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Full Length Research Paper

Effect of plant growth regulators (PGRs) on micropropagation of a vulnerable and high value medicinal plant *Hedychium spicatum*

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A complete micropropagation protocol was developed by applying different plant growth regulators (PGRs) of a vulnerable and high value aromatic medicinal plant, *Hedychium spicatum.* Three cytokinins, 6-benzyladenine (BA), kinetin (KN) and thidiazuron (TDZ) were used and among these, the lower concentration of TDZ (1.0 µM) was found to be the most effective treatment in relation to induction of high frequency shoot multiplication (83.33%), number of shoots per explant (3.86 shoots) and average number of shoots per flask (19.33 shoots). Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) were the used auxins in this study for *in-vitro* rooting. Among these used auxins, the lower concentration of IBA (2.5 µM) was the prominent plant growth regulator regarding *in vitro* rooting. Well rooted and healthy plantlets were obtained after 2 months of hardening and transferred to the field (1990 m) with 90.0% survival. On the basis of available literature, this is the first and significant study regarding the comparative effect of different PGRs on *in-vitro* propagation study of *H. spicatum*. This significant study could be useful for large scale propagation and *ex-situ* conservation of this vulnerable Himalayan species.

Key words: Cytokinins, thidiazuron, shoot multiplication, *in-vitro* propagation, *ex-situ* conservation.

INTRODUCTION

Hedychium is one of the plant genus which is commonly used in the preparation of indigenous medicine. There are 50 species of *Hedychium* in tropical Asia (Kirtikar and Basu, 1984), 37 species in India and 8 species in Western Himalaya. *Hedychium spicatum* (Zingiberaceae), commonly known as kapoor kachri, van- haldi or ginger lilly, is distributed in subtropical Himalayas in the states of Assam, Arunanchal Pradesh and Uttarakhand at an altitude of 1000 to 3000 m. The root stock (rhizome) is useful in asthma, pain, foul breath, hiccough, vomiting and laxative, stomachache, carminative, stimulant tonic to the brain, in liver complaints, diarrhea and pains (Kirtikar and Basu, 1984).

Rhizome extracts showed antimicrobial activity against gram-positive and negative bacteria (Bisht et al., 2006). Recently, the crude extract of the rhizome has been used in the preparation of an anticancerous drug, PADMA 28 (Nayab et al., 2004). Samant et al. (2007) reported that *H. spicatum* is commercially exploited from its natural area. According to the threat status of IUCN criteria, *H. spicatum* has become vulnerable (Samant and Pant, 2006). It is also listed in near threatened category of the essential oil bearing plants (Samant and Palni, 2000). Hence, prioritization of this species needs to be done for propagation and conservation. Therefore, the present study was conducted to see the effect of different PGRs on micropropagation of this species.

MATERIALS AND METHODS

Seeds of *H. spicatum* were collected in the month of October to November, 2008, from Nainital catchment forest (1990 m) and

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Table 1. Effects of cytokinins on *in vitro* shoot multiplication of *H. spicatum* using excised shoot tips in MS medium.

