An efficient in vitro shoot regeneration from immature inflorescence and ex vitro rooting of *Arnebia hispidissima* (Lehm). DC. - A red dye (Alkannin) yielding plant

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Abstract *Arnebia hispidissima*, which belongs to the family Boraginaceae, is an important medicinal and dye yielding plant. The alkannin, a red dye, are root-specific secondary metabolites of *A. hispidissima*. Shoots were regenerated from callus derived from immature inflorescence explants obtained from field grown plants. MS medium containing 4.52 μM 2, 4-D and 3.33 μM BAP was found to be most effective for the proliferation of callus, induced on medium containing 4.52 μM 2, 4-D. Maximum number (43.1 ± 0.25) with average length (5.2 ± 0.23) of shoots regenerated when callus was transferred to MS medium supplemented with 1.11 μM BAP, 1.16 μM Kin and 0.57 μM IAA. About 75.5 % of in vitro regenerated shoots were rooted on half-strength MS medium supplemented with 9.84 μM of IBA and 200 mg l^{-1} of activated charcoal. In comparison to in vitro, higher percent (90.2 %) of shoots were rooted under ex vitro conditions when treated with IBA (0.98 mM) for 5 min. Plantlets rooted in vitro as well as ex vitro were acclimatized successfully under the green house conditions. Ex vitro rooted plants exhibited higher survival percentage (75 %) as compared to in vitro rooted plantlets (60 %). Present study may be applicable in the large-scale root-specific red dye (alkannin) production via root induction under ex vitro condition.

Keywords Alkannin · Organogenesis · Secondary metabolites

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
IAA	Indole-3 acetic acid
IBA	Indole-3 butyric acid
Kin	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	Naphthalene Acetic Acid
PFD	Photon Flux Density
PGRs	Plant Growth Regulators
RH	Relative Humidity

Introduction

Arnebia hispidissima, which belongs to the family Boraginaceae, is an important medicinal and dye yielding plant. It grows generally in sandy soil and has the ability to tolerate drought stress. The alkannin and its optical isomer shikonin, a red dye, are root-specific secondary metabolites of *A. hispidissima* (Sharma et al. 2009). In addition, it has wide applications as a colorant in cosmetic, food and textile industries (Pal and Chaudhury 2010). Alkannin has an applicable pharmacological effect as an antibacterial agent, granulation tissue forming activity and anti-ulcer activity (Hayashi et al. 1969; Papageorgiou et al. 1999). Chemical investigations by Hamdard et al. (1988) found that the roots of *A. hispidissima* yield 3 new naphthaquinone which possess antibiotic and anticancerous properties. A flavonoid, characterised as vitexin, has been isolated from the fresh flowers of *A. hispidissima* (Hamdard et al. 1988). Hexane extract of root yielded a mixture of naphthoquinone: arnebin-1, arnebin-7, tiglic acid, alkannin, arnebinol and cycloarnebin-7 (Singh et al. 2004).

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A. hispidissima grows in a very specific habitat of arid and semi-arid region of Rajasthan, India (Bhandari 1990). During last 10–15 years, due to increasing human interference the wild status of this plant is drastically affected from several of its native places i.e. Bikaner, Barmer, Jodhpur, Nagaur and Sikar districts of Rajasthan. *A. hispidissima* is propagated through seeds. There is need for conservation and propagation of this plant through non-conventional methods for fulfilling demands of pharmaceutical industries. Also, there is need for selection of high yielding/superior genotypes for pigment production. Plant tissue culture is a useful technique for the conservation and rapid propagation of medicinally and pharmaceutically important plants and the production of bioactive compounds (Singh et al. 2009; Praveen et al. 2010; Phulwaria et al. 2013). Earlier, micro-propagation of *A. hispidissima* has been reported using nodal segments (Shekhawat and Shekhawat 2011) or shoot tip derived explants (Pal and Chaudhury 2010). These two reports mainly focused on pigment production from in vitro derived callus or shoot, not emphasized on higher number of shoots. In the present communication, we utilized immature inflorescence explants first time for shoot regeneration of *A. hispidissima*. To reduce labor, cost and time, efforts have been made to compare the optimum conditions for in vitro as well as ex vitro rooting in micropropagated shoots.

