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Full Length Research Paper

In vitro plant regeneration from seedling explants of *Hedychium coronarium* J. Koenig

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Although poor seed viability and low seed germination has been reported in *Hedychium coronarium* but a successful procedure was established in seed germination trials in the present study and about 80% germination has been recorded in Murashige and Skoog (MS) basal medium. Six to eight day old *in vitro* grown seedlings, cultured on MS medium supplemented with cytokinin Benzylaminopurine (BAP) or Kinetin (Kn) in the concentration range of 1.0 to 5.0 mgl^{-1} along with 0.5 mgl^{-1} Auxin Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) in different combinations. 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA was found to be the most effective plant growth regulator in producing maximum number of shoots. Roots developed *in vitro* in excised shoots upon transfer to MS medium supplemented with 0.5 mgl^{-1} NAA. Rooted plants, transferred to a mixture of forest soil, organic compost and sand in the ratio 1:1:1 (v/v) exhibited a survival rate of >65% which is an indication for the conservation and multiplication of this medicinally important plant.

Key words: Plant growth regulator, *In vitro*, medicinal plant.

INTRODUCTION

Hedychium coronarium J. Koenig is a monocotyledonous plant which belongs to the family Zingiberaceae. It is a perennial herb and is widely cultivated in tropical and subtropical regions of Japan, India, South China and South Asian countries including Bangladesh (Kiritikar and Basu, 1933). In India it has its origin from the Himalayan region of Nepal and North India where it is known as butterfly ginger (English) or dolan champa (Hindi). Traditionally it is used for the treatment of tonsillitis, infected nostrils, tumor and fever. It is also used as a febrifuge, tonic, excitant and anti- rheumatic in the Ayurvedic system of traditional Indian medicine (Jain et al., 1995). The essential oil extracted from leaves, flowers and rhizome of the plant have cercaricidal properties, molluscicidal activity, potent inhibitory action, antimicrobial activities, antifungal, anti-inflammatory, antibacterial and analgesic effects. In Amarkantak region of Chhattisgarh it is used as an eye drop to prevent motiabind (cataract) and is sold in the market by the name gulbakawali ark. Commercially the aerial stems constitute a useful raw material for making paper. The dried whole stems contain 43 to 48% cellulose. Due to over-exploitation for extraction of drugs, *H. coronarium* is rapidly disappearing from its natural habitat. So keeping in view the medicinal properties and rate of disappearance of this plant and such other medicinal plants a conservation and mass multiplication is the need of the hour. Since, very little information is available on the micropropagation studies of this plant. The aim of the present study is to develop a micropropagation protocol for *H. coronarium* using aseptically raised seedling explants as juvenile explants perform better in tissue culture than do mature explants.

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MATERIALS AND METHODS

Plant material

Seeds of *H. coronariium* were collected from the nursery situated in Lualli (A village near Pauri town) and the nursery of HAPPRC of HNB Garhwal University situated at Pothivasa a place at an altitude of 2.200 m above mean sea level (AMSL) between Ukhimath and Gopeshwar.

Establishment of aseptic cultures

Fresh seeds of *H. coronarium* were used to raise the seedlings under sterile conditions. Seeds were dipped in water and the seeds which settled down were selected and considered as viable. These seeds were presoaked overnight and washed for 30 min under running tap water to remove all the dust particles from the surface followed by second wash with liquid detergent (teepol) for another 15 min and then a proper and thorough wash to remove the detergent. These seeds were treated with Bavistin (fungicide) for about 20 min and finally washed thoroughly to remove the traces of fungicide. Seeds were surface sterilized with 0.1% $HgCl_2$ for 3 to 5 min constant shaking was done during the period to get thorough sterilization. Rinsing with sterile distilled water 4 to 5 times was necessary for the removal of sterilant from the seeds. These were then cultured on MS basal medium for germination. The seeds started germinating after 10 to 15 days of inoculation and the shoot tip part of the seedling was used as explant (Figure 1a). Initially, aseptic cultures were established by culturing shoot tip on MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones. BAP, IAA, IBA, Kn, NAA were used in the present study.

For all the experiments pH of the medium was adjusted to 5.86±0.1 prior to autoclaving at 15 lbs. at 121°C for 15 min. The culture conditions (temperature 25±2°C, 16 h photoperiod using cool white florescent light, and 3000 to 4000 lux light intensity) were maintained throughout the course of study. Rooted plantlets growing in rooting medium were gently removed from the culture media. Plantlets were thoroughly and gently washed with running tap water to remove agar from the roots, and planted in thermocol cups (5 cm dia.) having sterilized mixture of forest soil, organic compost and sand in the ratio 1:1:1 (v/v). These planted thermocol cups were kept in Environmental Chamber for two weeks and were irrigated with water during their growth in Environmental Chamber. Plantlets were additionally covered with polythene bags with small holes for air ventilation to ensure high humidity at initial stages. The polythene bags were removed after 2 weeks and the surviving plants were maintained in the growth chamber under 16 h photoperiod at 28 ±2°C. Plantlets were then transferred to earthenware pots (25 cm dia.) containing forest soil, organic compost and sand. Planted earthenware pots were kept in shade for two weeks and watered regularly. All the potted plants were then placed outdoors under full sun initially for a shorter period and gradually increasing the exposure after every third day for additional 1h.