Treatment (µM)	Number of shoot/ flask	Number of shoot/ explant	Percentage shoot multiplication	Average shoot length (cm)
Control (0.0)	05.00±0.00	-	-	1.80±0.65
BA (0.1)	05.66±0.88	1.13±0.17	53.33±6.66	3.92±0.30
(1.0)	07.00±0.57	1.40±0.11	53.33±6.66	3.653±0.52
(2.5)	08.66±1.45	1.73±0.29	43.33±23.33	3.214±0.41
(5.0)	07.00±1.54	1.40±0.23	40.00±11.54	2.568±0.07
(10.0)	05.66±0.33	1.13±0.06	40.00±11.54	2.632±0.36
(20.0)	05.66±0.66	1.13±0.13	40.00±11.54	2.236±0.34
KN (0.1)	07.66±1.76	1.46±0.40	76.66±8.81	4.94±0.36
(1.0)	07.33±0.88	1.46±0.17	53.33±6.66	4.00±0.35
(2.5)	07.00±1.52	1.40±0.30	73.33±13.33	4.54±0.95
(5.0)	07.66±1.45	1.46±0.35	56.66±20.27	3.90±0.17
(10.0)	07.33±0.33	1.13±0.26	40.00±11.54	3.52±0.23
(20.0)	05.33±0.33	1.15±0.07	26.66±6.66	3.59±0.83
TDZ (0.1)	10.66±1.20	2.13±0.24	83.33±12.01	1.52±0.08
(1.0)	19.33±4.91	3.86±0.98	83.33±12.08	1.10±0.15
(2.5)	16.66±0.88	2.66±0.54	63.33±14.52	2.01±0.34
(5.0)	14.00±6.50	2.80±1.13	50.00±20.81	1.41±0.08
(10.0)	08.33±1.76	1.16±0.35	56.66±20.27	1.53±0.03
(20.0)	10.33±2.90	2.06±0.58	40.00±11.54	1.99±0.16

Values are mean ± standard error, each treatment consisted of 15 explants (5 in each flask, 3 flasks per treatment). Data were recorded after 4 weeks of inoculation. - = no shoot multiplication.

brought to the laboratory. Seeds were separated from spikes, air dried for 3 days at room temperature and stored at 4°C in the dark. After 65 days, seeds were taken out and washed under running tap water along with a mild detergent and treated with a systemic fungicide (Bavistin, 1% w/v, 15 min). Following washing with double distilled water, seeds were transferred to laminar air flow cabinet. Surface sterilization was carried out by using mercuric chloride ($HgCl_2$, 0.1% w/v, 3 min) followed by washing with sterile distilled water (5 times). Seeds were inoculated (10 seeds per flask) on MS (Murashige and Skoog, 1962) basal medium containing agar (0.8% w/v) and transferred to the culture room. Three weeks after inoculation, shoot tips (size approximately 1.5 cm) were excised from *in vitro* germinated seedlings and inoculated on MS medium supplemented with different concentrations (0.1 to 20 µM) of 6-benzyladenine (BA), kinetin (KN) and thidiazuron (TDZ) (Table 1) for shoot multiplication. *In vitro* generated microshoots were used to induce rooting. These microshoots (2.5 to 5.0 cm in length) were inoculated on MS medium supplemented with different concentrations of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α- naphthalene acetic acid (Table 2).

All cultures were raised in 250 ml erlenmeyer flasks (100 ml medium in each flask). Sucrose (3.0%, w/v) was added as a carbon source and medium was gelled with agar (0.8%, w/v). The pH of the medium was adjusted to 5.8 before autoclaving (1.05 kg cm^{-2}, 121°C and 20 min). Cultures were maintained in a culture room at 25 ± 1°C in 16/8 h light/dark photoperiodic cycle, with irradiance by cool fluorescent tubes (Philips 40 W).

After rooting, the microshoots with well developed roots were taken out from the culture flasks and roots were then washed thoroughly with tap water to remove the adhering medium. Following washing, these plantlets were transferred to thermocole cups (8 cm height and 4 cm diameter) containing soil and farmyard manure (3:1, v/v). Transparent polythene bags were inverted over these thermocole cups (8 cm height and 4 cm diameter) to maintain the humidity and watered on alternate days. Initially, thermocole cups were kept inside a culture room for first four weeks and gradually removed to polyhouse (25 ± 1°C) for further growth. After 2 months, these hardened plantlets were transferred to field (near department nursery) to study the survival rate of these *in vitro* raised plantlets.