Material and methods

Explants selection and surface sterilization

A. hispidissima was collected from the various sites of Rajasthan (Kalyanpura, Barmer; Agolai, Jodhpur; and Nokha, Nagaur) during the months of October to January. Different types of explant viz. leaves, immature inflorescence, immature and mature seeds were used as explants for initiation of in vitro cultures. These explants were initially pretreated with 0.1 % of Bavistin (a systemic fungicide; BASF India Limited, Mumbai, India) for 15–20 min followed by surface sterilization with 0.1 % HgCl₂ for 3–5 min under aseptic condition in laminar air hood and rinsed 6–8 times with sterile double distilled water.

Induction and proliferation of callus

Explants were inoculated on MS (Murashige and Skoog 1962) medium + additives (283.8 µM ascorbic acid, 67.8 µM adenine sulfate, 143.5 µM arginine, 118.9 µM citric acid) and supplemented with various concentrations of 2, 4-D (0.0, 2.26, 4.52, 9.05, 13.57, 18.10 or 22.62 µM) or NAA (0.0, 2.68, 5.37, 10.74, 16.11, 21.48 or 26.85 µM) for induction of callus. Callus was further transferred to MS medium containing optimized concentration of 2, 4-D (4.52 µM) in

combination with different concentrations of cytokinins BAP (0.0, 1.11, 2.22, 3.33, or 4.44 µM) or Kin (0.0, 1.16, 2.32, 3.48, or 4.65 µM) and additives for proliferation. The cultures were incubated at 28±2 °C and 40–50 µmol⁻² s⁻¹ Photon Flux Density (PFD) light intensity for 12–14 h⁻¹ photoperiods provided by cool white fluorescent lamps (Philips, India) and 60 % relative humidity (RH).

Adventitious shoot induction and multiplication

Fast growing, globular white and green callus was transferred to various concentrations of BAP (0.0, 1.11, 2.22, 3.33 or 4.44 µM) alone or optimized concentration of BAP (1.11 µM) with various concentrations of Kin (0.0, 1.16, 2.32, 3.48 or 4.65 µM) for shoot regeneration. To evaluate the effect of auxins on shoot regeneration, callus along with shoots induced on optimized concentrations of shoot induction medium (MS+1.11 µM BAP+1.16 µM Kin) were transferred to medium containing IAA (0.0, 0.57, 1.14, 1.71 or 2.28 µM) or NAA (0.0, 0.54, 1.07, 1.61 or 2.15 µM).

Rooting of in vitro regenerated shoots

In vitro rooting

For the root induction under in vitro conditions, shoots (3–5 cm in length) were excised individually and transferred to half-strength MS medium supplemented with various concentrations of IBA (0.0, 4.92, 9.84, 14.76, 19.68 or 24.60 µM) or NAA (0.0, 5.37, 10.74, 16.11, 21.48 or 26.85 µM) and 200 mg l⁻¹ of activated charcoal.

Ex vitro rooting

For ex vitro rooting, in vitro regenerated shoots (3–5 cm in length) were excised and treated with various concentrations of auxins IBA (0.0, 0.49, 0.98, 1.48, 1.97 or 2.47 mM) or NAA (0.0, 0.53, 1.07, 1.61, 2.15 or 2.69 mM) for 5 min. The auxins treated shoots were transferred to glass bottles containing soil-rite (a mixture of horticulture grade perlite with Irish peat moss and exfoliated vermiculite supplied by Kel Perlite, Bangalore, India) and moistened with one fourth-strength of MS basal salts and kept under the green house near the pad section (high humidity and low temperature condition) at RH 80–90 % and 28±2 °C.

