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Influence of explant and plant growth regulators on callus mediated regeneration in *Lavatera cashmeriana*, Cambess

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Abstract
Plant growth regulators play a pivotal role in *in vitro* callus induction and callus growth habits and also determine the correlation of callus types with organogenesis for micropropagation purpose. Successful callus cultures of *Lavatera cashmeriana* were established in 7 explant types (root, node, petiole, leaf, petal, stamen and ovary) using auxins and cytokinins. Although, petal, anther and ovary explants were able to develop healthy friable callus even in hormone-free medium, highest callus induction % age, within 8-10 days was observed using auxins. 100% callus regeneration was observed in leaf explant followed by node (86.5%), petal (82%), ovary (78.5%), petiole (75.3%) and anther (75.1%) and the best growth substance for Callus induction was found to be 2, 4-D at the concentration of 2.0 mg/l. Using cytokinins+ auxins, the best percentage response of callus induction was obtained on BAP(1.0mg/l)+ NAA (1.0 mg/l)) in leaf explants(83.5%) followed by petal (79.2%), node (74%), petiole (71%), ovary(61%) and stamen(39.8%) on the same hormonal concentration within 19-27 days. Two forms of callus growth habits were observed, friable and compact calluses. In NAA + Kn cultures, 100% friable callus was obtained while as high frequency of green, compact organogenic calli were obtained from leaf, node, petal and petiole explant, on BAP(2.0mg/l)+NAA(1.0mg/l) supplemented medium. The organogenic calli developed shoots in shoot regeneration medium and maximum (16) number of multiple shoots were developed on leaf callus in BAP(2.0mg/l)+NAA(1.0mg/l), followed by node callus (13) and petal(6).Comparative callusing and shoot regeneration from different explants revealed that leaf discs are the best explant for *in vitro* studies in *L. cashmeriana*. The best rooting (62%) response was observed in ½ MS (half strength of salt) + IBA1.5 mg/l. Finally the well rooted plantlets were hardened in plastic mini pots containing soil + sand + vermiculites (1:1:1) plantlets and then transferred to fields for acclimatization with a survival frequency of 68%. This protocol could be used to micropropagate *L. cashmeriana* for conservation. Also, the high frequency callus production potential of this prized plant can be utilized for commercial secondary metabolite production in pharmaceutical industry.

Keywords: *In vitro*; friable callus; compact callus; micropropagation; plant regeneration, medicinal plants

Introduction
Lavatera cashmeriana (Malvaceae) a known medicinal herb endemic to Kashmir Himalaya and a principle source of Lavaterone, Lavaterepene, Lavateral, Lavaterosterol, Lavateronic acid (Hamid, 2002) [13] is under the severe pressure of overharvesting and has been categorized as endangered (Kaul, 1997) [20]. The herb is given as a mild laxative, leaves and flower extracts are used in many Unani medicinal preparations. Paste of dried flowers in milk is used for the treatment of mumps in children (Jeelani *et al*., 2013) [17]. Its roots are recommended in respiratory complaints (Rakhshanda *et al*., 2012) and throat problems (Ganie *et. al.*, 2013) and hence are collected in large quantities and sold as crude drug in market. Various plant extracts are also known for their anti-inflammatory, analgesic and antibacterial activity. Seeds are protease inhibitors, which have anti proliferative activity against human lung cancer cell lines (Rakhshanda *et al*., 2013b, Rakhshanda *et al*., 2013a). 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 (Hamid, 2002) [13].
Protocols for *in vitro* plant production via direct and indirect morphogenesis have many potential applications to any species particularly that of tremendous economic use and medicinal importance such as *Lavatera cashmeriana*. In general only few species of family Malvaceae have been studied *in vitro* and methods have been developed to induce callus from different tissues and to regenerate plants in some members of Malvaceae like Cotton (Gossypium spp.) Pushpa and Ravindren (2010) [32] *Hibiscus cannabinus*

(Samanthi *et al.*, 2013) [38] *Hibiscus cannabinus* (kenaf) (Ayadia *et al*., 2011: Pramanick *et al*., 2015: Ibrahim *et al*., 2015: Haque *et al*., 2017) [16] *Hibiscus sabdariffa* (Sie *et al*., 2010) *Hibiscus rosa sinensis*, (Kumari and Panday, 2010) [21] *Althaea rosea* (Munir *et al*., 2012) and there is a single reports on *in vitro* culture of *Lavatera cashmeriana* using shoot tip,leaf and node as explants (Parveen, 2012) [31]. In a rescent research study, successful callogenesis has been achieved from seed explants of *Lavatera cashmeriana* (Zahoor *et al*., 2016) [46].

The present study aimed to investigate the possibility of *in vitro* culture of this prized plant, and the objective was to optimize the production of callus from different explants, study the callus types for growth habits and determine the correlation of various callus types with organogenesis for micropropagation purpose.

Materials and Methods

Selection and preparation of explant

The explants for tissue culture purpose were excised from successfully established healthy *Lavatera cashmeriana* plants maintained in KUBG (Kashmir university botanical garden). Since each and every part of the plant has potential to regenerate a complete plant (Nagappa *et. al.,* 2008), in the present study 7 types of explants, which included explants from both vegetative (leaf, petiole, node, root) and flower (sepal, petal, anther and immature ovary) were used. Explants were washed under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (Laboline) for another 15-20 minutes and with tween twenty for 5 minutes respectively. The explant were again washed properly to remove the detergent and then transferred to the laminar air flow cabinet for surface sterilization. The most effective chemical sterilant for explants viz., leaf, node, petiole and root has been a solution of $HgCl_2$ (0.1%) treated for 5-6 minutes and for sepal, petal, stamen and ovary the most effective sterilant has been a solution of NaOCl (5%) treated for 10-12 minutes. These explants were 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. Explants were cut in appropriate sizes and inoculated on variously augmented MS media.

Selection of Culture media and growth regulators

During the present study MS medium supplemented with 3% (w/v) sucrose and pH (5.6-5.8) was used. The medium was solidified with 0.8 % agar, prior to autoclave at 121^0C at 15 lbs pressure for 15-20 minute. The growth regulators whose effects on callus production and subsequent organogenesis were studied, included auxins;2,4-dichlorophenoxy acetic acid (2,4-D), indole butyric acid (IBA),naphthalene acetic acid (NAA) and cytokinins; Benzyl amino purine (BAP) and kinetin (Kn). These were used both individually and in different combination and concentrations.

