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Induction of somatic embryogenesis in endangered butterfly ginger *Hedychium coronarium* J. Koenig

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An efficient protocol has been developed for regeneration of complete plants through somatic embryogenesis in *H. coronarium*. Creamish white, pale yellow and brown calli were obtained on MS medium supplemented with different concentrations of auxins [2, 4-Dichlorophenoxy acetic acid (2, 4-D), Indole-3 acetic acid (IAA) and 1-Naphthylacetic acid (NAA)] after 4 weeks. Creamy white calli developed on 0.5 mg L^{-1} 2, 4-D turned embryogenic when subcultured on basal medium and produced small globular somatic embryos after 6 weeks. Further growth of somatic embryos required their transfer to medium containing 6-benzylaminopurine (BAP) or kinetin (KN). BAP was more effective than KN in promoting shoot proliferation. Maximum shoot length was obtained with 0.5 mg L^{-1} BAP whereas maximum shoot number was obtained with 1.0 mg L^{-1} BAP. The plantlets thus formed were successfully hardened, and transferred to sand-soil and farm yard manure (1:1:1) with 95% survival.

Keywords: Callus culture, *Hedychium coronarium*, Medicinal plant, Somatic embryogenesis

Hedychium coronarium J. Koenig commonly known as butterfly ginger or Gulbakawali is an ornamental, medicinal and aromatic rhizomatous herb belonging to the family Zingiberaceae. It is cultivated in India, South East Asian countries, China, Japan[1]. The Himalayas are the probable centre of its origin[2]. This plant has tremendous medicinal properties and its various parts are used in traditional as well as modern medicine. The rhizome of the plant is used in the treatment of diabetes[1,3], headache, lancinating pain, contusion, inflammation and rheumatic pain[4]. The rhizome has anticancerous[5], antihypertensive, diuretic, leishmanicidal, antimalarial[6] activities.

The flowers of *H. coronarium* are widely cultivated for sweet perfume[15] and also provide essential oil borneol, methyl silicate, eugenol and methyl lanthranilate[7]. Furano diterpenes viz hedychenone[8] and labdane type diterpenes—coronarin A, B, C, D, E and F have been isolated from the rhizome of *H. coronarium*[4,9].

Due to restricted distribution of the plant, its over exploitation and harvest for medicine and trade, land clearing and lack of management practices it is fast disappearing[10]. As a result the plant has become endangered in Madhya Pradesh, Central India and other areas[11] and has become Red listed in Manipur[12]. Regeneration and reestablishment of plants through *in vitro* culture is one of the most effective biological techniques to conserve its diversity.

Monocotyledonous plants have always offered challenges towards *in vitro* plantlet regeneration, largely due to lack of sufficient areas of meristematic activity in them[13]. Due to good demand of the plant albeit difficult natural propagation, it is necessary to develop a suitable protocol for mass propagation from existing elite cultivars. The process of somatic embryogenesis is a suitable method of micropropagation and has the potential for mass production commercially at low cost.

Cell culture techniques for monocots enabling complete plant regeneration through somatic embryogenesis are available mostly for cereals and grasses. There have been few reports to date on somatic embryogenesis in the genus. Shoot apex derived callus of *Hedychium muluense*[14] and somatic embryogenesis in *H. bousigonianum*[15] has been the only *in vitro* report on this genus. To the best of our knowledge there is no report on *in vitro* regeneration in *H. coronarium.* The present communication reports first indirect somatic embryogenesis from rhizome explant in *Hedychium coronarium* J. Koenig.

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Materials and Methods

Plant material—The plant material was procured from the nurseries of Jawahar Lal Nehru Krishi Viswavidhyalaya (JNKVV), Jabalpur and State Forest Research Institute (SFRI), Jabalpur (M.P). Plants were identified by Institutional Botanist and voucher specimen (No. 1993) of healthy and vigorous plants were chosen for the study.

Establishment of cultures—Juvenile (1-2 month old) disease free plants were selected for the present work. Rhizome was excised and washed thoroughly under running tap water for 30 min to wash off the adhering soil particles. Roots and outer scales were removed and the rhizome was washed with 0.01% Labolene (Qualigens, India) for 10 min, followed by repeated washings with tap water. The explants were then transferred to laminar air flow hood where they were surface sterilized in 70% ethanol for 5 min after which they were washed with sterilized distilled water 2-3 times and finally treated with 0.1% $HgCl_2$ for 5 min. Explants were finally washed with sterile distilled water (3-4 times).