Acclimatization of the in vitro and ex vitro rooted plantlets

In vitro and ex vitro rooted plantlets were hardened in the green house. After initiation of roots, the caps of bottles were gradually opened over a period of 2–3 weeks and finally removed. The rooted plantlets were gradually shifted

Fig. 1 Shoot regeneration through callus induced from immature inflorescence and acclimatization of plants of *A. hispidissima*. **a** Immature inflorescence. **b** Callus induction on MS+4.52 μM 2, 4-D, after 2 weeks of inoculation. **c** Proliferation of callus on MS+4.52 μM 2, 4-D+3.33 μM BAP, after 4 weeks of subculture. **d** Shoots regenerated from callus on MS+1.11 μM BAP+1.16 μM Kin+0.57 μM IAA, after

4 weeks of subculture. **e** In vitro root induction in shoot on half-strength MS+9.84 μM of IBA+200 mg l^{-1} of activated charcoal, after 3 weeks. **f** Ex vitro rooted shoots treated with 0.98 mM IBA for 5 min, after 3 weeks **g** Hardening of micropropagated plants in soil-rite. **h** A well acclimatized plant in earthen pots, after 4 weeks

from the pad section of the green house towards the fan section (low humidity and high temperature) at $32 \pm 2^\circ\text{C}$ and 60–70 % RH. After 4 weeks, the hardened plantlets were transferred to the earthen pots containing organic manure, clay soil and sand (1:1:1) and kept near the fan section. Finally, the hardened plantlets were shifted to the nursery.

Experimental design and statistical analysis

All the experiments were conducted with minimum of 20 replicates per treatment. One replicate means one explant. Each experiment was repeated three times. The results are expressed as mean $\pm$ SD of three experiments. Observations

Table 1 Effect of auxins (2, 4-D or NAA) on callus induction of *A. hispidissima*

2, 4-D conc. (μM)	NAA conc. (μM)	Percent response for callus induction	Remarks
0.0	0.0	0.0	No callus induction
2.26	-	52.4 ^c	Slow growing callus
4.52	-	72.0 ^a	Creamy white, red, granular and fast growing callus
9.05	-	60.5 ^b	Fragile and watery callus
13.57	-	45.0 ^g	Slow growing and brown callus
18.10	-	32.5 ^j	Brown and fragile callus
22.62	-	48.4 ^e	Fast growing, fragile callus
-	2.68	43.0 ^h	Fragile and watery callus
-	5.37	47.5 ^{ef}	Creamy and watery callus
-	10.74	50.0 ^d	Granular and fast growing callus
-	16.11	36.3 ⁱ	Slow growing callus
-	21.48	27.5 ^k	Slow growing callus
-	26.85	22.6 ^l	Poor growth

Medium: MS+additives. Means in each column followed by same letters at superscript are not significantly different according to DMRT at $P < 0.05$

Table 2 Proliferation of callus on MS+4.52 μ M of 2, 4-D with various concentrations of BAP or Kin

BAP Conc. (μ M)	Kin Conc. (μ M)	Percent response for callus induction	Remarks
0.0	0.0	72.0 ^b	Creamy white, granular and fast growing callus
1.11	-	55.8 ^f	Slow multiplied
2.22	-	70.0 ^e	Slow growth but globular of callus
3.33	-	85.0 ^a	Fast growth and globular white & red callus
4.44	-	45.5 ^h	Slight callusing
-	1.16	60.5 ^e	Slow growth with friable callus is formed
-	2.32	65.5 ^d	Growth is good and white callus
-	3.48	50.0 ^g	Slow multiplied
-	4.65	40.2 ⁱ	Slight callusing

Medium: MS+additives. Means in each column followed by same letters at superscript are not significantly different according to DMRT at $P<0.05$

were recorded after 3–4 weeks of intervals. The data were analyzed statistically using one-way analysis of variance (ANOVA), and the significant differences between means were assessed by Duncan's multiple range test at $P<0.05$.

Results and discussion

Different types of explants i.e. leaf, immature inflorescence and mature and immature seeds were used for induction of callus in *A. hispidissima*. Out of these explants, only unopened immature inflorescence (Fig. 1a) was found suitable explants for callus induction. Other explants either failed to induce callus or responded poorly. Therefore, only immature inflorescence was used as explants for further experiments.