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. Cytokinins (BAP and Kn) for callus induction were added in 4 concentrations; 0.5, 1.0,1.5, 2.0 mg/l. 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. The media combinations used for each treatment combination was replicated five times.

Culture condition

Media containing culture vessels were exposed to UV light for 30 minutes under laminar air flow before inoculation. Inoculations were done in aseptic conditions under a laminar air flow cabinet in cultures tubes (150 X 25 mm) containing 20-25 ml medium and conical flasks containing 35-40 ml; plugged tightly with caps and non-absorbent cotton respectively. All culture tubes were incubated in a culture room at 250±20 ^{0}C under 16-18 hrs photoperiod at 3,000 lux light intensity (40W white fluorescent tubes, Philips, India.) and with 55-60 % relative humidity**.**

Investigation of callus

At the end of the culture period of 30 days, callus masses from different explant cultures were investigated for point of origin, colour, texture and organogenesis. This was done across the various auxin- cytokinin combinations. The callus induction frequency was calculated by using the following formula.

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

Sub culturing

After callus induction, calli were sub cultured within 20-28 days of interval on the fresh media for further proliferation. Since the callus formation showed the best results on 2,4-D,NAA, and BAP + NAA treatments combinations, all the further trials for callus proliferation were carried on, in only these treatments only. Watery, spongy brown and dead portion of calli were discarded during subcultures and only friable and nodular green calli were maintained to develop organogenic nature. The cultures were maintained by regular subculture at 30-40 days intervals on fresh MS medium.

Shoot regeneration

For shoot regeneration healthy callus masses, originating from different explant cultures were inoculated on shoot regeneration medium, that include Cytokinins (BAP :Kn) used individually and in combination with different concentrations of auxins. The data for the number of days taken for shoot primordial initiation, per cent callus cultures forming shoot primordia and number of shoots per callus were recorded after 60 days of culture. The regeneration frequency was determined by counting the number of calli forming shoots.

Shoot elongation and Multiplication.

Shoot buds induced indirectly on various explants, 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 30-40 days the number of shoots produced were counted and their length was measured.

Induction of rooting in shoots and hardening of plantlets

Full strength MS basal medium and half strength MS medium (½ MS) supplemented with different concentrations (0.5-3.0mg/l) of auxins (IBA, IAA and NAA) were used for root induction in regenerated shoots. 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. 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) and exposed to greenhouse conditions for hardening. Survival frequencies of *in vitro* regenerated plants were also recorded.

Observations and Results

Callus induction

Callus production from different explants using hormone free MS medium

When explants from vegetative parts of plant viz., leaf, petiole, node and root were inoculated on MS basal medium, no response was noticed in any of the explants, however the floral parts (petals, anther and ovary) were able to develop a healthy friable callus even in hormone-free medium within 10 days of culture. The petal explants showed the best response (60.5%) followed by immature stamen (40%) and then ovary (38.2%). Sepals neither responded to MS basal medium nor to the medium fortified with various hormones concentrations (Table1.a).

Callus production from different explants using auxins (2, 4-D/NAA/IAA/IBA)

For callus induction different explants when inoculated on MS medium fortified with auxins (2, 4-D /NAA/ IAA/ IBA) responded only in the following media concentrations (Table 1.a)

Table 1a: Effect of auxins (2, 4-D/NAA/IAA/IBA) on callus formation from explants excised from field grown plants of *L.cashmeriana*

Medium used/ Hormone Concentration mg/l	Callus formation after 30 days of culture						
	leaf	petiole	Node	Root	anther	Petal	ovary
MS	0	0	0	0	-Callus	*Callus	*Callus
2,4-D 0.5	*Callus	- Callus	0	0	*Callus	*Callus	*Callus
2,4-D 1.0	*Callus	*Callus	*Callus	0	**Callus	**Callus	**Callus
2,4-D 1.5	**Callus	**Callus	*Callus	0	**Callus	**Callus	**Callus
2,4-D 2.0	***Callus	***Callus	***Callus	0	***Callus	***Callus	***Callus
2,4-D 2.5	*Callus	0	0	0	0	0	0
2,4-D 3.0	*Callus	- Callus	- Callus	0	0	0	0
NAA 1.0	*Callus	*Callus	*Callus	0	0	0	0
NAA 1.5	**Callus	**Callus	**Callus	0	0	0	0
NAA 2.0	**Callus	**Callus	**Callus	0	0	0	0
NAA 2.5	*Callus	*Callus	*Callus	0	0	0	0
IBA 0.5	0	0	0	**Callus	**Callus	**Callus	0
IBA 1.0	*Callus	*Callus	*Callus	**Callus	*Callus	**Callus	-callus
IBA 1.5	0	0	0	*Callus	0	0	0

'***' Intense, (75-100%) '**' Moderate, (50-75%) '*' Meager (25-50%) '-'callus (below 25%) Number of replicates 30

MS+2, 4-D

2, 4-D was found to be most effective for callus induction as calli were initiated directly on the cut surfaces of different explants within 8-15 days in all the tested concentrations (except in 0.2mg/l). However, the higher number of calli were produced with 2.0 mg/l of 2, 4-D after 3 weeks of culture. Further, it was observed that the callus formed at 2.5- 3.0 mg/l of 2, 4-D turned brown, became necrotic and finally dried as a result of phenolic exudation. The best (100%) result of callus induction was obtained on 2.0 mg/l 2, 4-D in leaf explants followed by node explant (86.5%), petal explant (82%), pistil (78.5%), petiole (75.3%) and anther explants (75.1%) respectively on the same hormonal concentration (Fig1a-f:Fig 2a:fig 2h). Root explant did not responded for the callus induction on any concentrations of 2,4-D.

MS+NAA

In 1.0-2.5mg/l of NAA, meagre to moderate callus was produced on leaf, petiole and node explants within 15-25 days and no response was observed on sepal, petal, anther and ovary explants. Furhter increase in NAA concentrations did not induce any type of response and the explants dryed on the same medium.