Statistical analysis

Data were subjected to statistical analysis and standard error (SE) and ANOVA were calculated following Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Seeds started germinating within 15 days of inoculation on MS basal medium. After one week of germination (3 weeks after inoculation), the explants (shoot tips, size approximately, 1.5 cm) from *in-vitro* germinated seedlings were excised and inoculated on MS medium supplemented with different concentrations of BA, KN and TDZ which resulted in multiple shoot formation. However, number of shoots per explant, as well as number of shoots per flask varied with the treatments (Table 1). Each shoot tip when cultured on MS basal medium (without PGRs), developed into a complete plantlet with profuse root system. MS medium when supplemented

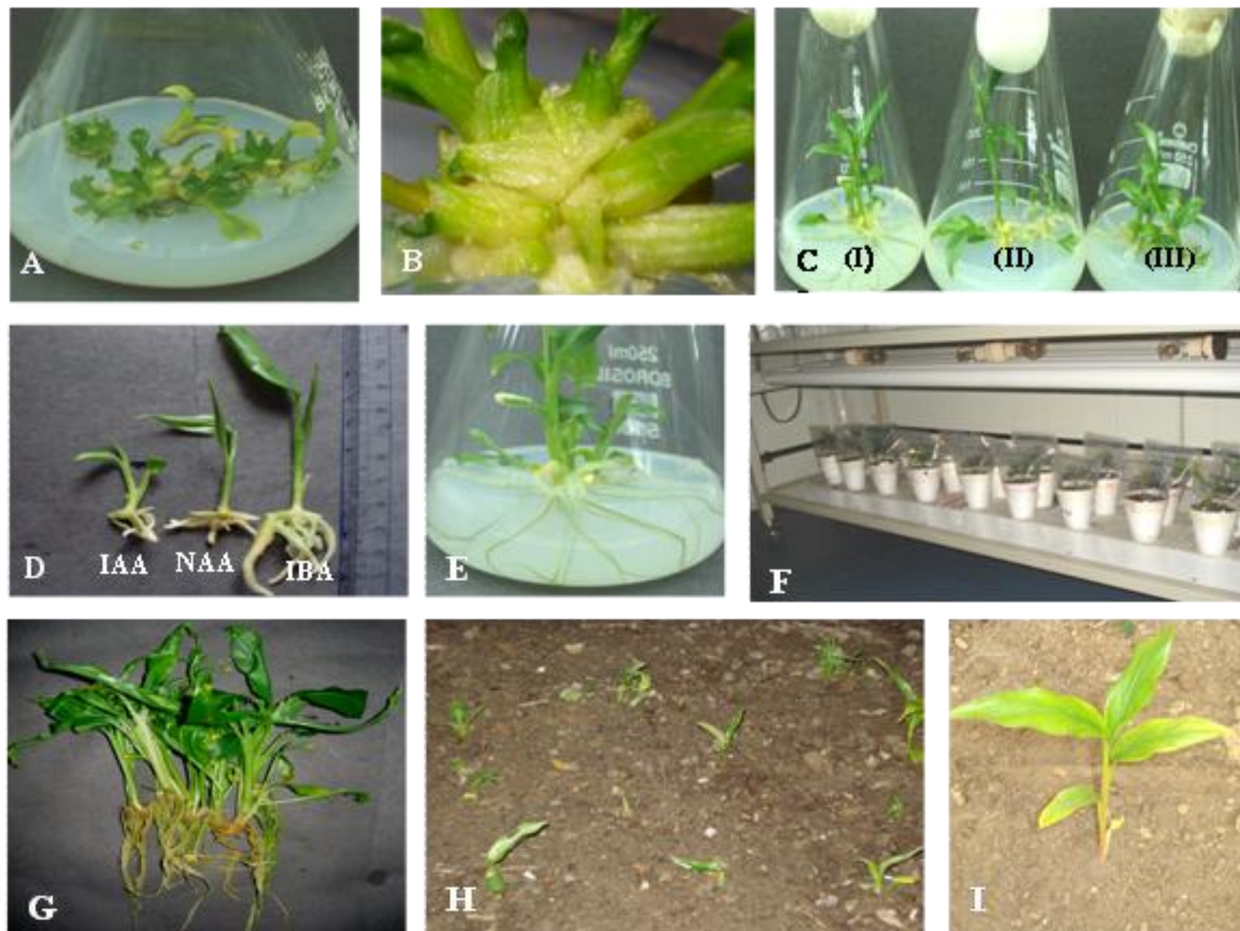

Figure 1. Micropropagation of *H. spicatum*. A and B, Lower concentration of TDZ (1.0 µM) showed high frequency of shoot multiplication after 4 weeks of inoculation in MS medium; C, effect of cytokinins on shoot length after 8 weeks of inoculation in MS medium: II (KN) > I (BA) > III (TDZ); D, effect of auxins on rooting after 4 weeks of inoculation in MS medium; E, rooting in lower concentration of IBA (2.5 µM) after 8 weeks of root initiation; F, hardening of plantlets in a culture room; G, well rooted plants after two months of hardening (photograph taken just before field transfer); H, field transfer of plantlets (at department nursery); I, well grown plant in field after 3 months of field transfer.

with different concentrations of BA had best response in 2.5 µM in terms of the number of shoots per flask (8.66) and number of shoots per explant (1.73). However, percent shoot multiplication was maximum (53.33%) with 0.1 and 1.0 µM BA, and the average shoot length was highest (3.92 cm) in lower concentration (0.1 µM) of BA. When KN was used for shoot multiplication, all the used concentrations (0.1 to 20 µM) had more or less same effect for three parameters (number of shoots per flask, number of shoots per explant and average