Immature inflorescences are highly meristematic and an excellent sources for the induction of regenerative/competent cultures as recommended by many workers in other studies (Kackar and Shekhawat 1989, 1991; Yadav et al. 2009; Głowacka et al. 2010; Gubisova et al. 2013). Low contamination and little browning of the culture medium are some other potential advantages of the immature inflorescence used as explants in culture initiation (Głowacka et al. 2010).

Induction and proliferation of callus

Out of two auxins tested, 2, 4-D was found more responsive than NAA for callus induction (Table 1). Callus derived from immature inflorescence on MS medium with 4.52 μ M of 2, 4-D was creamy white, red, granular and fast

Table 3 Influence of PGRs on shoot regeneration from callus of *A. hispidissima*

BAP conc. (μ M)	Kin conc. (μ M)	IAA conc. (μ M)	NAA conc. (μ M)	Response %	Shoot number (mean $\pm$ SD)	Shoot length (cm) (mean $\pm$ SD)
0.0	0.0	0.0	0.0	0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
1.11	-	-	-	40.0 ^{lm}	26.4 $\pm$ 0.42 ^{lm}	3.2 $\pm$ 0.22 ^{ij}
2.22	-	-	-	35.6 ⁿ	23.4 $\pm$ 0.24 ⁿ	2.5 $\pm$ 0.32 ^{mn}
3.33	-	-	-	25.5 ^o	20.2 $\pm$ 0.56 ^o	2.2 $\pm$ 0.23 ^o
4.44	-	-	-	15.0 ^p	18.4 $\pm$ 0.42 ^p	1.8 $\pm$ 0.45 ^p
1.11	1.16	-	-	65.3 ⁱ	34.2 $\pm$ 0.23 ^{ef}	3.8 $\pm$ 0.24 ^{cde}
1.11	2.32	-	-	62.0 ^j	32.1 $\pm$ 0.34 ^{gh}	3.5 $\pm$ 0.32 ^f
1.11	3.48	-	-	48.5 ^k	28.5 $\pm$ 0.44 ^j	3.0 $\pm$ 0.54 ^h
1.11	4.65	-	-	40.5 ^l	27.2 $\pm$ 0.22 ^l	2.8 $\pm$ 0.38 ^k
1.11	1.16	0.57	-	85.5 ^a	43.1 $\pm$ 0.25 ^a	5.2 $\pm$ 0.23 ^a
1.11	1.16	1.14	-	83.0 ^b	41.4 $\pm$ 0.48 ^b	4.9 $\pm$ 0.36 ^b
1.11	1.16	1.71	-	80.5 ^c	38.2 $\pm$ 0.37 ^c	3.9 $\pm$ 0.29 ^c
1.11	1.16	2.28	-	78.5 ^d	34.2 $\pm$ 0.45 ^e	3.6 $\pm$ 0.44 ^{cd}
1.11	1.16	-	0.54	74.4 ^e	36.2 $\pm$ 0.25 ^d	3.4 $\pm$ 0.22 ^g
1.11	1.16	-	1.07	70.5 ^f	32.5 $\pm$ 0.56 ^g	2.9 $\pm$ 0.52 ⁱ
1.11	1.16	-	1.61	69.2 ^g	28.9 $\pm$ 0.21 ⁱ	2.7 $\pm$ 0.33 ^l
1.11	1.16	-	2.15	67.0 ^h	27.8 $\pm$ 0.25 ^{jk}	2.4 $\pm$ 0.45 ^m

Medium: MS+additives. Means in each column followed by same letters at superscript are not significantly different according to DMRT at $P<0.05$

Table 4 Effect of auxins (IBA or NAA) on in vitro root induction from shoots of *A. hispidissima*