MS+IBA

Explants produced rhizogenic calli with white hairy roots after 15--20 day, when inoculated on medium supplemented with 0.5- 1.5 mg/l IBA.IBA1.0 mg/l was found to be most suitable concentration for callus production in all explants including roots (Fig 1.g). However, the best percentage response (67%) of callus induction on IBA 0.5mg/l was observed in petal explants followed by anther (52.3%) and roots (51%) respectively. It was also observed that the white hairy roots grew on all callus masses with more such roots on anther explant. (Fig 2.e-g)

Callus production from different explants using cytokinins (BAP: Kn) in combination with auxins (2, 4-D /NAA/ IAA/ IBA)

For callus induction different explants when inoculated on MS medium fortified with cytokinins (BAP and Kn) in combination with auxins (2, 4-D /NAA/ IAA/ IBA) produced the results only in the following media concentrations (Table1.b)

Table 1.b: Effect of cytokinin (BAP: Kn) in combination with auxins (NAA/IBA) on callus production from various explants of *L. cashmeriana*

BAP mg/l	Kn mg/l	NAA mg/l	IBA mg/l	Callus formation after 30 days of culture						
				leaf	petiole	Node	Root	anther	Petal	pistil
0.5		1.0	_	**Callus	*Callus	**Callus	0	0	**Callus	**Callus
		1.5	_	*Callus	*Callus	**Callus	0	0	*Callus	*Callus
		2.0	_	*Callus	Callus	*Callus	0	0	*Callus	*Callus
1.0		0.5	_	0	0	0	0	*Callus	*Callus	*Callus
		1.0	_	***Callus	**Callus	**Callus	0	***Callus	***Callus	**Callus

		1.5	_	**Callus	**Callus	**Callus	0	0	0	0
0.5		_	0.5	**Callus	**Callus	**Callus	**Callus	**Callus	**Callus	**Callus
		_	1.0	***Callus	**Callus	**Callus	**Callus	**Callus	**Callus	**Callus
		_	1.5	*Callus	*Callus	*Callus	*Callus	0	0	0
		_	2.0	0	0	0	0	0	0	0
1.0		_	0.5	**Callus	**Callus	**Callus	**Callus	0	0	0
		_	1.0	*Callus	*Callus	*Callus	*Callus	0	0	0
		_	1.5	Callus	Callus	*Callus	*Callus	0	0	0
		_	2.0	0	0	0	0	0	0	0
1.5		_	0.5	**Callus	**Callus	**Callus	0	0	0	0
		_	1.0	-Callus	-Callus	0	0	0	0	0
		_	1.5	-Callus	-Callus	0	0	0	0	0
	1.0	0.5	_	**Callus	0	**Callus	0	0	0	0
	1.5		_	*Callus	0	*Callus	0	0	0	0
	1.5	1.0	_	**Callus	0	***Callus	0	0	0	0
	2.0		_	*Callus	0	*Callus	0	0	0	0

***' Intense, (75-100%) '**' Moderate, (50-75%) '*' Meager (25-50%) '-'callus (below 25%)
Number of replicates 30

BAP+ NAA

Out of different Bap + NAA (0.2-3.0 mg/l) combinations callus was produced on BAP (0.5mg/l) + NAA (1.0-2.0mg/l) within 19-27 days in leaf, petoile, node, petal and ovary explants. (Fig 2.b-c:Fig 2 i-l) On BAP 1.0mg/l + NAA (0.5-2.0mg/l) combinations, callus formation occurred in all explants except root. The best percentage response of callus induction, using BAP + NAA combination was obtained on BAP[1.0mg/l + NAA (1.0mg/l] in leaf explants(83.5%) followed by petal (79.2%), petiole (71%), node (74%),ovary(61%) and stamen(39.8%) on the same hormonal concentration.

BAP + IBA

Callus with white hairy roots was formed within 30 days on almost all the explants in BAP(0.5mg/l) + IBA(1.0mg/l) combinations where as in rest of the combinations BAP[0.5-1.5mg/l) + IBA(0.5-2.0mg/l], callus was produced in explants from vegetative parts (Leaf, petiole and node) only. Best response of callus induction was produced in BAP (0.5mg/l) + IBA (1.0mg/l) by leaf explants (76.3%), followed by node (69.3%), petal (65.5%), pistil (59.1)%, petiole (55%), anther(53.5%) and root (51.7%) respectively. It was also observed that potentially organogenic callus masses were produced in leaf, node and petal explants in various BAP + IBA combinations and they could regenerate indirect shoots in many cultures when incubated for more than five weeks. Calli from the other explants proliferated further without any organogenic response.

Kn+NAA

Only leaf, and node explants responded to Kn + NAA combinations, where in callus was produced within 25 days of inoculation on Kn (1.0-2.0mg/l) + NAA(0.5-1.0mg/l) with best callus response of (76.3%) shown by node explant followed by leaf (71.5%) when inoculated on Kn [(1.5 mg/l) + NAA(1.0mg/l)](Fig 2d).

Callus proliferation

For callus proliferation, healthy calli from different explants, irrespective of their origin, were sub-cultured on the media that induced the best results on callus production, viz., (1.5-2.0) mg/l 2, 4-D, (1.5-2.0 mg/l) NAA,(1.0 mg/l) IBA,,(1.0 mg/l) BAP + NAA (1.0-1.5 mg/l),(1 BAP(0.5mg/l) + IBA(1.0mg/l) and(1.0-1.5mg/l) Kn + NAA(1.0mg/l). Generally, callus that was sub-cultured on growth media with (1.0 mg/l) BAP + NAA (1.0 mg/l) showed higher proliferation of friable callus (90-100%) as compared to other treatments. However, browning of the callus occurred after three cycles of sub-culture. Maintaining 2, 4-D concentration at 2.0 mg/l produced the best result whereas increasing concentration to 2.5 or 3.0 mg/l resulted in 50% and 40% callus proliferation respectively. This treatment (2.0 mg/l 2, 4-D) did not cause browning at all, while inducing a highest callus proliferation rate of 70%-95%.

Callus growth habits

Callus masses from different explant cultures were investigated for their growth habits to determine their correlation with organogenesis for micropropagation purposes. During the present study friable and compact calluses were recognised. These varied both in colour and texture and were categorised as compact and friable calli. Physically, the friable calli were very soft, brittle, cream or yellow in colour and watery. The compact calli on the other hand, were hard, white or light green in colour and required some pressure to break into pieces. In all tested concentrations of 2-4,D, IBA and NAA + Kn cultures, 100% friable calli were obtained for all the treatment combinationscv (Fig.2: a, d, g-h). Callus was generally watery and sticky on 2-4, D treatments and with no signs of organogenesis. With respect to NAA + BAP cultures various treatment combinations formed varying degrees of both compact and friable calli (Fig.2: b, c, e-f, i-l). The colour of various degrees of greening were also observed in some of the compact calli obtained on leaf, petiole and node explants (both compact and friable) showing signs of organogenesis. The friable callus in NAA +Kn cultures also developed green and white spots. Thus, greening (a sign of organogenesis) was not associated with a particular type of growth habit.