shoot length) (Table 1), but percent shoot multiplication was maximum (76.66%) in lower concentration of KN (0.1 µM). Among various concentrations of TDZ tested, 1.0 µM TDZ produced maximum number of shoots (19.33 shoots per flask, 3.86 shoots per explant) within four weeks (Table 1 and Figure 1A, B). Although other concentrations of TDZ also gave good shoot multiplication, the percent shoot multiplication was highest (83.33%) in lower concentration of TDZ (0.1 and 1.0 µM). The shoots formed in TDZ supplemented medium were short in length but the shoot length was quite good in KN supplemented MS medium (Figure 1C). Data were analyzed by using ANOVA which showed that among the used cytokinins, average number of shoots per flask, number of shoots per explant and average length of shoots were highly significant ($p < 0.01$ at 1%). However, only percent shoot multiplication was insignificant ($p < 0.01$ at 1%) within used concentrations of cytokinins (Table 2).

Effect of different auxins on *in vitro* induction of rooting of microshoots of *H. spicatum* is summarized in Table 3. All the used auxins (concentration ranges from 0.1 to 10 µM) were able to induce rooting in microshoots, including control (auxin free MS medium) also. However, rooting percentage in control was only 29.62% and it took 21 days to root in comparison with other treatments where the differentiation of roots on the elongated shoots

Table 2. ANOVA summary.

Source	Dependent variable	df	Mean square	F-value
Cytokinins	Average number of shoots per flask	2	215.73	16.25*
	Average number of shoots per explant	2	7.39	12.40*
	Percentage shoot multiplication	2	1220.63	2.51ns
	Average shoot length (cm)	2	22.46	37.39*
Concentration	Average number of shoots per flask	6	42.75	3.22 *
	Average number of shoots per explant	6	4.77	8.02*
	Percentage shoot multiplication	6	5039.68	10.37*
	Average shoot length (cm)	6	2.775	4.62*

*Significant at 1% ($p < 0.01$) and ns, not significant; no, number; df, degree of freedom

Table 3. Effect of different auxins on *in vitro* rooting of micro-shoots of *H. spicatum* on MS medium.