	IBA conc. (μM)	NAA conc. (μM)	Response (%)	Root number (mean $\pm$ SD)	Root length (cm) (mean $\pm$ SD)
	0.0	0.0	0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	4.92	-	50.2 ^{fg}	2.8 $\pm$ 0.33 ^{defg}	3.4 $\pm$ 0.39 ^{cd}
	9.84	-	75.5 ^a	3.8 $\pm$ 0.24 ^a	4.2 $\pm$ 0.25 ^a
	14.76	-	70.6 ^b	3.3 $\pm$ 0.40 ^b	3.5 $\pm$ 0.43 ^{bc}
	19.68	-	65.5 ^{cd}	2.9 $\pm$ 0.32 ^{cd}	3.1 $\pm$ 0.62 ^{cde}
	24.60	-	55.0 ^e	2.7 $\pm$ 0.44 ^{def}	2.9 $\pm$ 0.55 ^f
	-	5.37	45.2 ^h	2.6 $\pm$ 0.56 ^{de}	3.1 $\pm$ 0.69 ^{cd}
Medium: half-strength of MS+ 200 mg l ⁻¹ activated charcoal.	-	10.74	65.6 ^c	3.2 $\pm$ 0.44 ^{bc}	3.4 $\pm$ 0.57 ^b
Means in each column followed by same letters at superscript are not significantly different according to DMRT at $P<0.05$	-	16.11	55.0 ^e	2.7 $\pm$ 0.03 ^h	3.1 $\pm$ 0.17 ^g
	-	21.48	50.5 ^f	2.2 $\pm$ 0.21 ⁱ	2.6 $\pm$ 0.32 ^h
	-	26.85	40.0 ⁱ	1.9 $\pm$ 0.24 ^{ij}	2.2 $\pm$ 0.27 ⁱ

growing (Fig. 1b, c). Callus produced on 2, 4-D did not proliferate on the same culture medium, therefore, callus was transferred to MS medium containing various concentrations of cytokinins in combination with optimized concentrations of 2, 4-D. Out of different combinations, medium containing 4.52 μM 2, 4-D and 3.33 μM BAP was found to be most effective for the proliferation of callus. The combination of 2, 4-D and Kin was less effective in comparison to combination of 2, 4-D and BAP for callus proliferation (Table 2). In most cases, synthetic auxin 2, 4-D is only effective for the induction of unorganized callus growth or polarized embryo production; however, continuous treatment of 2, 4-D is inhibitory for callus proliferation or embryo development (von Arnold et al. 2002; Rathore et al. 2012). The probable reason behind this may be the higher accumulation of 2, 4-D in the cell. Furthermore, in comparison to other auxins, 2, 4-D is less metabolized by cells (von Arnold et al. 2002; Rai et al. 2010). Therefore, for callus proliferation, it is necessary to transfer the callus to medium without 2, 4-D or combination of 2, 4-D with low

concentrations of cytokinin. Similar to our observations, significant role of 2, 4-D in induction of callus and combination of 2, 4-D with cytokinins for callus proliferation has also been reported in many studies (Dang et al. 2011; Martin et al. 2011; Raghu et al. 2011; Rathore et al. 2011; Phulwaria et al. 2013).

Adventitious shoot regeneration and multiplication

Results of present study showed that shoots were regenerated from callus when transferred from callus proliferation medium to MS medium containing BAP alone or optimized concentration of BAP (1.11 μM) with combination of Kin, IAA or NAA. Maximum number (43.1 $\pm$ 0.25) and length (5.2 $\pm$ 0.23) of shoots were recorded on MS+1.11 μM BAP+1.16 μM Kin+0.57 μM of IAA (Fig. 1d; Table 3). The number of shoots produced in this study is considerable higher in comparison to previously published papers on the same plant species (Pal and Chaudhury 2010; Shekhawat and Shekhawat 2011).