Table 1.c: Effect of different plant growth regulators on callus growth habits

Hormone Concentration mg/l	Regeneration of callus (Days)	callus proliferation intensity	Callus colour	Morphology of callus
MS		-	Yellow	Brittle, Friable callus
0.5 2-4,D	10/14	-	Creamy yellow	soft, Friable callus
1.0 2-4,D	8/13	-	Creamy yellow	soft, Friable callus
1.5 2-4,D	8/14	+++	Creamy yellow	Watery, soft, Friable callus
2.0 2-4,D	12/14	+++	Creamy yellow	Watery, soft, Friable callus
2.5 2-4,D	12/14	-	Yellowish brown	Brittle Friable callus
3.0 2-4,D	9/15	-	Dark brown	Brittle Friable callus
1.5 NAA	21/23	++	White	Compact callus
2.0 NAA	19/22	++	White	Compact callus
2.5 NAA	15/20	-	yellow green	granular Friable callus
0.5 IBA	22/25	-	Yellow	Watery, Friable callus
1.0 IBA	20/23	++	Yellow	Watery, Friable callus
1.5 IBA	20/25	-	Yellow	Watery, Friable callus
0.5 BAP+1.0 NAA	20/22	++	Yellow with white spots	granular Friable callus
0.5 BAP+1.5 NAA	25/27	-	Brownish Yellow with White spots	granular Friable callus
0.5 BAP+2.0 NAA	26/27	-	Brownish Yellow with White	granular Friable callus
1.0 BAP+0.5 NAA	23/26	-	Brown and green	Compact callus
1.0 BAP+1.0 NAA	19/22	+++	Brown and green	granular Friable callus
1.0 BAP+1.5 NAA	22/23	++	yellow green	granular Friable callus
0.5 BAP+0.5 IBA	25/27	-	Creamy yellow	soft, Friable callus
0.5 BAP+1.0 IBA	23/25	+++	Creamy yellow	soft, Friable callus
1.5 BAP+1.5 IBA	24/26	-	Creamy yellow	
0.5 Kn+0.5 NAA	23/28	-	Yellow	Granular Friable callus
1.0 Kn +0.5 NAA	29/30	++	Yellow	Granular Friable callus
1.5 Kn +1.0 NAA	26/27	++	Brownish yellow with white spots	Granular Friable callus
2.0 Kn+ 1.0 NAA	19/28	-	yellow with white spots	Granular Friable callus

+++' Intense, (75-100%) '++' Moderate, (50-75%) '+' Meager (25-50%) '-'callus (below 25%)
Number of replicates 30

Shoot regeneration

The regeneration of adventitious shoots with varying shoot number and size, was observed on callus masses from leaf, node and petal explant 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, node and petal calli, when inoculated on the same regeneration medium. The responses of the callus masses to shoot regeneration medium is given as below.

Shoot regeneration from leaf callus using BAP in combination auxins (2, 4-D /NAA/ IAA/ IBA)

For shoot regeneration the leaf calli when inoculated on MS medium fortified with BAP (0.5mg/l-3.0mg/l)alone or in combination with Kn (0.5mg/l-3.0mg/l) and BAP in combination with auxins (2, 4-D /NAA/ IAA/ IBA) regenerated shoots only in the following media combinations (Table 2.a).

MS +BAP

Shoots of almost same length regenerated after 45 days on green nodular callus derived from in leaf explants in 1.0-1.5 mg/l of BAP supplemented medium. The number of shoots decreased with increase in concentration of BAP up to 2.0 mg/l but the length of shoots did not vary significantly. Above this level BAP did not induce any response. The maximum (8) number of shoots were recorded at 1.5 mg/l of BAP.

BAP + NAA

Leaf callus cultures in 1.0 mg/l BAP combined with 0.5 mg/l of NAA initiated shoot formation in 75% of the cultures after about 6 weeks. The number of shoot increased as the concentration NAA was increased to1.0mg/l and decreased with further increase in NAA. The maximum number of shoots (23) regenerated on medium fortified with 2.0 mg/l of BAP+ NAA1.0 mg/l (Fig 3 c). Any further increase in concentration of BAP resulted in decrease in number of shoots and increase in the concentration of NAA (1.5 mg/l) resulted in browning of cultures leading to their death. Maximum (3.46 cm) shoot length was observed 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.

BAP + IBA

The various combinations of BAP with IBA induced the formation of green friable callus masses on leaf explants in BAP(0.5-1.5mg/l) + IBA(0.5-2.0mg/l) within 30 days, 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 supplemented medium formed about 2.00 cm long shoots buds within 15-25 days with an maximum (6) number of shoots in about 73% of the cultures.

Shoot regeneration from leaf callus using Kn in combination auxins (2,4-D /NAA/ IAA/ IBA)

Kn +NAA

Shoot initiation was observed on green nodular calli developed from leaf explant, after fourth weeks of inoculation on combinations of Kn,(0.5-2.0 mg/l) and NAA(0.5-1.0 mg/l) supplemented medium (Fig 3d). Over a period of 7 weeks, 63% of leaf calli produced shoots, with a highest (9) number of shoots per explant in the medium containing 2.0 mg/l Kn and 1.0mg/l NAA while as the largest shoots with maximum shoot length 4.48 cm were obtained on 1.5 mg/l Kn and 0.5 mg/l NAA.

Table 2.a: Effect of BAP and Kn either alone or in combination with NAA/IBA/IAA on shoot regeneration from leaf callus of *L. Cashmeriana* (field grown)

BAP mg/l	Kn mg/l	IBA mg/l	NAA mg/l	%Culture response	Mean number of days taken for shoot regeneration	Average number of shoots(cm)	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
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	14.8	2.00
	-	-	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

Shoot regeneration from node callus using BAP alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA)

For shoot regeneration the node calli when inoculated on MS medium fortified with, BAP:Kn (0.5mg/l-3.0mg/l)alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA) regenerated shoots only in the following media combinations (Table 2.b).

MS+BAP

Multiple shoots were regenerated on calli from node explants, after about eight weeks of culture in 55 % cultures at 1.0-1.5mg/l of BAP. The maximum (5) number of shoots was observed in this range of concentration with maximum shoot length of 2.98 cm. The number of shoots decreased with increase in concentration of BAP upto to 1.5mg/l and further increase in BAP concentration did not induce any response.