Treatment (µM)	Percentage rooting	Time taken for root emergence (days)	Average number of root per shoot	Average length of root (cm)
Control (0.0)	29.62±3.70	21.00±1.15	1.15±0.03	1.86±0.03
IAA (0.1)	40.73±3.70	14.00±0.57	2.74±0.41	2.20±0.20
(1.0)	51.84±3.70	15.33±0.66	3.15±0.26	3.30±0.52
(2.5)	66.66±0.00	10.66±0.88	3.40±0.87	3.15±0.30
(5.0)	55.55±6.41	16.33±1.45	2.54±0.05	2.56±0.46
(10.0)	33.33±0.00	15.00±0.57	2.17±0.12	1.78±0.33
NAA (0.1)	44.44±6.41	11.66±1.45	3.12±0.43	2.94±0.10
(1.0)	62.95±3.70	10.35±0.47	3.07±0.29	3.74±0.19
(2.5)	81.47±3.70	10.5±0.57	3.83±0.16	3.92±0.18
(5.0)	85.17±3.70	10.44±0.59	3.78±0.18	3.42±0.46
(10.0)	37.03±3.70	11.77±0.39	2.59±0.02	2.54±0.22
IBA (0.1)	59.25±3.70	11.00±0.57	3.86±0.16	3.30±0.20
(1.0)	70.36±3.70	10.33±0.88	4.07±0.27	4.31±0.38
(2.5)	96.29±3.70	10.24±0.58	5.25±0.08	4.87±0.39
(5.0)	92.58±3.70	10.00±0.57	5.06±0.47	5.26±0.44
(10.0)	55.55±6.41	12.00±0.57	3.27±0.46	3.01±0.25

Values are mean ± standard error (SE). There are nine explants (three microshoots per flask) in each treatment.

occurred over a period of 1 to 2 weeks (Table 3). Trend for percent rooting was similar in all the three used auxins, which means percent rooting increased with increasing concentration of auxins up to certain extent and then it decreased with increasing concentration of auxin. In case of NAA, rooting percentage increased with increasing concentration up to 5.0 µM, and 5.0 µM NAA was able to induce 85.17% rooting, then rooting percentage decreased to 37.03% as the concentration increased to 10.0 µM. Similar trend was observed in microshoots which were inoculated in IAA and IBA supplemented MS medium. In these two auxin treatments, percentage rooting increased only up to 2.5 µM concentration and then it decreased with increasing concentration (Table 3). Among different concentration of IAA, 2.5 µM concentration enhanced maximum rooting percentage (66.66%) within 10 days of inoculation. The average number of roots per shoot was highest (3.40 roots/shoot) in 2.5 µM IAA, while 1.0 µM showed maximum average length of roots (3.3 cm). Percent rooting was improved up to 85.0% by using 5.0 µM of NAA within 10 days of inoculation, however, the lower concentration of NAA (2.5 µM) showed best response in relation to the average number of roots per shoot (3.83 roots) and average length of roots (3.92 cm). Among various concentrations of IBA, the lower concentration (2.5 µM) showed maximum

Table 4. ANOVA summary.

Source	Percentage rooting			Time taken for root emergence (in day)			Average number of root/shoot			Average length of root (cm)		
	DF	MS	F-ratio	DF	MS	F-ratio	DF	MS	F-ratio	DF	MS	F-ratio
Concentration	5	3791.0	75.37*	5	128.0	57.80*	5	10.3	52.9*	5	10.39	36.67*
Auxins	2	1982.0	39.40*	2	49.3	22.26*	2	7.40	38.0*	2	7.54	26.61*
Concentration x auxins	10	160.4	3.19*	10	6.0	2.74**	10	0.60	3.1*	10	0.64	2.28**
Error	36	50.29		36	2.2		36	0.19		36	0.28	

*Significant at 0.01 level (at 1%); ** significant at 0.05 level (5%).

percent rooting (96.29%) and average number of roots per shoot (5.25 roots), while the average length of roots was 5.26 cm in 5.0 µM IBA supplemented medium. Out of these three used auxins, IBA showed best response in relation to percent rooting; average number of roots per shoot and average length of roots (Table 3 and Figure 1D, E). Mean significance difference (at the 5% level) computed by applying ANOVA showed a significant difference among various parameters (percent rooting, time taken for root emergence (in days), average number of roots per shoot and average length of roots) (Table 4). One month after rooting, these plantlets were hardened under growth chamber for four weeks (Figure 1F). Well rooted and healthy plantlets were obtained after 2 months of hardening (Figure 1G) and transferred to the field condition (1990 m) with ninety percent survival rate (Figure 1H and I).