Table 5 Effect of auxins (IBA or NAA) on ex vitro root induction from shoots of *A. hispidissima*

	IBA conc. (mM)	NAA conc. (mM)	Response (%)	Root number (mean $\pm$ SD)	Root length (cm) (mean $\pm$ SD)
	0.0	0.0	0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	0.49	-	60.5 ^f	3.5 $\pm$ 0.53 ^{de}	3.5 $\pm$ 0.39 ^{de}
	0.98	-	90.2 ^a	4.5 $\pm$ 0.44 ^a	4.6 $\pm$ 0.25 ^a
	1.48	-	80.0 ^b	4.0 $\pm$ 0.70 ^b	3.9 $\pm$ 0.43 ^{bc}
	1.97	-	70.7 ^c	3.6 $\pm$ 0.44 ^d	3.8 $\pm$ 0.55 ^b
	2.47	-	65.1 ^{de}	3.1 $\pm$ 0.82 ^f	3.2 $\pm$ 0.37 ^{fg}
	-	0.53	65.3 ^d	3.2 $\pm$ 0.27 ^g	3.1 $\pm$ 0.39 ^{gh}
	-	1.07	70.7 ^c	3.9 $\pm$ 0.44 ^c	3.5 $\pm$ 0.47 ^d
	-	1.61	60.2 ^{fg}	3.0 $\pm$ 0.13 ^{hi}	3.1 $\pm$ 0.17 ^j
	-	2.15	55.0 ^h	2.8 $\pm$ 0.54 ^{gh}	2.4 $\pm$ 0.28 ^{ef}
	-	2.69	40.7 ⁱ	2.4 $\pm$ 0.19 ^j	2.2 $\pm$ 0.29 ⁱ

Treatment duration: 5 min.
Means in each column followed
by same letters at superscript are
not significantly different
according to DMRT at $P<0.05$

Rooting of in vitro regenerated shoots

Out of two auxins tested, IBA was found to be the best for in vitro rooting of shoots. About 75.5 % shoots were rooted with maximum number (3.8 ± 0.24) and highest average root length (4.2 ± 0.25 cm) on half-strength MS medium containing $9.84 \mu\text{M}$ of IBA and 200 mg l^{-1} of activated charcoal within 18–22 days (Fig. 1e). In comparison to IBA, NAA was found to be less effective for root induction (Table 4). The beneficial role of IBA in in vitro rooting has been reported in many other plants, such as *Vitex negundo* (Ahmad and Anis 2011), *Lawsonia inermis* (Ram and Shekhawat 2011) *Momordica dioica* (Shekhawat et al. 2011) and *Caesalpinia bonduc* (Cheruvathur et al. 2012).

In the present investigation, in vitro regenerated shoots were also subjected to the ex vitro rooting. In recent years, ex vitro rooting technique is more emphasized by many tissue culture specialists during the micropropagation of plants at commercial scale because it reduces the steps of micropropagation and saves cost, labor, resources and time. In addition, ex vitro rooted plantlets are easily hardened as compared to in vitro rooted plants which require additional steps, first in vitro for root induction and then ex vitro for acclimatization of plants (Pospisilova et al. 1999; Borkowska 2001; Pati et al. 2006; Annapurna and Rathore 2010; Yan et al. 2010; Phulwaria et al. 2011, 2012a, b; Shekhawat et al. 2012). In the present study, individually excised shoots were rooted ex vitro when treated with 0.98 mM of IBA for 5 min (Table 5). In comparison to in vitro rooting, higher percent (90.2 %) shoots were rooted via ex vitro method. Maximum 4.5 ± 0.44 roots with average length of 4.6 ± 0.25 cm were achieved within 18–24 days (Fig. 1f). NAA is less effective than IBA for ex vitro rooting. Similar results were also obtained in many plant species (Annapurna and Rathore 2010; Phulwaria et al. 2011, 2012a, b).

Hardening and acclimatization of plantlets developed from in vitro and ex vitro rooting

The in vitro and ex vitro rooted plantlets were acclimatized successfully under the green house condition (Fig. 1g). In comparison to in vitro rooted plantlets (60 %), percent survival of plants (75 %) those developed from ex vitro rooting (Fig. 1h) was significantly higher. The probable reason of higher percent survival of plants developed from ex vitro rooting may be due to the presence of lateral roots just like natural root system, root hair and higher root length (Yan et al. 2010; Phulwaria et al. 2012a).