Explants were cut into segments of 5-8 mm × 4-5 mm size, dried on sterile filter paper and aseptically transferred onto solidified MS[16] medium supplemented with different (0.1, 0.5, 1.0 and 5.0 mg L^{-1}) concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D), 1-naphthylacetic acid (NAA) and indole-3 acetic acid (IAA) supplemented with 3% (w/v) sucrose and 0.8% agar. The *p*H of the medium was adjusted to 5.6-5.8 (before adding agar) with 1 *N* NaOH and 1 *N* HCl prior to dispensing into culture tubes (15 × 150 mm). All the cultures were maintained at 25 ± 2 °C and photoperiodic cycle of 16 h light [approx 1500 lux] and 8 h dark provided by Philips cool white fluorescent tubes. The cultures were transferred onto fresh culture medium at every 4 weeks interval. All experiments were conducted in triplicates containing 12 explants each for each treatment.

Induction of callus—Callus was induced on the optimal callus induction medium with 0.5 mg L^{-1} 2, 4-D and was maintained on the fresh medium of the same composition by regular sub culturing after every 4 weeks. After one subculture friable callus was obtained. The embryogenic callus identified by anatomical studies on the basis of its creamy white colour and presence of small less vacuolated and densely filled cytoplasmic cells.

Induction of somatic embryos—The embryogenic callus obtained was sub cultured on basal medium (BM) for accelerating the induction of somatic embryos. After two weeks of subculture pale yellow, globular pro-embryoids were observed. The pro-embryoids matured on the same medium for further 2 weeks. Plants with healthy roots thus obtained were further subjected to hardening.

Histological studies—For histological studies the embryologic calli were fixed in FAA for 24 h, washed for 30 min with tap water, dehydrated by transferring through an ethanol –xylol series and then embedded in paraffin. Tissues were sectioned with 10µm thickness with microtome, mounted on glass slides, and stained with 0.5% safranin and 0.5% fast green[17].

Transfer of in vitro regenerated plant to soil—*In vitro* regenerated plants (3-4 months) containing well developed shoot and a root system were washed with tap water to remove agar without damaging the delicate root system and treated with 1% bavistin for 10 min. The plants were transferred to thermacol cups containing soil, sand and farmyard manure (1:1:1) for hardening. This mixture was sterilized by autoclaving for 20 min at 121 °C. To maintain the humidity, hardened plants were completely covered with plastic bags, which were removed progressively to aid adoption to normal environmental conditions and it was irrigated with sterilized distilled water. Plantlets were covered with plastic bags. After 2 weeks, polythene bags were perforated for gradual acclimatization. After 2 more weeks the plastic covers were removed for an hour daily. Subsequently exposure time was increased in the following weeks and after 7-8 weeks plastic covers were removed completely. After 8 weeks of hardening the plantlets were transferred to field.

Statistical analysis—All the experiments were conducted using completely randomized design with three replicates. The data were analyzed using analysis of variance (ANOVA) and means among treatments were separated by Duncan's multiple range test[18].

Results

Induction of callus- Explants induced callus after 6 weeks of culture on 2, 4-D supplemented callus induction medium. Callusing commenced at the cut ends of the explants leading to rapid cell division and subsequent callus formation. After 6 weeks of culture incubation, callus mass increased to a sizable proportion (Fig. 1a). Callus was mostly soft, sticky with pale yellow or creamish colour. Among different concentrations of auxins 0.5 mg L^{-1} 2, 4-D was found to be the best for induction of callus (Table 1).