BAP +NAA

The callus masses from nodal segments formed multiple shoots after about six weeks of culture, at BAP1.0-1.5mg/l, keeping the concentration of NAA constant at 1.0 mg/l (Fig 3a).No significant difference in the number and size of shoots was observed.

BAP +IBA

Callus cultures of nodal segments in different concentration and combinations of BAP +IBA developed multiple shoots and the maximum shoots were produced in the medium containing 2.0 mg/l BAP + 0.5 mg /l IBA, over a period of 7 weeks, in 54 % of cultures, with maximum(13) number of shoots. The size of shoots was observed to vary insignificantly. At higher concentrations of IBA (1.0-2.0) callus was produced only.

Shoot regeneration from node callus on Kn alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA)

For shoot regeneration the node calli when inoculated on MS medium fortified with Kn alone or in combination with auxins (2, 4-D /NAA/ IAA/ IBA) produced the results only in the following media combinations

MS+KN

Callus produced from nodal segments on MS medium supplemented with different concentration of Kn (0.5-2.5mg/l), formed multiple shoots within three to four weeks on 0.5-1.5 mg/l of Kn. The number and size of shoots decreased with increase in concentration of Kn with the highest number (7.2) and size (2.19cm) obtained at 1.0 mg/l in 38% calli. Further increase in Kn concentration resulted in callus regeneration only.

Kn +NAA

Callus induced in nodal segments on different concentrations and combinations of Kn(1.0-2.0) mg/l + NAA (0.5-1.0 mg/l), on further subculture on the same concentrations resulted in formation of multiple shoots, within six to seven weeks of culture at Kn(1.0-2.0) mg/l + NAA (0.5-1.0 mg/l) and maximum number(6.35) of shoots were produced on Kn 1.0 mg/l + NAA0.5 mg/l. Decreased trend of shoot formation was observed at Kn 1.5 mg/l with only callus differentiation on further increase in Kn concentrations. An increase in the concentration of NAA to 1.0mg/l resulted in few shoot buds which did not increase in size

Table 2.b: Effect of BAP and Kn either alone or in combination with NAA/IBA/IAA /2, 4-D on shoot regeneration from node callus of *L. Cashmeriana* (field grown)

BAP mg/l	Kn mg/l	IBA mg/l	NAA mg/l	%Culture response	Mean number of days taken for shoot regeneration	Average number of shoots	Average length of shoots(cm)
0.5		-	-	0	0	0	0
1.0		-	-	55	56	3.30	2.82
1.5		-	-	45	59	2.14	2.82
2.0		-	-	0	0	0	0
1.0		-	1.0	42	42	2.52	2.14
1.5		-	1.0	75	42	2.49	2.00
2.0		0.5	-	42	50	11.97	2.82
	0.5	-	-	47	41	3.33	1.97
	1.0	-	-	38	27	7.2	2.19
		-	0.5	45	50	6.35	1.37
		-	1.0	43	47	3.76	1.39
	1.5	-	-	59	32	4.64	1.87
		-	1.0	39	36	1.93	1.14
	2.0	-	1.0	50	42	1.67	1.09

Number of replicates 30

Shoot regeneration from petal callus using cytokinin (BAP) alone or in combination with auxins (2, 4-D /NAA/ IAA/ IBA)

For shoot regeneration the node calli inoculated on MS medium fortified with cytokinin (BAP) alone or in combination with auxins (2,4-D /NAA/ IAA/ IBA) responded only in the following media combinations (Table:2c)

BAP

Callus induced on petal explant in MS medium containing cytokinin (BAP 1.0-2.0 mg/l) was able to develope adventitious bud differentiation on BAP 2.0 mg/l in 65% cultures. The callus proliferated profusely on subculture in the same medium with the production of maximum (6) number of shoots with maximum (3.86cm) length. BAP concentration below 1.0 mg/l had no impact and above2.0 mg/l resulted in callus production only.

BAP +IBA

BAP (1.0-2.0 mg/l) with IBA (0.2 -0.5 mg/l) resulted in formation of shoot buds in petal callus within 18-28 days (Fig3b), which on further subculture on BAP 2.0 mg/l +0.5 mg/l proliferated with the production of roots and shoots in 60% of callus masses within 28 days of culture with maximum(7) root number and root length (3.30cm) on the same treatment. Further increase in IBA concentrations resulted in callus browning with necrotic patches.

Table 2c: Effect of BAP either alone or in combination with IBA on shoot regeneration from petal explant callus of *L. Cashmeriana* (field grown)

BAP mg/l	IBA mg/l	%Culture response	Mean number of days taken for shoot/root regeneration	Average number of shoots(cm)	Average length of shoots(cm)	Average number of roots	Average length of roots(cm)
1.0	-	65	38	4.20	0.60	0	0
	0.2	40	35	2.44	0.54	4.34	2.03
	0.5	30	35	2.91	0.16	3.21	2.46
1.5	0.2	33	29	1.72	0.33	2.00	2.07
	0.5	30	21	1.45	0.45	2.23	1.98
2.0	0.5	60	41	4.62	0.86	4.65	2.72

Number of replicates 30

Shoot multiplication

For shoot multiplication *in vitro* raised shoot clumps from leaf, node and petal cultures were separated and transferred on MS medium fortified with cytokinin (BAP and Kn) alone or in combination with auxins (2, 4-D /NAA/ IAA/ IBA).The shoot buds regenerated from petal callus did not produce any morphogenic response, instead the green buds lost their colour and deteriorated in few days. Rest of the shoot clumps produced the results only in the following media combinations (Table 3)

BAP +IBA

Multiplication of shoots, from leaf and node shoot buds, was observed on MS medium supplemented with BAP (1.0-2.0mg/l)+ IBA (0.5-1.0mg/l)(Fig4a,d).Maximum(26) number of shoots with maximum length (4.48cm) regenerated on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IBA. The increase in the concentration of BAP caused the browning and ultimately the death of shoots while as further increase in IBA concentration resulted in more callus formation.

BAP +NAA

Various combinations of NAA with BAP also promoted shoot multiplication and elongation, however, the shoot numbers was observed to be significatly less than BAP+IBA combinations with maximum (13) number and length (2.20cm) observed on 2.0 mg/l of BAP+ NAA1.0 mg/l (Fig4b).