In conclusion, we first time utilized immature inflorescence explants for high-frequency regeneration of *A. hispidissima*, an important red dye (secondary metabolite) yielding plant of arid and semi arid region of India. The ex vitro rooting provides not only more efficient micropropagation method

for this important plant species but also reduces cost, labor, resources and time. In addition, present study may also be applicable in the large-scale root-specific red dye (alkannin) production via root induction under ex vitro condition.

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References

- Ahmad N, Anis M (2011) An efficient in vitro process for recurrent production of cloned plants of *Vitex negundo* L. Eur J For Res 130:135–144
- Annapurna D, Rathore TS (2010) Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell Tissue Organ Cult 101:269–277
- Bhandari MM (1990) Flora of Indian desert. MPS Reports, Jodhpur
- Borkowska B (2001) Morphological and physiological characteristics of micropropagated strawberry plants rooted in vitro or ex vitro. Sci Hortic 89:195–206
- Cheruvathur MK, Britto J, Thomas TD (2012) Pulvinus: an ideal explant for plant regeneration in *Caesalpinia bonduc* (L.) Roxb., an important ethnomedicinal woody climber. Acta Physiol Plant 34:693–699
- Dang JC, Kumaria S, Kumar S, Tandon P (2011) Micropropagation of *Ilex khasiana*, a critically endangered and endemic holly of Northeast India. AoB Plant. doi:10.1093/aobpla/plr012
- Głowacka K, Jezowski S, Kaczmarek Z (2010) The effects of genotype, inflorescence developmental stage and induction medium on callus induction and plant regeneration in two *Miscanthus* species. Plant Cell Tissue Organ Cult 102:79–86
- Gubisova M, Gubis J, Zofajova A, Mihalik D, Kraic J (2013) Enhanced in vitro propagation of *Miscanthus × giganteus*. Ind Crop Prod 41:279–282
- Hamdard ME, Badar Y, Khan MSY (1988) Revised phytochemical study of *Arnebia hispidissima*. Pakistan J Pharm Sci 1:19–20
- Hayashi H, Coono M, Yoshinaga M, Muto M (1969) The role of SH-dependent protease and its inhibitor in Arthus type hypersensitivity reaction, with special reference to chemical mediation of Increased vascular permeability and leukocyte emigration. In: Bertelli A, Houck JC (eds) Inflammation biochemistry. Excerpta Medica Foundation, Amsterdam, p 34
- Kackar A, Shekhawat NS (1989) Regeneration of *Lasiurus scindicus* (Hern) from tissue culture. Ann Bot 64:455–458
- Kackar A, Shekhawat NS (1991) Plant regeneration from cultured immature inflorescence of *Cenchrus setigerus* and *Cenchrus ciliaris*. Indian J Exp Biol 29:62–64
- Martin KP, Pradeep AK, Madassery J (2011) High frequency in vitro propagation of *Trichopus zeylanicus* subsp. *travancoricus* using branch-petiole explants. Acta Physiol Plant 33:1141–1148
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
- Pal M, Chaudhury A (2010) High frequency direct plant regeneration, micropropagation and shikonin induction in *Arnebia hispidissima*. J Crop Sci Biotech 13:13–19
- Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC (1999) The chemistry and biology of alkannin,

