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In vitro studies in *Arnebia hispidissima Arnebia hispidissima* callus culture; the initiation, growth and organogenesis of callus derived from various explant sources

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Abstract
Callus was obtained from internode segments, shoot meristem and axillary buds *of Arnehia hispidissima*. *2.5* mg/1 2,4-D containing MS medium proved to be the best among different concentration of 3 different growth regulators, used for callus induction. Shoot meristem showed significantly highest callusing (80%) with earliest callus initiation (8 days) among internode (75%, 10 days) and axillary bud (35%, 12 days). No callus could be obtained from leaf segments. Calli derived from these explants were successfully maintained by subculture on the same medium. After 25 days of callus induction, calli were transferred to MS medium supplemented with different growth regulators in 32 different combinations for regeneration. Highest regeneration was obtained on MS medium supplemented with 1.5 mg/1 kinetin + 2.5 mg/1 NAA in shoot meristem (79.2%) and axillary buds (32.5%), while highest regeneration (73.4%) was obtained on 2.0 mg/1 BAP with 0.5 mg/1 NAA containing MS medium in internode cultures.

Keywords: *Arnebia hispidissima, in vitro* culture, shoot meristem, intemode, axillary bud and leaf

Introduction
Arnebiahispidissima, a medicinal plant belonging to family of Boraginaceae, is a herbaceous weed of 25-30 cm height and found throughout India in dry and semi arid areas [1]. *A. hispidissima* has tremendous medicinal value for its use in the treatment of tongue and throat ailment in Indian traditional medicine [2]. Vitexin, a flavanoid, isolated from *A. hispidissima* showed potent hypotensive, anti-inflammatory and anit-spasmodic properties [4]. Phytochcmical constituents of the plant like *Shikonin* and *Arnebin* exhibit anticancerous, antimicrobial and antifungal activities, tested against Gram positive and Gram negative bacteria and fungi [2, 3]. Shikonin has a high value both for its colouring properties as natural red dye are root specific secondary metabolite of A.hispidissima [11-12] and medicinal as crude drug [5] with the magic property of accelerating wound healing in ulcers and burns [6]. Shikonin is permitted for use as dye in food stuf by Government of India [7] isolated from *Arnebia* roots. In addition it has wide applications as a colorant in cosmetics, food and textile industries [13]. The magenta dyes in the roots of the *A. hispidissima* (Arabian primrose) were also used by women as dyes and pastes to enhance their natural colours or accentuate their features. Plants must grow for five to seven years before shikonin concentration in their roots reaches one to two per cent, therefore, an efficient source, from the plants unable to meet demands. These difficulties of supply for this valuable natural product motivated research to produce it from plant cell cultures. As un differentiated callus tissues *of A. hispidissima* are capable of synthesizing shikonin which are normally formed in the root cells [6], it was left to biotechnologists to develop plant cell culture dependent manufacturing process. Improvement of the culture process in *Arnebia hispidissima* was the subject of intense research but very little work has so far been done on this plant. To develop a reliable and efficient protocol by using different explants and growth regulators in different combinations is the major motive of present study.

Material and Methods
Arnebia hispidissima plants were collected from the campus of Guru Jambeshwar University, Hisar. Internode segment of 4th to 6th from tip to branch, axillary buds of 3rd-4th leaf and shoot meristem of young parts of plants were excised. All the explants were washed with detergent labolene (0.5%) followed by running tap water,

These were then sterilized with 0.1% aqueous $HgCl_2$, for 4-6 minutes and subsequently washed three times with sterile double-distilled water to remove traces of $HgCl_2$. The surface sterilized shoot, leaf and internode explants were cut into small segments of 0.5-1.0 cm while axillary buds were inoculated as a whole. 4-6 segment of each explant were inoculated in each flask, containing MS basal medium with three different auxins (IAA, 2, 4-D and NAA) at different concentration for callus induction. Two media solidifying substance agar and guargum in all combinations were tested in present experiment. But agar was found better solidifying agent than guargum so further only agar were used as binding agent in all the media. Callus developed from all the explants but no callus was obtained from leaf segments. Callus was subcultured twice, after a week interval on same medium. After 25 days of callus induction, calli were transferred on MS media for regeneration.

Culture media and conditions of incubation

The basic culture medium was that of MS [8] supplemented with three different growth regulators in different concentrations for callus induction while 32 combinations of different growth regulators for regeneration media were used. All the cultures for callusing and regeneration both were kept in a culture room under a continuous light and a temperature of 22±2°C. All the media were adjusted to pH 5.8±1°C before autoclaving.

Statistical analysis

About 90 explants of each explant were used on each 9 callus induction media for callus induction in three replication and 30 explants were considered an unit of replication. Data were analysed by using completely Randomized Design (CRD) after angular transformation of the data.