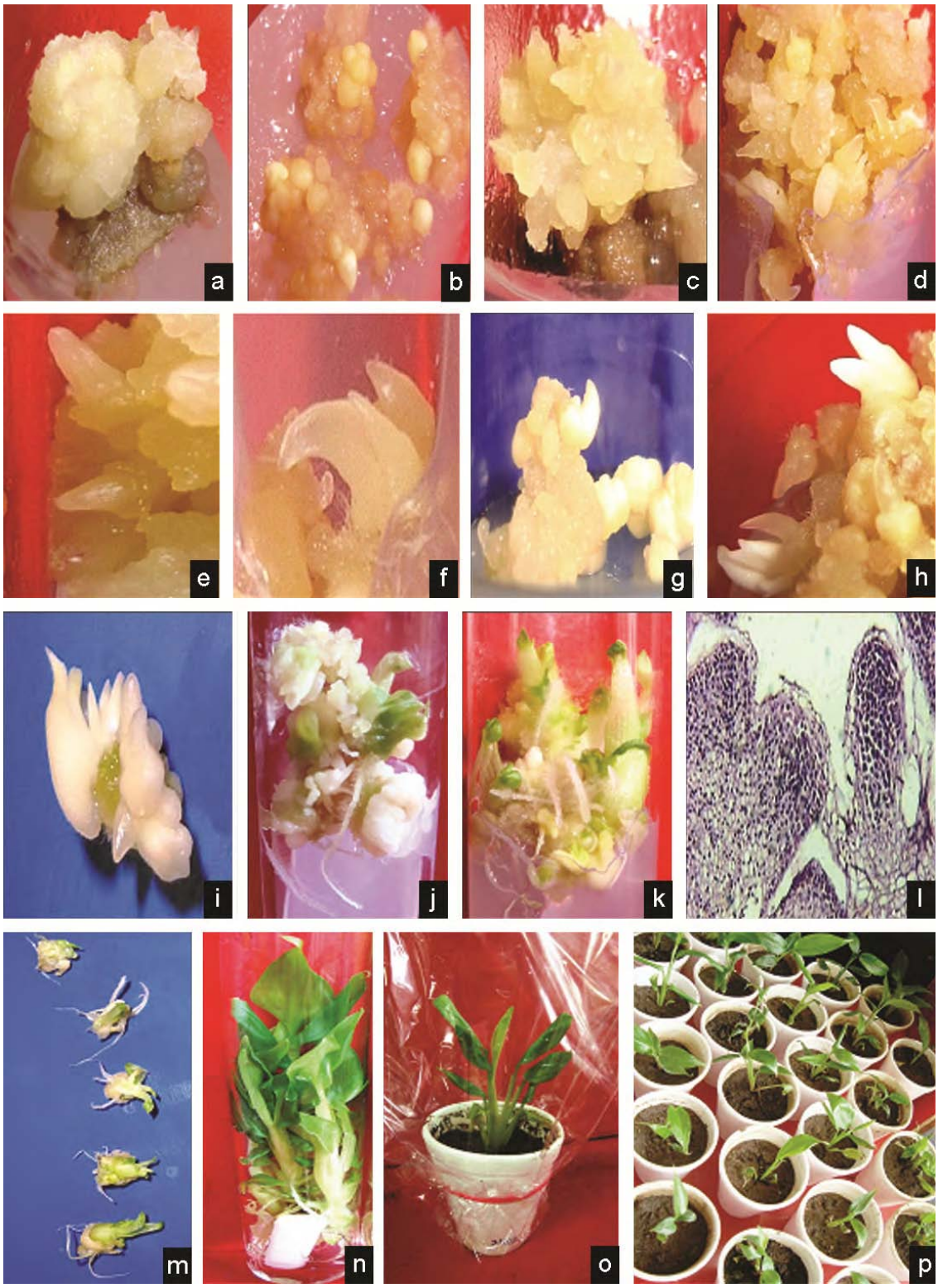

Fig 1—Callus proliferation and somatic embryogenesis in *H. coronarium*. (a) sticky callus, (b) white globular embryoids on the peripheral region of the callus on medium supplemented with 2, 4-D (0.5 mg L^{-1}), (c-e) club shaped somatic embryos, (f) scutellum shaped embryos, (g-i) close-up view of somatic embryo, (j) somatic embryo with cotyledon and scutellum initiation, (k) mature green somatic embryos on BAP (1.0 mg L^{-1}), (l) histological slide of globular somatic embryos. (m) somatic embryos of different sizes, (n) proliferation of plants from somatic embryo, (o-p) acclimatization of plantlets.

Induction of somatic embryos—After 6 weeks of incubation the embryogenic calli obtained from the rhizome were excised and transferred to plant growth regulator free medium (BM). After 2 weeks of sub-culturing on BM creamish white sectors were seen. Numerous globular somatic embryos (Fig. 1b) appeared on the surface of callus on BM. This process continued in the subsequent sub cultures leading to the successive production of globular, torpedo and scutellum shaped somatic embryos (Fig. 1c-i). Fully developed somatic embryos consisted of a scutellum, coleoptile (12 weeks), shoot apex and coleorhiza (Fig. 1j-l).