- shikonin, and related naphthazarin natural products. *Angew Chem Int Ed* 38:270–300
- Pati PK, Rath SP, Sharma M, Sood A, Ahuja PS (2006) In vitro propagation of rose-a review. *Biotechnol Adv* 24:94–114
- Phulwaria M, Rai MK, Harish, Gupta AK, Ram K, Shekhawat NS (2012a) An improved micropropagation of *Terminalia bellirica* from nodal explants of mature tree. *Acta Physiol Plant* 34:299–305
- Phulwaria M, Ram K, Gahlot P, Shekhawat NS (2011) Micropropagation of *Salvadora persica*- a tree of arid horticulture and forestry. *New Forest* 42:317–327
- Phulwaria M, Ram K, Harish, Gupta AK, Shekhawat NS (2012b) Micropropagation of mature *Terminalia catappa* (Indian almond) - a medicinally important forest tree. *J Forest Res* 17:202–207
- Phulwaria M, Shekhawat NS, Rathore JS, Singh RP (2013) An efficient in vitro regeneration and ex vitro rooting of *Ceropegia bulbosa* Roxb. - a threatened and pharmaceutical important plant of Indian Thar Desert. *Ind Crop Prod* 42:25–29
- Pospisilova J, Ticha I, Kadlec P, Haisel D, Plzakova S (1999) Acclimatization of micropropagated plants to ex vitro conditions. *Biol Plant* 42:481–497
- Praveen N, Naik PM, Manohar SH, Nayeem A, Murthy HN (2010) In vitro regeneration of brahmi shoots using semisolid and liquid cultures and quantitative analysis of bacoside A. *Acta Physiol Plant* 31:723–728
- Raghu AV, Unnikrishnan K, Geetha SP, Martin G, Balachandran I (2011) Plant regeneration and production of embelin from organogenic and embryogenic callus cultures of *Embelia ribes* Burm. f.- a vulnerable medicinal plant. *In Vitro Cell Dev Biol-Plant* 47:506–515
- Rai MK, Asthana P, Jaiswal VS, Jaiswal U (2010) Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. *Tree Struct Funct* 24:1–12
- Ram K, Shekhawat NS (2011) Micropropagation of commercially cultivated Henna (*Lawsonia inermis*) using nodal explants. *Physiol Mol Biol Plant* 17:281–289
- Rathore JS, Rai MK, Shekhawat NS (2012) Induction of somatic embryogenesis in gum arabic tree [*Acacia senegal* (L.) Willd.]. *Physiol Mol Biol Plant* 18:387–392
- Rathore MS, Chikara J, Shekhawat NS (2011) Plantlet regeneration from callus cultures of selected genotype of *Aloe vera* L.-An ancient plant for modern herbal industries. *Appl Biochem Biotechnol* 163:860–868
- Sharma RA, Singh B, Singh D, Chandrawat P (2009) Ethnomedicinal, pharmacological properties and chemistry of some medicinal plants of Boraginaceae in India. *J Med Plant Res* 3:1153–1175
- Shekhawat MS, Shekhawat NS (2011) Micropropagation of *Arnebia hispidissima* (Lehm) DC. and production of alkannin from callus and cell suspension culture. *Acta Physiol Plant* 33:1445–1450
- Shekhawat MS, Shekhawat NS, Harish, Ram K, Phulwaria M, Gupta AK (2011) High frequency plantlet regeneration from nodal segment culture of female *Momordica dioica* (Roxb.). *J Crop Sci Biotechnol* 14:133–137
- Shekhawat NS, Mohnot S, Phulwaria M, Harish, Shekhawat S (2012) Micropropagation of *Salvadora oleoides* - an oil yielding tree of arid forests. *J Sustain For* 31:620–632
- Singh B, Sahu PM, Jain SC, Singh S (2004) Estimation of naphthoquinones from *Arnebia hispidissima* (Lehm.) DC. In vivo and in vitro I. anti-inflammatory screening. *Phytother Res* 18:154–159
- Singh SK, Rai MK, Asthana P, Sahoo L (2009) An improved micropropagation of *Spilanthes acmella* through thin cell layer culture. *Acta Physiol Plant* 31:693–698
- von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell Tissue Organ Cult* 69:233–249
- Yadav CB, Jha P, Mahalakshmi C, Anjaiah V, Bhat V (2009) Somatic embryogenesis and regeneration of *Cenchrus ciliaris* genotypes from immature inflorescence explants. *Biol Plant* 53:603–609
- Yan H, Liang C, Yang L, Li Y (2010) In vitro and ex vitro rooting of *Siratia grosvenorii*, a traditional medicinal plant. *Acta Physiol Plant* 32:115–120