-D /NAA/ IAA/ IBA)

For shoot regeneration the leaf explants as well as the leaf calli were inoculated on MS medium fortified with cytokinin(BAP) alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA).However the results were obtained only in the following media concentrations.

3.3.1. MS +BAP

When different concentrations of BAP ranging from 0.2-3.0 mg/l were used, it was noticed that BAP at lower concentration (0.2-0.5 mg/l) did not induce any shoot formation, however within 20 days of inoculation green nodular callus was produced in leaf cultures and shoots of almost same length regenerated after 45 days on calli in 1.0-1.5 mg/l of BAP supplemented MS medium. The number of shoots decreased with increase in concentration of BAP up to 2.0 mg/l and above this level BAP did not induce any response. The length of shoots did not vary significantly and ranged from 2.55cm-2.80cm. The maximum number of shoots (8) were recorded at 1.5 mg/l of BAP.

3.3.2. BAP + NAA

When the effect of BAP with NAA was assayed for regeneration of shoots from leaf explants it was observed that 1.0 mg/l BAP combined with 0.5 mg/l of NAA initiated indirect shoot formation in 75% of the leaf cultures after about 6 weeks of culture. The number of shoot increased as the concentration of both BAP and NAA was increased. The maximum number of shoots (23) regenerated on medium fortified with 2.0 mg/l of BAP+ NAA1.0 mg/l.Any further increase in concentration of BAP(2.5 mg/l onwards) resulted in decrease in number of shoots. More trials were performed with increased concentration of NAA (1.5 mg/l) combined with different concentrations of BAP but the shoots started to turn brown leading to their death. The length of shoots showed varied response in BAP + NAA combinations with the maximum shoot length (3.46 cm) at BAP 1.0mg/l combined with NAA 0.5mg/l. Sub cultured leaf callus also produced shoot buds but they did not regenerate further (Fig. 1 C).

3.3.3. BAP + IBA

The various combinations of BAP with IBA induced the formation of green friable callus masses within 30 days on leaf explants in BAP(0.5-1.5mg/l) + IBA(0.5-2.0mg/l) which after sub culture proliferated but did not regenerate shoots. However, the leaf callus sub cultured in BAP 2.0mg/l + IBA 0.5mg/l formed about 2.00 cm long leaf like shoots buds within 15-25 days with an average number of shoots(4.33) in about 73% of the cultures.

3.3.4. BAP + Kn

The combined effect of BAP + Kn (0.2-3.0 mg/l) had no effect in any of the leaf explant except the combination of BAP(1.5-2.0 mg/l) + KN (0.5-2.5 mg/l),which generated brownish yellow , friable callus after 15-18 days having no morpogenic potential.

3.4. Shoot regeneration from leaf explant using Kn alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA)

Leaf explants as well as the leaf calli were inoculated on MS medium fortified with cytokinin (Kn) alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA). However the results were obtained only in the following media concentrations.

3.4.1. MS+Kinetin(Kn)

Leaf explants when cultured on different concentrations of Kn (0.2-3.0 mg/l) produced callus only on Kn (1.0-2.0 mg/l) with no significant effect above and below these concentrations. However, the calli subcultured on Kn (1.0-3.0 mg/l) proliferated further into friable yellow callus masses with no morpogenic potential.

3.4.2. Kn + NAA

It was observed that calli developed from the leaf expalnts on media containing Kn , (0.5-2.0 m/l) and NAA (0.5-1.0 m/l) combinations, after about 3weeks of subculture, gradually developed several green spots that changed to profuse light green to dark green nodular calli, within the fourth week of culture. Generally, shoot initiation was observed after four weeks of inoculation on all combinations of Kn, (0.5-2.0 m/l) and NAA (0.5-1.0 m/l) supplemented medium. The shoot like structures could be distinguished by the presence of green, opaque and compact nodules. Over a period of 7 weeks, (63%) of leaf explants produced indirect shoots, with a highest number of shoots (11) per explant in the medium containing Kn (2.0 m/l) and NAA (1.0mg/ l) while as longest shoots(maximum shoot length 4.39 cm) were obtained on Kn (1.5 mg/l) and NAA (0.5 mg/l) in 45% cultures.