Germination of somatic embryos—The somatic embryos obtained failed to germinated on auxin (2, 4-D) supplemented medium and required sub culture to cytokinin supplemented medium. The mature somatic embryos of 12 weeks were transferred to BM and somatic embryo regeneration medium containing different (0.1, 0.5, 1.0, 5.0 mg L^{-1}) concentrations of cytokinins such as 6-benzylaminopurine (BAP) and kinetin (KN). The mature embryos on BM and BAP supplemented medium turned green after 1 week of culture. After 2 weeks the coleoptile became green and shoots differentiated with one or two leaf plumules at one end and white, hairy roots at the other end. At this stage the somatic embryos started germinating and develop shoots (Fig. 1k). Shoots attained a height of 12-15 cm within 17 weeks (Fig. 1m). Germination of somatic embryos was observed in all the plant growth regulators (PGR) concentrations attempted and germination percentage varied from 20-30% on KN, 50% on BM and 60% on BAP.

Of the two cytokinins tested, BAP was more effective than KN in supporting shoot proliferation. On medium containing KN, only three to four plants developed per explant. Among the PGR concentrations attempted the optimum shoot length was obtained on 0.5 mg L^{-1} BAP whereas maximum shoot number was obtained on 1.0 mg L^{-1} BAP (Fig. 2a and b). Among all the concentrations attempted 0.5 mg L^{-1} BAP produced maximum root number and root length (Fig. 2c and d). High conversion frequencies of somatic embryogenesis on 0.5 mg L^{-1} BAP rich medium were obtained (Fig. 3b). Somatic embryogenesis on BAP was found

Table 1—Callus induction from rhizome of *H. coronarium* after 6 weeks (12 explants treatment).

Growth regulators (mg L^{-1})	Explants inducing callus (%)	Degree of callus formation
2, 4-D		
0.1	-	-
0.5	80	***
1.0	70	**
5.0	40	**
10.0	30	*
NAA		
0.1	-	-
0.5	30	*
1.0	45	**
5.0	80	***
10.0	25	*
IAA		
0.1	-	-
0.5	20	*
1.0	35	*
5.0	35	*
10.0	40	**

(-) indicates no response, (*) slight callusing, (**) considerable callusing,
(***) profuse callusing.

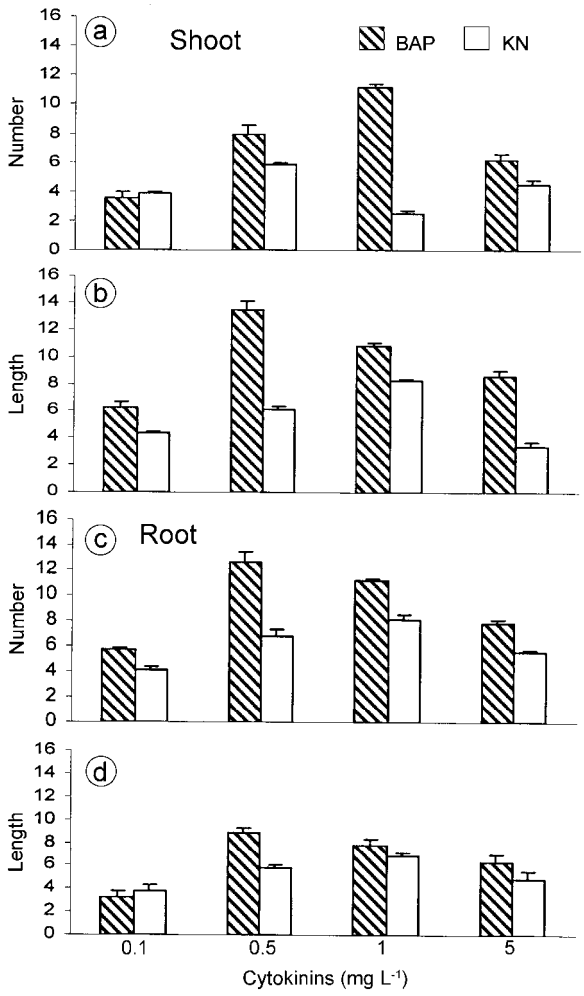

Fig. 2—Effect of cytokinins on somatic embryo derived shoot number (a), length (b), root number (c) and length (d).