Kn+NAA

Little response of shoot multiplication was observed in Kn (1.0 -2.0mg/l) +NAA (0.5-1.0mg/l) combinations with maximum (8) number of shoots regenerated on 1.5mg/l Kn +.0.5 mg/l NAA) (Fig4c).

Table 3: Effect of BAP + Kn and BAP or Kn in combination with NAA/IBA on shoot multiplication

BAP mg/l	Kn mg/l	IBA mg/l	NAA mg/l	%Culture response	Mean number of days taken for shoot multiplication /elongation	Average number of shoots	Average length of shoots(cm)
1.0		0.5		46	25	8.5	1.53
1.5				47	27	13.19	1.72
2.0				32	29	21.6	3.23
1.0		1.0		42	36	6.52	2.25
1.5				50	36	6.49	2.12
2.0				66	38	6.37	2.61
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	3.97	2.49
	1.5			59	44	7.43	2.12
	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

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.

Rooting

Once the shoots (3-4 cm in length) developed 4–5 leaves, they were transferred to the rooting medium, i.e. half and full salt strength MS basal medium alone and in combination with IAA, IBA and NAA. The microshoots developed roots only in IBA (1.0-1.5 mg/l) (Fig. 5:d-e) and NAA(1.0 mg/l) (Fig. 5:a-c) treatments while as no response was observed on IAA treatments. The best rooting was observed on ½ MS medium +1.5 mg/l IBA with maximum (6) number of roots and maximum root length (2.32 cm) obtained in 62% of micro shoots (Table 4).

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

MS basal salt	NAA mg/l	IBA mg/l	% Response	Mean Number of days taken for rooting	Average Number of roots	Average Length of roots
Full strength	0	0	40	24	3.69	1.73
	1.0	0	33	20	1.43	1.00
	0	1.0	0	0	4.11	1.89
	0	1.5	0	0	6.35	2.22
Half strength	0	0	70	27	3.93	1.98
	1.0	0	59	22	3.99	1.47
	0	1.0	45	27	6.75	2.04
	0	1.5	62	29	8.48	2.31

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 plant lets were transferred to a mixture of sand: soil: vermiculite (1:1:1) in the greenhouse conditions. Finally the hardened plantlets were transferred to field where they survived with a frequency of 68%. The *in vitro* regenerated plants were observed to be morphologically similar to wild plants. (Fig 5f-h)

Discussion

Results of the present study provide evidence that, each and every plant part has the capacity to regenerate and the exogenous application of PGRs induces callus in various plant parts *in vitro*. The varied responses of various explants (root, leaf, node, petiole, petal, anther and ovary) regarding callus induction as presented in Table 1, reflect the existence of a large inter-explant variability in producing any *invitro* response (Zouine and El-Hadrami, 2004; Dhar and Joshi, 2005; Zouzou *et al.*, 2008) [47, 8, 48].

The process of callus regeneration at the basal end of explants particularly in floral explants as observed by us, indicates that morphogenetic processes at the basal parts is probably due to associated meristems in these parts. A similar course of regeneration at the bases of flower parts was also observed in petal cultures of *Dianthus caryophyllus* (Kakehi 1979; Simard *et al*. 1992) [18] and *Araujia sericifera* (Torne *et al*. 1997), in stamen cultures of *Brassica campestris* (Choi *et al*. 1998) [7] and in pistil cultures of *Citrus deliciosa* and *C. tardiva* (Carimi *et al*. 1999) [6]. The formation of callus can be attributed to the action of endogenous auxin accumulated on the cutting edge of the baseline, which stimulates cell proliferation, especially in media enriched with cytokinins (Affonso *et al*., 2007) [2].

The best (82%) callus response observed on petals confirms the superiority of petal explant (highest regeneration potential) over anther and pistil. Similar results have been reported by Miller *et al.* 1991 [24]; Nugent *et al.* 1991 [30] and

Wojciechowicz (2007) [44] while investigating the regeneration potential of different explants from flower buds (petals, stamens and pistil). Petals of one carnation cultivar regenerated plants from 100% of explants (Nugent *et al*. 1991) [30].

So far as the effect of growth regulators on callus induction is concerned, auxin and cytokinin have been by far the most extensively used and studied hormones in the context of callus formation and subsequent organ regeneration (Akiyoshi *et al*. 1983). During the present study, It was observed that 2, 4-D was the most effective hormone for callus induction with 2.0 mg/l of 2, 4-D, as the optimum concentration for best callusing. Another auxin found effective in callus formation next to 2, 4-D was NAA. The effectiveness of 2, 4-D and NAA in inducing callus, might be due to their role in DNA synthesis and mitosis as reported by Samanthi (2013) [38] in *Hibiscus cannabis*. 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 was successful for callus formation. The reports of Memon (2010) [23] are also in accordance to our results who achieved the induction of callus in anther and ovule culture in upland cotton (*Gossypium hirsutum* L.) using various auxin concentrations with best results in 2.4-D.

In our study two types of calli were recognised (compact and friable callus) and their formation was dependent over a range of auxin- cytokinin combinations of the culture medium. The marked structural differences observed between the friable and compact calli indicates that the loosely arranged nature of friable calluses may have been the reason for their more water retention capacity and hence very soft, and watery texture and the hard texture of compact calluses on the other hand, indicates their tightly arranged nature (Reddy and Narayana (1974). Friable calli obtained in 2, 4 D treatments showing no signs of organogenesis is in accordance with the callus formation in *Hibiscus rosa sinensis* with no organogenesis observed in explants inoculated on medium containing 2, 4-D (Kumari 2011) [21]. Calli (both friable and compact) obtained in other PGR combinations developed green colour in some compact calli showing signs of organogenesis. This superiority of compact calluses in inducing organogenesis has been reported earlier (Zamora *et al*., 1987) [46] and is attributed to cell aggregation and integrity favouring chloroplast formation (George and Sherrington, 1984) [11].

In accordance to the world literature, BAP in different concentration and combination with auxins was observed to be most effective hormone for shoot regeneration and the best response for shoot production was recorded from leaf and node explants on different concentration and combination of BAP (1.0-2.0 mg/l) in combination with auxins NAA and IBA. The combined effect of higher concentration of cytokinin with lower concentration of auxin induced better shoot formation, was reported by various workers in different plant species of family *Malvaceae* (Rudra and Juwarkar, 2002., Mishra *et al.,* 2004 and Vidya *et al*., 2005) [41, 25, 37].