Shoot regeneration from shoot tip explants

Shoot tip were cultured on MS medium with 3% (w/v) sucrose and 0.8% (w/v) Agar (Hi-Media) fortified with various levels (1.0 to 5.0 mgl^{-1}) of different cytokinins BAP or Kn. Similarly, 0.5 mgl^{-1} of different auxins (IAA, IBA or NAA) were also added in combination with cytokinin for selecting optimal medium for direct multiple shoot regeneration from shoot tip explants. Growth response of cultured shoot tip explant was examined after every 10 days of culture.

***In vitro* rhizogenesis in micro- shoots**

In vitro regenerated shoots were excised from the cluster and transferred on half strength MS medium supplemented with different auxins viz. IAA, IBA or NAA (0.5 to 1.0 mgl^{-1}) and were kept in dark. Frequency of root induction was examined after every 15 days of culture.

Analysis of data

In all the experiments standard deviation was calculated by using following formula:

$$\frac{\sqrt{n\sum \chi 2 - (\sum \chi)}}{n\,(n-1)}$$

Where, n= number of replicates observed; ∑= summation; χ= observation of the replicate.

RESULTS AND DISCUSSION

Shoot regeneration from shoot tip

During 50 days of culture on MS medium shoots developed directly from meristematic explants (shoot tips). Shoot formation was maximum in 3.0 mgl^{-1} BAP whereas Kn exhibited favourable response towards shoot formation at initial stages as compared to BAP but the response became slower as the days progressed (Table 1). Number of leaves and shoot length was also found highest in BAP concentrations. Sripichitt et al. (1987) also observed that BAP was more effective than Kn in inducing shoot formation on MS medium. BAP at the concentration of 3.0 mgl^{-1} exhibited the most promising results with the induction of 5.76 shoots/explant (Figure 1b). This is coinciding with the previous observations of Franklin et al. (1998); Sujatha and Reddy (1998); Alam et al. (2010) who obtained maximum shoots in 3.0 mgl^{-1} BAP through shoot tip explants. Among different combinations of the growth regulators, a high concentration of cytokinin with a low concentration of auxin was proven to be the best for direct organization of seedling explants by many workers (Joshi and Kothari, 2007; Mezghani et al., 2007; Rahman et al., 2008; Ashrafuzzaman et al., 2009; Guadalupe et al., 2009; Otroshy et al., 2011).

In these studies, it was reported that regeneration only took place using high concentrations of BAP with low concentrations of auxins. Supplementation of lower levels of NAA in the medium synergized the morphogenic response. Higher number of shoots per explant (7.90), and shoot length (6.04) was obtained in BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) combination in the present study (Table 2 and Figure 1c). Therefore, this was opted as the most suitable plant growth regulators (PGR) combination for shoot multiplication. Similarly Joshi et al. (2003) reported that MS medium supplemented with BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) was ideal condition for the induction of shoots in *Foeniculum vulgare*. Several workers (Amin et al., 2002; Kalamani and Micheal, 2002 and Pant and Manandhar, 2007) have tried the similar combination of these growth regulators for shoot regeneration in different plants. Replacing NAA with IAA or IBA was not found very effective in inducing shoot proliferation in the present case. 1.0 to 3.0 mgl^{-1} BAP with0.5 mgl^{-1} IAA was found to increase the shoot proliferation but a gradual reduction was noticed in higher concentration of BAP (Table 2). Similarly Hussain et al. (1999) and Dabauza and Peria, (2001) reported the use