Table 1 Effect of BAP and Kn either alone or in combination with NAA/IBA/IAA on shoot regeneration from field grown leaf explant callus of *L. Cashmeriana*

BAP mg/l	Kn mg/l	IBA mg/l	NAA mg/l	% Culture response	Number of days taken for shoot regeneration	Average number of shoots	Average length of shoots (cm)
0.5	-	-	-	0	0	0	0
1.0	-	-	-	68	35	4.70	2.80
1.5	-	-	-	80	27	6.90	2.82
2.0	-	-	-	75	32	3.80	2.55
2.5	-	-	-	62	32	0	0
1.0	-	-	0.5	53	38	6.20	3.32
	-	-	1.0	75	42	8.11	2.00
	-	-	1.5	80	32	4.32	1.60
	-	-	2.0	60	35	2.77	1.45
2.0	-	-	0.5	42	38	7.70	2.06
	-	-	1.0	75	42	15.38	2.80
	-	-	1.5	72	32	5.32	1.14
	-	-	2.0	0	0	0	0
2.5	-	-	0.5	49	45	2.14	2.04.
	-	-	1.0	47	45	1.86	1.98
	-	-	1.5	33	50	1.03	2.09
	-	-	2.0	0	0	0	0
-	1.0	-	0.5	42	50	1.97	2.82
	1.5	-	0.5	45	55	2.78	4.32
	1.5	-	1.0	49	43	4.44	2.33
	2.0	-	1.0	63	49	7.90	1.28
	2.5	-	1.0	0	0	0	0
2.0	-	0.5	-	73	18	4.33	2.0

Number of replicates 30

3.5. Shoot multiplication

The *in vitro* raised shoot clumps were separated and transferred on MS medium fortified with BAP+Kn (1.0-2.0 m/l) and BAP or Kn in combination with IBA and NAA for shoot multiplication. Repeated sub culture after four weeks interval, resulted in degeneration and browning of shoot buds in all combinations of BAP+Kn. Multiplication of shoots was observed on MS medium supplemented with BAP (1.0-2.0m/l)+ IBA (0.5-1.0m/l) with maximum number (22.8) of shoots with maximum length (4.48cm), regenerated on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IBA (Fig. 1D). The increase in concentration of BAP caused the browning and ultimately the death of shoots while as further increase in IBA concentration resulted in more callus formation. The fortification of MS medium with Kn (1.0-2.0mg/l) +IBA (0.5-1.0 mg/l) did not induce any response so far as shoot elongation and multiplication is concerned. The effect of NAA with BAP or Kn on *in vitro* raised shoot clumps also promoted shoot multiplication and elongation, however, the shoot numbers was observed to be significantly less than BAP+IBA combinations with minimum number (0.43) of

shoots observed on 2.0mg/l Kn + 1.0 mg/l NAA and maximum number (3.76) observed on 1.0 mg/l KN +0.5mg/l NAA (Table 2). Regenerated shoots were excised from callus and allowed to grow for 7 to 10 days in the same hormone combination before transfer to the rooting medium.

Table 2 Effect of BAP + Kn and BAP or Kn in combination with NAA/IBA on shoot multiplication and elongation in of *L. Cashmeriana*

BAP mg/l	Kn mg/l	IBA mg/l	NAA mg/l	% Culture response	Number of days taken for shoot multiplication /elongation	Average number of shoots	Average length of shoots(cm)
1.0		0.5		47	23	9.5	1.53
1.5				45	29	13.19	1.72
2.0				32	29	21.6	3.23
1.0		1.0		42	36	10.52	2.24
1.5				51	36	6.49	2.12
2.0				67	38	6.37	2.62
1.0			0.5	47	31	4.43	2.37
1.5				58	37	4.12	2.19
2.0				45	39	6.35	2.47
1.0			1.0	43	47	8.76	1.39
1.5				59	32	8.64	1.87
2.0				39	36	11.93	1.14
	1.0		0.5	52	42	2.97	1.49
	1.5			59	44	2.43	1.12
	2.0			52	41	1.43	0.87
	1.0		1.0	50	45	1.05	0.98
	1.5			48	33	0.76	0.65
	2.0			31	47	0.63	0.81

Number of replicates 30

3.6. Rooting

Once the shoots developed 4–5 leaves (4 cm in length), they were transferred to the rooting medium, i.e. half and full strength MS basal medium alone and in combination with IAA, IBA and NAA. These *in vitro* grown shoots formed thin adventitious roots successfully only on MS half strength and full strength medium, with more number of roots on half strength MS medium within 4 weeks. On combining different auxins with basal media, rooting was induced in IBA (1.0-1.5 mg/l) and NAA (1.0 mg/l) treatments while as no response was observed on IAA treatments. The best rooting was observed on MS half strength medium at 1.5 mg/l IBA with maximum number of roots(6) and maximum root length (2.32 cm) obtained in 62% of micro shoots (Fig. 1E).