Results and Discussion

Highest callus induction (80%) with earliest callus initiation (8 days) took place in shoot meristem among internode (75%, 10 days) and axillary buds (35%, 12 days) on MS medium supplemented with 2.5 mg/1 2,4-D, followed by 2.0 mg/12,4-D containing medium (50%, 8 days) and 2.0 mg/1 IAA (50%, 10 days) in shoot meristem (Table 2). In internode it was followed by 2.0mg/l 2,4-D (58%, 9 days) and 1.0 mg/1 IAA containing medium (35%, 14 days) while in axillary buds it was followed by 1.0 mg/1 and 2.0 mg/1 2,4-D supplemented medium respectively. However, MS medium supplemented with NAA of different concentration in all explants induced poor callusing. Callus was green, compact and nodular in stem meristem and axillary bud (Fig. 1). While internode form white and nodular callus (Fig. 2). Callus initiation observed from cut region of explant in the begining and gradually extended all over the explants. There was significant differences among explants and growth regulators for callusing as well as for regeneration. Shoot regeneration was observed after 10-12 days in shoot meristem calli, while in internode after 14-16 days and in axillary buds regeneration started after 20-25 days. MS medium supplemented with 1.5 mg/1 kinctin + 2.5 mg/1 NAA was best for regeneration in shoot meristem calli (79.2%), and axillary bud (32.5%), followed by 1.5 mg/1 BAP + 2.5 mg/l IAA and 1.5 mg/1 kinetin + 2.0 mg/1 IAA supplemented MS medium for shoot meristem calli. While for axillary bud 1.5 mg/1 BAP + 2.0 mg/1 IAA and 1.5 mg/1 kinetin + 2.0 mg/1 NAA containing media was good. Internode derived calli showed highest regeneration (73.4%) on 2.0 mg/1 BAP +0.5 mg/1 NAA containing medium followed by 2.0 mg/1 BAP + 1.5 mg/1 IBA (70.8%) and 1.5 mg/1 kinetin + 2.5 mg/1 NAA (69.2%) among 32 different combinations of different growth regulators (Table 3). Formation of leaves and floral buds were observed in shoot meristem calli as well as internode calli (Fig. 3). While formation of large number of creamish yellow flower from cultured axillary buds through organogenesis were also observed (Fig. 4). The present results revealed that shoot meristem proved the best explant among the internode, axillary bud and leaf. Shoot meristem is characterized as dome of totipotent cells. Their juvenile vegetative phase are considered to be the most appropriate explant by Anju Sharma [9] also. Cells of axillary buds also divide from all the sidcsto induce rcgcncrablc callus but in present study it showed poor callusing and regeneration in *A. hispidissima.* /In present study fully developed calli rendered reddish brown colour to the medium due to shikonin secretion because pigments were released from the cells in to culture media in the late culture stage (after three weeks) (Fig. 1). Vassilios [6] also suggested that pigment formation occurred when callus cultures were grown in presence of agar in medium, as effect arose from a triggering of the secondary metabolism by a polysaccharide present in the agar and cultures produced large quantities of shikonin. Thus we can produce shikonin in large amount within short period through tissue culture.

Present study revealed that the medium containing IAA was good for callusing and regeneration of *Arnebia* cultures. Vassilios [6] also reported that the IAA, also stimulated pigment production. Variable response of explanls to different growth regulatorswas reported in the present study. Similar was reported by Gupta [10] in *Capsicum annum.* However, a little work has so far been done on this plant but in present study we developed a reliable plant tissue culture system for *A. hispidissimtifim* elite and rare plant. The fruits of these studies are a highly effective commercial production method of shikonin and other derivates from callus cultures. As in present study we report callus induction up to 80 percent. It is in demand in Japan also. The entire supply of it in Japan had to be imported from china and Korea.

Table 1: Analysis of variance and the mean squares for callus induction and plantlet regeneration from various explants of *A. hispidissima.*

S.O.V.	Callus induction		Regeneration	
	d.f.	M.S.	d.f.	M.S.
Factor A	2	1624.40	2	6364.55
Factor B	8	749.46	31	690.43
Factor AxB	16	76.04	62	277.56
Error	54	12.24	192	2.76

A - Explants
B - Media

Table 2: Callus induction from various explants in A.hispidissima

Auxin mg/1	Internode callusing (%)	Shoot meristem callusing (%)	Axillary bud callusing (%)
IAA 0.5	25 (29.99)	30 (33.11)	15 (22.77)
1.0	35 (36.23)	25 (29.74)	12 (20.21)
2.0	30 (33.20)	50 (45.00)	10 (18.36)
2,4-D 1.0	32 (34.44)	40 (39.21)	30 (33.14)
2.0	58 (49.82)	50 (44.99)	25 (29.98)
2.5	75 (60.42)	80 (63.52)	35 (36.26)
NAA 1.0	33 (35.03)	30 (33.14)	10 (18.40)
2.0	20 (26.52)	20 (26.52)	10 (18.32)
2.5	20 (26.56)	30 (33.18)	15 (22.76)
Total mean	36.11 (36.91)	39.44 (38.71)	18.00 (24.47)

CD
Factor A 1.91
Factor B 3.31
Factor AxB 5.73
Note: Figures in parenthesis are angular transform values.