Therefore, on the basis of results obtained in this experiment, TDZ was found to be the most effective cytokinin for shoot multiplication, however, shoot lengths was better in KN supplemented medium. On the basis of literature, it is known that TDZ act as potent regulator for *in vitro* propagation system and as an effective mean of induction of adventitious shoots in a number of plant species (Huettman and Preece, 1993; Lu, 1993). It has been known to promote cytokinins-like activity similar to that of N^6 substituted adenine derivatives and has been used in tissue culture studies since 1982 (Mok et al., 1982; Siddique and Anis, 2007). TDZ stimulate cytokinins activity and it is a better substitute of other cytokinins like BA and KN that are generally used in tissue culture work (Kannan et al., 2007; Vinayak et al., 2009). Sujatha and Reddy (1998), also reported that a higher shoot multiplication rate was obtained on TDZ supplemented media. However, it concomitant with a mark reduction in shoot length. Mok et al. (1987) showed that lower concentration of TDZ (0.3 µM) was very effective in multiplication of Broccoli (*Brassica oleracea)* and higher concentration (10.0 µM) induced abnormal growth and vitrification. TDZ stimulated shoot regeneration in a concentration-dependent manner, and higher concentration significantly reduced the height of newly formed shoot (Tomsone et al., 2004). Present study indicates that by using lower concentration of TDZ (1.0 µM), the shoot multiplication frequency was the best. The high efficiency of TDZ may be attributed to its ability to induce cytokinin accumulation (Victor et al., 1999) or enhance the accumulation and translocation of auxin within the tissue (Murthy et al., 1998). The growth promoting activity of thidiazuron was accompanied by high acid-phosphate level (Mok et al., 1987) and is stable and biologically active at low concentration than other cytokinins (Genkov and Ivanova, 1995).

This significant study concluded that the lower concentration of IBA was the prominent PGRs regarding i*n vitro* rooting. In the present study, the superiority of IBA over other auxins regarding *in vitro* rooting is supported by the result of other studies like Sreekumar et al. (2000) in *Hemidesmus indicus*, Mala and Bylinsky (2004) in *Daphne cneorum,* Kannan et al. (2006) in *Caesalpinia bonduc* and Gantait et al. (2009) in *Dendrobium chrysotoxum*. The present investigation suggests that the IAA showed least response in root formation. In comparison with other auxins, similar observation was reported by Kannan et al. (2007). However, it was clear that increasing the concentration of these auxins did not responded well in the present study. The variation in effectiveness of different auxin sources may be attributed to their differential affinity to auxin receptors involved in the rooting process, which may be cultivar specific (Tereso et al., 2008) and depend on the plant type (Nandgopal and Kumari, 2007). A disadvantage of micropropagation is the high mortality rate during the transfer of *in vitro* plantlets to *ex vitro* (Lovato et al., 1999). But in the present study, a very good survival rate (90.0%) was achieved in comparison to earlier studies like Badoni et al. (2010), where they got only 40 to 50% survival rate, and Koul et al. (2005) that reported 80 to 85% survival rate. There were no previous literatures available on the comparative study that applied these three different auxins on *in vitro* rooting of this species. This significant study shows that the lower concentration of IBA was the prominent plant growth regulator regarding *in vitro* rooting.

On the basis of available literature regarding micropropagation study of *H. spicatum* (Koul et al., 2005; Badoni et al., 2010), there was no such type of comparative study using different plant growth regulators and

this would be the first report on this species. This significant study concluded that, by using lower concentrations of TDZ (1.0 μM) and IBA (2.5 μM), it could be able to achieve a number of *in vitro* raised plantlets within a limited period of time. Beside propagation, this study would also become an *ex situ* conservation strategy of this vulnerable Himalayan species.

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