Table 3 Effect of different concentrations and combinations of auxins on root induction in full strength MS basal medium fortified with IBA and NAA

NAA mg/l	IBA mg/l	% Response	Number of days taken for rooting	Average Number of roots	Average Length of roots
0	0	40	24	3.69	1.73
1.0	0	33	20	1.43	1.00
0	1.0	0	0	0	0
0	1.5	0	0	0	0

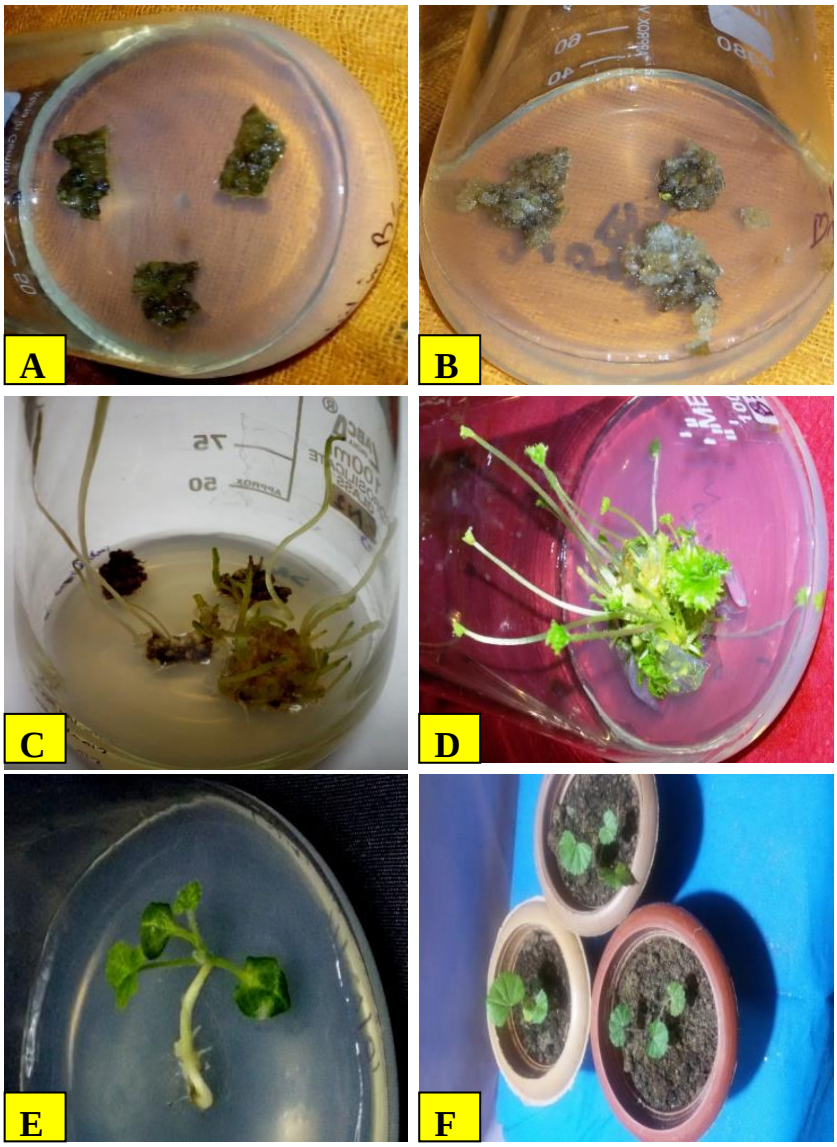

Fig.1: A. Leaf explant showing callus initiations; B. Callus culture; C. Shoot induction and regeneration from callus; D. Shoot proliferation. E. Root formation; F. Hardening of plants

Table 4 Effect of different concentrations and combinations of auxins on root induction in half strength MS basal medium fortified with IBA and NAA

NAA mg/l	IBA mg/l	% Response	Number of days taken for rooting	Average Number of roots	Average Length of roots
0	0	70	27	3.93	1.98
1.0	0	59	22	3.99	1.47
0	1.0	45	27	2.75	2.04
0	1.5	62	29	4.48	2.11

3.7. Hardening and acclimatization

The rooted plantlets were subsequently transplanted to small pots containing sterilized soil. The plantlets were covered with polyethylene bags perforated gradually and kept in the culture room. The pots were placed on petri plates carrying some water for

the uptake by the plantlets. After 15 days the polyethylene bags were removed and well established plants were transferred to pots containing a mixture of sand: soil: vermiculite (1:1:1) and kept in the greenhouse conditions. The survival frequency of *in vitro* regenerated plantlets was 50-60%. The regenerated plantlets did not show any morphological difference from plants growing in natural conditions (Fig. 1F).