The superiority of BAP over Kinetin in shoot formation as observed in our study, has earlier been reported by Bajaj *et al*.(1986) in *G. arboreum* and *G. hirsutum*; Gupta *et al*. (1997) [12] in *G. hirsutum*; Bhalla *et al*. (2009) [5] in *Hibiscus rosa sinensis* and is attributed to group localized at N6 position of this cytokinin Murashige (1974) [29]. Our results disagree with Troncoso *et al.* (1997) [41] who reported the death of explants of *L. maritama* on MS fortified with BAP and Munir *et al*., 2012 who reported only callus formation in *Althea rosa* using BAP.

The addition of cytokinins (BAP or Kn) to the regeneration medium increased the *in vitro* shoot height up to certain concentrations only and further increase in the concentration of these PGRs declined or deterioted the shoot growth. These results are similar to the reports of Wareing & Philips (1981) [43] and Hu Wang (1983) [15], 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.

So far as regeneration potential of leaf explant is concerned, our results are contradictory to the findings of Parveen (2012) [31] who reports low regeneration potential of field grown explants especially leaf explants. However our study is supported by Samanti *et al., (*2013) who, by obtaining *in vitro* shoot regeneration from leaf explant of *Hibiscus cannabinus* established that vegetative plant parts especially leaves are desirable explants for *in vitro* propagation because regeneration from these explants would preserve the genetic homozygosity of the parent genotype.

Conclusion

This experiment was basically focused on the study of callus formation and subsequent morphogenesis using different explants and PGRs. From the various experiments we could conclude that each and every part of a plant has the capacity to regenerate *in vitro* and vegetative plant parts especially leaves and nodes are desirable explants for *in vitro* culture and propagation of *L. cashmeriana*. Through the study we have been able to standardize an efficient reproducible plant regeneration protocol for micropropagation of *L.cashmeriana* using leaf explant, collected from the field grown plants and the most suitable plant growth regulator for achieving maximum shoot formation from this explant was BAP 2.0mg/l+1.0 m/l NAA. Additionally, the ability of petal explant to undergo indirect organogenesis seems to be a valuable material for further studies, especially for highlighting the ornamental value of *L.cashmeriana* in addition to its medicinal use.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Akiyoshi DE, Morris RO, Hinz R, Mischke BS, Kosuge T, Garfinkel DJ, et al. Cytokinin/ auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proceedings of National Academy of Science USA. 1983; 80:4007-411
2. Affonso VR, Bizzo HR, Lima SS, Esquibel MA, Sato A. Solid Phase Microextraction (SPME) analysis of volatile compounds produced by *in vitro* shoots of *Lantana camara* L. underhe influence of auxins and cytokinins. J Braz. Chem. Soc. 2007; 18:1504-1508.
3. Ayadi A, Hamrouni L, Hanana M, Bouzid S, Trifi M, Khouja L. *In vitro* propagation and regeneration of an