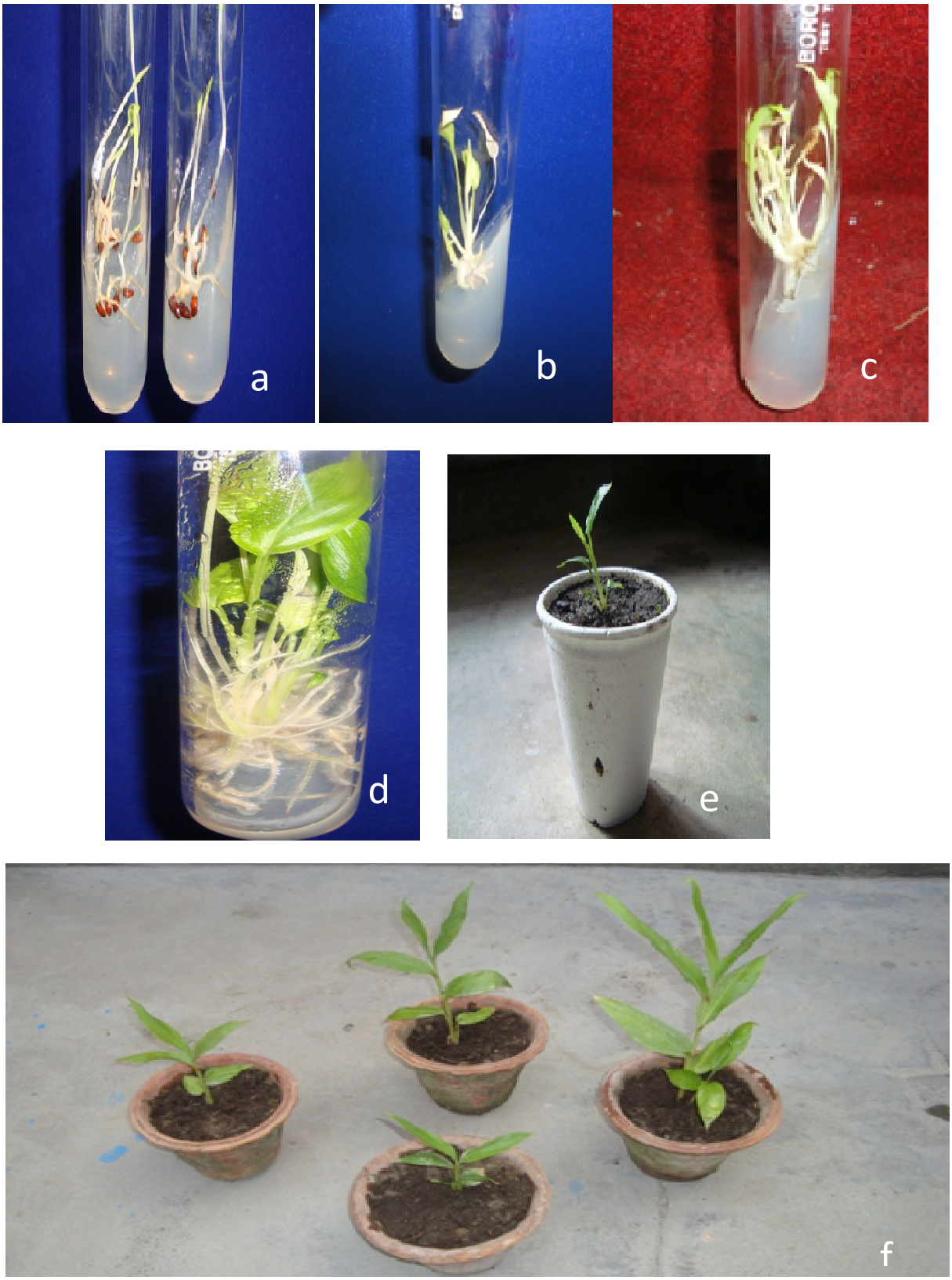

Figure 1. *In vitro* seed germination of *H.coronarium*; **a** multiple shoot regeneration in *H.coronarium* in MS medium supplemented with: 3.0 mgl^{-1} BAP; **b** 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA; **c** Rooting in half strength MS medium supplemented with 0.5 mgl^{-1} NAA; **d** Rooted plantlets successfully transferred to thermocol cup; **e** Hardened plants in pots; **f**.

Table 1. Growth response of cultured shoot tip explants of *H.coronarium* in MS medium after 50 days of culture under the influence of BAP and Kn in various concentrations.

BAP (mg l^{-1})	Kn (mg l^{-1})	Total no. of shoots/explant	Shoot length (cm)
1.00	-	3.21±0.86	2.10±0.18
2.00	-	4.66±0.98	3.12±0.12
3.00	-	5.76±0.57	3.48±0.14
4.00	-	4.19±1.09	2.84±0.32
5.00		4.08±0.94	2.15±0.18
-	1.00	2.10±0.15	1.98±0.57
-	2.00	2.50±0.18	2.74±1.10
-	3.00	2.75±0.25	2.96±0.57
-	4.00	3.18±0.14	3.02±0.54
-	5.00	3.60±0.23	3.19±1.08

± Standard deviation.

Table 2. Effect of BAP and different auxins (NAA, IAA and IBA) on number of shoots per explant and shoot length (cm) in MS medium after 50 days of culture.