4. DISCUSSION

During the present study, it was observed that leaf explant excised from the plants growing in natural conditions was able to develop healthy calli. It was further observed that 2, 4-D was most effective for callus induction followed by NAA. The effectiveness of cytokinin and auxin to promote callus formation has been reported earlier (Akiyoshi et al. 1983). The effectiveness of 2, 4-D and NAA might be due to their role in DNA synthesis and mitosis in inducing callus as reported by Samanthi (2013) in *Hibiscus cannabis*. Also the best hormone for callus induction of *Althea rosea* was found to be 2, 4 D at a concentration of 0.03 mg/l (Munir 2012) Similar result was shown by Kamal (2011) who proposed the use of high auxin and low cytokinin concentration for callus induction in cotton (*Gossypium* sp.) and studied that a medium with the addition of 2.4-D including (no cytokinin) was successful for callus formation.

Our results estimated 2.0 mg/l of 2,4-D as the optimal concentration for callus formation and a further increase in the concentrations of 2,4-D(2.5mg/l - 3.0 mg/l) resulting in browning of callus as a result of phenolic exudation. Also the callus obtained in 2, 4 D treatments did not show any signs of organoenesis. Similar trend which led to necrosis of the callus was observed by Kumari (2011) in *Hibiscus (rosa sinensis*).She obtained callus from leaf explant on medium containing 2, 4-D (0.5mg/l to 5mg/l) the concentration of which above 5mg proved inhibitory. There was only callusing and no organogenesis in media containing 2,4-D (Kumari 2011).

Indirect regeneration of shoots was found in all concentrations of BAP (0.5mg/l-2.05mg/l) during the present study, although the rate was not high. The medium and shoot production recorded from leaf explants on different concentration and combination of plant growth regulators like BAP 1.0-2.0 mg/l in combination of auxins has been observed in our study. Similar results were obtained for *Solanum americanum* where BAP (2.0 mg/l) + 2, 4- D (1.0 mg/l) + IAA (1.0 mg/l) stimulated production of multiple number of shoots from leaf explants (Ramar *et al*., 2014) .Also, the results of Bajaj *et al*.(1986) who in *G. arboreum* and *G. hirsutum* obtained multiple shoot formation using MS with BAP; Gupta *et al* .(1997) in *G. hirsutum* using MS+BAP; Bhalla *et al*.(2009) in *Hibiscus rosa sinensis* after culturing leaf and nodal segments on MS and BAP, are in agreement to our results. Such superiority of BAP over Kinetin has also been reported by Murashige (1974) who attributed it to group localized at N6 position of cytokinin. Our results are not in agreement with Mushtaq *et al*.(1994) who reported only callus formation from various explants of *Althea rosa* on MS+BAP; Troncoso *et al*.(1997) who reported the death of explants of *L. maritama* after culturing them on MS +BAP; Munir *et al*., 2012 also reported only callus formation in *Althea rosa* on MS +BAP +NAA.

The addition of PGRs to the regeneration medium in our study contributed to increase in *in vitro* shoot height up to certain concentrations only and further increase of PGR concentrations decline the shoot growth. These results are similar to the reports of Wareing & Philips (1981) and Hu Wang (1983), according to whom the inhibitory effect of cytokinin is expected, as cytokinins are known to inhibit stem elongation. Therefore, the present study leads to presume that *in vitro* shoots require a substantial amount of exogenous supply of cytokinin and auxins for shoot initiations and elongation. Also the results obtained from leaf explants as reported by Parveen (2012) is contradictory to our results .During her study little response or no response in terms of shoot formation was observed in *L. cashmeriana* when field grown leaves were used as explants and mostly callus formation was recorded. In our study leaf explant has been observed to be having a good potential so far as shoot regeneration is concerned. Our results are supported by Samanthi (2013), who by developing *in vitro* shoots from leaf explant of *Hibiscus cannabinus* established that vegetative plant parts especially leaves are desirable explants for *in vitro* improvement because regeneration from these explants would preserve the genetic homozygosity of the parent genotype.

5. CONCLUSION

In vitro requirement is an efficient means of *ex situ* conservation of plant diversity and it assists in sustainable maintenance of the present day dwindling germplasm on long term basis, especially for endangered and endemic medicinal plants. The study established the *in vitro* propagation of *L. cashmeriana* and high proliferation rate achieved with uniform and vigorous growth. The present protocol achieved high shoot propagation and proliferation from leaf explant from field grown plants. This high proliferation rate could only be achieved using micro propagation rather than the traditional methods because; at present the rate of propagation in nature is far less than the rate of exploration. The highest number of shoots could be obtained on MS medium supplemented with 2.0 mg/l BAP+ 1.0 mg/l NAA and the appropriate medium for rooting was half strength MS medium fortified

with 1.5 mg/l IBA. This fruitful protocol set up through multiple shoot induction from leaf explant could be exploited for commercial propagation and conservation of this valuable, endangered and endemic medicinal plant.

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