Table 3: Regeneration frequency of calli derived from different explants of A.hispidissima, on MS medium supplemented with different growth regulators in various combinations

	Medium Code	Kinetin	BAP	IAA	NAA	IBA	Internode	Shoot meristem	Axilliary bud
1.	MS-K_1	1.0	-	-	-	-	22.4 (28.21)	16.8 (23.96)	12.4 (20.61)
2.	MS-K_2	1.5	-	-	-	-	20.5 (26.91)	12.6 (21.34)	15.1 (22.86)
3.	MS-K_3	2.0	-	-	-	-	42.0 (40.13)	11.5 (19.59)	14.2 (22.11)
4.	MS-K_4	2.5	-	-	-	-	50.4 (45.23)	18.9 (25.70)	13.1 (21.20)
5.	MS-B_1	-	0.5	-	-	-	15.4 (23.09)	17.5 (24.68)	10.9 (19.23)
6.	MS-B_2	-	1.0	-	-	-	17.9 (25.02)	19.4 (26.13)	11.2 (19.54)
7.	MS-B_3	-	1.5	-	-	-	18.0 (25.04)	15.9 (23.49)	13.4 (21.46)
8.	MS-B_4	-	2.0	-	-	-	47.8 (43.73)	17.9 (25.01)	9.5 (17.94)
9.	MS-1_1B_1	-	1.0	-	-	0.0	15.6 (23.26)	16.8 (24.18)	4.5 (11.99)
10.	MS-1_1B_2	-	1.0	-	-	0.5	16.2 (23.71)	15.6 (23.26)	4.5 (12.03)
11.	MS-1_1B_3	-	1.5	-	-	1.0	54.0 (47.30)	19.8 (26.40)	14.9 (22.69)
12.	MS-1_1B_4	-	2.0	-	-	1.5	70.0 (56.84)	20.0 (26.79)	25.6 (30.39)
13.	MS-$1A_1B_1$	-	1.0	1.0	-	-	14.6 (22.50)	25.6 (30.36)	16.1 (23.64)
14.	MS-$1A_2B_2$	-	1.0	1.5	-	-	18.0 (25.8)	19.4 (26.12)	17.2 (24.49)
15.	MS-$1A_3B_3$	-	1.5	2.0	-	-	25.0 (29.97)	44.5 (41.84)	29.1 (32.64)
16.	MS-$1A_4B_4$	-	1.5	2.5	-	-	45.0 (42.13)	69.4 (56.42)	14.9 (22.69)
17.	MS-N_1B_1	-	0.5	-	0.5	-	37.4 (37.70)	22.5 (28.31)	41.5 (12.22)
18.	MS-N_2B_2	-	1.0	-	0.5	-	46.0 (42.70)	21.8 (27.81)	4.5 (12.18)
19.	MS-N_3B_3	-	1.5	-	0.5	-	68.4 (56.01)	52.6 (46.49)	4.5 (12.13)
20.	MS-N_4B_4	-	2.0	-	0.5	-	73.4 (58.97)	56.4 (48.68)	14.1 (22.01)
21.	MS-I_1K_1	1.0	-	-	-	1.0	5.6 (13.61)	16.8 (24.19)	4.5 (12.13)
22.	MS-I_2K_2	1.5	-	-	-	1.5	7.4 (15.76)	19.7 (26.30)	4.5 (12.18)
23.	MS-I_3K_3	1.0	-	-	-	2.0	6.2 (14.31)	20.8 (27.11)	4.5 (12.24)
24.	MS-I_4K_4	1.5	-	-	-	2.5	4.5 (12.20)	25.4 (30.25)	4.5 (12.05)
25.	MS-IA_1K_1	1.0	-	0.5	-	-	12.8 (20.92)	28.9 (32.47)	4.5 (12.20)
26.	MS-IA_2K_2	1.0	-	1.0	-	-	12.0 (20.26)	49.5 (44.71)	4.5 (12.17)
27.	MS-IA_3K_3	1.5	-	1.5	-	-	14.8 (22.61)	55.6 (48.24)	4.5 (12.16)
28.	MS-IA_4K_4	1.5	-	2.0	-	-	16.2 (23.69)	69.2 (56.32)	4.5 (12.20)
29.	MS-N_1K_1	1.0	-	-	1.0	-	48.9 (44.37)	12.4 (20.58)	18.1 (25.17)
30.	MS-N_2K_2	1.0	-	-	1.5	-	52.6 (46.49)	59.1 (50.24)	17.6 (24.80)
31.	MS-N_3K_3	1.5	-	-	2.0	-	46.8 (43.16)	75.4 (60.27)	28.1 (31.99)
32.	MS-N_4K_4	1.5	-	-	2.5	-	69.2 (56.30)	79.2 (62.90)	32.5 (34.50)
33.							31.71 (33.04)	32.10 (33.75)	11.21 (19.31)

CD
Factor A 0.47
Factor B 1.54
Factor AxB 2.67

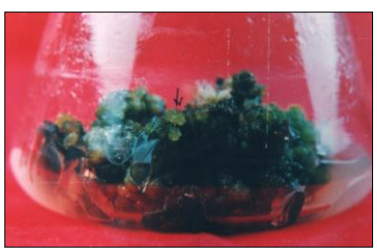

Fig 1: Shoot Meristem derived calli in A hispidissima

Fig 2: White and nodular callus from internode

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