industrial plant kenaf (*Hibiscus cannabinus* L.) Industrial crops and products. 2011; 33:474-480
4. Bajaj YPS, Manjeet S, Gill M. Micropropagation and germplasm preservation of cotton (*Gossypium* spp) through shoot tip and meristem culture. Indian Journal of Experimental Biology. 1986; 24:581-583.
5. Bhalla S, Abdullah J, Sreeraman S, Karuthan C. Shoot induction from Hibiscus rosa sinensis nodal explants using N6 –benzylaminopurine (BAP). Research Journal of Agriculture and Biological Sciences. 2009; 5(4):403-410
6. Carimi F, Pasquiliqui F. Somatic embryogenesis and plant regeneration from pistil thin layers of citrus. Plant cell, tissue, organ culture. 1999; 54:183-185
7. Choi PS, Min SR, Ahin MM, Liu JR. Somatic embryogenesis and Plant regeneration in immature zygotic embryo, filament and another culture of Chinese cabbage. Scientia Horticulture. 1998; 72:152-155.
8. Dhar U, Joshi M. Efficient plant regeneration protocol through callus for Saussurea obvallata (DC.) Edgew (Asteraceae): effect of explant type, age and plant growth regulators. Plant Cell Reporter. 2005; 24:195-200.
9. Endangered species from Grazalema Natural park. Lagascalia. 19(1-2):703-710.
10. Ganai AH, Teli BA, Rathar AM. An Ethanobotanical study in Budgam district of Kashmir Valley. International research Journal of Pharmacy. 2013; 4(1):2230-8407
11. George EG, Sherrington PD. Plant Propagation by Tissue Culture. Handbook and directory of commercial operations Exegetics Ltd., Basingstoke. 1984, 709.
12. Gupta SK, Srivastava AK. Singh PK, Tuli R. *In vitro* proliferation of shoots and regeneration of cotton. Plant Cell Tissue Organ Culture. 1997; 51:149-152.
13. Hamid H. Phytochemical investigation of some plants used in Indian system of Medicine. Ph.D. Thesis Jamia Hamdard University, New Delhi, 2002.
14. Haque MS, Biswas T, Islam MS, Hossain MS. *In vitro* regeneration and PCR-RAPD based detection of somaclonal variation in Kenaf (*Hibiscus cannabinus*). Progressive Agriculture. 2017; 28(2).
15. Hu CY, Wang PJ. Handbook of plant cell culture. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds.) Meristem shoot tip and bud culture. New York, Macmillan. 1983, 177-227.
16. Ibrahim AM, Kayat FB, Susanto D, Ariffullah M, Kashiani P. Callus Induction from Ovules of Kenaf (*Hibiscus cannabinus* L.). Biotechnology. 2015; 14:72-78
17. Jeelani SM, Wani PM, Kumari S, Gupta R, Siddique MAM. Ethnobotany of some polypetalous plants from the Kashmir Himalaya. Journal of Medicinal Plants Research. 2013; 7(36):2714-2721.
18. Kakehi M. Studies on the tissue culture of carnation induction of dedifferentiated plant from petal tissue. Bulletin of Hiroshima Agricultural College. 1979; 6:159-166.
19. Kamal GB. The study of callus induction in cotton (*Gossypium* sp.) under tissue culture conditions. International Journal of Agriculture and Crop Sciences. 2011; 3(1):6-11.
20. Kaul MK. Medicinal plants of Kashmir and Ladakh, Temperate and Cold Arid Himalaya. Indus Publishing Co., New Delhi. 1997, 173.
21. Kumari and Pandey RK. Micropropogation of *Hibiscus rosasinensis*. International Journal of Biosciences. 2010; 6(2):335-337.
22. Kumari AV, Anita Gnana, Palavesam J, Anbu Jeba Sunilson, Anandarajagopal M, Vignesh J. Parkavi. Preliminary phytochemical and antiulcer studies of *Hibiscus rosa-sinensis* Linn. Root extracts. International Journal of Green Pharmacy. 2010, 41-46.
23. Memon S. Induction of Callus through Anther and Ovule Culture in Upland Cotton (*Gossypium hirsutum* L.) World Applied Sciences Journal. 2010; 8:76-79
24. Miller RM, Kaul V, Hutchinson JF, Mahesvaran G, Richards D. Shoot regeneration from fragmented flower buds of carnation (*Dianthus caryophyllus*). Annals of Botany. 1991; 68:563–568.
25. Mishra P, Datta SK, Johri IK, Singh HB, Srivastava A. An improved method for in vitro large scale propogation of piper betle. Plant Biochemistry and Bioechnology. 2004; 13:161-164.
26. Mishra P, Datta SK, Johri IK, Singh HB, Srivastava A. An improved method for in vitro large scale propogation of piper betle. L.J. Plant Biochemistry and Bioechnology. 2004; 13:161-164.
27. Mishra P, Datta SK, Johri IK, Singh HB, Srivastava A. An improved method for in vitro large scale propogation ofmpiper betle. L.J. Plant Biochemistry and Bioechnology. 2004; 13:161-164.
28. Munir M, Hussain A, Haq I, Qureshi R, Munazir M, Arshad M, Leghari MK. Callogenesis potential of cotyledonary explants of Althaea rosea from Pakistan. Pakistan Journal of Botany. 2012; 44:271-275.
29. Murashige T. Principles of rapid propagation in propagation of Higher plants through Tissue Culture (Hugesh SW. Henke, R.R. andm Consantin, M.J. Eds.) Technical Information Centre, U.S. Department of Energy. Springfield 14, 1978.
30. Nugent G. Wardley-Richardson T, Lu CY. Plant regeneration from stem and petal of carnation (*Dianthyus carypylous* L.) Plant cell Reporter. 1991; 10:477-480.
31. Parveen S. In vitro studies on some medicinal plant species of western Himalaya, Ph.D.Thesis Submitted to University of Kashmir, Srinagar, 2012.
32. Pushpa and Raveendran TS. Optimization of shoot apex based cotton regeneration system. Electronic Journal of Plant Breeding. 2010; 1(4):1235-1240.
33. Rakashanda S, Ishaq M, Masood A, Amin S. Antibacterial activity of a trypsin-chymotrypsin-elastase inhibitor isolated from *Lavatera cashmeriana* camb. seeds. Journal of Animal and Plant Science. 2012; 22:983-986.
34. Rakashanda S, Mubashir S, Qureshi Y, Hamid A, Masood A, Amin S. Trypsin inhibitors from *Lavatera cashmeriana* camb. seeds: Isolation, characterization and in-vitro cytoxic activity. International Journal of Pharmacology and Scientific Invention. 2013b; 2:55-65.
35. Rakashanda S, Qazi AK, Majeed R, Rafiq S, Dar IM, Masood A. Anti-proliferative Activity of *Lavatera cashmeriana*-Protease Inhibitors towards Human Cancer Cells. Asian Pacific Journal of Cancer Prevention. 2013a; 14:3975–3978.
36. Reddy, Murthy KBS, Narayana R. Nutritional requirements of Vigna sinensis callus cultures. Physiology Plant. 1974; 32:1-9.
37. Rudra J, Juwarkar AS. In vitro multiplication of Punica granatum Linn. CV GBG – 1 (Ganesh) through exillary bud culture. Advances Plant Science. 2002; 15(1):5-10.
38. Samanthi PW, Puad AM, Suhaimi N, Kumar SM, Noraini NS. *In Vitro* Shoot Regeneration from Leaf

Explants of Kenaf. Annals of Plant Sciences. 2013; 3(1):582-587.
39. Sie G, Charles HF, Sakhanokho Y, Toueix Y, Djei A. Sangare, Branchard M. Protocols for callus and somatic embryo initiation for *Hibiscus sabdariffa* L. (Malvaceae): Influence of explant type, sugar, and plant growth regulators. AJCSI. 2010; 4(2):98-106.
40. Torné JM, Rodriguez P, Manich A, Claparols I, Santos MA. Embryogenesis induction in petals of Araujia sericifera. Plant Cell Tissue Organ Culture. 1997; 51:95-102
41. Troncoso A, Zarate R, Cantos M. Conservation via in vitro propagation of Vidya, S. M., Krishna, V., Nanjumatha, B. K. and Shankarmurthy, K. 2005. Micropropogation of Entada Pursaetha DC – An endangered medicinal plant of Western Chats. Indian J. Biotechnology. 1997; 4:561-564.
42. Vidya SM, Krishna V, Nanjumatha BK, Shankarmurthy K. Micropropogation of Entada Pursaetha DC – An endangered medicinal plant of Western Chats. Indian Journal Biotechnology. 2005; 4:561-56.
43. Wareing PF, Phillips IDJ. The control of Growth and differentiation in plants. Pergamon Prees. New York. Ed. 3rd, 1981.
44. Wojciechowicz MK. Comparison of regenerative potential of petals, stamens and pistils of five Sedum species *in vitro*. Biodiversity, Research and Conservation. 2007; 8:87-94.
45. Zahoor A, John S, Shukla PK, Nissar S, Misra P. Optimization of phytohormone combinations for *in vitro* callogenesis in *Lavatera cashmeriana*: An endemic medicinal plant of Kashmir. International Journal of plant sciences. 2016; 11(1):93-97.
46. Zamora AB, Gruezo SS, Damasco OP. Callus induction and plant regeneration from internode tissue of Dendrocalamus latiflorus cv. Machiku. In Rao AN, Yusoff AM (eds): Proceedings of the seminar on tissue culture of forest species. 15-18th June, 1987, Kuala Lumpur. pp 76-82. Forest Research Institute, Malaysia and International Development Research Centre, Singapore, 1987.
47. Zouine J, Hadrami I. Somatic embryogenesis in *Phoenix dactylifera* L.: Effect of exogenous supply of sucrose on proteins, sugar, phenolics and peroxydases activities during the embryogenic cell suspension culture. Biotech. 2004; 3:114-118
48. Zouzou M, Kouakou TH, Koné M, Amani NG, Kouadio YJ. Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.) Australian Journal Crop Science. 2008; 2(1):1-9.