BAP (mg l^{-1})	NAA (mg l^{-1})	IAA (mg l^{-1})	IBA (mg l^{-1})	Total no. of shoots/explant	Shoot length (cm)
1.0	0.5	-	-	7.90±0.78	6.04±0.76
2.0	0.5	-	-	6.71±0.62	5.23±0.12
3.0	0.5	-	-	6.30±0.81	5.00±1.16
4.0	0.5	-	-	6.13±0.28	4.66±0.48
5.0	0.5	-	-	5.21±0.97	3.86±0.32
1.0	-	0.5	-	2.80±0.23	2.16±0.10
2.0	-	0.5	-	3.66±0.14	2.40±0.98
3.0	-	0.5	-	4.66±0.26	3.62±0.36
4.0	-	0.5	-	1.10±0.12	1.08±0.04
5.0	-	0.5	-	2.33±0.19	2.10±0.17
1.0	-	-	0.5	1.32±0.11	1.10±0.08
2.0	-	-	0.5	1.96±0.19	1.26±0.10
3.0	-	-	0.5	2.33±0.18	1.50±0.20
4.0	-	-	0.5	3.54±0.25	2.90±0.22
5.0	-	-	0.5	2.90±0.32	2.00±0.18

± Standard deviation.

of BAP and IAA in similar range for shoot multiplication in *Capsicum annum*.

Root induction in regenerated shoots

Data recorded periodically after every 15 days of inoculation of *in vitro* regenerated shoots revealed that amongst all the auxins that is, IAA, IBA or NAA tested, only NAA and IBA could induce root on MS half strength basal media whereas IAA did not respond. Similarly, the promotory effect of reducing the salt concentration of MS on *in vitro* rooting of shoots has been reported in several studies (Constantine, 1978; Skirvin et al., 1980; Baskaran and Jayabalan, 2005). Reducing MS salt with varying concentrations (0.5 to 1.0 mgl^{-1}) of auxin (NAA or IBA) was used in the present study. 0.5 mgl^{-1} NAA reflected high frequency of root formation than 0.5 mgl^{-1} IBA (Table 3).

Similar response was observed by several workers (Heng et al., 1998; Sarma and Rogers, 1998; Syamala and Devi, 2003). However, IAA was used for root induction by Saradamani et al. (2003); Baskaran and Jayabalan (2005).

Root branching was noticed in IBA as well as NAA concentrations in MS half with best branching in 0.5 mgl^{-1} NAA. An average number of 12.20 roots/shoot was obtained with an average length of 4.68 cms in 0.5 mgl^{-1} NAA concentration (Figure 1d) against an average number of 8.60 roots/shoot with an average length of

Table 3. Periodic variation in root number (RN) and root length (RL) in half strength MS medium supplemented with varying levels of NAA and IBA.

MS ½ + NAA (mg l^{-1})	Growth parameter	Number of days			
		15	30	45	60
0.5	No. of roots/shoot	6.74±1.62	8.32±1.73	10.18±1.57	12.20±2.63
	Root length (cm)	1.64±0.16	2.08±0.57	3.06±0.29	4.68±1.03
	Root branching	-	++	+++	++++
1.0	No. of roots/shoot	4.38±0.16	5.75±0.98	6.66±1.01	8.60±2.61
	Root length (cm)	1.08±0.12	1.86±0.66	2.62±0.84	3.37±1.40
	Root branching	-	-	++	+++
MS ½ + IBA (mg l^{-1})					
0.5	No. of roots/shoot	1.67±0.45	2.49±0.36	3.82±0.46	5.12±0.48
	Root length (cm)	0.74±0.32	0.96±0.19	1.16±0.16	2.96±0.22
	Root branching	-	+	++	+++
1.0	No. of roots/shoot	1.04±0.24	2.11±0.57	2.96±0.57	3.74±0.54
	Root length (cm)	0.61±0.29	0.78±0.18	1.08±0.20	1.75±0.51
	Root branching	-	-	+	++

- = No response, + = poor, ++ = good, +++ = better, ++++ = best, ± standard deviation

3.37 cms in 1.0 mgl^{-1} NAA concentration. Root branching was also recorded in both the concentrations of NAA. Similarly in IBA concentration the maximum (5.12±0.48) and minimum (3.74±0.54) number of roots were recorded in 0.5 and 1.0 mgl^{-1} of IBA, respectively at 60 day stage where no root branching was observed. Controlled conditions given at initial stages, keeping plantlets in shade for longer period, regular irrigation, use of organic compost, sand and forest soil in the ratio 1:1:1 (v/v) and periodically gradual exposure to sunlight are the essential conditions to decrease the mortality rate and to achieve higher survival of the plantlets (Figure 1e and f). 65% survival was achieved following these conditions in the present study. Therefore present study was undertaken keeping in mind to devise methods for possible recovery of this over-harvested medicinally important plant species.

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