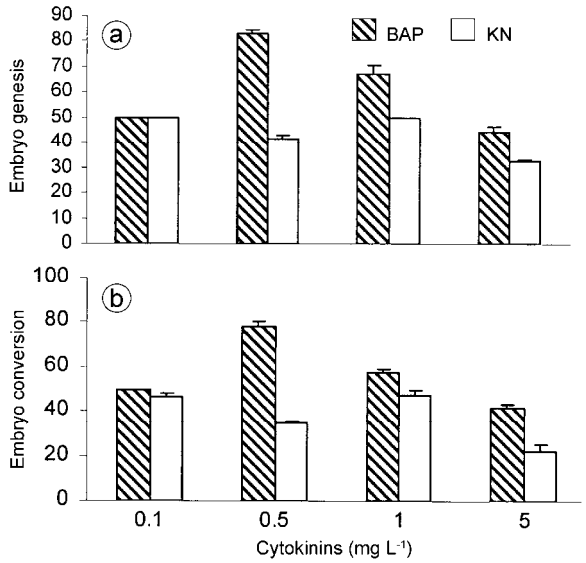

Fig. 3—Effect of cytokinins on somatic embryogenesis (a) frequency of somatic embryo conversion into plants

to be repetitive (Fig. 1j) and supported all stages of indirect somatic embryogenesis from *Hedychium corornarium.*

Acclimatization of tissue culture raised plants—Well developed plantlets were transferred to thermocol cups containing sterile sand, soil and farm yard manure (1:1:1). Approximately 95% of hardened plantlets survived (Fig. 1n and o). After 4 weeks of hardening the plantlets was transferred to field.

Discussion

In recalcitrant monocotyledonous species, efficient and reproducible regeneration protocols have been developed using mostly immature cells and tissues such as immature embryos[14]. Also the lack of seed set in Zingiberaceae family makes conventional breeding methods inapplicable. The age of explants plays a major role in somatic embryogenesis[19]. Low frequency of embryogenesis and embryogenic induction related to the age of explants suggest that the intrinsic physiological stage of explant plays a decisive role in the induction of embryogenesis. Such observations were reported in somatic embryogenesis from zygotic embryo culture in many plant species[20].

Somatic embryogenesis has already been used as a method for conserving overexploited and endangered plant species. In monocotyledons, auxins as promoters of cell differentiation have been found to induce indirect somatic embryogenesis. The calli induced by NAA and IAA were shown to be inappropriate for somatic embryo induction in the present study, while the one induced by 2, 4-D was suitable. In Zingiberaceae species, 2, 4-D has been used for somatic embryo induction through callus, such as *Zingiber officinale*[21] and *Kaempferia galanga*[22].

Growth regulators play an important role in the initiation of somatic embryogenesis. Successful plantlet regeneration from somatic embryos was observed in the present study with BAP showing better rooting and shooting response than KN. The sole use of BAP in the culture medium seems better for shoot elongation. Among the various concentrations of BAP tested, the highest number of shoots per explant was observed on medium containing 1.0 mg L^{-1} BAP. Similarly BAP was found more efficient for germination of somatic embryo in *Boesenbergia rotunda*[23]. Lower concentrations of 1.0 mg L^{-1} BAP resulted in successful shoot induction from ginger rhizome derived calli[24]. BAP is reported to stimulate shoot multiplication in different explants of *Saccharum officinarum*[25]. Complete plant regeneration through embryogenesis was observed from different explant of *Allium sativa*[26] and *Oryza sativa*[27] using BAP. The positive effect of PGR free medium (BM) on somatic embryo development has been reported frequently, especially for the members of Liliaceae and Iridaceae families[28,29].

In the present study, shoots obtained on BAP were healthy, bearing long and thick green leaves and healthy roots. Plantlets obtained on BM had long shoots with white hairy roots, but leaves were viscous and yellowish green in colour. However, shoot formation response on KN supplemented media was considerably low and shoots obtained on KN were weak, plants were small in size with reduced shoot length. On this medium, frequency of embryo conversion into shoots and roots was also low. The quality of shoots obtained on BAP was better than shoots obtained on BM and KN.

BAP was nearly twice as effective as KN in number of shoot buds regenerated per explants. The superiority of BAP over KN has been reported earlier[26,30]. The somatic embryos in monocots followed the typical developmental stages such as globular, club shaped and the plumule stage. The monocotyledon embryo is cylindrical and shoe shaped with a slightly pointed distal end and broad blunt coleorhizal end. Similar pattern of embryo development in other monocots like *Hemerocallis*[31] has been reported.

Conclusion

The *in vitro* techniques help in multiplication of plant species, which have limitations of conventional

propagation. The present study has established an effective protocol of plant regeneration through somatic embryogenesis in *Hedychium coronarium*. The optimum concentration of BAP (0.5 mg L^{-1}) was found to be effective for somatic embryo formation. This would help the large scale propagation of *H. coronarium* through *in vitro* regeneration.

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